Effect of different substrates on functional performance and kinase activation during reperfusion after ischaemia in hearts from obese insulin resistant rats

by

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DECLARATION

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Abstract

Obesity is an important risk factor for the development of insulin resistance, the metabolic syndrome and diabetes and has also been implicated as one of the major risk factors for coronary heart disease. Ischaemic heart disease impacts on both cardiac metabolism and function.

During early reperfusion after ischaemia, several protein kinases are specifically activated, including PI3K/PKB, MAPKs (ERK, JNK and p38 MAPK), and the tyrosine kinases. Activation of PKB and ERK, the so-called reperfusion injury salvage kinase (RISK) pathway, is associated with a reduction in infarct size and/or improvement in functional recovery. PKB is an enzyme central also to insulin signalling and glucose uptake. Activation of the JNK signaling pathway has been suggested to be a prerequisite for PKB activation; however, its role in ischaemia/reperfusion (I/R) injury remains controversial.

We hypothesize that obesity-induced insulin resistance will affect infarct size, functional recovery and interactions between PKB, JNK, ERK, p38MAPK and PTEN activation during reperfusion after exposure to ischaemia. The aim of the study was therefore to assess the effects of hyperphagia-induced obesity and insulin resistance in rats on the response of the heart to I/R injury, with particular attention to the intracellular signalling pathways during early reperfusion. To further elucidate the role of JNK, we used SP600125, a specific inhibitor of JNK.

Methods: Insulin resistance was induced by feeding rats a high caloric diet for 16 weeks (DIO). Hearts from DIO and age-matched controls (C) were perfused in the working mode (preload 15cm H_2O; afterload 100cm H_2O) and subjected to (i) 15 min global ischaemia followed by different reperfusion times for evaluation of functional recovery and freeze-clamping of tissues for Western blot or (ii) 35 min regional ischaemia followed by 2 hours reperfusion for infarct size determination (IS), using tetrazolium staining. Substrates were glucose (G) (10mM), glucose (10mM) plus BSA (3%) (G+B), and glucose (10mM) plus fatty acid (1.2mM palmitate / 3% BSA) (G+FA). The JNK inhibitor, SP600125, was administered either before ischaemia or during reperfusion after ischaemia. Infarct size, functional recovery as well as expression and activation of PKB, ERK, JNK, p38MAPK and PTEN were used as endpoints.

Results: (I) In the presence of glucose alone as substrate, the hearts from DIO rats exhibited an improved tolerance to ischaemia/reperfusion (I/R) injury as reflected by an increase in functional recovery (after exposure to 15 min global ischaemia) as well as a reduction in infarct size (after 35 min regional ischaemia) compared with the age-matched controls. This was associated with early activation of PKB and JNKp54/p46 at 10 min reperfusion, with down regulation of activation of these kinases after 30 min reperfusion.

(II) Contrary to expectations, the combination of a high concentration of fatty acids and glucose as substrates (G+FA) afforded significantly more protection against I/R injury in hearts from both DIO and control rats, when compared with the respective groups perfused with glucose alone as substrate. This improved protection in both groups was associated with increased activation of the PKB pathway. Interestingly, perfusion with glucose and a high concentration of fatty acid maintained PKB activation throughout the reperfusion phase, in contrast to the transient activation seen with glucose alone as substrate.

(III) SP600125 (10 uM), administrated either before ischaemia or during early reperfusion after ischaemia, almost completely inhibited the JNK pathway and exacerbated myocardial I/R injury, particularly in hearts from DIO rats.

Conclusion: Our study demonstrates, in contrast to several other studies, that dietary-induced obesity and high perfusate fatty acid concentrations, increase the tolerance of the ex vivo myocardium to I/R injury. It was also found that, contrary to expectations, a high concentration of circulating fatty acid was not detrimental to hearts of normal rats during I/R, indicating the beneficial actions of fatty acids on the outcome of I/R injury. This protection was shown to be associated with activation of PKB and JNK during early reperfusion.

Administration of the selective JNK inhibitor, SP600125, before or after myocardial ischaemia indicates that JNK and its downstream signalling pathways are critical in mediating protection against I/R in our study. SP-induced effects were also associated with lower activation of PKB. Our results suggest that the cross-talk between the JNK and PKB pathways in the post-ischaemic myocardium may be a major contributing factor to the outcome of I/R injury.

The data presented here, although seemingly dichotomous, actually solidify the hypothesis that JNK signalling specifically and simultaneously modulates pro- and antiapoptotic effector mechanisms within cardiomyocytes. They also reflect an extraordinary complexity of the heart's metabolic, functional, and structural changes in obesity. In addition, the results obtained showed that moderate hyperphagia-induced obesity does not have a harmful effect on the ischaemic-reperfused heart and in fact, reduced the sensitivity of the heart to I/R damage. This was further substantiated by the beneficial effects of fatty acids in the perfusate.

Taken together, our results are potentially of clinical significance, and confirm the importance of events during early reperfusion as possible therapeutic targets.

Abstrak

Vetsug is ‘n belangrike risikofaktor in die ontwikkeling van insulienweerstandigheid, die metaboliese sindroom en diabetes en word beskou as een van die hoof risikofaktore van koronêre hartvatsiektes. Iskemiese hartsiekte op sy beurt, affekteer beide miokardiale metabolisme en funksie.

In die vroeë fase van herperfusie na miokardiale iskemie word verskeie proteïen kinases soos byvoorbeeld PI3K/PKB, die MAPKs (ERK, JNK en p38 MAPK), asook tirosien kinases, geaktiveer. Aktivering van PKB en ERK, die sogenaamde herperfusie-besering herwinningspad (RISK), word met ‘n vermindering van infarktgrootte en/of ‘n verbeterde funksionele herstel, geassosieer. PKB staan ook sentraal aan insulienseintransduksie en glukose opname. Aktivering van die JNK seintransduksiepad is voorgestel om ‘n voorvereiste vir die aktivering van PKB te wees maar die rol van hierdie pad in iskemie/herperfusie (I/H) besering, is tans kontroversieël.

Ons hipotese is dat vetsug-geïnduseerde insulienweerstandigheid miokardiale infarktgrootte, funksionele herstel asook die interaksie tussen PKB, JNK, ERK, p38MAPK en PTEN aktivering gedurende herperfusie na iskemie, sal beïnvloed. Die doel van hierdie studie was dus om die effek van hiperfagie-geïnduseerde vetsug en insulienweerstandigheid in rotte op die respons van die hart op I/H besering te bepaal met besondere aandag aan die intrasellulêre seintransduksiepaaie tydens vroeë herperfusie. Om die rol van JNK uit te lig en te evalueer, is van ‘n spesifieke inhibitor van JNK, SP600125, gebruik gemaak.

Metodes: Insulienweerstandigheid is ontlok deur rotte vir 16 weke ‘n hoë-kalorie dieet te voer (DIO). Harte van die DIO en ouderdomsgepaarde diere (C) is volgens die werkhartmetode geperfuseer (voorbelading 15cm H_2O; nabelading 100cm H_2O) en blootgestel aan (i) 15min globale iskemie gevolg deur verskillende herperfusietye vir die evaluering van funksionele herstel asook vriesklamping van weefsel vir Western klad analises of (ii) 35min streeksiskemie gevolg deur 2 uur herperfusie vir die bepaling van infarktgrootte (IS) met behulp van tetrazolium kleuring. Substrate gebruik: glukose (G) (10mM), glukose (10mM) plus BSA (3%) (G+B) en glukose (10mM) plus vetsure (1.2mM palmitaat/3% BSA) (G+FA). Die JNK inhibitor, SP600125, is of voor iskemie of gedurende herperfusie na iskemie toegedien.

Infarktgrootte, funksionele herstel asook uitdrukking en aktivering van PKB, ERK, JNK, p38MAPK and PTEN is as eindpunte gebruik.

Resultate: (I) In die teenwoordigheid van slegs glukose as substraat kon die harte van DIO rotte I/H besering beter as die ouderdomsgepaarde kontroles weerstaan, aangedui deur ‘n verbeterde funksionele herstel (na blootstelling aan 15min globale isgemie) sowel as kleiner infarktgrootte (na 35min streeksiskemie). Dit is gekenmerk deur vroeë aktivering van PKB en JNKp54/p46 na 10min herperfusie asook afregulering van die aktivering van hierdie kinases na 30min herperfusie.

(II) In teenstelling met wat verwag is, het die kombinasie van ‘n hoë konsentrasie versure met glukose as substrate (G+FA) beduidende verhoogde beskerming teen I/H besering verleen in harte van beide DIO en kontrole rotte, in vergelyking met die ooreenstemmende groepe wat slegs met glukose as substraat geperfuseer is. In beide groepe is hierdie verbeterde beskerming met verhoogde aktivering van die PKB pad geassosieer. Dit is ook interessant dat perfusie met glukose en ‘n hoë konsentrasie vetsure, die aktivering van PKB tydens die hele herperfusiefase kon onderhou, in teenstelling met die verbygaande aktivering waargeneem met glukose alleen as substraat.

(III) Toediening van SP600125 (10uM) voor iskemie of gedurende die vroeë fase van herperfusie na iskemie, kon die JNK pad feitlik heeltemal onderdruk en het I/H besering, veral in die harte van DIO rotte, vererger.

Gevolgtrekking: Hierdie studie, in teenstelling met verskeie ander studies, toon aan dat dieet-geïnduseerde vetsug asook hoë konsentrasies vetsure in die perfusaat, die weerstandigheid van die ex vivo miokardium teen I/H besering, kan verhoog. Dit is ook gevind dat, in teenstelling met wat verwag is, ‘n hoë sirkulerende vetsuurkonsentrasie nie nadelig vir harte van normale rotte, blootgestel aan I/H, is nie, inderdaad ‘n voordelige effek van vetsure op die uitkoms van I/H besering aantoon. Hierdie beskerming het gepaard gegaan met die aktivering van beide PKB en JNK gedurende vroeë herperfusie.

Toediening van die selektiewe JNK inhibitor SP600125 voor of na miokardiale iskemie, het aangetoon dat, in ons studie, JNK en sy geassosieerde seinstransduksiepaaie krities belangrik as bemiddelaar van I/H besering is. Hierdie effekte het gepaard gegaan met laer aktivering van PKB. Ons resultate dui dus

daarop dat ‘n interaksie tussen die JNK en PKB seintransduksiepaaie in die post-iskemiese miokardium, ‘n belangrike bydraende faktor in die uitkoms van I/H besering mag wees.

Alhoewel die data wat hier aangebied word, teenstrydig mag voorkom, ondersteun dit juis die hipotese dat JNK seintransduksie spesifiek en tergelykertyd pro- en anti-apoptotiese meganismes in kardiomiosiese mag moduleer. Dit reflekteer ook die uitsonderlike kompleksiteit van die hart se metaboliese, funksionele en strukturele veranderinge in vetsug. Die resultate dui ook daarop dat matige hiperfagie-geïnduseerde vetsug nie nadelige effekte op die iskemies/herperfuseerde hart het nie maar eintlik die sensitiwiteit van die hart teenoor I/H beskadiging verminder. Hierdie aanname is verder onderskryf deur die voordelige effekte wat met vetsure in die perfusaat waargemee, is.

Wanneer saamgevat, het die resultate van hierdie studie potensiëel klinies belangrike implikasies en bevestig die belangrikheid van gebeurtenisse tydens vroeë herperfusie as moontlike terapeutiese teikens.

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List of Abbreviations

ACC: acetyl-CoA carboxylase

AIF: apoptosis-inducing factor

AMPK: AMP activated protein kinase

Apaf-1: apoptotic protease activating factor 1

ASM: acid soluble metabolites

ATF: activator protein-1 (AP-1) transcription factors

ATP: Adenosine triphosphate

BSA: Bovine Serum Albumin

CAP: Cbl associated protein

CAT: carnitine translocase

Cbl: casitas b-lineage lymphoma

CPT-1: carnitine palmitoyl transferase-1

CREB: cAMP response element binding protein

CRP: C-reactive protein

CVD: cardiovascular diseases

DG: diglycerides

Diablo: direct inhibitor of apoptosis protein (IAP) binding protein with low pI

eNOS: endothelial nitric oxide synthase

ER: endoplasmic reticulum

ERK: extracellular signal regulated kinase

ET-1: endothelin-1

ETC: electron transport chain

FA: fatty acid

FABPs: plasma membrane FA binding proteins

FADD: Fas-associated death domain

FADH-2: flavin adenine dinucleotide hydrogen 2

FAT: FA translocase

FATP: FA transport protein

FLIPL: Flice (caspase-8) inhibitory protein

FOXO: Forkhead Box subclass O

G-6-P: glucose-6-phosphate

GLUT: glucose transporter

GPCR: G protein-coupled receptors

HB-EGF: heparin-binding epidermal growth factor-like growth factor

HBP: hexosamine biosynthetic pathway

HSL: hormone sensitive lipase

HSP90: heat shock protein 90

HUVEC: human umbilical vein endothelial cells

ICAM-1: intracellular adhesion molecule-1

IGF: insulin-like growth factor

IKKβ : inhibitor-kappa-B kinase β

IL-6: interleukin 6

ILK: integrin-linked kinase

IRS: insulin receptor substrates

JNK: C-jun-N-terminal kinase

LC acyl-CoA: long-chain fatty acyl-CoA

LPL: lipoprotein lipase

LSP-1: lymphocyte-specific protein 1

MAPK: mitogen-activated protein kinase

MAPKK: MAPK kinase

MCD: malonyl-CoA-decarboxylase

MCP-1: monocyte chemoattractant protein-1

MCT: monocarboxylate transporter

MDM: murine double minute

MIF: macrophage migration inhibitory factor

mPTP: mitochondrial permeability transition pore

mTOR: mammalian target of rapamycin

NADH: nicotinamide adenine dinucleotide hydrogen

NF-κB: nuclear factor-kB

NO: nitric oxide

PARP-1: poly (ADP-ribose) polymerase-1

PC: preconditioning

PDGF: platelet-derived growth factor

PDH: pyruvate dehydrogenase

PDHK: PDH kinase

PDHP: PDH phosphatase

PDK: phosphoinositide-dependent protein kinase

PFK-1: phosphofructokinase-1

PHLPP: PH domain leucine-rich repeat protein phosphatase

PI3K: phosphoinositide 3 kinase

PKB: protein kinase B

PKC: protein kinase C

PP: protein phosphatases

PPARα: peroxisome proliferator-activated receptor α

PTB: phosphotyrosine binding

PTEN: phosphatase and tensin homologue deleted on chromosome 10

PUMA: p53 up-regulated modulator of apoptosis

RAGE: receptor for advanced glycation end-products

RAS: renin angiotensin system

RBP-4: retinol binding protein-4

ROS: reactive oxygen species

S6K: small subunit ribosomal protein 6 kinase

SAPK: stress-activated protein kinases

SGEs: advanced glycation end-products

Shc: SH2-containing collagen-related proteins

Smac: second mitochondrial-derived activator of caspase

TCA: tricarboxylic acid

TAG: triacylglycerols

TLR: Toll-like receptors

TNF: tumor necrosis factor

TNFR: TNF receptor

TRADD: TNFR-associated death domain

TRAF: TNFR-associated factor

TRB: Tribbles

UCP3: uncoupling protein 3

UPR: unfolded protein response

VCAM: vascular cell adhesion molecule

VLDL: very-low density lipoproteins

VSMC: vascular smooth muscle cell

Chapter I

Introduction

1.1. General introduction

Obesity has reached global epidemic proportions in both adults and children and is associated with comorbidities, including development of the metabolic syndrome (1). The metabolic syndrome, in turn, is characterized by central adiposity, insulin resistance, dyslipidemia and hypertension, which significantly increase all-cause as well as cardiovascular mortality in humans (2,3,4). Patient as well as animal studies have indicated that insulin resistance can decrease glucose uptake, alter lipid metabolism and impair protein kinase B (PKB)-dependent signalling in both metabolic and vascular insulin target tissues (4-8). In view of the dependence of the myocardium on glycolysis for its energy needs during oxygen deficiency, it is expected that insulin resistance exacerbates the harmful effects of ischaemia on the heart.

PKB is an enzyme central not only to insulin signalling and glucose uptake, but also to myocardial survival during reperfusion after ischaemia (9-11). Inhibition of its activation during early reperfusion has been shown to enhance apoptosis, cell death and contractile failure (11,12). It has recently been suggested that activation of the stress kinase C-jun-N-terminal kinase (JNK) is essential for PKB phosphorylation at the onset of reperfusion (13): activation of JNK phosphorylates PKB on Thr450, demonstrated to be a prerequisite for the phosphorylation of PKB at Thr308 and Ser473 to be fully active. Thus, these observations suggest that JNK activation during early reperfusion is a prerequisite for cardioprotection.

However, despite the above convincing data (10,11,13), the role of JNK activation in cell survival is not clear. For example, it has been reported that pharmacological inhibition of JNK activation during early reperfusion is cardioprotective, indicating that this kinase is pro-apoptotic (14). JNK has been shown to phosphorylate the 14-3-3 scaffolding proteins, thereby releasing BAX to translocate to the mitochondria where it mediates release of cytochrome C and activates apoptosis (15,16). In addition, JNK is known to be overexpressed in insulin resistance or diabetic states (17,18).

However, this kinase is surprisingly under-researched in the phenomenon of ischaemia/reperfusion, particularly in the case of insulin resistant hearts.

The phosphorylation and thus activation of PKB is also regulated upstream by phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (19). PTEN has been suggested to be involved in cardioprotection (20,21) and pharmacological inhibition of this phosphatase has been reported to be associated with reduced ischaemia/reperfusion injury (22,23).

Despite the overwhelming evidence that obesity is an important cardiovascular risk factor, several large clinical studies documented a so-called obesity paradox, in which overweight and obese people, even type 2 diabetic obese people, have a better prognosis than normal weight or thin individuals after suffering a heart attack (24,25). In view of the many adverse effects of obesity and its clinical consequences in humans, it was decided to study the effect of hyperphagia-induced obesity and insulin resistance in rats on the response of the heart to ischaemia/reperfusion injury, with particular attention to the intracellular signalling pathways during early reperfusion. Since (i) increased fatty acid oxidation rates at the expense of glucose oxidation during reperfusion have been proposed to impair functional recovery (26-28) and (ii) the serum free fatty acid concentrations of the hyperphagia-induced obese rats were increased at least twofold (29,30), the hearts were perfused with glucose alone, as well as with a combination of glucose plus a high concentration of fatty acid (palmitic acid) to simulate the in vivo conditions. Infarct size, functional recovery as well as activation of the so-called reperfusion injury salvage kinase pathway (RISK) were used as endpoints.

1.2. Regulation of fatty acid and glucose metabolism in the heart

1.2.1. Overview of the fatty acid and glucose metabolic pathways in heart

Myocardial energy metabolism is tightly regulated, as the heart has a very high energy and oxygen demand but a relatively low ATP content (~5 μmol/g wet wt, 10 mM, enough for only a few beats) and a small capacity for anaerobic metabolism. The myocardium, even at the resting heart rate, consumes approximately 75% of the oxygen delivery (31,32) to continually generate ATP at a high rate to maintain its

intracellular ATP levels for contractile function, basal metabolic processes, and ionic homeostasis (32-40)

Myocardial metabolism is extremely plastic in that overall ATP synthesis changes rapidly in response to alterations in substrate supply, hormonal and neural signals or specific enzyme reactions etc. (41-44). In the normal healthy adult heart, almost all (95%) of the ATP generated is derived from mitochondrial oxidative phosphorylation, with the remainder generated by glycolysis and GTP formation in the tricarboxylic acid (TCA) cycle (37,41,45-48). Among them, mitochondrial fatty acid (FA) β-oxidation accounts for 60–90% of the total energy production (in the form of ATP) (33-35,39,41,46,49,50), the remaining 10–40% is generated from the oxidation of carbohydrates, mainly glucose under normal physiological conditions (38,49,51,52). For a particular physiological environment, the heart selects the most efficient substrate for energy production, for example, in the postprandial state, when blood glucose and insulin levels are elevated, glucose utilization is dominant, whereas in the fasted state, FA are preferentially metabolized (35,38,53,54). Therefore, fuel selection is a characteristic feature of the heart.

Insulin is the hormone that plays a major physiological role in coupling metabolic and cardiovascular homeostasis under physiological conditions.

1.2.2. Insulin signalling pathways regulating cardiovascular physiology

1.2.2.1. General features of cardiovascular actions of insulin

Since its discovery by Banting and Best (10,55) in the early 1920s, insulin has been studied extensively (56,57). However, it was not until 1949 that insulin-induced glucose uptake was experimentally demonstrated (58) whereas the insulin-sensitive glucose transporter 4 (GLUT4) was only discovered in the 1980s (59).

The important physiological actions of insulin in metabolism and homeostasis include stimulation of glucose transport, protein and glycogen synthesis, inhibition of lipolysis, regulation of gene transcription and translation, cell growth and proliferation, contractility, vascular tone and apoptosis (57,60-65). Over the last 20 years, much progress has been made in understanding the metabolic actions of insulin, however, the full identification of the molecular signal transduction pathways involved in its actions, is still in progress.

Myocardial excitation is associated with transmembrane movement of extracellular calcium (Ca^{2+}) into the cardiac myocytes through activated Ca^{2+} channels and reversed Na^{+}/Ca^{2+} exchange. This influx of Ca^{2+} stimulates additional release of Ca^{2+} from the sarcoplasmic reticulum via the ryanodine receptors, which results in myofilament activation and contraction.

Studies in isolated human cardiac myocytes suggest that insulin enhances Ca^{2+} influx through activation of L-type Ca^{2+} channels and reverse-mode Na^{+}/Ca^{2+} exchange (66,67). Insulin also enhances myofilament Ca^{2+} sensitivity in isolated human cardiac muscle (67), and increases cardiac contractility in vivo in humans and in isolated animal cardiac muscle (57,60). Increased cardiac contractility, in turn, enhances myocardial work and oxygen consumption (68).

Moreover, insulin increases cardiac nitric oxide (NO) production through the phosphoinositide 3 kinase (PI3K) / PKB / endothelial nitric oxide synthase (eNOS) pathway (69) which may contribute to the inotropic effects of insulin (70). The PI3K inhibitors wortmannin or LY294002 inhibit the inotropic actions of insulin (71,72), while inhibition of NOS also inhibits the effects of insulin on intracellular Ca^{2+} (73).

Because myocardial blood flow and oxygen consumption are tightly coupled and regulated, it is difficult to evaluate the direct actions of insulin on the coronary vasculature in vivo.

1.2.2.2. Insulin signal transduction pathways

Insulin increases energy storage by inducing glucose uptake and glycogen synthesis in liver and muscle, and FA synthesis in liver and adipose tissue.

The biological effects of insulin can generally be divided into two major pathways, namely (i) the PI3K/PKB pathway which is responsible for the metabolic actions of insulin; (ii) the RAS (Rat Sarcoma) / mitogen-activated protein kinase (MAPK) kinase (MAPKK or MEK) / extracellular signal regulated kinase (ERK) pathway (RAS/MEK/MAPK) which mediates vascular smooth muscle cell mitogenesis, release of endothelin-1 (ET-1) and pro-inflammatory cytokines (Fig 1) (73-75). These two major insulin signal transduction pathways are arranged in highly complex networks that regulate cardiovascular homeostasis by multiple feedback loops and cross-talk between the two signalling pathways (56,74-79).

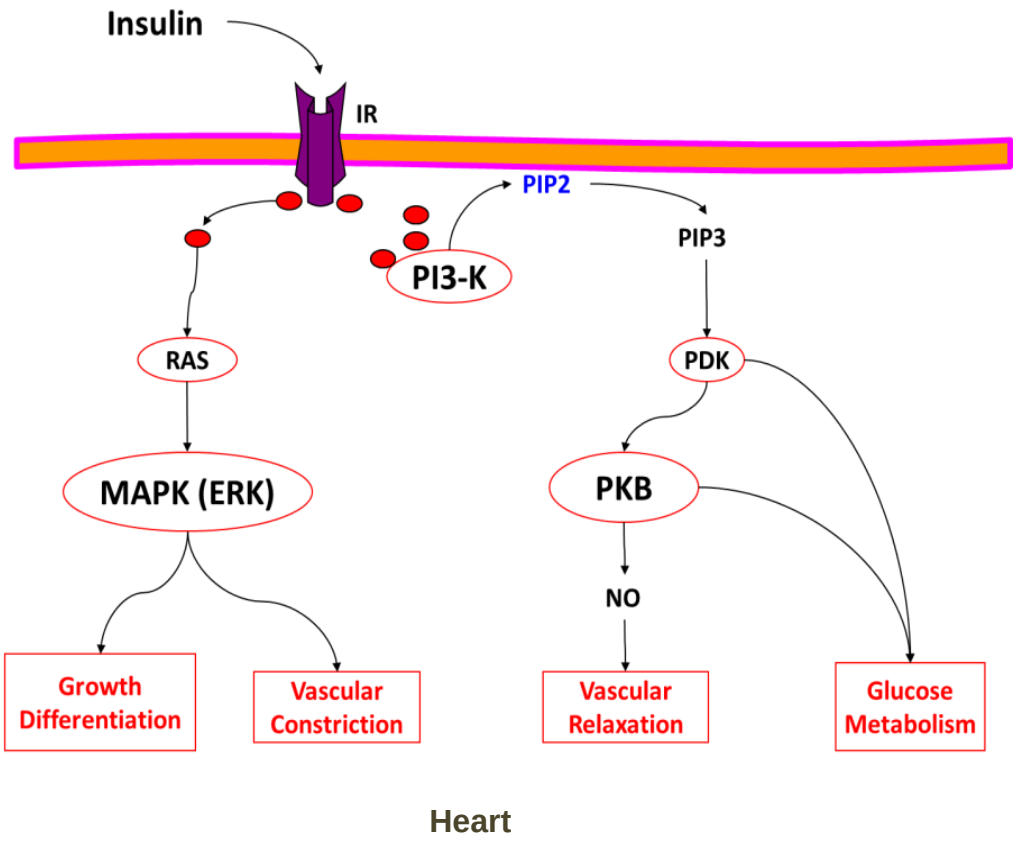


Fig 1. General features of insulin signal transduction pathways: PI3K/PKB and Ras/MAPK branches. The PI3K/PKB pathway is responsible for the vascular relaxation and glucose metabolism of insulin. The RAS/MAPK branch mediates vascular constriction and growth differentiation. Modified from Ranganath Muniyappa et al. Endocrine Reviews 28(5):463–491, 2007.

1.2.2.3. Insulin PI3K/PKB signalling pathways

Insulin receptor

The biological actions of insulin are mediated by specific cell surface insulin receptors which were first described in 1971 (80). Physiological concentrations of insulin (100–500 pM) selectively bind to its receptors on insulin sensitive tissues, such as muscle, liver and adipose tissue, mediating the complex signal transduction networks that regulate diverse cellular functions, including the rapid stimulation of glucose uptake into its target tissues (81-83).

Insulin receptors are expressed on nearly every cell in the body, for example, insulin receptors in the heart are expressed at levels of about 10,000 to 100,000 receptors

per cardiomyocyte (83). Molecular cloning of the insulin receptor in 1985 (84,85) allowed for investigations into the signal transduction mechanisms underlying insulin action in both cellular and physiological contexts.

The insulin receptor, which is encoded by a single gene located on the short arm of chromosome 19 and contains 22 exons and 21 introns (86,87), is a α2β2 heterotetrameric enzyme comprising two extracellular α-subunits (extracellular agonist binding domain) and two transmembrane β-subunits, each composed of a short extracellular domain, a transmembrane domain and an intracellular cytoplasmic domain flanked by 2 regulatory regions (a juxtamembrane region (JM) and a C-terminal tail (CT)), the ATP binding site and autophosphorylation sites (88,89).

These α2β2 subunits are disulfide-linked in a β–α–α–β configuration. The disulfide bridges stabilize the interactions between the 2 α- subunits, and between the α- and β-subunits (90). The intracellular domain of the β subunit of the insulin receptors possesses a series of intermolecular trans-autophosphorylation reactions that generate the intrinsic tyrosine kinase activity involved in signal transduction (85,89,91). In the absence of an agonist, unoccupied α-subunits on the cell surface inhibit the intrinsic tyrosine kinase activity of the cytoplasmic domain of the β-subunit, and hence function as critical regulatory subunits of the catalytic intracellular subunits (88,92,93). Binding of insulin to the extracellular α subunits on the cell surface results in a conformational change in the juxtapositioned cytosolic β-subunits and induces the initiation step of the tyrosine autophosphorylation mechanism in which one β subunit tyrosine kinase domain phosphorylates the adjacent β subunit on several tyrosine residues resulting in the activation of the intrinsic substrate kinase activity of the insulin receptors (18,93-96). Tyrosine phosphorylation at residues 1146, 1150, and 1151 in the kinase domain relieves pseudosubstrate inhibition, further enhancing tyrosine kinase activity of insulin receptors.

Thus, after tyrosine kinase activation by autophosphorylation, a family of soluble adaptors or scaffolding molecules, such as the insulin receptor substrates (IRS), SH2-containing collagen-related proteins (Shc), casitas b-lineage lymphoma (Cbl) or Cbl associated protein (CAP) can be recruited to the insulin receptor for participation in the signalling cascade (56,97-99).

Insulin receptor substrates (IRS)

The insulin receptor phosphorylates at least nine intracellular signalling molecules including four intracellular IRS proteins (IRS-1, -2, -3, -4). Both IRS1 and IRS2 contain a pleckstrin homology (PH) and a phosphotyrosine binding (PTB) domain at the N-terminus. The PTB domain of IRS is located in a NPXY motif of the juxtamembrane region of insulin receptors (100-102). According to studies on transgenic mice, IRS-1 is a major substrate for the insulin receptor tyrosine kinase in the heart (82,101). Insulin binding to the insulin receptor phosphorylates the tyrosine sites of IRS1/2 as positive regulatory sites to activate the PI3K/PKB pathway involved in the anabolic actions of insulin. In addition, insulin also increases the phosphorylation of several serine sites of IRS-1 having negative or both positive and negative effects on insulin signalling (102-105). A phosphorylation pattern is postulated where the positive regulatory sites (such as tyrosine) are phosphorylated before the negative regulatory sites (such as serine) (106,107). In physiological conditions, insulin maintains the balance between the phosphorylation of positive and negative regulatory sites of IRS, however, in pathophysiological conditions, insulin signalling may be impaired by the imbalance occurring where phosphorylation of the negative regulatory sites (such as serine) disrupts the interaction between the insulin receptor and IRS-1 or the interaction between IRS-1 and downstream effectors (108). For example, in obesity induced insulin resistance, several inducers promote the phosphorylation of the negative regulatory sites (such as serine) of IRS-1 by activation of c-Jun N-terminal kinase (JNK), inhibitor-kappa-B kinase β (IKKβ), mammalian target of rapamycin (mTOR) / small subunit ribosomal protein 6 kinase (S6K), ERK, and protein kinase C (PKC) isoforms (108,109).

PKB

PKB (also called Akt), is a 57 kDa serine/threonine kinase located at the centre of the insulin and insulin-like growth factor 1 (IGF1) signalling pathway, mediating the effects of insulin on glucose transport, glycogen synthesis, protein synthesis, lipogenesis and suppression of hepatic gluconeogenesis (Fig 1). PKB is conserved from invertebrates to mammals, exhibiting a high degree of homology with protein kinases A and C, emphasizing its pivotal role in development, cell proliferation and metabolism (110,111). There are three known isoforms of PKB (PKB1/PKBα, PKB2/PKBβ and PKB3/PKBγ) identified in mammals consisting of a conserved

domain structure: a N terminal pleckstrin homology (PH) domain, a central T-loop kinase domain (KD) and a C-terminal regulatory domain (RD) which contains the hydrophobic motif (111). Among them, the PKB2/β isoform's function appears to be specifically required for translocation of the insulin-stimulated glucose transporter 4 (GLUT4) in both adipocytes and striated muscle (112-116).

In unstimulated cells, PKB is located in the cytoplasm and exhibits a low basal activity. When stimulated, PKB is translocated to the plasma membrane via its N-terminal PH domain (117-119). Membrane-associated PKB is fully activated by phosphorylation of its two regulatory sites, Threonine-308 by phosphoinositide-dependent protein kinase-1 (PDK1) (118,120,121) and Serine-473 (in the case of PKB1/PKBα) by the integrin-linked kinase (ILK) in association with mammalian target of rapamycin (mTOR) (122-124) (Fig 2).

Activated PKB exerts its biological effects by phosphorylating downstream substrates at various sites within the cell, some located in the nucleus, by an unknown mechanism related to gene expression (119,125-127). PKB substrates include Bad, caspase-9, IkB-kinase, and Forkhead Box subclass O (FOXO) which are associated with survival, and murine double minute 2 (MDM2), p21, p27, and Myt1 (a dual-specificity protein kinase) which are involved in progression of cell cycle (124). PKB also regulates glucose metabolism by phosphorylating MDM2, and AS160 (PKB/Akt substrate of 160 kDa) (128,129). GSK-3 in turn, mediates multiple actions of PKB in both cell cycle and protein synthesis.

Insulin is a very potent activator of PKB in the heart (130,131). Activation of PKB by insulin is mediated via the insulin receptor and IRS-1/2 in insulin sensitive tissues such as skeletal and heart muscle (131-133). PKB also plays a key role in regulating cardiomyocyte growth (134).

PKB is dephosphorylated and inactivated by protein phosphatases (PP). Protein phosphatase 2A (PP2A) is associated with dephosphorylation of T308 and PH domain leucine-rich repeat protein phosphatase (PHLPP) is predominantly involved in dephosphorylation of S473 (135,136) (Fig 2).

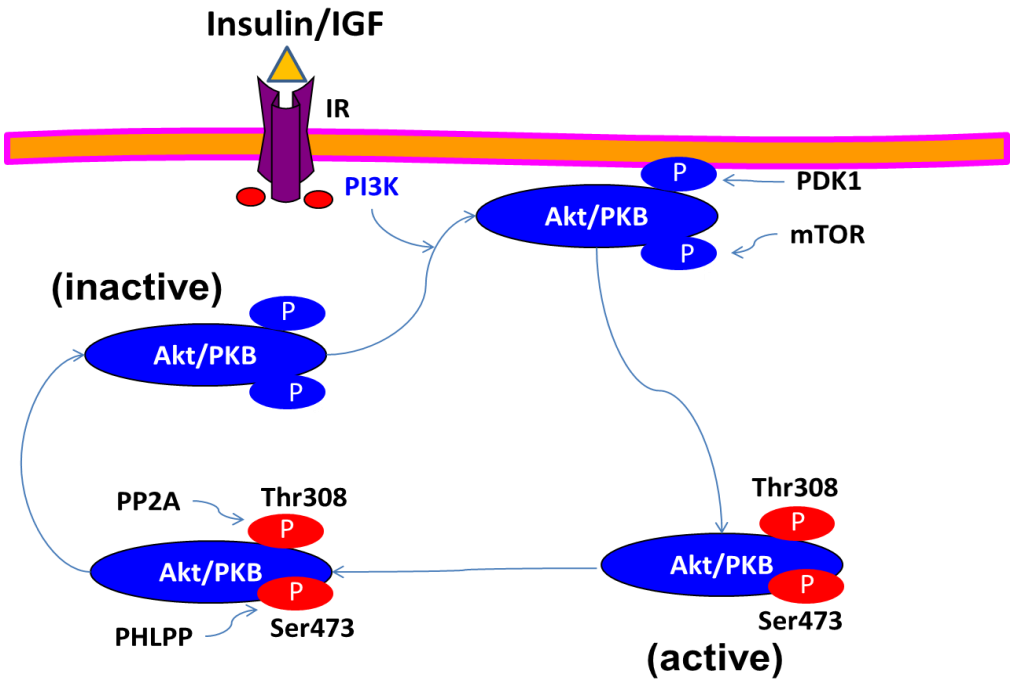


Fig 2. Regulation of PKB activity. Upon insulin/IGF stimulation, PKB is recruited to the plasma membrane via its N-terminal PH domain, and activated by phosphorylation at T308 (by PDK1) and S473 (by mTOR). Active PKB translocates to various sites within the cell and phosphorylates downstream substrates. PKB activity is then down-regulated by dephosphorylation of the two regulatory sites by protein phosphatases (T308 by PP2A, and S473 by PHLPP). Modified from Ichiro Shiojima and Kenneth Walsh. Genes Dev. 20: 3347-3365, 2006.

An additional mechanism for regulating PKB activity has recently been identified. The interacting protein, a Drosophila Tribbles homolog 3 (TRB3) has been shown to inhibit hepatic PKB activation by insulin (137-139). Moreover, it was suggested that functional polymorphism(s) of TRB3 might be associated with insulin resistance and related clinical outcomes (139).

Regulation of the activity of the PI3K/PKB pathway by the phosphatase PTEN will be discussed in 1.4.3

eNOS/ nitric oxide (NO) and the insulin signalling pathway

Among the most important cardiovascular actions of insulin is the stimulation of increased production of the potent vasodilator NO by the vascular endothelium (140-143).

Classical vasodilators, including acetylcholine, via G protein-coupled receptors (GPCR), stimulate an increase in intracellular Ca^{2+} that promotes the binding of calcium/calmodulin to eNOS. In the presence of a variety of cofactors, this results in dissociation of eNOS from caveolin-1 with subsequent dimerization and activation of the enzyme (144,145).

Insulin employs a phosphorylation-dependent mechanism to stimulate NO production which is completely distinct, separate, and independent from classical calcium-dependent mechanisms (146-148). Pretreatment of cells with the Ca^{2+} chelator BAPTA does not inhibit the ability of insulin to stimulate phosphorylation of eNOS at Ser1179 or enhance eNOS activity (146). In addition, insulin treatment does not alter intracellular Ca^{2+} levels in endothelial cells (148). Insulin can directly increase eNOS activity via the PI3K/PKB pathway in vascular endothelium. This in turn catalyzes the conversion of the substrate L-arginine to NO and L-citrulline (144,149,150). PKB directly phosphorylates and activates human eNOS at Ser1177 (equivalent to Ser1179 in bovine eNOS) (150), leading to increased production of NO (83,140,145,146). Pretreatment with N (G)-nitro-l-arginine methyl ester (NOS inhibitor) attenuates insulin-enhanced capillary volume by 50 to 70%, suggesting that these effects are partially NO-dependent (151,152). It appears that PKB-1 is the predominant isoform in the vasculature and endothelial cells and the PKB phosphorylation site on eNOS is absolutely essential for its activation: PKB-1 KO mice have significantly lower levels of active eNOS, lead to impaired vascular maturation (145,147,153,154). Overexpression of dominant inhibitory mutant PKB proteins in human umbilical vein endothelial cells (HUVEC) nearly completely inhibits production of NO in response to insulin (83). Cells expressing a mutant eNOS with a disrupted PKB phosphorylation site (alanine substituted for serine at position 1179) are unable to produce NO in response to insulin (148). These studies suggest that insulin-stimulated production of NO is calcium-independent and mediated by activation of PKB.

Although PKB is an essential signalling molecule for insulin-stimulated activation of eNOS, its activation per se is not sufficient to activate eNOS. For example, treatment of endothelial cells with either insulin or platelet-derived growth factor (PDGF) results in comparable phosphorylation and activation of endogenous PKB. Nevertheless, only insulin (but not PDGF) treatment results in phosphorylation and activation of eNOS at the PKB phosphorylation site Ser1179 with consequent production of NO (140,146,147). Moreover, although insulin-induced eNOS activation is calcium-independent, insulin stimulates calmodulin binding to eNOS (148). One potential mechanism underlying this specificity may be that insulin (but not PDGF) elicits the formation of a ternary eNOS-heat shock protein 90 (HSP90)-PKB complex which facilitates eNOS phosphorylation by PKB (145,147). This suggestion is supported by the finding that association of heat shock protein 90 (HSP90) with eNOS is critically important for eNOS-mediated NO production (147).

1.2.2.4. Insulin RAS/MEK/MAPK(ERK) signalling pathways

In addition to PI3K-dependent insulin signalling, another major insulin signalling branch is the RAS/MEK/MAPK(ERK) pathway which generally regulates biological actions related to growth, mitogenesis and differentiation, and controls secretion of ET-1 in vascular endothelium (147), but is not involved in insulin-stimulated glucose transport or glycogen synthesis or direct metabolic actions (Fig 1, see p 3).

RAS

RAS proteins (H-, N-, and K-RAS) are key regulators in essential cellular processes and its pathways have drawn the attention of many investigations. The functions of RAS proteins are associated with plasma membranes and include endomembranes like the endoplasmic reticulum (ER) and the Golgi complex (GC) (145,147,148,155). Importantly, Ras proteins are known to translocate between cellular compartments and their sublocalization appears to depend on their activation status (156).

Binding of the adapter protein SHC to the SH2 domain of the growth factor receptor-bound protein-2 (Grb-2) results in activation of the pre-associated GTP exchange factor SOS (81,157). This converts the inactive RAS form (RAS-GDP) to the active form (RAS-GTP), which subsequently recruits and activates RAF to phosphorylate and activate the MAPK kinase (MAPKK/MEK) and MAPK (ERK) signalling cascade

(10,155,157,158). This particular pathway (RAS/MEK/MAPK(ERK)) is not involved in insulin mediated NO production. This has been demonstrated in a study where down-regulation of RAS in endothelial cells has little effect on NO production by insulin, suggesting that PI3K/PKB signalling is required for insulin mediated NO production in endothelial cells (83).

Extracellular signal regulated kinase (ERK)

The ERK cascade belongs to the classic MAPK family. Acting as serine and threonine protein kinases, MAPKs regulate a wide range of processes: cell growth, migration and differentiation, gene expression, mitosis, cell motility, metabolism, cell survival and apoptosis, and embryogenesis (see review articles 159-161).

The classic MAPK family consists of four subfamilies: ERK1/2, c-Jun N-terminal kinase (JNK1/2/3), p38 MAPK and ERK 5 (159-161). The MAPK signalling pathway is a three-tiered cascade: the MAPK kinase kinase (MAPKKK or MEKK or MAP3K) are activated by upstream signalling proteins (e.g., small GTPases) and phosphorylate MAPK kinase (MAPKK or MEK or MAP2K). MAPK are the third layer of the cascade, and activated by MAPK kinase (159-161) (Fig 3).

ERK1/2 is expressed in all tissues, include the heart. Although ERK 3-8 have been identified, their function and regulation are less well characterized (162-164). More than 150 proteins have been identified as substrates of ERK1/2. These include transcription factors, protein kinases, protein phosphatases, cytoskeletal proteins, scaffolding proteins, receptors, signalling molecules as well as apoptosis-related proteins (163).

Conventionally, ERK1/2 can be activated by a cascade comprised of small G protein Ras-Raf family members (Raf-1, A-Raf, B-Raf) followed by MEK1/2 (growth factors, serum, cytokines, transforming growth factors, osmotic stress, and microtubule disorganization) (160,165).

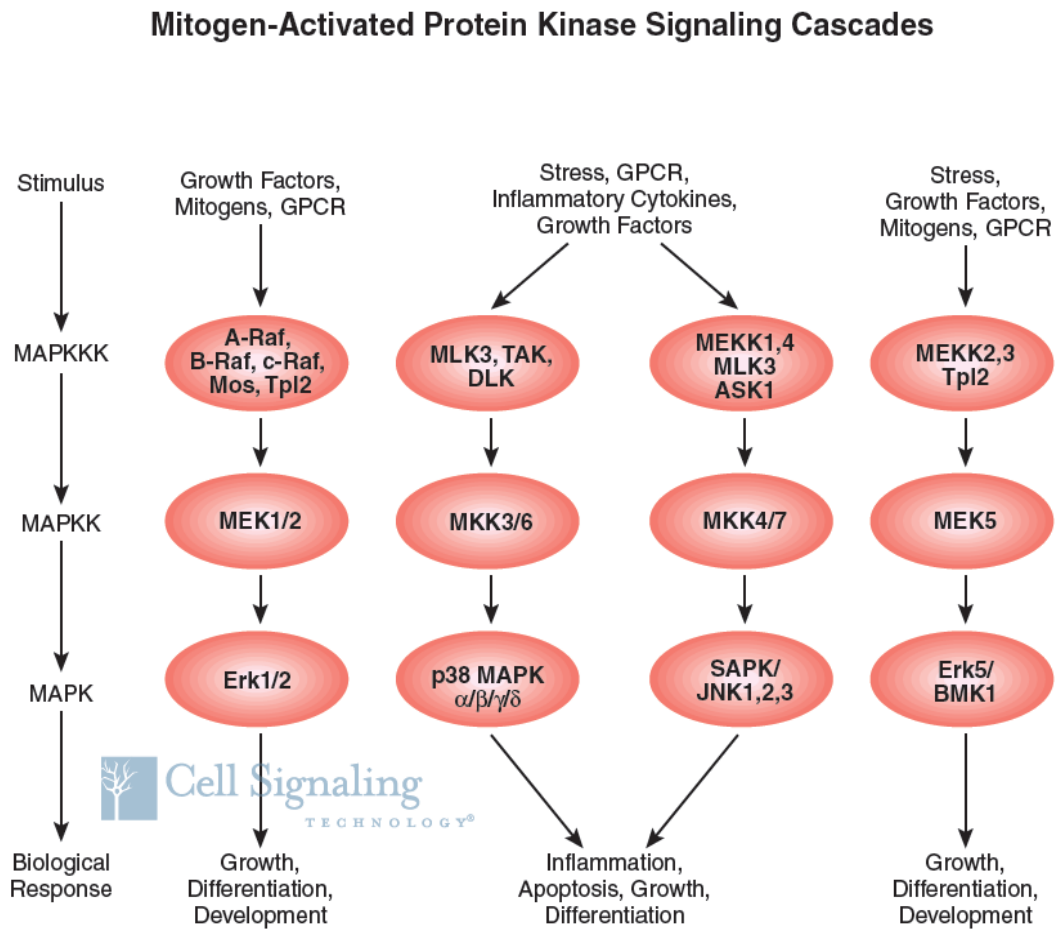


Fig 3. The different stimuli activate the three-tiered cascade of MAPK pathways from upstreams to downstreams: MAPKKK, MAPKK, MAPK. The MAPK pathways are responsible for the biological response, such as growth, differentiation, inflammation and apoptosis. MAPKKK: MAPK kinase kinase (MEKK or MAP3K), MAPKK: MAPK kinase (MEK or MAP2K), MAPK: mitogen-activated protein kinase. For more details see Cell Signalling website. Cell Signalling.www.cellsignal.com

Under resting conditions, ERKs are present in the cytoplasm, as a consequence of their interaction with several types of cytoplasmic anchors. Upon stimulation, phosphorylated ERKs lose their affinity for their anchors and undergo a rapid relocalization. Half of the activated ERKs remains in the cytoplasm (166) and processes extra-nuclear actions, such as the formation of cell-matrix contacts (167), adhesion (168), endosomal traffic (169), Golgi fragmentation (170) and anti-apoptotic signalling (171). Some of the activated ERKs are translocated into the nucleus where they phosphorylate multiple nuclear proteins to regulate transcription, DNA replication, chromatin remodeling, and miRNA synthesis (172,173). Interestingly, within the

nucleus, ERKs may interact with lamin A at the nuclear envelope to release c-Fos from its inhibitory interaction with lamin A, and promote rapid, mitogen-dependent AP-1 activation (174).

JNK and p38 MAPK will be discussed in section 4.

Endothelin-1 and adhesion molecules in insulin pathways

In addition to vasodilator actions of insulin discussed above, insulin also has effects that oppose the vasodilator actions of NO, such as the stimulation of secretion of the vasoconstrictor endothelin-1 (ET-1) from vascular endothelium (75). ET-1 (a 21-amino-acid peptide) is a paracrine factor secreted by endothelial cells. The plasma concentrations of ET-1 are less relevant than local concentrations and do not predict ET-1 activity in the vascular milieu (75).

Endothelial expressions of cellular adhesion molecules include intercellular adhesion molecule-1, vascular cell adhesion molecule (VCAM-1), and E-selectin. Among them, E-selectin is critical in modulating cell-cell interactions between circulating inflammatory cells and vascular endothelium.

Insulin and other hormones acutely stimulate the secretion of ET-1 and expression of VCAM-1 and E-selectin on endothelium using MAPK-dependent (but not PI3K-dependent) signalling pathways (78,79,175,176)

In conditions of insulin resistance, decreased PI3K signalling and increased MAPK signalling in response to insulin may lead to a shift in the balance between vasoconstrictor and vasodilator actions of insulin resulting in decreased production of NO and increased secretion of ET-1, characteristic of endothelial dysfunction. The insulin-stimulated, MAPK-dependent secretion of ET-1 and its receptor binding are associated with a vasoconstrictor effect in the vascular endothelium (184). Inhibition of MAPK blocks the vasoconstrictor effects of insulin in rat skeletal muscle arterioles (185). Vasodilator actions of insulin are potentiated by ET-1 receptor blockade in animals (186) and humans (187). In the presence of ET-1 receptor blockade, intra-arterial insulin infusion causes measurable vasodilation (187).

The ET family has three peptides (ET-1, ET-2, and ET-3). As the distribution and properties of these peptides are different, each peptide is believed to play specific

physiological roles. ET has two types of receptors: ETA- and ETB. The ETA receptor with a high affinity for ET-1 and ET-2 is mainly located on muscle cells, whereas the ETB receptor with an affinity for all three peptides, is expressed on endothelial, epithelial, endocrine, and nerve cells. Both subtypes on vascular smooth muscle cells mediate vasoconstriction, whereas the ETB-receptor subtype on endothelial cells contributes to vasodilatation and ET-1 clearance.

Stimulation of the ETB1 receptor leads to the release of vasodilators such as nitric oxide (NO) and prostaglandin I2 and clearance of ET-1 from the circulation within the lungs, kidneys, and liver (188-192). On the other hand, although another ETB-receptor subtype (ETB2), located on VSMCs, exerts vasoconstriction, it has become clear that ETB2 receptor–induced vasoconstriction is negligible under normal conditions but becomes more important in certain diseases such as atherosclerosis and essential hypertension (193-195).

In endothelial cells, the insulin stimulated ET-1 secretion (not by IGF-I) is inhibited by genistein, a broad inhibitor of tyrosine kinases. The insulin mediated ET-1 secretion is also suggested to occur via the insulin receptor (177). This is supported by the observation that in mice with targeted deletion of the insulin receptors in vascular endothelium (vascular endothelium insulin receptor knockout (VENIRKO) mice), expression of both eNOS and ET-1 is significantly diminished (178).

ET-1 induces pro-atherogenic effects such as vasoconstriction (179), increased vascular permeability (180), and vascular smooth muscle cell (VSMC) proliferation (181), increased production of interleukin 6 (IL-6) by endothelial cells and monocytes (182,183), and increased proteoglycan synthesis by VSMCs.

Under normal pressure, the coronary vasculature is kept relaxed by the combined impact of NO and ETA receptor activation, with the latter exerting a negative control on ETB2 rather than a direct effect on muscle (196).

ET-1-mediated coronary vasoconstriction, interacting with the direct myocardial depressant effect of NO, contributes to myocardial depression in hearts isolated from lipolysaccharide (LPS)-treated rats (197).

Although ET-1 and its receptors are part of the etiology or precipitating factors in various cardiovascular diseases (CVD) (198,199) and selective ETA- or nonselective ETA/ETB-receptor antagonisms have been suggested as potential strategies for the

treatment of several CVD based on clinical and animal experiments, it remains unclear which antagonists are suitable for individuals with CVD because upregulation of the nitric oxide system via the ETB receptor is responsible for vasoprotective effects such as vasodilatation and opposition of cell proliferation (198,199).

Interaction between NO and ET-1

In physiological conditions, vascular nitric oxide (NO) and endothelin-1 (ET-1) are balanced, but in pathophysiological conditions, the reduction in NO bioavailability concomitant with increased ET-1 expression leads to an imbalance between these two mediators which is a characteristic feature of endothelial dysfunction and vascular disease (184).

Under normal physiological conditions, a fundamental role of NO in blood vessels may be to tonically inhibit the vasoconstrictor actions of ET-1 within the vasculature. However, the importance of the interaction between these two mediators is still not clear (for a review, see 200).

Some studies showed a key mechanism of interaction between NO and ET-1 in that NO inhibits ET-1 release via a cGMP-dependent mechanism. Importantly, these studies implicate cGMP signalling within the endothelium and not within the VSMC (201-205).

A critical point is that the results obtained in vivo are different from those obtained ex vivo, probably because of the removal of local, neural, and humoral factors that regulate vascular tone when vessels are isolated from an intact animal. For example, nitrergic innervation, as well as signals that promote ET-1 expression and release are absent in isolated vessels. Therefore, the importance of the interaction between NO and ET-1 may be underestimated using ex vivo experimental approaches.

1.2.3. Myocardial fatty acid and glucose metabolism

1.2.3.1. Source of fatty acids and glucose for heart

Fatty acids

The importance of FA and lipids for mammalian metabolic homeostasis is well recognized. The main source of FA for the body is dietary lipid which typically comprises 30–40% of the energy intake, and consists mostly of long-chain FA esterified in triacylglycerols (TAG). Oral and pancreatic lipases hydrolyze these TAG into monoacylglycerol and FA, which are taken up by jejunal and ileal enterocytes, reesterified into TAG, and incorporated with other lipids, lipid-soluble vitamins, and apolipoproteins into chylomicrons for subsequent secretion into the circulation. Under physiological conditions, when the amount of energy entering the body exceeds the immediate energy expenditure, the excess energy is stored in adipocytes in the form of TAG. The release of FA from adipose tissue is well regulated so that appropriate amounts of FA are released to meet the energy requirements of tissues, including the heart. A part of FA is synthesized de novo by the liver.

FA are transported in the body via the lymphatic and vascular system. Basically, FA are transported in blood in esterified (mono-, di- and triacylglycerols, phospholipids and cholesteryl esters) and non-esterified forms. The main circulating lipoproteins, such as chylomicrons, are carrying exogenous lipids, while very-low density lipoproteins (VLDL) are transporting endogenous lipids. After hydrolysis of the triacylglycerols (TAG) by lipoprotein lipase (LPL) located at the surface of the capillaries, the FA released are delivered to peripheral tissues (Fig 4).

Due to their low solubility in aqueous solutions, FA are bound to binding sites on albumin for bulk transport from fat cells in adipose tissue to FA-consuming cells like cardiac and skeletal myocytes. The main source of FA for the heart is the FA derived from the lipolysis of adipose tissue and which is bound to albumin in the blood. FA released from TAG contained in chylomicrons and VLDL probably accounts for $\leqslant$ 20 – 25% of the cardiac FA consumption (41,48,206,207).

Normal circulating FA concentrations range between 0.2 and 0.6 mM (38). However, these levels can vary dramatically from very low concentrations in the fetal circulation (208) to over 2 mM during severe stresses such as myocardial ischaemia, chronic obesity and uncontrolled diabetes (209-211).

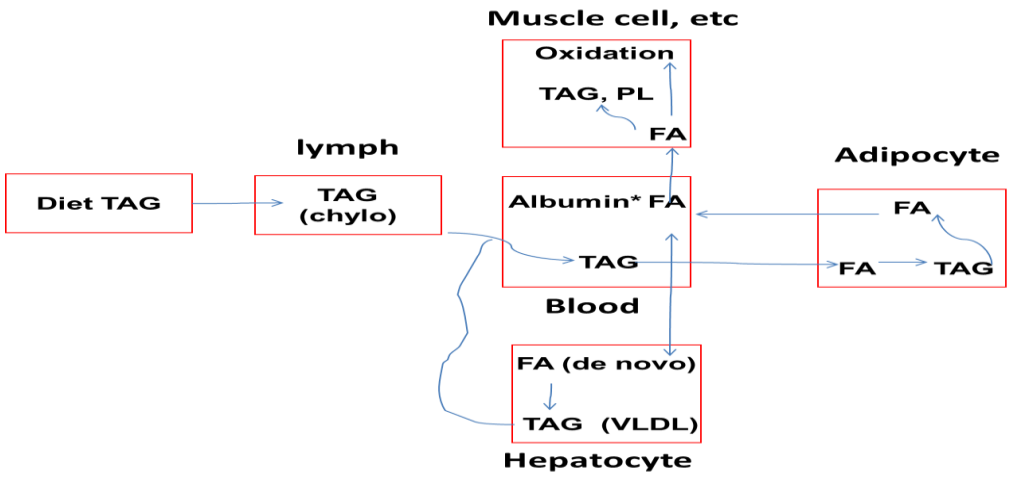


Fig 4. Simplified schematic of flow of fatty acids from diet triacylglycerols to parenchymal cells such as skeletal and cardiac muscle cells, and hepatocytes. After diet, resynthesized triacylglycerols (TAG) incorporate in chylomicrons and transport through the basal membrane of the epithelium to the lymphatic system. Under resting conditions, the bulk of chylomicrons reach adipose tissue. FA in adipose tissue diffuse back to the capillary lumen and are transported via the blood stream binding with albumin to organs such as heart and skeletal muscle to fulfill their energy requirements. Excess of circulating FA is taken up by the liver, incorporated as TAG in very low density lipoproteins (VLDL) and released to the blood compartment. A part of FA is synthesized de novo by the liver. Ger J. van der Vusse. Drug Metab. Pharmacokinet. 24 (4): 300–307, 2009. TAG: triacylglycerols, Chylo: chylomicrons, PL: protein lipase, VLDL: very-low density lipoproteins

Glucose

For the body, glucose is supplied by ingested carbohydrate or by stored glycogen. Glucose homeostasis is maintained by a hormonal network in which insulin and glucagon are the main agents (see 38, 212 for reviews). In humans, blood glucose levels are kept constant in a narrow range from 4 to 7 mM, despite variable supply due to the alternation between feeding and fasting. Because the brain cannot use FA as energy substrate, one main danger of prolonged hypoglycemia is acute brain damage. At the other end of the scale, acute hyperglycemia is a serious complication of decompensated diabetes mellitus.

1.2.3.2. Myocardial fatty acid and glucose uptake

Myocardial fatty acid uptake

The exact mechanism of FA uptake at the endothelial and cardiomyocyte membrane is not yet fully understood (213). Two mechanisms are thought to play an important role in cardiomyocyte FA transport (Fig 5): diffusion and transport via vehicles (214-216). These mechanisms depend on both FA concentration in the blood and the regulation of the transport vehicles (transporters) (217,218). Diffusion can be defined as the absorption of FA onto the cardiomyocyte membrane followed by its translocation and subsequent movement into the cytoplasm. The albumin receptor acts as a docking place to dissociate FA from albumin in the circulation resulting in an increased unbound FA pool in the cardiomyocyte membrane, thus enhancing its diffusion into the cytoplasm (219,220). FA-transport vehicle systems are associated with three transmembrane proteins (219,221,222): (I) the plasma membrane FA binding proteins (FABPs), one in the peripheral (plasma) membrane (FABPpm) and another in the cytoplasm (FABPc) (223) (II) the FA transport protein (FATP) and (III) the FA translocase (FAT/CD36)(221-224). Since these three proteins display most features of a classic transport system, they may interact with each other to facilitate FA uptake, for example, interactions between FABPpm and FAT/CD36, and between FAT/CD36 and FATP, have been identified in controlling FA uptake (213,214,216,222). However, FABPpm and FAT/CD36 seem to play key roles in transmembrane transport of FA, albeit in an indirect manner (222).

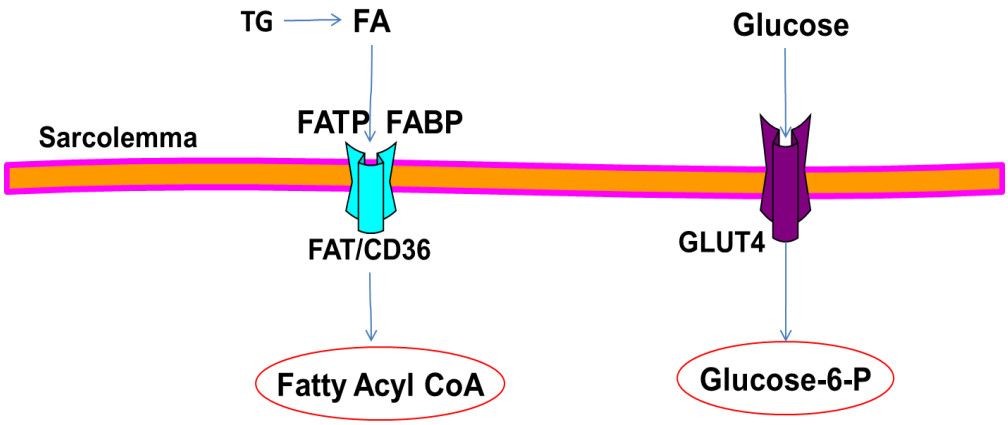


Fig 5. In myocardial metabolism, FA uptake is associated with FA transporter system with three transmembrane proteins: the FA binding proteins (FABPs), the FA transport protein (FATP) and the FA translocase (FAT/CD36). Once transported across the sarcolemma, FA are converted to cytoplasmic long-

chain fatty acyl-CoA (Fatty acyl-CoA) by fatty acyl-CoA synthetase. Glucose is uptaken by predominantly GLUT4 in insulin-dependent manner. Once entering the myocyte, glucose is phosphorylated to glucose-6-phosphate (G-6-P) in the non-oxygen dependent glycolytic pathway. Edited from Aaron KF Wong, et. al. Clinical Science 116: 607–620, 2009.

Myocardial glucose uptake

Early work of Opie et al. (225) showed that myocardial glucose uptake depends partly on its arterial concentration and partly on energy demand. Subsequently, Gould and Holman (226) demonstrated that the glucose transporters (GLUT1 and predominantly GLUT4) play an important role in glucose uptake by myocytes (Fig 5) (38).

The GLUT family (also called solute carriers 2A (SLC2A)) is diverse and 13 isoforms have been identified thus far (227-229).

GLUT1 is functioning primarily as a regulator of basal glucose transport in cardiac myocytes. It is the dominant myocardial isotype during fetal life and undergoes a rapid regression after birth. In the adult heart, GLUT4 is the dominant myocardial isotype in a GLUT4/GLUT1 ratio of 3:1 (228,230-232). GLUT4 is a high-affinity, insulin-responsive transporter that is highly expressed not only in striated muscle (including heart) but also in adipose tissue (233). It is responsible for the postprandial removal of glucose from the circulation (232,234-237). In the basal state, GLUT4 undergoes a slow but continuous recycling between the plasma membrane and several intracellular compartments, with only 5% of the total GLUT4 protein pool localized in the plasma membrane. In response to acute insulin stimulation (2-3 min), however, the rate of GLUT4 exocytosis markedly increases concomitant with a small decrease in endocytosis, so that approximately 50% of the GLUT4 protein is relocated to the cell surface for glucose uptake (234,238,239). Although GLUT1 is insulin-independent, insulin stimulation of glucose transport by this transporter in vascular cells appears to occur in a similar manner as GLUT4 in metabolic cells, namely via the PI3K/PKB pathway, except that GLUT1 is less dynamically translocated (112). Interestingly, in ischaemic preconditioning of the heart, the

increased glucose uptake is mediated through GLUT1 in an insulin-independent manner (39).

Recently, PI3K/PKB-independent glucose uptake by GLUT4 was described. Binding of insulin to its receptor finally activates the small G-protein TC10 via the scaffolding protein, Cbl-associated protein (CAP), resulting in GLUT4 translocation and enhanced glucose uptake (240-242). This mechanism seems to regulate the intracellular insulin responsive vesicle storage compartments of GLUT4 to the plasma membrane. Contraction-mediated GLUT4 translocation from the intracellular compartments to the sarcolemma may also contribute significantly to myocardial glucose uptake independent of PI3K/PKB pathway (242). In addition, increased AMP activated protein kinase (AMPK) activation, e.g. during ischaemia, stimulates GLUT4 translocation to the sarcolemma also in a PI3K/PKB-independent manner (243).

1.2.3.3. Cytoplasmic control of myocardial fatty acids and glucose metabolism

Fatty acid metabolism

In the heart, upon entering the myocyte, FA are directed towards one of three major metabolic fates: (I) oxidation in mitochondria for energy generation, (II) conversion to glycerolipids, including TAG, diglycerides (DG), and major membrane phospholipids, and (III) conversion to sphingolipids, including sphingomyelin and ceramide. When the uptake of FA exceeds the rate of β-oxidation, intramuscular lipids can accumulate, leading to lipotoxicity. This is known to activate kinases involved in the downregulation of insulin signalling and its actions (see review in 18).

Glucose metabolism

In the heart, glucose can either be oxidized or stored as glycogen, or to a lesser extent as fat (via de novo lipogenesis). Early studies using indirect calorimetry in combination with femoral vein catheterization and the euglycemic-insulin clamp suggested that for its disposal, nonoxidative glucose metabolism was the major pathway in healthy subjects (244,245). About 75% of insulin-dependent postprandial glucose disposal occurs in the skeletal muscle (246).

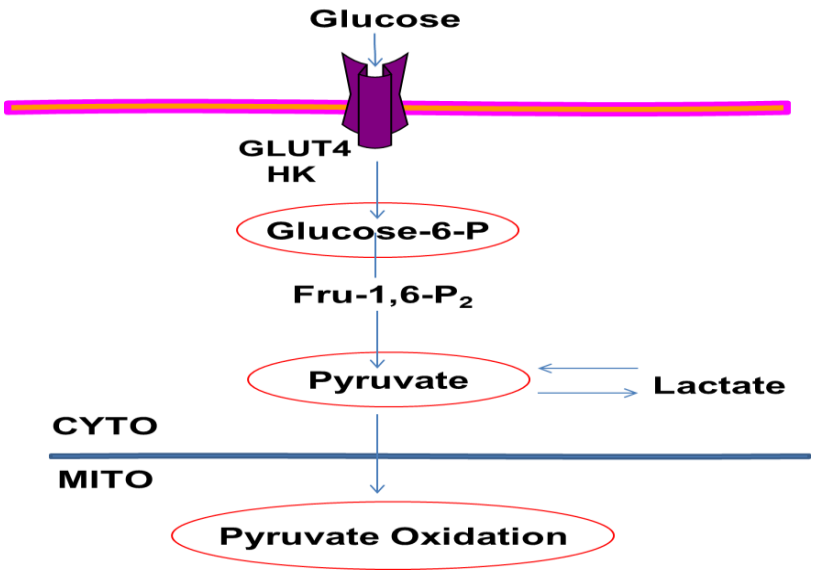


Fig 6. Glucose metabolism. The glucose uptaken by GLUT4 enters the non-oxygen dependent glycolytic pathway in the myocyte. Glucose is phosphorylated to glucose-6-phosphate, and then converted to fructose-6-phosphate, fructose 1,6-bisphosphate, eventually formed pyruvate which can either enter the mitochondria for oxidation or be reduced to lactate in the cytosol. Edited from Louis Hue and Heinrich Taegtmeyer. Am J Physiol Endocrinol Metab 297: E578–E591, 2009.

Once entering the myocyte, glucose is phosphorylated to glucose-6-phosphate (G-6-P) in the non-oxygen dependent glycolytic pathway. Subsequently glucose-6-phosphate is converted to fructose-6-phosphate and irreversibly into fructose 1,6-bisphosphate via phosphofructokinase-1 (PFK-1) eventually to form pyruvate which is the end product of glycolysis (Fig 6) (247). Pyruvate can either enter the mitochondria for oxidation or be reduced to lactate in the cytosol depending on oxygen availability.

1.2.3.4. Myocardial mitochondrial fatty acid and glucose metabolism

Mitochondrial fatty acid uptake and oxidation

The cytoplasmic long-chain fatty acyl-CoA (LC acyl-CoA) converted from FA can either be esterified to triglyceride by glycerolphosphate acyltransferase (41,48,248) or transported into the mitochondria to undergo β-oxidation (41). In the healthy normal heart 70–90% of the fatty acids entering the cell are oxidized in mitochondria (a small

extent in peroxisomes) (249,250) and 10–30% enter the intracardiac triglyceride pool (41,43,251).

Since the mitochondrial outer membrane is impermeable to acyl CoA derivatives, the transport process is facilitated by a carnitine-dependent transport system. This system includes carnitine palmitoyl transferase-1 (CPT-1), carnitine translocase (CAT), and carnitine palmitoyl transferase-2 (CPT-2) (307,308), which maintains the balance of acyl-CoA moieties between cytoplasm and mitochondria (Fig 7).

CPT1 has two cytoplasmic binding sites: a substrate site for LC acyl-CoA and a regulatory site for malonyl-CoA (252-256). CPT-1 governs the entrance of LC acyl CoA into the mitochondria and is the rate limiting enzyme for mitochondrial FA uptake and β-oxidation (257,258). Malonyl-CoA is a potent endogenous inhibitor of CPT-I, regulating mitochondrial FA uptake and oxidation (259,260). Malonyl-CoA can be converted into acetyl-CoA by malonyl-CoA-decarboxylase (MCD) resulting in reduced malonyl-CoA levels, which relieves its inhibitory effect on CPT-1 and promotes FA uptake and β-oxidation (reviewed in refs 18,259,261). In contrast, increased malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACC) activation, inhibits CPT-1 resulting in decreased β-oxidation. Thus, CPT-1 is the rate limiting enzyme of mitochondrial FA uptake and β-oxidation. However, recent studies suggest that this may not always be the case, for example, etomoxir-induced partial CPT-I inhibition in vivo does not alter cardiac FA uptake and β-oxidation (262), and in db/db mice, the malonyl CoA levels are increased by a reduction of AMPK activity, while myocardial FA β-oxidation remains elevated (263,264). These observations indicate that other mechanisms independent of malonyl CoA and CPT-1, may be of significance in mitochondrial FA uptake and oxidation.

CPT-2 transfers the acyl group of acylcarnitine across the inner mitochondrial membrane, after which carnitine is released and LC acyl-CoA is formed again in the mitochondria. CPT-2 is only loosely associated with the inner membrane and insensitive to inhibition by malonyl-CoA (265).

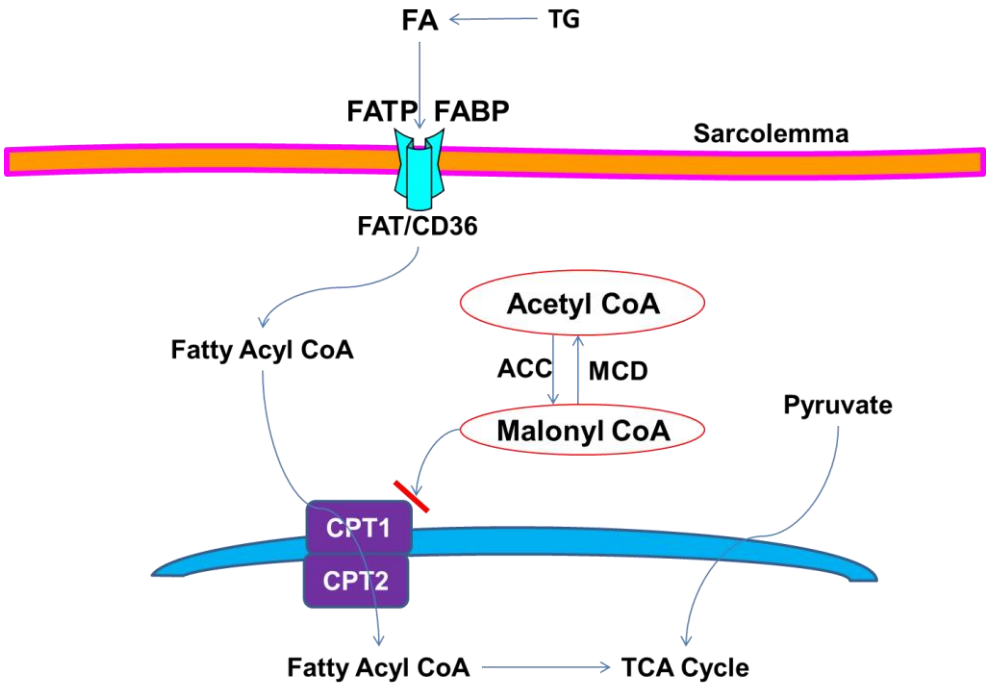


Fig 7. Regulation of mitochondrial FA uptake in myocardial metabolism. The FA uptake is facilitated by the carnitine palmitoyl transferase-1 (CPT-1) and carnitine palmitoyl transferase-2 (CPT-2) transport system. Malonyl-CoA inhibits CPT-I, regulating mitochondrial FA uptake and oxidation. Malonyl-CoA can be converted into acetyl-CoA by malonyl-CoA-decarboxylase (MCD) and increased from acetyl-CoA by acetyl-CoA carboxylase (ACC). Edited from Aaron K F Wong, et cl. Clinical Science 116: 607–620, 2009.

Mitochondrial glucose uptake and oxidation

Pyruvate generated by glycolysis is transported into the mitochondria by the monocarboxylate transporter (MCT). Studies from both human and rat showed the presence of large amounts of the MCT-1 isoform in heart (266,267), the expression of which is increased in response to exercise in rats (268).

After transport into mitochondria, pyruvate is converted into acetyl CoA by the pyruvate dehydrogenase (PDH) complex, for oxidation in the TCA cycle. The PDH complex is a key regulating enzyme complex in mitochondria for the conversion of pyruvate to acetyl-CoA. This step is considered to be irreversible in carbohydrate oxidation. The PDH complex is tightly regulated by two enzymes: PDH kinase (PDHK), a phosphorylating enzyme, and PDH phosphatase (PDHP), a

dephosphorylating enzyme (269). PDH is phosphorylated and inactivated by PDHK 1–4 (269), the latter being the dominant isoform in the heart (270). PDHK activity can be inhibited by coenzyme A (CoA), nicotinamide adenine dinucleotide (NAD+), ADP and pyruvate, which leads to reduced PDH phosphorylation, therefore increased activity (Fig 8) (for review, see 38).

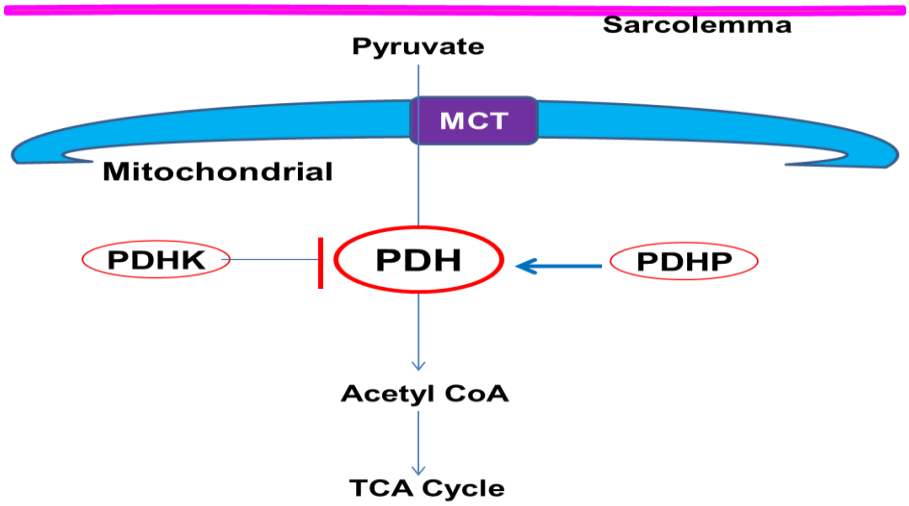


Fig 8. Regulation of mitochondrial glucose uptake and oxidation in myocardial metabolism. Pyruvate is transported into the mitochondria by the monocarboxylate transporter (MCT). After uptake, it is converted into acetyl CoA in mitochondria by the pyruvate dehydrogenase (PDH) complex. The PDH complex is tightly regulated by PDH kinase (PDHK) and PDH phosphatase (PDHP). PDH is phosphorylated and inactivated by PDHK and it is dephosphorylated and activated by PDHP. Edited from Gary D Lopaschuk, et cl. Physiol Rev 90:207-258, 2010.

When the energy status of the cardiomyocyte is high or when FA and ketone bodies are the predominant utilised substrates, acetyl-CoA and nicotinamide adenine dinucleotide hydrogen (NADH) can positively stimulate PDHK activity, resulting in PDH inactivation by phosphorylation (negative feedback) (271,272). In contrast, PDHP can also be activated by increased levels of Ca^{2+} and Mg, resulting in increased PDH activation by dephosphorylation (273,274). PDHP can also be acutely activated by insulin mediated protein kinase Cδ in muscle and liver (275). Insulin can directly activate PDH, but this is restricted to cells which are capable of lipogenesis, such as fat cells (276).

1.2.3.5. Interaction between fatty acid and glucose metabolism

Under physiological conditions, myocardial ATP production is derived from the mitochondrial oxidation of different substrates, with FA (60–70%) being predominant over glucose (20-30%) and lactate (10%). This phenomenon of substrate preference was first described by Philip Randle and colleagues in the 1960s and has been termed the 'Randle Cycle' (269). The Randle Cycle postulated that increased FA oxidation can cause elevated mitochondrial acetyl CoA to CoA (acetyl CoA:CoA) and NADH to NAD+ (NADH:NAD+) ratios. These changes subsequently suppress glucose metabolic pathways at the level of the PDH complex, to a lesser extent on glycolysis and glucose uptake. This leads to the accumulation of intracellular glucose which further prevents glucose uptake (277). Although, the Randle Cycle has been clearly demonstrated in the heart, it is not a true metabolic cycle like Krebs' urea cycle or the citric acid cycle. Rather the phenomenon describes the complex interactions between carbohydrates and FA, the two main classes of energy producing substrates. However, any strategy to stimulate FA β-oxidation needs to consider the possible inhibitory effects of FA on glucose metabolism according to the Randle Cycle.

There are, however, opposite arguments to certain aspects of the traditional view of the Randle Cycle. One study using TAG and heparin infusion showed that a reduction in glucose uptake in the presence of increased FA availability is not due to the increase in FA oxidation, but rather to a main defect in glucose uptake causing a secondary defect in glucose oxidation (278,279). The authors suggested that the rate of glycolysis, determined by the intracellular availability of glucose-6-phosphate, is the predominant factor determining the rate of glucose oxidation.

In contrast to the above, other studies provided evidence to support the Randle Cycle and the inhibitory effect of increased plasma FFA on whole body glucose uptake and glucose storage (279-282). Decreases in mitochondrial fatty acid uptake and β-oxidation result in an increased glucose oxidation as well as an increase in insulin-stimulated glucose uptake during hyperinsulinemia. The existence of the Randle Cycle in skeletal muscle as well as consideration of the opposite perspective to certain aspects of the traditional view of the Randle Cycle, should therefore be taken into account when considering approaches aimed at stimulating fatty acid β-oxidation to treat insulin resistance.

1.2.3.6. Cardiac efficiency: role of substrates

The logic of metabolism is based on the First Law of Thermodynamics— the Law of Energy Conservation—which states that energy can neither be created nor destroyed. The first law of thermodynamics forms the basis for the stoichiometry of metabolism and the calculation of the efficiency of cardiac performance (277). The heart depends on a constant arterial O_2 supply to produce enough energy to maintain essential cellular processes. Efficiency can be defined as the ratio between generated work and energy input, and the latter is measured in the heart either as the rate of substrate utilization or the rate of O_2 consumption. The efficiency of ATP production is conventionally expressed as the ratio of ATP synthesis rate to O_2 consumed (P:O ratio). The O_2 consumption averages 60–150 µl/min/g in the resting heart and can increase fivefold during exercise (283,284). For oxidation of glucose only (a condition that occurs only in severe ischaemia), the P:O ratio is 15% higher than during oxidation of FA only, indicating a greater efficiency with glucose utilization. Increased FA utilization can also decrease cardiac efficiency via the futile cycling of FA intermediates, since more ATP is consumed for noncontractile versus contractile purposes. The cycling of FA and TAG is ATP dependent, and it has been reported to contribute to 30% of total cellular energy consumption in isolated non-contracting cardiac myocytes suggesting a significant amount of futile cycling (285). The process of FA anion export from the mitochondrial matrix by uncoupling protein 3 (UCP3) and re-entering into the mitochondrial matrix by prior conversion to an acyl CoA ester, consumes the equivalent of two molecules of ATP, which represents another futile cycle (286). In addition, high concentrations of FA can also activate sarcolemmal Ca^{2+} channels that would increase the entry of extracellular Ca^{2+} into the cytosol and increase the rate of ATP hydrolysis required to maintain normal cytosolic Ca^{2+} homeostasis (287). But on a molar basis, however, one molecule of FA can form 129 molecules of ATP, while one molecule of glucose can generate only 38 molecules of ATP, indicating that much more ATP is produced from FA oxidation than from glucose utilization (288).

1.3. Fatty Acid and glucose metabolism in obesity

1.3.1. Obesity: general information

Obesity and overweight are most often defined by body mass index (BMI) (289,289a,290), which is subdivided into underweight (20 kg/m^2), normal (20–25 kg/m^2), overweight (25–30 kg/m^2), class I obesity (30–35 kg/m^2), class II obesity (35–40 kg/m^2), and class III obesity (> 40 kg/m^2).

Obesity is an important risk factor for the development of diabetes (D), hypertension, hyperlipidemia, coronary artery disease, ventricular dysfunction, congestive heart failure, stroke and cardiac arrhythmias (289-294). Obesity-induced insulin resistance and diabetes are worldwide disorders, becoming a growing health crisis of epidemiological proportions (289a,290,295,296). Life style plus a genetic predisposition can cause obesity. Although, epidemiological evidence has shown the relationship between obesity and type 2 diabetes (T2D) with inflammation for more than 100 years (297), the molecular mechanisms underlying these conditions only started to become clear in the late 1990s (298,299). Both human and animal studies show that obesity is associated with cardiac structural and functional changes (300). Many of these changes, such as left ventricular (LV) hypertrophy, left atrial (LA) enlargement, and subclinical impairment of LV systolic and diastolic function are believed to be precursors to more overt forms of cardiac dysfunction and heart failure (294).

Life style, such as high-energy feeding, is a major cause of obesity-induced insulin resistance. This is characterized by a decreased tissue reaction to the biological effects of insulin, such as, an inability of muscle to utilize and store carbohydrate, along with an inability of the adipose tissue and liver to store fat and curb glucose output, respectively. Accumulation of visceral fat in obesity may be a key role player in development of the systemic proinflammatory state associated with insulin resistance (10,301-303). Obesity also appears to induce lipid accumulation in “ectopic sites,” such as the liver and skeletal muscle, and possibly in pancreatic cells and the kidney. Ectopic fat accumulation is also associated with insulin resistance (304,305).

Insulin resistance is a central factor in the metabolic syndrome, a disorder involving a cluster of metabolic abnormalities that leads to many severe diseases including T2D

and cardiovascular disease. The National Cholesterol Education Program's Adult Treatment Panel III (ATPIII) and the World Health Organization (WHO) have rigorously defined the components of the metabolic syndrome in recently published/updated criteria (306,307).

The pathophysiology of insulin resistance involves the same complex network of insulin signalling pathways in target tissues (e.g. muscle, liver, and adipose tissue) (308). A key feature of insulin resistance is pathway-specific impairment with decreased PI3K-dependent and increased RAS/MAPK-dependent signalling (5,56). This imbalance in insulin pathways leads to endothelial dysfunction and insulin resistance that contributes to metabolic and cardiovascular diseases (10,56). Genetic studies and therapeutic interventions in both animals and humans support these concepts. The progression of such a subnormal response in pre-diabetic conditions is usually insidious, with affected individuals living subclinically for years with glucose levels nearly normal due to hypersecretion of insulin which may precede the development of T2D by many years (309).

However, despite current knowledge regarding different aspects of the phenomenon of insulin resistance, its mechanism still remains to be fully elucidated.

1.3.2. Mechanisms of obesity-induced insulin resistance

There is compelling evidence showing that exposure of adipocytes to several types of stressors (oxidative stress, inflammatory cytokines, elevated concentrations of FA) induces abnormal cellular responses mediated by kinases, including MAPK (ERK, JNK and p38 MAPK), inhibitor of NFkB kinase β (IKK-β), mammalian target of rapamycin (mTOR), and various conventional and atypical protein kinase C (PKC) isoforms.

1.3.2.1. Alterations in circulating fatty acids in the setting of obesity

High circulating levels of FA are common in obesity and insulin-resistant conditions (308). Exposure of the vasculature, myocardium, and skeletal muscle to high levels of FA affects multiple cellular processes including impaired insulin signalling (310,311), increased oxidative stress (312,313), alterations in the local renin

angiotensin system (RAS) (314), and enhanced VSMC adrenergic sensitivity (315). All of these abnormal processes may contribute to cardiac, vascular, and metabolic insulin resistance (10,37). In adipocytes, obesity-induced insulin resistance, in turn, leads to increased hormone sensitive lipase (HSL) activity, which increases the breakdown of TAG, resulting in further release of FA and thus exacerbating metabolic insulin resistance (37).

Intracellular lipid metabolites in insulin resistance

Studies on obese animals (316) and human studies (317) have shown convincingly that the elevated circulating FA and TAG cause an imbalance between the uptake and oxidation of FA. This may lead to accumulation of intracellular lipids such as TAG, DAG, LC acyl CoA, and ceramide, a process frequently referred to as “cardiac lipotoxicity” (37,318,319). The presence of these metabolites in the intracellular environment can activate serine kinases such as PKC, IKK and JNK, which in turn can phosphorylate the serine sites of IRS-1, impairing insulin signalling (304,320).

Despite the accumulation of TAG within the myocardium, a rapid rate of turnover of the endogenous TAG pool can occur in the presence or absence of high concentrations of FA (321,322). This is associated with increased oxidation of FA (322).

The role of TAG in insulin resistance is controversial. Studies performed on Zucker rats showed that the accumulation of intramyocardial TAG in response to increased circulating FA reduced their ability to upregulate FA oxidative capacity, contributing to lipotoxicity (323,324). Despite these findings, ongoing observations postulate that intramyocardial TAG accumulation itself is not responsible for defects in muscle insulin signalling (325). Currently, it is believed that intramyocardial TAG may provide a protective effect by storing fat to limit lipid metabolite levels, thereby maintaining insulin sensitivity (325).

DAG has been shown to accumulate in rodents on a high-fat-diet (HFD) and in obese humans, and may be involved in development of insulin resistance (329,330). Infusion of lipid and heparin caused insulin resistance in muscles associated with accumulation of intracellular DAG (326). The serine kinases activated by lipid

metabolites include PKC, IKK and JNK, which can phosphorylate the serine sites of IRS-1 to impair insulin signalling (327,328).

Long chain fatty acyl-CoA is increased in obesity. Studies on HFD animals or obese insulin-resistant Zucker rats showed that the high availability of LC acyl-CoA in muscle may increase FA β-oxidation, but the downstream pathways such as the TCA cycle or the electron transport chain activity may not increase accordingly, leading to incomplete oxidation (18,318,331). However, to date, a direct target of LC acyl-CoA in the insulin signalling pathway has not been identified.

1.3.2.2. Inflammatory signalling and cytokines in obesity-induced insulin resistance

Recent studies have demonstrated that obesity leads to increased circulating inflammatory cytokines in a pro-inflammatory state that may contribute to insulin resistance (332). These cytokines are believed to directly or indirectly affect the pathophysiology of various disorders and biologic processes that are involved in metabolic and vascular homeostasis (297,334,335).

In obesity and the metabolic syndrome, the inflammatory state has a peculiar presentation, as it is not accompanied by infection or signs of autoimmunity or massive tissue injury, and the dimension of the inflammatory activation is not large. This state differs from the classic inflammation which is associated with other pathologies, such as those caused by viral and bacterial infections (337). Thus the inflammatory state in obesity is often called “low-grade” chronic inflammation, also referred to as metainflammation (metabolic inflammation), or “parainflammation” (an intermediate state between basal and inflammatory states)(337,338).

Currently, it is well established that adipose tissue (and infiltrated resident macrophages) behave not only as a simple lipid storage depot but also as immune cells and an active endocrine organ, secreting a plethora of pro-inflammatory peptide hormones (335,339,340). Adipokines include leptin, adiponectin, tumor necrosis factor (TNF-α), plasminogen activator inhibitor type 1 (PAI-1), interleukin (IL) 1β, IL-6, IL-8, IL-10, IL-18, IL-33, monocyte chemoattractant protein-1 (MCP-1), C-reactive protein (CRP), macrophage migration inhibitory factor (MIF), resistin, retinol binding protein-4 (RBP-4), angiotensinogen and visfatin (333,334). Most of these adipokines

are positively involved in the development of insulin resistance (334). However, adiponectin was found to be negatively correlated with adipocyte size and insulin resistance (344).

The circulating cytokines released from adipocytes, may elicit significant actions on multiple organ systems, including the heart (341). Among adipose tissue, visceral fat is apparently more susceptible to lipolysis than subcutaneous adipose tissue (342) and is associated with a higher production of TNF-α (342,343), PAI-1 (344), IL-6 and CRP (345), and seems to be an independent predictor of insulin sensitivity (346,347). Increased adiposity in target organs is associated with an accumulation of macrophages, which are a major source of TNF-α (348,349). In addition to adipocytes, the pro-inflammatory cytokines are also expressed in other cells, such as infiltrating macrophages or stromal cells. Recent studies also show that macrophages directly infiltrate skeletal muscle, potentially contributing to local inflammation of this tissue (318).

The inflammatory process has its own unique features, and its mechanisms are far from being fully understood (333,334). Pro-inflammatory cytokines may contribute to insulin resistance by impairing insulin signalling and endothelial function.

1.3.2.2.1. Role of cytokines on insulin signalling pathways in obesity-induced insulin resistance

Many reports indicated that the various pro-inflammatory cytokines may play a role in the myocardial remodelling process by directly influencing aspects such as hypertrophy, apoptosis, fibrosis, and ultimately contractility (154,350). In obesity, the most extensively studied pro-inflammatory cytokines in the development of insulin resistance are TNF-α, leptin, adiponectin and resistin. TNF-α induces insulin resistance at a molecular level and is associated with activation of a variety of serine kinases including JNK, IKK, and IL-1 receptor-associated kinase that directly or indirectly reduce IRS-1/2 activation via serine 307 phosphorylation, impairing the insulin signalling pathway (336,351).

TNF-α binding to its TNF receptor (TNFR) superfamily on the cell surface can activate the TNFR-associated factor (TRAF) proteins followed by JNK activation via MAPKKK (352-354)(Fig 9). Studies from gene knockout animals targeting MKK7

showed that JNK activation by TNF-α involves MKK7 while its full activation requires the basal activity of MKK4 in response to TNF-α (354). It was also reported that the activation of JNK is mediated by endoplasmic reticulum stress through TRAF2 (355). Furthermore, TNF-α can not only specifically suppress the insulin PI3K/PKB/NO vasodilator pathway but also simultaneously increase the insulin Ras/MAPK/ET-1 vasoconstriction pathway in skeletal muscle arterioles (356,357). TNF-α also disrupts glucose uptake by directly reducing GLUT4 expression (82,308,358). A key role for TNF-α was demonstrated by the observation that insulin sensitivity was improved in both dietary and genetic (ob/ob) obese mice lacking TNF-α or TNFR (338). Interestingly, TNF-α is also involved in the expression of CRP which is an important marker of vascular inflammation and its plasma levels correlate with risk of cardiovascular disease. For example, CRP can inhibit insulin-dependent NO production by phosphorylation of IRS-1 on Ser 307 (359,360) and by decreasing expression of eNOS in the vascular endothelium (360-362).

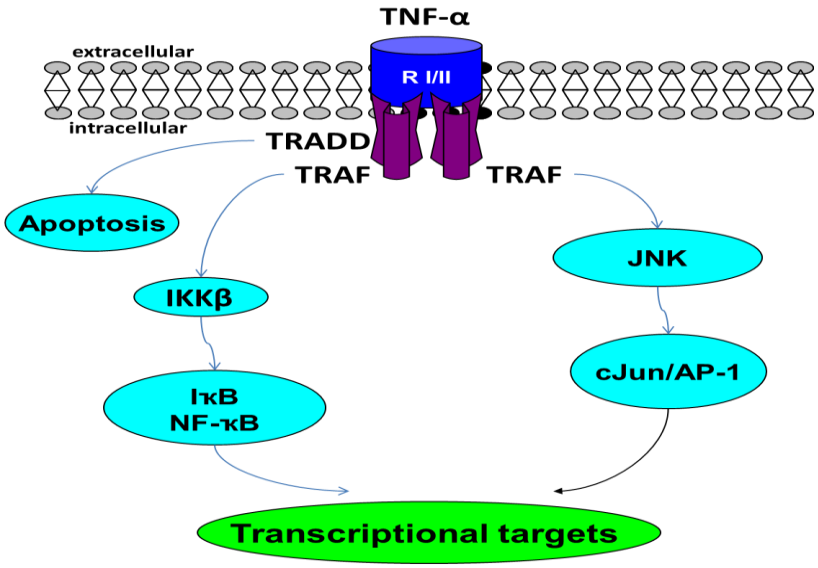


Fig 9. TNF-α receptor signalling cascade. Binding of TNF-α to its cognate receptor (TNFR) can initiate downstream signalling pathways. The TNF receptor-associated death domain (TRADD) associates with apoptotic signaling cascade. The activation of the TNFR-associated factor (TRAF) proteins can lead to the nuclear factor kappa B (NFκB) avtivation via IkB kinase (IKK) and JNK activation via MAPKKK and MAPKK. These signalling cascades can result in activation/repression of key transcriptional targets and/or alterations in cellular physiology and viability. Edited from Keigan M Park, et al. Cellular Signalling 22: 977–983, 2010.

1.3.2.2.2. Role of intramuscular nuclear factor-kB

Nuclear factor-kB (NF-κB), a family of nuclear transcription factors, are the central mediators of inflammatory signalling in the development of insulin resistance (363,364).

Under normal conditions, NF-kB predominantly resides in the cytoplasm, bound to its inhibitory protein IkBα (members of the IkB family) in an inactive state (364)(Fig 9). As a consequence, when IkBα is degraded, mostly by IkB kinase (IKK), and NF-kB is liberated from its inhibitory protein and translocates to the nucleus, it results in transcription of inflammatory genes. Numerous inflammatory cytokines and ultimately adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (365) cause a potent feed-forward production of pro-inflammatory transcripts (17). These adhesion molecules are reactive oxygen species (ROS) dependent and facilitate the attraction, adhesion and infiltration of white blood cells into sites of inflammation, leading to vascular dysfunction (365). Furthermore, pharmacological inhibition of NF-kB nuclear translocation, prevented palmitate-induced insulin resistance in L6 myotubes (366), suggesting that nuclear translocation and subsequent NF-kB-dependent gene expression are associated with FA induced insulin resistance in skeletal muscle.

Skeletal muscle NF-kB activation has also been associated with insulin resistance: this was demonstrated by studies inhibiting IKKβ/NF-kB signalling via heterozygous knockout in rodents (299) or high doses of salicylate in rodents (299,367) and humans (368). Several other studies in HFD or acute hyperlipidemia (lipid infusion) models have shown that (i) an increased IKKβ activity and a reduction of IkBα levels in rat skeletal muscle are associated with reduced insulin signalling (17) and (ii) long chain saturated FA-induced insulin resistance is associated with activation of the NF-kB pathway (17).

An acute elevation in plasma free fatty acids (FAs) in humans induced insulin resistance and this coincided with accumulation of DAG, an increase in PKC activity, and a reduction in IkBa in skeletal muscle (320). The latter is a sign of increased NF-kB activation and suggested that accumulation of DAG could lead to insulin resistance via activation of the NF-kB pathway.

However, FA-induced NF-kB activation per se is not sufficient to cause insulin resistance as indicated by the following studies. Several unsaturated FA failed to induce insulin resistance in spite of activating the NF-kB pathway in muscle cells (369). In an in-vivo study by transgenic expression of the IkBα super-repressor (MISR mice) to inhibit muscle-specific NF-kB-dependent gene expression, this did not protect against the development of HFD-induced insulin resistance (370). Muscle-specific expression of a constitutive active IKKβ mutant protein in mice did not result in muscle insulin resistance, indicating that IKKβ activation per se is not sufficient to induce insulin resistance in muscle (369,370). Altogether, these studies suggest that insulin resistance does not require muscle NF-kB activation.

NF-kB was shown to negatively regulate the mitochondrially encoded cytochrome c oxidase III and cytochrome b in response to TNFα stimulation (371).

Moreover, several studies on rodents demonstrated that FAs can activate intramyocellular inflammatory signalling pathways via activation of the Toll-like receptors (TLR) or after accumulation of intramyocellular lipid metabolites. Activation of cytokine receptors, TLR receptors, and the intracellular accumulation of lipid metabolites can all lead to the activation of the NF-kB pathway.

In addition, NF-kB exerts its antiapoptotic effects by inducing antiapoptotic genes thereby promoting cell survival and proliferation. It antagonizes the proapoptotic functions of p53 and NF-kB has been shown to negatively regulate p53 stability by modulating the p53 E3 ubiquitin ligase, Mdm2 levels (372).

1.3.2.2.3. Role of c-Jun NH2-terminal kinase in insulin resistance

The JNK family of protein kinases, also known as stress-activated protein kinases (SAPK), are members of the MAPK family (373,374). JNK initially was described in the early 1990s, 10 years after the discovery of ERK.

Three highly related JNK proteins: JNK1, JNK2 and JNK3 have been identified. JNK1 and JNK2 are broadly expressed while JNK3 is predominantly expressed in neurons. These kinases are activated via a three-tiered kinase cascade by a range of stress stimuli (373,374)(Fig 2). Members of the MKKK (MAP3K) that activate JNKs are MEKK1, MEKK2, and MEKK3, as well as mixed lineage kinase 2 and 3 (MLK2 and MLK3) (375). These kinases in turn activate MKK4 and MKK7 by

phosphorylation on specific serine or threonine residues within their activation loop (S257 and T265 for MKK4; S271 and T275 for MKK7). MKK4/7 then activates JNK by phosphorylation on a conserved loop Thr-Pro-Tyr (TXY) motif (T183 and Y185 in JNK1).

Similar to the other MAPKs, JNK has the ability to shuttle between the cytoplasm and the nucleus in response to specific cellular stimuli. JNK has more than 25 nuclear and more than 25 nonnuclear substrates, including activator protein-1 (AP-1) transcription factors (ATF), to regulate the expression of a number of stress-responsive genes for any specific stimulus (376,377).

c-Jun and activating transcription factor 2 (ATF-2) are the two primary transcription factors that are phosphorylated by JNK to control specific gene expression (376,378). The phosphorylation of c-Jun at serine 63 and 73 sites by JNK increases c-Jun stability in response to UV irradiation and other stress stimuli (353,376). Mutations of c-Jun phosphorylation sites at serine 63 and 73 by substituting alanines lead to anti-apoptotic action in neurons (377). c-Jun phosphorylation activated by all stress stimuli and cytokines is lost by immunodepletion of JNK from cell extracts (353). Interestingly, JNK and IKK (through activation of AP-1 and NF-kB) inhibit insulin-stimulated expression of eNOS (379). JNK also phosphorylates and activates JunB, JunD and Ets dome in protein (ElK1), which are all AP-1 proteins and involved in induction of the early gene expression. Cytokine-induced JNK signalling appears to have a significant role in chronic inflammatory diseases, such as rheumatoid arthritis and atherosclerosis (370).

In obesity-induced insulin resistance, JNK is activated by multiple factors including increased lipid metabolites, Toll-like receptors (TLR), cytokine receptor activation and TNF-α in insulin-sensitive tissues, such as the liver, muscle, and adipose tissues (17,381). The involvement of JNK in the development of insulin resistance is indicated by the following: (i) JNK activation is associated with inactivation of IRS-1 by serine phosphorylation to impair insulin signalling (17,297, 382), and also inhibits glucose-induced insulin production in β-cells (383); (ii) Disruption of the JNK signalling pathway in animal models has been shown to reduce or prevent insulin resistance (17). Furthermore, suppression of the JNK pathway, restored β-cell function and insulin sensitivity by improving glucose tolerance in obese type 2 diabetic mice (384).

1.3.2.2.4. Toll-like receptors and insulin resistance

In obese individuals, innate immune receptors such as TLR4 and TLR2 are increased in adipose tissue (385,386), and FA binding to innate immune receptors such as TLR4, leads to the activation of NF-kB signalling and JNK, which in turn, results in the subsequent induction of pro-inflammatory factors linked to the development of inflammation in states of hyperlipidemia (387,388). Activation of TLR results in synthesis of pro-inflammatory factors such as TNF-α, IL-6, and chemokines (385,388).

1.3.2.2.5. Role of reactive oxygen species (ROS) in insulin resistance

Although usually regarded as toxic by-products of metabolism, ROS are signalling molecules involved in physiologic processes (389), for example, short-term exposure to low levels of ROS triggers activation of specific pathways resulting in insulinomimetic effects (390). However, chronic exposure to ROS causes potential tissue damage by activating stress-signalling pathways in key target organs, such as the vasculature and pancreas (338).

Numerous stress-sensitive kinase pathways contribute to ROS generation (338,389,389a). Two primary sources of ROS in the vasculature are nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (313) and the mitochondrial electron transport chain (ETC) (310). NADPH oxidase, a major source of superoxide generation, is found in a variety of cells, including adipocytes, vascular smooth muscle cells (VSMC), endothelial cells, fibroblasts and monocytes／macrophages (313,392). In nutrient excess conditions, the surplus of mitochondrial effluxed protons reduces the ETC kinetics, enhancing the production of ROS, such as superoxide (393). In obesity, the metabolic overload-increased demand for nutrient oxidation, inflammation, endoplasmic reticulum (ER) stress and the unfolded protein response (UPR), and dysregulated hormonal and growth factors regulation may lead to the accumulation of ROS and the development of oxidative stress (394). Since the mitochondrion lacks a robust repair system, the increased ROS production and oxidative stress render mitochondrial DNA susceptible to oxidative damage and thus contributes to vascular dysfunction in insulin resistance (395-397).

In obesity, oxidative stress activates kinases such as JNK, PKCθ, p38 MAPK and IKK that may directly interfere with insulin signalling or indirectly via induction of NF-kB, contributing to insulin resistance (386,389,398). In addition, ROS inhibits insulin-stimulated eNOS/NO production (312) and also decreases expression of antioxidant enzymes, which all decrease NO bioavailability in the vascular endothelium (10,312,399,400). Furthermore, ROS production activates the hexosamine biosynthetic pathway (HBP) and increases the formation of advanced glycation end-products (AGEs) (400-403). All of these mechanisms may independently impair the insulin signalling pathway.

Studies have shown that the generation of mitochondrial ROS is increased in the diabetic heart (404,405). In the heart, excess oxidative stress stimulates myocardial growth, matrix remodeling, and cellular dysfunction, which cause myocardial remodeling, contractile dysfunction and structural alterations. Hyperglycemia induced by streptozotocin exaggerates LV remodeling and failure after MI in experimental studies (406,407). Similar to type 1 diabetes, LV remodeling and failure after MI were exacerbated also in high-fat diet-induced type 2 diabetes (408,409).

ROS can activate downstream kinases and transcription factors which are associated with hypertrophy (412). ROS-mediated DNA and mitochondrial damage and activation of proapoptotic signalling kinases also contribute to remodeling and dysfunction (413,414). ROS-induced DNA damage can elicite the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1) activation, which regulates the expression of a variety of inflammatory mediators and correlates with the progression of cardiac remodeling (410,414,416).

ROS impair prosurvival signalling pathways such as PKB in diabetic hearts and activates proinflammatory and cell death pathways (410,411).

Finally, ROS is directly involved in excitation-contraction coupling (263,416,417). This includes modification of critical thiol groups (SH) on the ryanodine receptor to enhance its open probability, the suppression of L-type calcium channels, and oxidative interaction with Ca^{2+} ATPases in the sarcoplasmic reticulum to inhibit Ca^{2+} uptake, leading to cytoplasmic Ca^{2+} overloading. However, the significance of these effects of ROS in the contractile dysfunction characteristic of the diabetic heart remains to be established.

1.3.3. Obesity: myocardial fatty acid supply, uptake, and β-oxidation

1.3.3.1. Fatty acid supply in obesity

Adipose tissues store the excess lipid when overconsumption of food perturbs the balance between energy demand and supply. Due to the increased adipocyte size under these conditions, circulating FA and TAG are elevated by spillover of lipids in adipocytes (263). Both human and animal studies have shown that obesity is associated with an elevation in circulating FA and TAG (418).These elevated FA levels can also accelerate VLDL-TAG synthesis in the liver, further contributing to hyperlipidemia (419). It also appears that insulin-resistant animals have an enlarged coronary LPL pool (420). Streptozotocin-induced acute and chronic diabetes are associated with increased heparin-releasable LPL activity (421,422).

1.3.3.2. Fatty acid uptake

In obesity-induced insulin resistance, the elevated FA supply to the heart is associated with an increased cardiac FA uptake. In addition, a greater expression and sarcolemmal localization of FA transporters may occur in cardiac myocytes. For example, increases in translocation of FAT/CD36 to the sarcolemma were observed in db/db mice (264) and in the obese insulin-resistant Zucker rat with no change in total cellular content (423,424). Hyperinsulinemia in obesity-induced insulin resistance could contribute to the increased translocation of FAT/CD36 to the sarcolemma of rat cardiac myocytes (418). In addition, total protein and sarcolemmal content of FABPpm were also elevated in cardiac myocytes in association with increased FA uptake (425,426).

1.3.3.3. Mitochondrial fatty acid uptake

As described before, malonyl-CoA has a potent endogenous inhibitory effect on CPT-I and modification of malonyl CoA levels plays an important role in the transport of FA into the mitochondria and subsequent FA β-oxidation. Malonyl-CoA can be converted to acetyl-CoA by malonyl-CoA-decarboxylase (MCD) resulting in a reduction in its levels, which relieves its inhibitory effect on CPT-1, promoting FA uptake and β-oxidation (427,428). It has been shown that elevated FA concentrations by HFD or

fasting increase the expression and activity of cardiac MCD (418), thereby reducing malonyl-CoA levels and indirectly elevating fatty acid β-oxidation at the expense of glucose oxidation (418,429). However, recent studies suggest that this may not be the case, for example, in db/db mice, the malonyl CoA levels are increased by a reduction of AMPK activity, while the myocardial FA β-oxidation remained increased (264). As stated in 1.2.3.4, these findings indicate that other mechanisms, independent of malonyl CoA, may also affect FA uptake and oxidation in mitochondria in obesity.

1.3.3.4. Fatty acid β-oxidation

Controversy exists as to whether the observed accumulation of intramyocardial lipid metabolites (TAG, DAG, LC acyl CoA, and ceramide) in obesity is primarily due to an excessive FA supply or to an impaired ability of the myocardium to oxidize the available FA (18). A number of experimental studies suggested that decreased FA β-oxidation plays a major role in the accumulation of intramyocardial lipid metabolites (18). Evidence supporting this concept is based on the observation that the activity of enzymes involved in FA β-oxidation in muscle, the size and number of mitochondria, as well as the activity of proteins in the respiration chain are all reduced in obese insulin-resistant humans, rodents (429), or humans with T2D (429). The predominant view in the literature suggests that lipid accumulation in insulin-resistant muscle may be attributable to lower rates of fatty acid β-oxidation, higher rates of FA uptake, or both (323,430). In contrast, recently, the preponderance of existing evidence from both human and rodent insulin-resistant models has shown that cardiac FA β-oxidation is increased, despite increased intramyocardial TAG levels for storage, as opposed to an impaired FA β-oxidation (18,418,429). In addition, direct measurements of myocardial FA β-oxidation have shown that it is accelerated in most situations of insulin resistance (430,434,435). Furthermore, different strategies to inhibit fatty acid β-oxidation in heart and skeletal muscle have been shown to increase insulin sensitivity (431,432,433). These findings imply that besides increased FA uptake, enhanced FA β-oxidation occurs in insulin resistance (418,429). Therefore, it seems highly unlikely that FA β-oxidation is reduced in obesity-induced insulin resistance. While increased FA uptake and β-oxidation occur due to the increase in FA supply to the heart in obesity and diabetes, it is clear that additional

mechanisms should also be present. However, while it is clear that increased FA oxidation exacerbates insulin resistance, this may lower the lipid intermediate levels. This, in turn, could alleviate insulin resistance. Further research is required to solve this discrepancy.

1.3.3.5. Incomplete fatty acid β-oxidation in obesity

It is postulated that obesity-induced insulin resistance can only induce the expression of genes related to FA uptake and oxidation but not the genes related to TCA cycle and ETC. For example, the increased FA uptake and supply in DIO models serve as endogenous ligands for the activation of peroxisome proliferator-activated receptor α (PPARα) in response to induction of the genes related to increased FA β-oxidation (437,438). In contrast, exercise causes not only the induction of PPAR related genes, but also increases in TCA cycle and ETC related genes (438). In obesity, the mismatch between oxidation and TCA cycle and ETC activity causes incomplete FA oxidation (438,439). However, it is clear that the increased FA β-oxidation in obesity contributes to this incomplete oxidation in mitochondria. This is supported by studies in which acid soluble metabolites (ASM), markers of incomplete β-oxidation, accumulated in insulin-resistant conditions (such as obesity and type II diabetes), indicating the failure of the muscles to completely oxidize FA (433,438). Incomplete FA β-oxidation can create an unfavourable microenvironment in the mitochondria, such as a change in ATP/ADP ratio resulting in an increased proton gradient, which facilitates the production of ROS (440) leading to the development of oxidative stress (439). Recent studies showed that decreased products of incomplete FA β-oxidation are associated with improved insulin sensitivity (433,441), suggesting that the products of incomplete FA β-oxidation may contribute to muscle insulin resistance (433). Thus it is clear that in obesity, incomplete FA β-oxidation is associated with insulin resistance. Therefore, it is logical to speculate that further enhancing FA β-oxidation without coupling of the downstream TCA cycle and ETC, will not increase insulin sensitivity. Rather, lowering FA β-oxidation to correct the “mismatch” between increased FA β-oxidation and the downstream TCA cycle and ETC may alleviate insulin resistance.

1.4. Alterations in fatty acid and glucose metabolism and signalling pathways in the setting of ischaemic heart disease

1.4.1. General

Cardiovascular disease is the leading cause of death and disability in developed countries worldwide, accounting for 16.7 million deaths per annum (442,443). Among them, ischaemic heart disease is responsible for more than 50% of total mortality and is predicted to be the major global cause of death by the year 2020 according to the World Health Organization (444,445).

Ischaemic heart disease develops when coronary blood flow is inadequate due to partial or complete coronary artery occlusion, and hence, the oxygen supply to the myocardium is insufficient to meet the oxygen demand. The history of ischaemic heart disease is relatively brief, the first clinical study describing myocardial infarction appeared in 1910 and the precise diagnosis was only possible after the introduction of the electrocardiogram into clinical practice in the 1920s (446,447).

In the clinical treatment of acute myocardial infarction, it is well-established that early, effective restoration of normal myocardial blood flow (termed reperfusion) using either thrombolysis or primary percutaneous coronary intervention, has proved to be the most powerful intervention for limiting myocardial infarct size (446,448). However, it was observed both in animal and human studies, that reperfusion after ischaemia may contribute to further tissue damage that extends the injury which occurred during the ischaemic period, a phenomenon known as “reperfusion injury” (449). Most cardiovascular surgeons are aware of the existence of the potentially adverse effects associated with reperfusion (450). However, the concept of reperfusion injury has been a subject of debate for the past three decades: some investigators believe that all injury occurs during the ischaemic period only; whereas others argue that blood reflow extends tissue injury. In recent years the discovery of post-conditioning has bolstered the concept of reperfusion injury (451,452).

It should be noted that the progress in the prognosis, diagnosis and therapy of ischaemic heart disease is the result of very close collaboration between theoretical and clinical cardiologists, and in almost every instance, these advances came from interdisciplinary and international collaborations (449). Although the cardiovascular

health status of our population has improved substantially causing a decline in cardiovascular mortality in recent years, we are still far from the ideal situation.

Two different aspects during the development of myocardial injury should be concentrated on: (i) factors responsible for ischaemic damage and myocardial cell death and ways to prevent it; and (ii) positive and negative consequences of myocardial reperfusion. Ischaemic heart disease impacts on both cardiac metabolism and function. Amongst others, several protein kinase pathways including the PKC isoforms, the MAPK (ERK, JNK and p38 MAPK), PI3K/PKB, and the tyrosine kinases are activated by myocardial ischaemia/reperfusion (I/R). These kinases are all associated with mitochondrial oxidative phosphorylation which is the main supply of ATP (453). At the level of the myocyte, dysfunction by impaired excitation-contraction coupling, electrical instability, altered ionic homeostasis and a shift from aerobic to anaerobic metabolism, on the one hand, and irreversible myocyte loss, on the other, are believed to contribute to disease progression.

1.4.2. Injury in ischaemia/reperfusion

1.4.2.1. Injury in the ischaemic phase

In the ischaemic phase, due to the energy deficiency, several injurious (damaging), intracellular alterations and self-amplifying loops and propagation via diverse injurious pathways may occur directly or indirectly, as discussed by Opie (see his book in ref 36).

In view of the numerous review articles that have appeared on this topic, it will only be briefly discussed (see for example refs 449,453,454). In summary, during ischaemia, due to the lack of oxygen, breakdown of creatine phosphate and ATP occurs associated with accumulation of Pi, ADP, lactic acid, and a rapid decline in intracellular pH (454,455). The increase in intracellular H^+ during ischaemia also reverses the Na^+/H^+ exchanger resulting in Ca^{2+} overload, which causes osmotic swelling contributing to eventual disruption of the plasma membrane.

Acidosis further suppresses ATP generation from glycolysis (457). Simultaneously, increased ROS production from mitochondrial electron transfer complexes I and III occurs during ischaemia (456).

The glycolytic pathway converts glucose 6-phosphate and NAD to pyruvate and NADH and generates two ATP for each molecule of exogenous glucose in the cytosol under anaerobic or aerobic conditions, respectively.

The availability of higher energy generating FA in aerobic perfused hearts lower glucose utilization at several steps in the glycolytic pathway, i.e., blocked glucose transport, inhibition of hexokinase by its product glucose-6-P, phosphofructokinase (PFK) by citrate, and pyruvate dehydrogenase (PDH) by the ratios of acetyl-CoA/CoA and NADH/NAD^+ which activates PDH kinase (457-460). During ischaemia, diminished O_2 supply for respiration and oxidative phosphorylation cause a decrease in mitochondrial energy production (ATP synthesis, oxidative phosphorylation) and thus a fall in cellular energy (ATP) content, leading to rapid decline of ATP and PCr. The concomitant increase in Pi, as a consequence of PCr hydrolysis, stimulates anaerobic ATP generation via an increase in glycolysis and lactate production (461,462). Under hypoxic or anoxic conditions the heart switches primarily from FA to glucose as substrate; but under ischaemic conditions, this process is limited due to shortage of substrate, and a hypoxia/ischaemia-induced rise in NADH which inhibits glyceraldehyde-3-P dehydrogenase, thus restricting glycolysis (33,463). Therefore cell function is progressively compromised by ischaemic injury.

The enzyme phosphofructokinase-1 (PFK-1) is a key regulatory site in the glycolytic pathway and catalyzes the first irreversible step (464).

PFK-1 utilizes ATP to produce fructose 1,6-bisphosphate and is activated by ADP, AMP, and Pi. It can also be stimulated by fructose 2,6-bisphosphate (F2,6BP), which is formed from fructose 6-phosphate by the bifunctional enzyme phosphofructokinase-2 (PFK-2) (465,466). F2,6BP also decreases the inhibitory effects of ATP on PFK-1. Synthesis of F2,6BP is a feed forward activator of the PFK-1 enzyme (467).

PFK-2 activity is controlled by three main mechanisms: I) allosteric modulation: PFK-2 is allosterically inhibited by citrate, II) phosphorylation control: a number of hormones that activate glycolysis, including insulin, glucagon, epinephrine, norepinephrine, and thyroid hormone, exert phosphorylation control on PFK-2 (467). In addition, AMPK can also phosphorylate PFK-2 (468). Phosphorylation and activation of PFK-2 by AMPK is an attractive mechanism to explain AMP-induced

acceleration of glycolysis (469), and III) transcriptional control of enzyme activity (470,471).

PFK-1 is inhibited by ATP, citrate, and protons (464), it can also be inhibited by fructose 1,6-bisphosphate and by a fall in pH. Inhibition of PFK-1 depends on the ATP levels, with the inhibition being greatest when ATP levels are high (see Ref. 467). As AMP accumulates, the sensitivity of PFK-1 to $[H^+]$ decreases, thus accelerating flux through glycolysis when the phosphorylation potential falls.

Citrate is a negative allosteric regulator of PFK-1 and links changes in mitochondrial oxidative metabolism to glycolysis. High rates of fatty acid oxidation result in increased cytosolic citrate concentration which contributes to the decrease in glycolysis by inhibiting PFK-1 and PFK-2 in various tissues (457,460,473,474).

Studies assessing the effect of inhibition of glycolysis suggest that glycolytically generated ATP is perferentially used by the sarcoplasmic reticulum to fuel Ca^{2+} uptake (476) and by the sarcolemma to maintain ion homeostatis (475,477,478). Furthermore, inhibition of glycolysis impairs relaxation in ischaemic and postischaemic reperfused myocardium, suggesting that glycolytic ATP may be essential for optimal diastolic relaxation (479-481).

1.4.2.2. Injury in the reperfusion phase

As described above, the phenomenon of “reperfusion injury” can lead to exacerbation of ischaemic damage. Thus, cell injury upon reperfusion, especially in the early reperfusion phase, may be a direct consequence of intracellular alterations that occurred in the ischaemic phase (453). Four initial factors were suggested to cause the immediate reperfusion injury: (i) re-energization, (ii) increased ROS generation, (iii) rapid normalization of tissue pH and (iv) rapid normalization of tissue osmolality (453,472).

During early reperfusion, protons are eliminated, which leads to increased intracellular Na^+ via the Na^+/H^+ exchanger. To compensate for this increase in intracellular Na^+, the Na^+/Ca^{2+} exchanger is stimulated, leading to increased intracellular Ca^{2+}. Repolarization of mitochondrial $\Delta\Psi$ coupled with the increased cytosolic Ca^{2+} leads to an increase in mitochondrial Ca^{2+} content. Reperfusion and the concomitant re-introduction of oxygen are also associated with generation of

mitochondrial ROS (453,482). The increased Ca^{2+} overload and ROS generation further disrupt ionic homeostasis (448). The mitochondrial permeability transition pore (mPTP) is a voltage-dependent, high-conductance channel located in the inner mitochondrial membrane. At present, it is widely accepted that during early reperfusion, ROS accumulation, pH normalization and Ca^{2+} overload, create an ideal scenario to open the mPTP, resulting in the release of pro-apoptotic factors contributing to the loss of cell viability and irreversible I/R injury (for review see 448,483,484). While ischaemia causes some cell death on its own, reperfusion is associated with accelerated apoptotic cell death (485,486).

Many findings support the physiological significance of excess mitochondrial ROS production in cardiac injury during reperfusion (487). The metabolic changes that occur during I/R also impair the endogenous antioxidant defence systems of cardiomyocytes. The first line of defence against the deleterious effects of mitochondrial ROS is the reduced glutathione (GSH) / oxidized glutathione disulphide (GSSG) system, which is directly linked to the NADPH:NADP+ ratio via glutathione reductase. Because NADPH is not produced during ischaemia, the normal metabolic mechanism for regenerating GSH, namely GSSG reductase, does not function. Mitochondrial membrane depolarization and the mPTP are sensitive to decreased GSH and NADPH levels. The depletion of glutathione increases ROS formation, oxidative stress, and Ca^{2+} overload (488). Moreover, hearts from glutathione peroxidase (GSHPx) null mice displayed increased levels of apoptosis in response to I/R compared to wild-type controls, whereas hearts from transgenic mice overexpressing GSHPx were more resistant to I/R injury (488). Similarly, overexpression of manganese-superoxide dismutase reduced myocardial I/R injury in transgenic mice (489), whereas hearts from Cu/Zn-superoxide dismutase knockout mice were more susceptible to I/R injury compared with wild type (490). Thus, the formation of ROS during reperfusion occurs when the heart cell's endogenous defence mechanisms are compromised.

In the reperfusion phase, activation of various signalling pathways, such as PKB, ERK, JNK or nuclear factor-kB (NF-kB) pathway occurs.

1.4.2.3. RISK pathway

During early reperfusion after ischaemia, a group of survival protein kinases are specifically activated, such as PKB and ERK, the so-called reperfusion injury salvage kinase (RISK) pathway (Fig 10). This pathway exerts its protective effects via transcriptional, translational, and post-translational mechanisms, and has been observed during reperfusion after ischaemic pre- or post-conditioning or pharmacologic pre- or post-conditioning (482) and is associated with a reduction in infarct size and/or improvement in functional recovery. The RISK pathway can be activated via specific G-protein coupled receptors (GPCR) or via non-receptor mediated mechanisms. Known triggers via a GPCR mechanism in the RISK pathway include adenosine (492,493), bradykinin (494,495), catecholamines (496) and opioids (497). There are also other triggers, such as adrenomedullin (a vasodilating peptide) binding to the calcitonin gene-related peptide like receptor (498,499), urocortin (a peptide related to corticotrophin-releasing factor) (500,501), glucagon-like peptide-1 (GLP-1) (a gut incretin hormone) (502), isoflurane (acts via the β2-adrenergic receptor) (503,504) and natriuretic peptides (505). A stimulus via the non-receptor mediated mechanism to activate the RISK pathway, include the 3-hydroxy-3-methylglutaryl CoA reductase inhibitor (simvastatin) (506). However, the mechanism in this non-receptor mediated cascade is currently unclear.

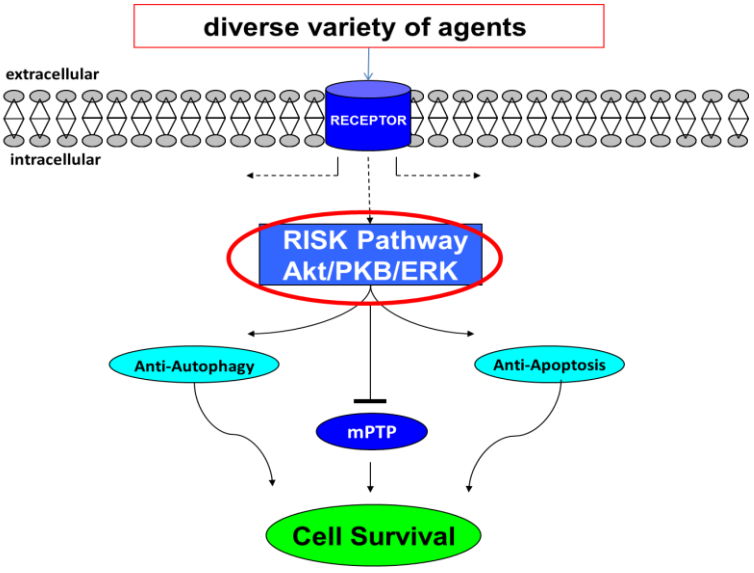


Fig 10. The Reperfusion Injury Salvage Kinase (RISK) pathway. The diverse variety of agents binding its receptor can activate the PKB and ERK, the so-called RISK pathway, associates with anti-autophagy, anti-apoptotic mechanisms and inhibition of the mitochondrial permeability transition pore

(mPTP), mediating cell survival. Edited from Derek J. Hausenloy, Derek M. Yellon. Heart Fail Rev 12:217–234, 2007.

The activation of the RISK pathway by ischaemic pre- or post-conditioning or pharmacologic agents, mediates cell survival through various pathways. These include various anti-apoptotic mechanisms such as the phosphorylation and inhibition of the pro-apoptotic proteins BAX and BAD, the inhibition of caspase 3 activation, and the phosphorylation and activation of p70s6K (which acts to inhibit BAD (507) and the phosphorylation and activation of the antiapoptotic protein Bcl-2 (9).

The mitochondrial permeability transition pore (mPTP) has been identified as a downstream target of the RISK pathway (508-511). Inhibition of the mPTP by the RISK pathway provides a common cardioprotective pathway recruited at the time of myocardial reperfusion.

It is proposed that I/R promotes mPTP opening in two phases:

(i) During ischaemia, mPTP pore opening is minimized and occurs as a progressive inner mitochondrial membrane (IMM) leak, accompanied by depressed ETC function in the setting of FA accumulation, and loss of cytochrome *c* and ROS scavengers;

(ii) Reperfusion after more prolonged ischaemia, results in impaired recovery of $\Delta\Psi m$ and myocardial energetics. These mediate cell death by uncoupling oxidative phosphorylation and inducing mitochondrial swelling (484,511,512), leading to increased matrix Ca^{2+}, Pi, ROS (513) and long-lasting mPTP pore opening. Sustained and prolonged opening of the mPTP can lead to excessive H_2O entry into the matrix, matrix swelling, and increased outer mitochondrial membrane (OMM) permeability (via oligomerization of Bid or Bax) or rupture of the outer mitochondrial membrane channels. Adenine nucleotides, Mg^{2+}, and matrix H^+ restrict the pore from opening (514-518).

However, the mechanism through which activation of the RISK pathway inhibits the opening of the mPTP is unclear, although there are several hypotheses:

(a) GSK-3β, a downstream target of the RISK pathway, has been linked to the inhibition of mPTP opening in the context of cardioprotection (510,519); IPC protection for example, can be mediated by the phosphorylation and inhibition of GSK-3β, a downstream target of PKB, thereby, inhibiting mPTP opening in part by binding to the adenine nucleotide transporter (ANT) (520). Cyclophilin D (CypD) was

first identified as an ANT-binding protein that mediated the inhibitory effect of cyclosporin A (CsA) on mPTP.

(b) eNOS, another downstream target of the RISK pathway has the potential for inhibiting mPTP opening either through the PKG/PKC(ε)-Mitochondrial Potassium ATP (mKATP) channels signalling pathway (521-524) or it may suppress mPTP opening through the generation of nitric oxide (525);

(c) The inhibition of BAX translocation to mitochondria (526) and/or the activation of mitochondrial hexokinase II (527,528) may act in concert to inhibit mPTP opening;

(d) PI3K activation by insulin can reduce calcium uptake by the sarcoplasmic reticulum, which may in turn act to inhibit mPTP opening at the time of myocardial reperfusion (529).

Despite the abundance of experimental data demonstrating effective cardioprotection associated via activation of the RISK pathway, clinical studies are still limited (482).

PKB

Numerous in vivo and in vitro studies demonstrated that activation of the PKB pathway protects the heart against I/R injury (see review 519). As described before, PKB is located downstream of PI3K and three known isoforms of PKB (PKB1/PKBα, PKB2/PKBβ and PKB3/PKBγ) have been identified in mammals. Activation of PKB by various growth and survival factors involves two distinct pathways to promote cell survival: (I) interaction with Bcl-2 family proteins to preserve mitochondrial integrity and to inhibit apoptosis (530), and (II) activation of the NF-kB pathway (530).

There is abundant evidence showing that PKB localized in various cellular compartments, confers protection against short-term and long-term stress (530). The most studied downstream effector of PKB activation in I/R is GSK-3β. It is well known that PKB inhibits GSK-3β activity by its phosphorylation at Ser9 (531, 562, 532a). A study using transgenic mice also showed that phosphorylation of GSK-3β at Ser9 is cardioprotective in the post-conditioned heart (533). However, it is currently unclear whether the cardioprotection conferred by PKB activation occurs via the inhibition of GSK-3β and prevention of mPTP opening (533). A study using GSK-3α(S21A) and GSK-3β(S9A) double knock in mice showed that insulin could still prevent mPTP opening, suggesting that targets of PKB other than GSK are involved in the prevention of this pore opening (533). In addition, it was demonstrated that neither

GSK-3α nor GSK-3β phosphorylation at the known PKB sites, were required for pre- and post-conditioning mediated cardioprotection (533). Furthermore, the work of Matsuda T (534) indicated that the phosphorylation of GSK-3β at Ser9 is pro-apoptotic whereas that of GSK-3α at Ser21 is protective.

The mechanism by which inhibition of GSK-3 reduces mPTP opening is unclear, for example, maintaining S9 of GSK-3β in an unphosphorylated form prevented cardiac decompensation during pressure overload; on the other hand, maintaining S21 of GSK-3α in an unphosphorylated form aborted the compensatory activation of cell proliferation in the heart. It is plausible that GSK-3 alters mPTP by altering phosphorylation of target substrates (536).

Obviously, with so many substrates, it will be challenging to define the mechanisms involved in the cardioprotection afforded by inhibition of GSK-3. The subcellular localization and the substrate specificity of the S9/S21 should be characterized extensively and the roles of GSK-3 phosphorylation in myocytes and nonmyocytes clarified.

Other possibilities are that PKB-induced cardioprotection against I/R is accompanied by increased glucose uptake via enhanced sarcolemmal Glut-4 expression (535). PKB activation induces eNOS/NO and PKG activation, also known to be associated with cardioprotection (9). In addition, the PKB/mTOR/p70S6K complex is protective by promoting, among others, the post-ischaemic synthesis of contractile proteins (375).

Extracellular signal regulated kinase (ERK)

A plethora of studies have shown that activation of the ERK pathway during reperfusion is associated with cardioprotection (for reviews see refs 375,482,537). Interestingly, ERK1/2 activation has been shown to compensate for loss of PKB activity in the post-infarct myocardium and promote cardioprotection in response to erythropoietin (538). Similar to PKB, ERK1/2 activation can also induce eNOS/NO and PKG activation associated with cardioprotection against I/R injury (539,540).

The effects of Ca^{2+} channel blockers and β-adrenergic receptor blockers, two classes of drugs commonly used to treat cardiac related diseases, have been reported to be mediated in part through ERK1/2 activation (541). The cardioprotection in Ang II-mediated pre-conditioning is also due in part to ERK1/2 dissociating from caveolin

(542,544). In neonatal ventricular myocytes, ERK1/2 exerted its cardioprotective effects by phosphorylating and activating the transcription factor GATA4 to increase the expression of anti-apoptotic proteins (542). However, recent work in adult cells showed that GATA4 is not a downstream effector of ERK1/2 signalling in response to the β-adrenergic receptor activated survival pathway (543).

ERK1/2 may also promote survival of cardiomyocytes by interacting with other signalling pathways, for example, IL-10 mediated ERK1/2 activation was shown to inhibit TNFα induced apoptotic signalling by blocking IKK phosphorylation and subsequent NF-kB activation (545). Finally, ERK1/2 activation has been found to suppress gap junction permeability in response to mitoK_{ATP} channel opening during I/R, thus reducing myocardial damage (552). Multiple mechanisms may therefore exist for the prosurvival effects of ERK1/2.

1.4.2.4. Survivor Activating Factor Enhancement (SAFE) pathways

Recent studies with ischaemic post-conditioning demonstrated that protection can occur independently of the activation of the RISK pathway, therefore confirming the existence of multiple protective pathways (546,547)

The activation of the Survivor Activating Factor Enhancement (SAFE) pathway, is involved in the activation of TNFα and the transcription factor, signal transducer and activator of transcription 3 (STAT3) (548,549). The 'RISK-free' pathway also can confer protection in ischaemic pre-conditioning (548-551).

The upstream and downstream activators of the SAFE pathway have been poorly studied. Many pharmacological agents capable of mimicking ischaemic pre- or post-conditioning may confer their cardioprotective effect via the SAFE pathway. A link between the RISK pathway and the SAFE pathway has also been suggested (9,548,549)

1.4.3. Phosphatase and tensin homologue deleted on chromosome ten (PTEN)

Phosphatase and tensin homologue deleted on chromosome ten (PTEN), (also called mutated in multiple advanced cancers (MMAC1) or TAGF regulated and epithelial cell-enriched phosphatase (TEP-1)), is a dual protein–lipid phosphatase

discovered relatively recently. It is expressed ubiquitously in cells (553), and can be upregulated by increased synthesis and downregulated by phosphorylation, oxidation and proteasomal degradation (554-556).

PTEN is the main downregulator of the prosurvival PI3K/PKB pathway by dephosphorylating the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3) produced by PI3K, to its precursor phosphatidylinositol (4,5) bisphosphate (PIP2), thereby interrupting the downstream activation of PKB (19,557,558). In contrast to the overwhelming evidence of the importance of upregulation of the PI3K/PKB pathway in myocardial survival following I/R, relatively little is known about the role of PTEN in this scenario.

PTEN has been shown to be involved in cell survival, including that of cardiomyocytes in I/R (559,564), but the mechanisms through which this occur, are complex and not yet elucidated completely (21). Using an isolated perfused rat heart as model, a reduction in PTEN activity in ischaemic pre-conditioning, associated with protection has been reported (20). Similarly, pharmacological inhibition of PTEN elicits cardioprotection (562,563). PTEN has also been shown to be associated with hypertrophy and remodelling, as well as regulation of the L-type calcium currents and contractile function in cardiomyocytes (564-566). It has been demonstrated that PTEN can be inhibited by vanadium compounds to protect against ischaemia (23,567), for example, sodium orthovanadate was shown to increase the tyrosine phosphorylation of PTEN leading to protection against cerebral ischaemia (23). In addition to I/R injury, it is also reported that homozygous PTEN knockout mice are not viable whereas the heterozygous animals develop numerous tumors (20). In humans, many tumor types are characterized by deficient PTEN expression (568).

It would seem that most data support the hypothesis that the PTEN downregulation is an endogenous protective mechanism.

1.4.4. JNK and p38 MAPK in ischaemia/reperfusion

1.4.4.1. JNK in ischaemia/reperfusion

Activation of the JNK pathway occurs in response to a number of different stimuli. As a stress-activated protein kinase, JNK responds most robustly to inflammatory cytokines and cellular stresses such as heat shock, hyperosmolarity, ischaemia-reperfusion, UV radiation, oxidant stress, DNA damage, and ER stress (569,573,574).

Activated JNK has a large number of downstream substrates, including nuclear and cytoplasmic proteins. Amongst others, JNK has been shown to phosphorylate transcription factors such as c-Jun at the NH2-terminal Ser63 and 73 residues and AP-1 in response to UV irradiation and other stress stimuli (373,569-572). Similar to the other MAPKs, JNK has the ability to shuttle between the cytoplasm and the nucleus to exert its effects depending on the specific cellular stimuli. The diversity of JNK signalling is conferred by signalling via more than 25 nuclear substrates and more than 25 nonnuclear substrates for any specific stimulus (376). As a stress-induced signalling pathway, JNK has both protective and pathological roles in different cell types.

Numerous in vitro and in vivo studies have shown that JNK is activated during reperfusion after ischaemia (575-579) while ischaemia alone did not result in activation (579,580-583). The role of the JNK pathway in I/R injury remains controversial, perhaps reflecting the complexity of the multistage, multitargeted signalling networks involved in this process.

The possible harmful effects of JNK, suggesting a detrimental role, can be summarized as follows:

(i) JNK activity is widely reported to increase reperfusion injury in different cells including heart, brain, kidney, liver, gastric mucosa, and lung (reviewed in refs. 591-596).

(ii) In myocardial I/R, JNK activity contributes to the detrimental effects of a number of proteins including the receptor for advanced glycation end-products (RAGE) (597,598), PKC isforms (599), β-adrenergic receptors (600), uncleaved heparin-binding epidermal growth factor-like growth factor (HB-EGF) (601), Rho-kinase (589,580), and poly(ADP-ribose) polymerase (589). JNK activation is probably associated with mitochondrial pro-apoptotic factors (584-587). JNK is known to directly phosphorylate pro-apoptotic Bcl family members such as Bak and Bid, increasing cleaved caspase-9, caspase-3, and Bax promoting apoptosis (588), and to induce the expression of pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 (584).

(iii) In addition, JNK mediates apoptosis-inducing factor (AIF) translocation from the mitochondria to the nucleus (589,590). Most recently, JNK activity has been shown to

promote apoptosis during I/R via atrogin-1, an E3 ubiquitin ligase (602). Atrogin-1 targets MAPK phosphatase- 1 (MKP-1) for degradation, resulting in a sustained activation of JNK.

(iv) Mice models with reduced JNK activity in the heart were found to have less ischaemia/reperfusion injury and less apoptosis (603). Sun et al. (604) reported that hypoxia-reoxygenation resulted in activation of JNK and p38MAPK; post-conditioning reduced apoptosis in cardiomyocytes and also reduced activation of JNK and p38MAPK. Furthermore, addition of anisomycin, a JNK/p38MAPK activator, eliminated the inhibition of apoptosis by post-conditioning.

(v) Studies using different JNK inhibitors showed reduced apoptosis in hepatocytes (586) and in cardiomyocytes in a rat cardiac I/R model (14), and reduced myocardial ischaemia-reperfusion injury and infarct size in vivo (606).

In contrast, a number of other studies demonstrated a critical role for JNK in myocyte survival and cardioprotection (382,607-611), for example, JNK provides an essential function in protecting the heart against reperfusion injury if the period of ischaemia is brief, but it increases cell death and injury when the period of ischaemia is extended (13,603,612-616). Further evidence for a protective function of JNK is the following:

(i) Sustained JNK activation obtained by generating mice with increased MKK7 (the kinase that phosphorylates JNK) in the heart, protected the hearts against ischaemia/reperfusion injury (604).

(ii) JNK is reported to interact with proapoptotic Bax and Bad on the mitochondrial membrane (585,617). However, other prosurvival pathways, including PKB, also are targeted by JNK (13).

(iii) JNK has been viewed as antiapoptotic in response to nitric oxide (NO) in vitro (618). Similarly, blocking JNK activity increased apoptosis and the activity of both caspase-9 (613) and caspase-3 (619) in another in vitro I/R model. This has been proposed to be mediated by the interaction of JNK with Apaf-1 to form a complex with the apoptosome and delay the activation of caspase-9 (614).

(iv) Most studies report that PC results in activation of JNK, but the effects of JNK in the heart appear to be complex (11).

(v) It has recently been suggested that part of JNK's cardioprotective effect is due to reactivation of PKB by JNK (13). This study showed that activation of JNK is essential for PKB phosphorylation at the onset of reperfusion (13): activation of JNK phosphorylates PKB on Thr450, demonstrating that JNK activation is a prerequisite for the full PKB activation by phosphorylation at Thr308 and Ser473.

However, convincing as these data are, the complexity of the system is probably best exemplified by Kaiser et al. who reported enhanced myocyte survival after IR with both JNK activation and inhibition (603).

This dichotomy also was observed in cardiomyocytes. These seemingly contradictory and confusing results underscore the complexity of the JNK pathway in cell death regulation in the heart.

1.4.4.2. p38 MAPK in ischaemia/reperfusion

As mentioned before, the p38 MAPK pathway is a subgroup of the MAPK family of signalling pathways, which plays an important role in myocardial I/R injury as well as in a variety of other biological processes, including inflammation, cell growth and differentiation, regulation of cardiac gene expression, myocyte hypertrophy, energy metabolism, contractility, proliferation and apoptosis (353,620-625).

p38 MAPK is composed of two domains: a N-terminal domain and a C-terminal domain. The catalytic site lies at the junction between the two domains (626-628). Four isoforms of p38 MAPK, α, β, γ and δ, have been identified and share structural homology. Expression of p38α/β MAPK is prevalent in the heart, p38γ MAPK expression is restricted to muscle and p38δ MAPK is predominantly found in the lungs and glomeruli (375,626,629).

p38 MAPK is phosphorylated on threonine (Thr180) and tyrosine (Tyr182) for activation. MKK3 is associated with activation of p38α and p38β isoforms while MKK6 is involved in phosphorylation of all p38 MAPK isoforms (622,630,632,633). p38 MAPK can be activated by various physical and chemical stresses, such as ischaemia, oxidative stress, heat shock, UV irradiation, hypoxia, and exposure to pro-inflammatory cytokines (IL-1 and TNF) (353,634). In resting cells, p38 MAPK resides in both the cytoplasm and nucleus. Upon activation, it can translocate to the nucleus (630-633,635). The first identified substrate of p38α MAPK is MAPK-activated protein

kinase 2 (MK2) which phosphorylates various substrates including heat shock protein 27 (HSP27) (636-638), lymphocyte-specific protein 1 (LSP1) (638) and cAMP response element binding protein (CREB) (639,640). In the nucleus, a broad range of transcription factors are phosphorylated by p38 MAPK.

The role of p38 MAPK activation in I/R injury is controversial, it has been shown to be both protective as well as detrimental. Many factors such as animal species, time of administration of drugs and experimental protocol etc. may affect the outcome.

Many reports showed that p38 MAPK activation during myocardial ischaemia enhances lethal injury (160,641-644) and inhibition of its activation protects against it (642,645,646). Studies from our laboratory, demonstrated the detrimental effect associated with p38 MAPK activation during ischaemia and reperfusion in IPC and β-adrenergic PC protection (647). Similarly, mice heterozygous for a p38α MAPK null allele, with reduced levels of myocardial p38α MAPK, are resistant to infarction (648,649). However, there is also evidence to suggest that p38 MAPK activation confers protection to the heart (650). For example, it has been reported that ischaemic pre-conditioning (IPC) of rabbit hearts increases p38 MAPK activity during ischaemia, and protects the heart against I/R injury (651). By using adenoviral-mediated co-expression of p38 α and β MAPK in neonatal rat cardiac myocytes, it could be demonstrated that the α isoform of p38 MAPK has pro-apoptotic effects, whereas overexpression of its β isoform results in a hypertrophic phenotype (650). This perhaps explains why pharmacological inhibition of p38 MAPKs during pre-conditioning blocks protection (since the β isoform is the dominant form), while the inhibition of p38 MAPKs during lethal ischaemia causes protection (when the α isoform is activated) (652). The evidence presented to date certainly supports the concept that the different isoforms of p38 MAPK may determine the controversial outcomes obtained regarding p38 MAPK activation in I/R injury.

1.4.5. Mechanisms of apoptosis in myocardial I/R

It is well-established that myocardial I/R results in cell loss and consequently, a reduction in contractile function. Cell loss in myocardial I/R is caused by two different mechanisms: necrosis and apoptosis (653).

Necrosis is an irreversible process characterized by cell swelling and disruption of the cell membrane (653). The ensuing release of cytoplasmic contents into the extracellular space provokes inflammation causing damage to neighbouring cells. Apoptosis is a distinct form of cell death without an inflammatory response. This process is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing, and formation of apoptotic bodies (654). In myocardial I/R, the distinction between necrosis and apoptosis is blurred (655,656), sarcolemmal integrity may be lost in excessively energy-starved cells before the process of apoptosis is complete, resulting in necrotic cell death (657). The relative proportion of each form is still open to debate. During the past several years, another form of cell death, autophagic cell death, has also drawn considerable attention (658). Autophagy is an intracellular phenomenon in which a cell digests its own constituents to remove the “biological wastes”, such as defective mitochondria, thus maintaining cellular homeostasis (659).

For more details regarding the above processes, please see references (654,659).

Accumulating evidence from in vivo and in vitro studies strongly suggest that apoptosis may play an important role in the pathogenesis of several cardiovascular diseases. Apoptosis has been detected in cardiac myocytes exposed to hypoxia/reoxygenation (660), mechanical stretch (661), as well as in animal models of cardiac I/R injury (657,662). It has also been observed in myocardial samples obtained from patients with end-stage congestive heart failure (663), arrhythmogenic right ventricular dysplasia (664), and myocardial infarction (655).

Apoptosis can be activated through the death receptor signalling (extrinsic) and the mitochondrial (intrinsic) apoptotic pathways (including activation of initiator and effector caspases and of Bcl-2 family members).

1.4.5.1. Death receptor pathway in apoptosis

In apoptosis, the death receptor pathway is one of the best characterized pathways (Fig 12). This pathway is mediated by the death receptors on the cell membrane. These receptors belong to the TNFR gene superfamily and contain a distinct conserved cytoplasmic death domain (666,667).

When stimulated, death receptors can form a homotrimeric complex, and recruit adaptor proteins for interaction via their death domains. One group of adaptor proteins are the Fas-associated death domain (FADD) adaptor proteins, the other is TNFR-associated death domain (TRADD) adaptor proteins (668,669). Both caspase-8/10 (670,671) and NF-κB (659) are downstream of the death receptor pathway and subsequently lead to activation of caspase-3, culminating in cell death by apoptosis (672-675). On the other hand, the NF-κB pathway may also induce the expression of survival genes and counteract apoptotic cell death (676-678).

1.4.5.2. The mitochondrial pathway in apoptosis

The other well-characterized apoptotic pathway is the intrinsic mitochondrial pathway. Numerous studies in intact hearts, cardiac myocytes, and isolated cardiac mitochondria (679-688) have demonstrated that different apoptotic stimuli, such as I/R (689,690), hypoxia (691), serum and glucose deprivation (681), cocaine (688), and ROS (682,684,686,692) can lead to the release of pro-apoptotic factors into the cytosol from mitochondria. These include cytochrome c, apoptosis-inducing factor (AIF), second mitochondrial-derived activator of caspase (Smac) / direct inhibitor of apoptosis protein (IAP) binding protein with low pI (Diablo), endonuclease G (endo G), and procaspases. Smac/Diablo is highly expressed in the heart (696,697).

Cytochrome c released from mitochondria is a critical step in the execution of apoptosis. It forms a macromolecular complex with Apaf-1, dATP and caspase-9, known as the apoptosome, to participate in the caspase pathway by triggering the activation of caspase-3 and apoptosis (693-695). However, it is possible that the caspase pathway actually can be further activated in the mitochondrial intermembrane space by the release of mitochondrial Smac/Diablo which will bind and sequester the IAP proteins. Therefore Smac/Diablo will reverse caspase inhibition causing activation. Released AIF translocates from the mitochondria to the nucleus and causes chromatin condensation and large-scale DNA fragmentation (698).

Apoptosis through the mitochondrial pathway is partly regulated by the Bcl-2 family proteins.

1.4.5.3. Bcl-2 protein family in apoptosis

Expression of Bcl-2 family proteins has been described in regulating apoptosis in the cardiovascular system in both developing and adult cardiac myocytes (691,682,699-701) and hearts exposed to I/R (700). The activation of Bcl-2 proteins is complex and may be regulated by subcellular localization, proteolytic cleavage, transcription, and phosphorylation (702-706).

Bcl-2 proteins share up to four conserved regions known as Bcl-2 homology domains (BH1, BH2, BH3, and BH4). According to their BH domains, the proteins of the Bcl-2 family are divided into three classes: (i) anti-apoptotic proteins containing four (1-4) BH domains (Bcl-2, Bcl-XL, Bcl-W, and Mcl-1, A1); (ii) pro-apoptotic Bax-like proteins contain three (1-3) BH domains (Bax, Bak, and Bok); (iii) pro-apoptotic BH3-only proteins containing the BH3 domain only (Bim, Bad, Bid, p53 up-regulated modulator of apoptosis (PUMA), and Noxa (705,706). These proteins exert their effect by protein-protein interactions primarily at the level of mitochondria (706). For example, upon an apoptotic stimulus, BH3-only proteins are activated to repress the anti-apoptotic Bcl-2 family members, and to activate pro-apoptotic Bax-like proteins (708-711). These lead to oligomerization of Bax and Bak at the mitochondrial membrane, release of cytochrome c, and subsequent activation of the caspase apoptotic pathway.

1.4.5.4. Reactive oxygen species in apoptosis

Apoptosis occurs during events such as aging and I/R, which are associated with the production and release of ROS (682,684), for example, it has been reported that oxidative stress can induce apoptosis in cardiac myocytes (682,684) and that excessive ROS production can cause mitochondrial damage and dysfunction (712). Reperfusion after an ischaemic period is associated with a burst of free radical production within the first few minutes of reperfusion and apoptotic cell death. (485,486).

Hearts from transgenic mice overexpressing glutathione peroxidase (GSHPx) were more resistant to ischaemia/reperfusion injury (488) while hearts from glutathione peroxidase (GSHPx) null mice showed increased levels of apoptosis in I/R.

Similarly, overexpression of manganese-superoxide dismutase reduced myocardial ischaemia/reperfusion injury in transgenic mice (489), whereas hearts from Cu/Zn-superoxide dismutase knockout mice were more susceptible to ischaemia/ reperfusion injury compared with wild type mice (712a).

Antioxidants reduce oxidative stress by removing free radicals from the cell, and significantly decreased cell death and apoptosis in I/R, confering protection against ischaemia/reperfusion injury (713-715).

1.4.5.5. PKB in apoptosis

PKB activation protects against apoptosis through the Bcl-2 family proteins after a wide variety of stimuli, including the withdrawal of growth factors, UV irradiation, matrix detachment, cell cycle disturbance, DNA damage, and treatment of cells with anti-Fas antibody (reviewed in 716-719).

PKB phosphorylates, sequesters, and/or inactivates several pro-apoptotic proteins including Bad, Bax, and caspase-9 (9,690). Upon the phosphorylation at Ser-136 by PKB, Bad is inhibited and sequestered by the 14-3-3 family proteins (720). Bax is inactivated by phosphorylation at Ser-184 by PKB while PKB also promotes dissociation of Bad from the Bcl-XL protein and inhibits its translocation to mitochondria (12,526,719,721-728). The harmful effects of Bax are further demonstrated by the fact that deletion of the Bax gene decreases cardiac I/R injury (729). PKB has also been suggested to be involved in the protective effect of heat shock proteins (HSP) on the Bcl-2 family. Inhibition of Bax by HSP27 and Bad by HSP20 occurs in a PI3K/PKB-dependent manner in the heart (730-732). In addition to modulation of activity of pro-apoptotic proteins through phosphorylation, PKB also regulates the expression level of Bcl-2 family proteins. For example, in pre-conditioning, PKB activation prevents the decrease of Bcl-2 expression level induced by I/R (719).

1.4.5.6. JNK in apoptosis

JNK signalling in regulation of the apoptotic pathway is well established (15), and is associated with both the pro- and anti-apoptotic Bcl-2 family members. JNK mediates

its effects on apoptosis through (I) its essential role in modulating the functions of pro- and anti-apoptotic proteins located in mitochondria (585,733,734), and (II) its effects on transcription of genes leading to the upregulation of pro-apoptotic and/or downregulation of anti-apoptotic factors (583,585,736,737). However, the mechanism is controversial and appears to be stimulus and tissue specific (15).

Evidence for a role of JNK in the induction of apoptosis is the following:

(i) Normally, the pro-survival kinases such as PKB, PAK-1, and PKA inhibit the pro-apoptotic activity of BAD by phosphorylating it at serine-136, serine-112, or both. The Ser112/136-phosphorylated BAD is sequestered by the 14-3-3 family of proteins (15). JNK appears to ensure pro-apoptotic signalling by specifically phosphorylating Ser128 of BAD and Ser184 of 14-3-3ζ protein (16,738-740), inhibiting their interaction so that 14-3-3 releases the sequestered BAD to antagonize the anti-apoptotic Bcl2 proteins, thereby promoting apoptosis (16,720,740).

(ii) JNK induces pro-apoptotic proteins by cleaving Bid (caspase-8 independent). The resultant 21 kDa fragment of Bid (jBid) lead to apoptosis (741,742),

(iii) JNK is involved in the release of Smac/Diablo by Bid cleavage and disrupting the TRAF2-cIAP1 complex with caspase-8, thereby mediating apoptotic signalling (735,741);

(iv) JNK is involved in the activities of some of the other pro-apoptotic BH3–only subgroup of Bcl2 family of proteins, such as Bim and Bmf (743). The phosphorylation of Bim and Bmf by JNK releases them from the hold of the sequestering dynein and myosin V motor complexes (743), causing translocation to mitochondria and activation of Bax and/or Bak to initiate apoptosis (744,745). Alternatively, the phosphorylated Bim can bind and neutralize the anti-apoptotic activities of Bcl2 and Bcl XL, thereby promoting apoptosis (746-749);

(v) JNK phosphorylates and inhibits the anti-apoptotic proteins Bcl-2 and Bcl-xL (587,734,750-752), although there is evidence against the involvement of these proteins as substrates of JNK-induced apoptosis in vivo (750,753);

(vi) JNK is involved in the degradation of Flice (caspase-8) inhibitory protein (FLIPL)(a caspase-8 inhibitor), indirectly inducing apoptosis (754);

(vii) JNK is also reported to play a role in a histone 2 variant (H2AX), which has been thought to be essential for DNA fragmentation in apoptosis (755);

(viii) Most recently, it was demonstrated that JNK promotes apoptosis during IR by atrogin-1, an E3 ubiquitin ligase (23);

(ix) JNK phosphorylates the cellular homologue of avian myelocytomatosis virus oncogene (c-Myc) at two sites (Ser62 and Thr71). c-Myc plays a potential role in pro-apoptotic signalling;

(x) JNK directly induces the release of cytochrome c from the mitochondria and mediates apoptosis-inducing factor (AIF) translocation from the mitochondria to the nucleus (589,590);

(xi) The tumor suppressor p53, may be another potential target of pro-apoptotic JNK signalling. JNK was reported to destabilize p53 by promoting ubiquitin-mediated degradation (757,758). Conversely, activation of JNK due to stress has been shown to inhibit ubiquitin-dependent degradation of p53 thereby stabilizing it. Caspase 3 can amplify activation of JNK, as it is able to cleave and activate MEKK1, a kinase upstream of JNK (759).

As described above, the role of JNK in induction of apoptosis is well established. However, it has also been viewed as an anti-apoptotic kinase contributing to survival: (i) JNK has been shown as anti-apoptotic in response to NO in vitro (618) and in a model of hypoxia/reoxygenation in adult cardiac myocytes (613); It has also been suggested that part of JNK's cardioprotective effect, is due to activation of PKB resulting in an anti-apoptotic effect (13).

(ii) The interaction of JNK with apoptotic protease activating factor 1 (Apaf-1) can delay the activation of caspase-9 by the apoptosome (614), demonstrating an anti-apoptotic role. Similarly, in another in vitro I/R model, blocking JNK activity was associated with increased activity of both caspase-9 (613) and caspase-3 (619), resulting in apoptosis. This has been proposed to be mediated by the interaction of JNK with Apaf-1.

The opposing effects of JNK on apoptosis may depend on the duration or magnitude of the activation of the anti-apoptotic pathway, for example, prolonged activation of JNK has been shown to mediate apoptosis, whereas transient activation has been shown to promote cell survival (760,761). Differences in cell culture may also play a

role in the opposing effects of JNK, for example, an anti-apoptotic role of JNK was shown in neonatal cardiac myocytes and isolated cardiac myocytes (13,613), whereas the pro-apoptotic effect of JNK was demonstrated in isolated perfused rat hearts and adult cardiac myocytes (762-764).

In view of the above information, the role of JNK in apoptosis is still open to debate and remains to be investigated further.

1.4.6. Ischaemia-induced alterations in fatty acid and glucose pathways

1.4.6.1. FA concentrations in I/R

It is well-established that plasma FA levels increase dramatically during and following ischaemia due to the release of catecholamines. For example, increased activity of the sympathetic nervous system elicited by I/R injury (209,210,765,766), causes a significant increase in circulating FA resulting primarily from β-adrenoceptor-mediated stimulation of hormone-sensitive lipase activity in adipose tissue (209). The resulting elevated concentration of circulating plasma FA leads to an increased delivery to the myocardium and changes their metabolism during both the ischaemic and post-ischaemic periods (49,209,211,767). Chronically elevated levels of circulating FA in obesity and diabetes are also important determinants of the high rates of FA uptake and β-oxidation observed in these pathophysiological states.

It is important to recognize that the changes in circulating FA levels can have different impacts on the outcome of I/R when considering the effects of FA on the myocardium. It should be noted that in severe ischaemia, high levels of FA can aggravate lactate and H^+ production during and after ischaemia, but there is little evidence to support a detrimental effect of high concentrations of FA on hearts exposed to hypoxia or very mild ischaemia, because the potentially harmful metabolic by-products can be rapidly removed from the affected region(s) of the myocardium (768).

1.4.6.2. Fatty acid β-oxidation and glucose oxidation in I/R

In the normal heart, energy metabolism and cardiac function are exquisitely matched, however, in myocardial I/R, the increased entry of fatty acyl CoA moieties into the

mitochondrial matrix, causes continued contribution of FA β-oxidation to residual oxidative ATP generation (463,769-771) with no increase in the relative contribution of carbohydrate oxidation (772,773). Especially, during reperfusion, the rates of FA β-oxidation recover rapidly to pre-ischaemic values and can account for 90% of myocardial energy production, but this negatively influences cardiac efficiency and function at the expense of glucose oxidation and uncouples glucose metabolism. This leads to disproportionate high levels of glycolysis compared to the subsequent pyruvate oxidation, aggravating intracellular acidosis and altering ionic homeostasis (27,774-776). Thus, disturbances in the balance between the oxidation of FA and glucose result in a decreased control of FA oxidation. This is further exacerbated during reperfusion, when rates of FA β-oxidation are accelerated, further inhibiting glucose oxidation (777-781).

It should be noted that with global ischaemia, there is an accumulation of reducing equivalents in the form of NADH and flavin adenine dinucleotide hydrogen 2 (FADH2) causing the inhibition of FA β-oxidation (768) since both FA β-oxidation enzymes, namely acyl CoA dehydrogenase and 3-hydroxyacyl CoA dehydrogenase, are sensitive to the redox state of the matrix (NAD/NADH and FAD/FADH2 ratios)(33). The inhibition of FA β-oxidation can result in the accumulation of FA intermediates in distinct cellular compartments.

Thus, in the setting of ischaemic heart disease, the general premise for the optimization of cardiac energy metabolism is to either stimulate the more efficient oxidation of glucose or reduce FA β-oxidation (212).

1.4.6.3. Subcellular control of fatty acid and glucose oxidation in I/R

AMP activated protein kinase (AMPK) is considered to play an important role in regulating both FA and glucose metabolism in stress conditions (468,777,781-786).

AMPK is rapidly activated during ischaemia, and its activation persists into reperfusion (468,777,781-786,791). Activation of AMPK in the ischaemic heart can accelerate mitochondrial FA uptake and β-oxidation by removing the inhibitory effect of malonyl-CoA on CPT1. This is achieved by decreased cardiac malonyl CoA levels through the AMPK-induced inhibition of ACC by phosphorylation (780,790-792). AMPK can also stimulate cardiac glucose uptake by regulating GLUT4 translocation

(793) and indirectly activate phosphofructokinase-1 to stimulate glycolysis (468,794). In this regard, AMPK activation would be beneficial in I/R injury by increasing glucose uptake and metabolism. However, the inhibition of AMPK by insulin administration in I/R can reduce FA β-oxidation and increase glucose oxidation to alleviate myocardial acidosis, which would benefit the aerobically reperfused heart after ischaemia, but high concentrations of FA in the perfusate can interfere with insulin's ability to inhibit AMPK. Thus the role of AMPK in I/R is still controversial. Studies in isolated working hearts from transgenic mice by expressing a dominant negative (DN) 2-subunit of AMPK (AMPK-2 DN) showed nearly a complete loss of myocardial AMPK activity. These hearts were unable to increase GLUT4 translocation and glucose uptake (795,796), and had significant contractile dysfunction during I/R (793). However, recently studies (469,797) using hearts from AMPK-2 KO (KO) mice have shown that, although AMPK-2 deficiency accelerated the appearance of contracture during ischaemia, there was no functional depression during reperfusion after ischaemia, suggesting that inhibition of AMPK is not detrimental to the heart (797). It may be highly dependent on substrate availability and on the balance of the effects of AMPK on glucose and FA metabolism.

The above data indicate the complex regulation of AMPK in I/R injury (790). However, there is insufficient evidence to substantiate a role for AMPK in the protection of the ischaemic myocardium (777)

1.4.7. Obesity paradox in I/R

It is important to emphasize that obesity has been implicated as one of the major risk factors for type 2 diabetes (T2D), coronary heart disease (CHD) and hypertension (HTN) (154). From the standpoint of prevention, reducing levels of obesity should decrease the overall burden of cardiovascular disease in terms of prevalence and outcomes. Although, obesity is well known as a major risk for cardiovascular disease (CVD), several studies from clinical cohorts of patients with established CVD indicated an "obesity paradox" where such patients tended to have a more favourable short- and long-term prognosis (24,25). An explanation for these conflicting findings regarding the impact of obesity on I/R injury has not yet been provided. The body mass index (BMI), as defined by the World Health Organization, is commonly used to predict the development of cardiovascular disease and the majority of reports

describing the effects of obesity on mortality were based on BMI. It is possible that the distribution of fat may more accurately predict outcome, and consensus regarding the most suitable measure of obesity for epidemiological studies has not yet been obtained (798). One recent study has suggested that overweight and obese individuals were in fact protected from short-term death yet have a long-term mortality risk that is similar to that of normal-weight individuals (799). Moreover, given that most of the studies that suggest an “obesity paradox” have been retrospective and cross-sectional, a direct mechanistic link between obesity and improved myocardial outcomes following acute cardiovascular events, remains to be elucidated. The conclusion that obesity may both elicit cardiac disease and protect from cardiovascular death clearly requires further mechanistic analyses at cellular, molecular, and systematic levels.

1.4.8. Aims of the study

As stated in the literature review, obesity-related insulin resistance is an important contributor to metabolic disturbances. Understanding of the association between obese insulin resistance and ischaemic heart disease is complicated by the multifaceted interplay between various hemodynamic, metabolic, and other physiological factors that ultimately impact on the myocardium. The transition from normal to insulin resistance leads to changes in the myocardium that may affect its sensitivity to ischaemia and reperfusion.

A growing body of evidence indicates involvement of the MAPKs in metabolic adaptation, and many studies have causally linked these kinases to the development of insulin resistance. The MAPK and PI3-K/PKB signalling systems have been suggested to play a pivotal role, not only in insulin signalling, but also in the outcome of myocardial ischaemia/reperfusion. However, little is known about the role of insulin resistance in ischaemia/reperfusion.

The Reperfusion Injury Salvage Kinase (RISK) pathway, relayed by PKB and ERK, confers powerful cardioprotection when specifically activated at the time of myocardial reperfusion (9). However, despite the abundance of preclinical data demonstrating effective cardioprotection with a variety of different agents given at the time of myocardial reperfusion to activate the RISK pathway, clinical studies in this regard are limited.

The JNK pathway appears to be a regulator that triggers the oxidative-inflammation cascade that can become chronic and cause abnormal glucose metabolism. This can lead to insulin resistance and dysfunction of the vasculature. Numerous in vitro and in vivo studies have shown that JNK is activated during reperfusion after ischaemia (575-579). Some studies support the deleterious role of JNK in ischaemic injury in different cells including heart, brain, kidney, liver, gastric mucosa, and lung (reviewed in refs. 591-596). In contrast, a number of other studies demonstrated a critical role for JNK in myocyte survival and cardioprotection (13,382,607-611, 617,618).

As discussed in detail in the preceding literature review, the role of the JNK pathway in I/R injury remains controversial, reflecting the complexity of the multistage, multitargeted signalling networks involved in this process. On the basis of the observed interaction between JNK and PKB/Akt during early reperfusion, we hypothesize that JNKs may play an important role in the impairment of PI3K/PKB(Akt) signaling in the insulin-resistant state, and thus contribute to the reduced postischaemic survival of such hearts (Fig 11).

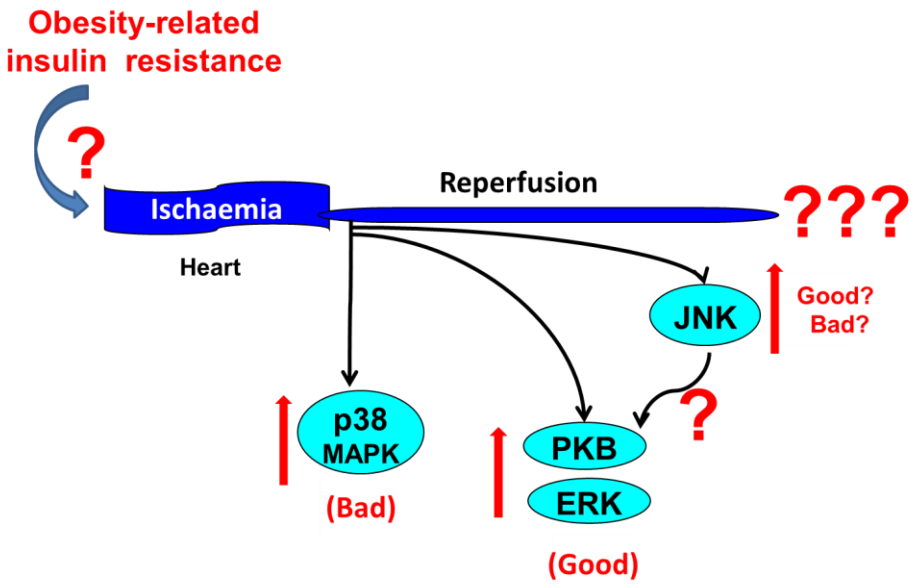


Fig 11. Aims of the study. The effect of hyperphagia-induced obesity and insulin resistance in rats leads to changes in the myocardium that may affect its sensitivity to ischaemia and reperfusion. The Reperfusion Injury Salvage Kinase (RISK) pathway, relayed by PKB and ERK, confers powerful cardioprotection when specifically activated at the time of myocardial reperfusion. The role of JNK in ischaemic injury remains controversial in this process. JNKs may play an important role in I/R of such hearts from insulin-resistant rats.

It was decided to study the effect of hyperphagia-induced obesity and insulin resistance in rats on the response of the heart to ischaemia/reperfusion injury in the presence of different substrates, with particular attention to the intracellular signalling pathways during early reperfusion. Since (i) increased fatty acid oxidation rates at the expense of glucose oxidation during reperfusion has been proposed to impair functional recovery (26-28) and (ii) the serum free fatty acid concentrations of the hyperphagia-induced obese rats were increased at least twofold (29,30), the hearts were perfused ex vivo with glucose alone, as well as with a combination of glucose plus a high concentration of fatty acid (palmitic acid). Palmitic acid was chosen because gas-chromatographic analysis of serum showed this to be the FA most elevated in the obese vs control rats.

The broad objective of this study is therefore to evaluate the role of obesity induced insulin resistance in ischaemia/reperfusion injury and to establish a framework for further defining the role of insulin resistance in cardiovascular disease.

The specific aims are the following:
(1). Assessment of the effects of obesity on baseline parameters:
In vitro: Baseline myocardial mechanical function; expression and activation patterns of kinases (PKBs473, ERKp44/p42, JNKp54/p46 and p38 MAPK) and PTEN when perfused under control, normoxic conditions in the presence of different substrate combinations.
(2). Assessment of the effects of obesity on the response of the heart to ischaemia/reperfusion injury in the presence of different substrate combinations:
The following parameters will be evaluated: post-ischaemic functional recovery, infarct size, expression and activation patterns of PKBs473, ERKp44/p42, JNKp54/p46 and p38 MAPK and PTEN.

(3). Investigation into the significance of JNK activation during ischaemia/reperfusion of hearts from obese insulin resistant and control animals. Numerous in vitro and in vivo studies have shown that JNK is activated during reperfusion after ischaemia (575-579) while ischaemia alone did not result in activation (579,580-583). The role of the JNK pathway in I/R injury remains controversial.

This will be done by using the specific JNK inhibitor, SP600125, administered either

before ischaemia or during reperfusion after ischaemia. The parameters evaluated will be the same as described above.

Exception for investigation of the insulin effects on I/R in this study

Firstly, for experimental protocols, the hearts from control and DIO rats were perfused with three different substrates: (i) glucose (ii) glucose plus high fatty acid (iii) glucose plus low fatty acid. If only one dose of insulin will be used in this study, it would be somewhat supraphysiologic since the hearts from both control and DIO rats were perfused in three different substrates in this study.

Secondly, under physiological conditions, myocardial ATP production is derived from the mitochondrial oxidation of different substrates, with FA (60–70%) being predominant over glucose (20-30%) and lactate (10%) (269). The myocardium rapidly adjusts to fluctuations in circulating substrate concentrations, giving the heart the metabolic flexibility needed for feeding, fasting, and intense exercise. The heart switches its substrate preference toward glucose during stress conditions such as ischemia and FA during reperfusion. Insulin may play a different role in ischaemia and reperfusion in the hearts from control and DIO insulin resistant rats.

Thirdly, in all groups, hearts were stabilized for 40 min (15 min retrograde perfusion, 15 min working heart mode, 10 min retrograde perfusion), then subjected to 15 min sustained global ischaemia followed by 5, 10 or 30 min reperfusion. The protocol will be 85 min per heart. For dermination of infarct size, hearts were stabilized for 40 min, the regional ischaemia was 35 min, and the reperfusion was 120 min. The protocol will be 195 min per heart. For Western blots, each kinase and phosphatase (ERK, p38 MAPK, JNK, PKB and PTEN) included phosphorylation and total protein at 5 min, 10 min and 30 min reperfusion interval. Except above experimental protocols, for JNK inhibitor (SP600125), the protocols included pretreatment and post-treatment groups in differen substrates. **These experimental protocols in this study were extremely extensive**.

In consideration of above mentioned conditions, we decided that the insulin effects on I/R injury were not included in this study.

Chapter II

Materials and methods

2.1. Animals:

Male Wistar rats (200±5g body weight at onset of experimentation) were fed for 16 weeks with a high calorie diet containing 65% carbohydrate, 19% protein, and 16% fat (800a) of which each rat consumed ~30 g per day (570±23 kJ/day), which is designed to induce hyperphagia (800b). In contrast, the age matched control group received a diet consisting of 60% carbohydrate, 30% protein, and 10% fat of which each rat consumed ~20 g per day (371±18 kJ/day). Thus the DIO rats consumed a diet containing more carbohydrate and fat than the controls. However, because of the larger consumption of food by the DIO animals, the actual protein consumption was similar in these two groups. Animals were housed in a temperature- and humidity-controlled environment under a 12-h light/12-h dark cycle.

The rats were allowed free access to food and water until the time of experimentation. The project was approved by the Ethics committee of the University of Stellenbosch (Faculty of Health Sciences) and the investigation conforms to the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

2.2. Chemicals:

The primary antibodies for PTEN, PKB, ERK, JNK and p38 MAPK as well as phospho-PTEN (Ser380/Thr382/383), phospho-PKB (Ser473), phospho-ERKp42/p44 (Thr202/Tyr204), phospho-JNKp54/p46 (Thr183/Tyr185) and phospho-p38 MAPK (Thr180/Tyr182) were purchased from Cell Signalling Technology (Beverly, MA, USA). Horseradish peroxidase-labelled secondary antibody, ECL and the ECL detection reagents were obtained from Amersham Pharmacia Biotech. Routine chemicals were of Analar grade and obtained from Merck, RSA. Palmitic acid and sodium carbonate were purchased from Sigma–Aldrich Chemical GmbH (Germany). Bovine serum albumin (BSA) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Spectra/Por® dialysis membrane tube (MW cut-off 6000–8000) was purchased from Spectrum Laboratories, Inc (USA).

2.3. Perfusion systerm

2.3.1. Basic perfusion buffers

The basic buffer used for perfusion of the working heart in this study was the Krebs–Henseleit bicarbonate buffer (KHB) containing (in mM): NaCl 119; $NaHCO_3$ 25; KCl 4.75; KH_2PO_4 1.2; $MgSO_4$ 0.6; Na_2SO_4 0.6; $CaCl_2$ 1.25; Glucose 10, pH 7.4.

Buffers containing fatty acid: fatty acids in the BSA contributed 0.3 mM to the fatty acid concentration, and the rest of the fatty acids were added in the form of palmitate.

2.3.2. Fatty acid/BSA containing perfusion buffer

Because the lipophilic nature of FA and their transport in blood with albumin or lipoproteins as described in the introduction, FA are also not easily dissolved in the perfusion buffer in vitro, so FA bound to albumin were used in the perfusion buffer in the isolated working heart model of this study (778). For each heart, 200 ml FA/BSA containing KHB solution was perfused and recirculated.

The FA/BSA containing KHB solution was prepared on the day prior to experimentation as the FA needed to be prebound to the albumin.

For each heart, 6 g of BSA (the final volume of 200 ml buffer of 3% BSA) was weighed out and added to 80 ml of KHB solution (without glucose) in a beaker (200 ml) stirred at a low heat (Note: the solution at this point in time was greater than 3% BSA). It is important to avoid excessive heating as this may cause the BSA solution to gel, rendering it useless. While the BSA was dissolving in solution, approximately 20cm of dialysis tubing (MW cut-off 6000–8000) was rolled up and placed in a small beaker (100 ml) of distilled water to soften up. For the concentration of 1.2 mM palmitate in the buffer, in theory, a 1:1 molar ratio of sodium carbonate to palmitate can be used to provide a source of Na^+ to form the Na^+/palmitate complex which is water soluble, but it is better to use a slight excess of sodium carbonate, so in this study, 1.5 mM Na_2CO_3 was used. The weighed palmitate and Na_2CO_3 were mixed with 2 ml of 95% ethanol and 5 ml of double distilled water in a small beaker, and then boiled continuously. Once the ethanol was boiled off, palmitate/ Na_2CO_3 solution was quickly poured into the warm dissolved 80 ml BSA containing KHB solution. After the mixture, the FA/BSA containing buffer was poured into the dialysis tubing, and then dialyzed overnight for 16h to allow the calcium binding sites on the albumin to become occupied and dialyze out any ethanol that may remain in the solution. At the experimental day, 0.36 g of glucose (final concentration of 10 mM glucose in 200 ml buffer) was added to the dialyzed FA/BSA containing KHB buffer and made up to final 200ml volume (palmitate 1.2 mM/3%BSA, 10 mM glucose) for each heart.

2.3.3. 3%BSA containing perfusion buffer

The only 3%BSA containing perfusion buffer was prepared same as FA/BSA containing KHB solution as described above, but without adding palmitate and Na_2CO_3 in the KHB solution (3%BSA, 10 mM glucose). For each heart, 200 ml 3%BSA containing KHB solution was perfused and also was recirculated.

2.3.4. Heart perfusion technique

At the end of the 16 week feeding programme, the rat was weighed and anaesthetized by intraperitoneal injection of 100 mg/kg sodium pentobarbital until deep anaesthesia, as evidenced by the lack of reaction to a foot pinch. The thoracic cavity was opened through a bilateral thoracotomy and the heart excised by cutting through the descending aorta, therefore with the brachiocephalic-, common carotid- and left subclavian artery intersections visible. The heart was immediately immersed in ice-cold KHB solution. The branching of these 3 arteries was cut open to render an opening slightly broader than the ascending aorta. Within 1 min of removal, the heart was mounted via the aorta onto the aortic cannula of a Morgan working heart perfusion apparatus, hereby allowing Langendorff perfusion. The KHB was conituously gassed with 95% O_2/5% CO_2, (37^{o}C, pH 7.4), delivered through an inverted fritted glass filter. After trimming away excess tissue and fat, the left atrium was also cannulated via the pulmonary vein to allow perfusion in the working heart mode (preload 15 cm H_2O, afterload 100 cm H_2O). After stabilization, the perfusion mode was switched from retrograde to working heart (Fig 12).

During heart perfusion, a temperature probe inserted into the right ventricular cavity through a small incision was used for monitoring of perfusion buffer temperature, which was maintained at 37°C during experimentation.

When perfusing with FA/BSA or glucose/BSA as substrate, hearts were initially perfused for 2-3 min with glucose-containing KHB buffer to wash out all blood, before switching to perfusion in a recirculating manner with 200 ml of the BSA-containing buffer. Fatty acids in the BSA contributed 0.3 mM to the fatty acid concentration in the buffer, and the rest of the fatty acids were added in the form of palmitate. The fatty acid concentrations employed in the present study were based on those previously described in a study to investigate the effects of high and low fatty acid concentrations on fatty acid oxidation in normal hearts (29).

A temperature probe was used for constant monitoring of myocardial temperature which was maintained at 36.5°C during sustained global or regional ischaemia.

Normothermic, zero-flow global ischaemia was induced by simultaneous clamping of both the aortic and left atrial cannulae while regional ischaemia was achieved by ligation of the left anterior descending coronary artery (LAD). Reperfusion was initiated by unclamping of the tube to the aortic cannula or by removal of the LAD ligature.

Intra-aortic pressure and heart rate were monitored via a pressure transducer (Viggo Spectromed) inserted into the aortic cannula, while the coronary and aortic flow rates were measured manually. Mechanical activity was monitored before and after sustained global ischaemia. Work performance was calculated according to the formula described by Kannengieser et al. (533): 0.002222 × (aortic pressure − 11.25) × cardiac output.

At the time of sacrifice, intraperitoneal fat was dissected out and weighed.

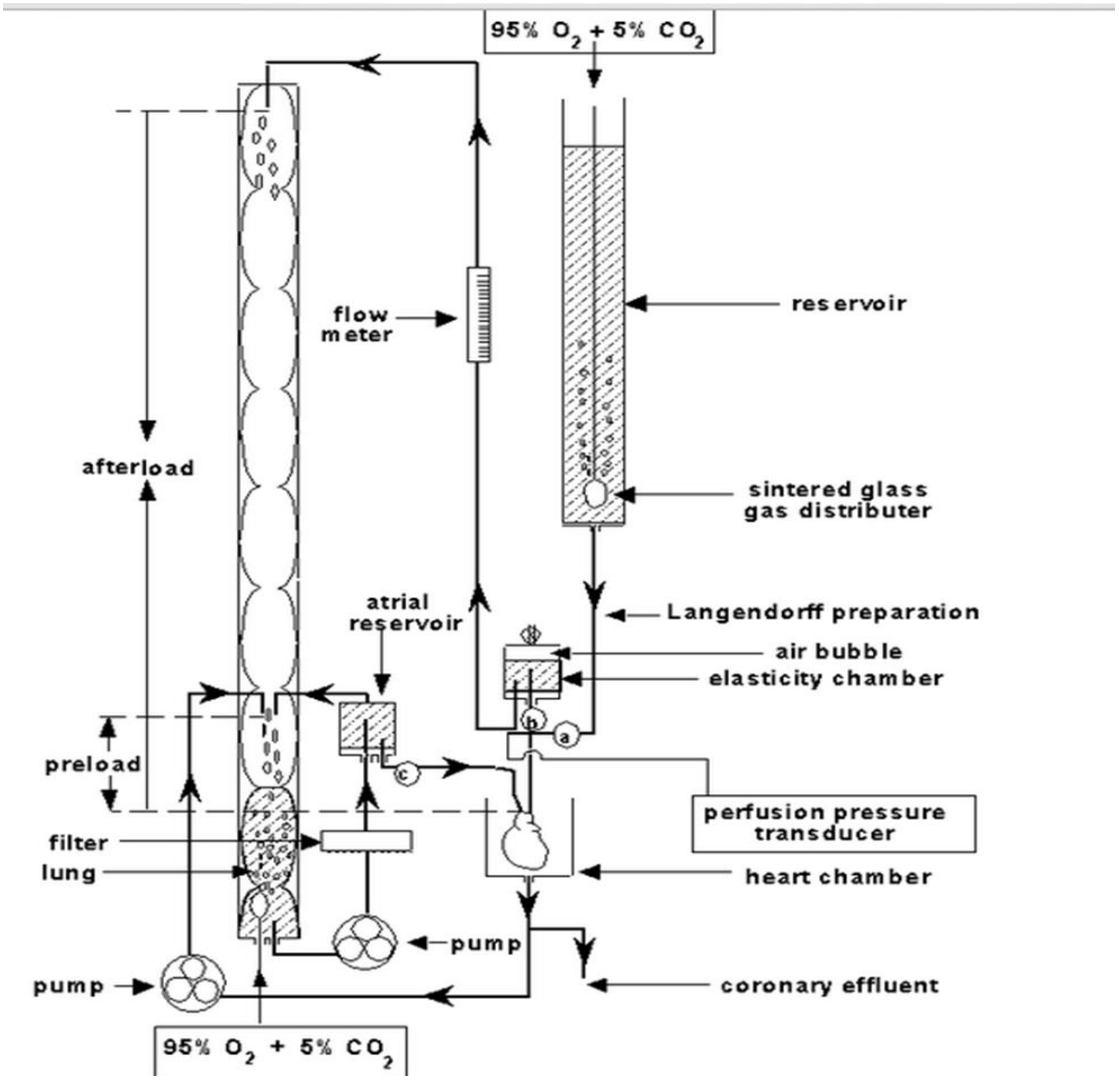


Fig 12: Heart perfusion apparatus in working heart model.

2.4. Determination of infarct size

After the isolated heart was stabilized for 40 min in the working heart model, a suture was passed around the main branch of the left anterior descending coronary artery, and the ends were pulled through a small vinyl tube to form a snare. The coronary artery was occluded by tightening the snare and regional ischaemia confirmed by a reduction in coronary flow. Reperfusion was started by opening the tightened snare. In this study, the regional ischaemia was 35 min, and the reperfusion was 120 min.

Myocardial infarct size was determined as previously described (804). Briefly, at the end of the reperfusion period, the silk suture around the coronary artery was securely tied and ~1 ml of a 0.5% Evans Blue suspension was slowly injected via the aorta cannula to delineate the area at risk. Hearts were frozen overnight, and then cut into 2-mm-thick slices. After defrosting, the slices were stained with 1% wt/vol triphenyltetrazolium chloride in phosphate buffer containing (in mM): NaH_2PO_4-$2H_2O$ 20, Na_2HPO_4 80, pH 7.4 at 37°C for 15 min. After staining, the viable tissue in the ischaemic area appeared red (tetrazolium positive) distinguishing it from the infarct tissue that was pale and white (tetrazolium negative). The heart slices were then fixed in 10% v/v formaldehyde solution. The left ventricle area at risk (R) and the area of infarcted tissue (I) were determined using computerized planimetry (UTHSCSA Image Tool program, developed at the University of Texas Health Science Center at San Antonio, Texas). UTHSCSA ImageTool has functions for displaying, editing, analyzing, processing, saving and printing images in grayscale or color. The size of the triphenyltetrazolium chloride-defined infarct in each cardiac slice was measured by planimetry from outlines of left ventricular slices obtained by manually tracing the two-dimensionally projected computer-displayed image. Triphenyltetrazolium chloride infarct size for each slice was expressed as a percent of infarct area to total left ventricular area on the two-dimensional display (planimetric infarct size). The infarct size was expressed as a percentage of the area at risk (I/R %).

2.5. Western blots

(See addendum for buffer- and PAGE compositions)

At different times during reperfusion after global ischaemia, hearts were freeze-clamped with pre-cooled Wollenberger tongs, and plunged into liquid nitrogen. Ventricular tissue (~60 mg) from the freeze-clamped heart was pulverized with a pre-cooled mortar and pestle and homogenized in 800 μL lysis buffer with a Polytron

PT10 homogenizer, 2x4 seconds at setting 4. The lysis buffer contained (in mM): Tris–HCl 20 (pH 7.5); EGTA 1; EDTA 1; sodium orthovanadate 1; sodium pyrophosphate 2.5; NaCl 150; β-glycerophosphate 1; 1% Triton X-100; phenylmethyl sulphonyl fluoride (PMSF) 0.3; aprotinin 10 µg/ml and leupeptin 10 µg/ml. Samples were centrifuged at 1,000 g for 10 min. The protein content in the supernatant was determined using the Bradford technique (801). The tissue lysates were diluted in Laemmli sample buffer, boiled for 5 min and microfuged for 5min to thoroughly mix the samples. A volume of sample containing the following amounts of protein: 20 µg of protein for ERK and p38 MAPK; 40 µg for JNK, PTEN and PKB, was loaded separately onto polyacrylamide gels (12% for ERK and p38 MAPK; 10% for JNK, PTEN and PKB) using the Bio-RAD Mini-PROTEAN III System. The separated proteins were transferred to a PVDF membrane (Immobilon®P, Millipore) and fixed by washing in methanol and air-drying the membrane. Afterwards, the dry membrane was stained with Ponceau Red reversible stain for visualization of proteins. The Ponceau Red was removed by washing with distilled water. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline–0.1% Tween 20 (TBST) for 1-2 hours at room temperature with gentle shaking. This was followed by copious washing with TBST. The amounts of protein as well as activated enzyme were visualized with the appropriate primary antibody. The membranes were probed overnight at 4^{o}C with polyclonal primary antibodies (1:1,000 dilution in TBST). Membranes were subsequently washed with large volumes of TBST (2 × 1 min and then 3 × 5 min) and the immobilized primary antibody conjugated with TBST-diluted horseradish peroxidase-conjugated antirabbit antibodies (1:4,000 dilution) for 1 hour at room temperature. After thorough washing with TBST, membranes were covered with ECL^{TM} detection reagents for 1min and exposed to an autoradiography film (Hyperfilm ECL) using suitable casettes and working in a dark room, to detect light emission via a non-radioactive method. Films were densitometrically analyzed by laser scanning and suitable software (UN-SCAN-IT, Silkscience). For the blots, the same samples were loaded to two gels on the same day on the same system: one gel was probed with Ab against the phosphorylated protein and the other one with Ab against the total protein. These 2 blots were exposed below each other on 1 film to minimize variation. In some blots, antibody binding was stripped using 0.2N NaOH for subsequent probing with the corresponding antibody against the phosphorylated protein or beta-tubulin, the latter to substantiate equal protein loading. All results were expressed as the ratio between phospho/total arbitrary densitometry units (AU).

NB: all values from control animals, baseline conditions, were normalized to one or as indicated in the text.

2.6. Experimental protocols

The hearts from control and DIO rats were perfused with the following substrates: (i) glucose (10mM) alone (ii) glucose (10mM) plus a high concentration of fatty acid (1.2mM palmitate) prebound to bovine serum albumin (3%BSA) (iii) glucose (10mM) plus bovine serum albumin (3%BSA). The BSA contributed 0.3mM to the total fatty acid concentration of the buffer. These solutions will be referred to as (i) glucose, (ii) high fatty acid (1.5mM) and (iii) low fatty acid (0.3mM).

In all groups, hearts were stabilized for 40 min (15 min retrograde perfusion, 15 min working heart mode, 10 min retrograde perfusion). For Western blotting, hearts were subsequently subjected to 15 min sustained global ischaemia followed by 5, 10 or 30 min reperfusion, or subjected to 35 min regional ischaemia followed by 120 min reperfusion for determination of infarct size. For baseline, the hearts were only perfused for 30 min (15 min retrograde perfusion, 15 min working heart mode). Measurements of mechanical activity were made at 30 min (15 min retrograde perfusion, 15 min working heart mode) before subjected to ischaemia and at 30 min during reperfusion after ischaemia.

2.7. Experimental protocols for JNK inhibitor (SP600125)

Pretreatment was induced by administering the JNK inhibitor, SP600125 (10uM) for 10 min without wash out before 15 min global ischaemia.

Post-treatment was induced by administering the JNK inhibitor, SP600125 (10uM) for the first 10 min of reperfusion after 15 min global ischaemia.

2.8. Statistical analysis

All analyses were performed using GraphPad prism version 5. All values were expressed as mean ± standard error (S.E). Multiple comparisons were made by one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. When two groups were compared, Student's t-test was used. Statistical significance was set at $p < 0.05$. A minimum of 3-8 individual hearts were analysed for every time-point investigated.

Chapter III

Results: effects of obesity

3.1. Effects of obesity on baseline parameters

3.1.1. In vivo

After 16 weeks feeding with a high caloric diet, rats (DIO) gained significantly more weight than their control counterparts fed normal rat chow (C). The body weights (BW) of the DIO rats in the present study were 19.6% higher with 79.8% greater visceral fat mass (VF) compared to their age matched controls (D/C: BW 511±13/427±49; VF 30.2±1.7/16.8±0.9, $p < 0.05$, Fig 13).

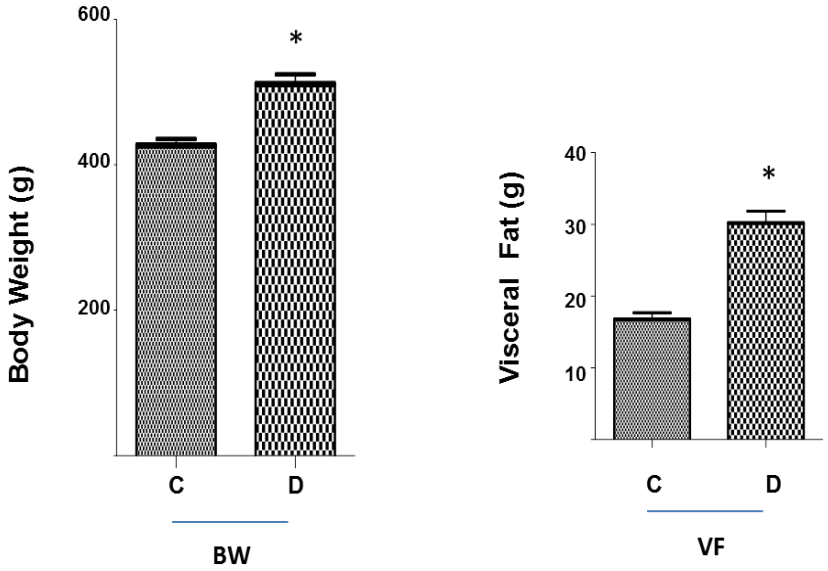


Fig 13. Effect of diet on body weight (g) and visceral fat (g): comparison between control (C) and DIO rats (D). Data are expressed as means ± SE. * $p < 0.05$ vs C. n = 14 per group.

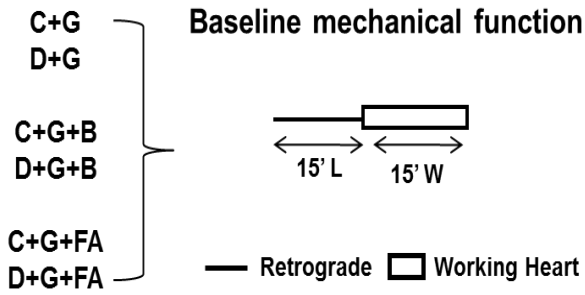
We have previously shown that the plasma triglyceride and nonesterified free fatty acid concentrations as well as the homeostasis model assessment (HOMA) index were significantly higher in the DIO rats, but that fasting glucose levels were within the normal range (29,30). In our laboratory, there were a few different studies used the same hyperphagia-induced obesity rat model. For this study, in the same time, while another study in our laboratory had showed that the rats were obese insulin resistance, we did not repeat the plasma triglyceride and nonesterified free fatty acid concentrations as well as the homeostasis model assessment (HOMA) index because of the large numbers of rats used and the expense involved. These animals

in this study showed the significant increase in body weights (BW) and greater visceral fat mass (VF) of the DIO rats compared to their age matched controls.

3.1.2. In vitro

At the end of the 16 week feeding program, the rats were anaesthetised by intraperitoneal injection of sodium pentobarbital (100 mg/kg). The hearts were rapidly excised and mounted on a perfusion rig within 1 min of excision and perfused in the working heart mode as described in Materials and Methods. The substrates in the perfusate were as decribed above: glucose alone (D+G, C+G), low fatty acid (D+G+B, C+G+B) or high fatty acid (D+G+FA, C+G+FA). To obtain baseline values, the hearts were perfused for 30 min (15 min retrograde, 15 min working heart) for measurement of mechanical function, then the hearts were freeze-clamped at the 30 min perfusion time point for analyses of the expression and activation of kinases of interest and the phosphatase PTEN (see protocol I).

Protocol I



C: control
D: diet induced obesity (DIO)

C+G: the hearts from control rats perfused with glucose (G)(10mM) alone
D+G: the hearts from DIO rats perfused with glucose (10mM) alone

C+G+B: the hearts from control rats perfused with glucose (10mM) plus BSA (B)(3%)
D+G+B: the hearts from DIO rats perfused with glucose (10mM) plus BSA (3%)

C+G+FA: the hearts from control rats perfused with glucose (10mM) plus fatty acid (FA) (1.2mM palmitate/3%BSA)
D+G+FA: the hearts from DIO rats perfused with glucose (10mM) plus fatty acid (FA) (1.2mM palmitate/3%BSA)

3.1.2.1. Baseline mechanical function before sustained global ischaemia

As described in Table 1, all parameters of baseline mechanical function (coronary flow (CF), aortic output (AO), cardiac output (CO), heart rate (HR), peak systolic pressure (PSP) and total work (TW)) at 30 min perfusion were similar in hearts from DIO and control rats regardless of the substrate present (Table 1).

Table 1

Baseline mechanical function

		CF	AO	CO	PSP	HR	TW
15'L 15'W	C+G	18.5±1.2	46.4±1.7	64.9±2.5	98±2	283±11	14.17±0.64
	D+G	19.1±1.1	43.2±1.3	62.3±2.1	99±1	285±9	13.67±0.47
	C+G+B	18.8±0.8	48.0±1.4	66.8±1.9	101±2	282±8	13.38±0.21
	D+G+B	20.5±1.3	47.3±1.8	67.8±2.5	106±3	264±14	14.23±0.08
	C+G+FA	21.0±1.0	45.8±1.5	66.8±2.3	103±2	254±14	15.31±0.56
	D+G+FA	21.0±1.0	43.3±1.5	64.3±2.3	105±2	248±17	15.40±0.32

Data are expressed as means ± SE. n= 5-8 per group.

CF: coronary flow (ml/min) AO: aortic output (ml/min) CO: cardiac output (ml/min)
PSP: peak systolic pressure (mmHg) HR: heart rate (beats/min) TW: total work (mW)

3.1.2.2. Baseline kinase and PTEN expression and activation patterns before sustained global ischaemia

All values obtained were normalized to those of control hearts.

When the hearts were perfused with glucose alone or glucose plus FA as substrates for 30 min as described in Materials and Methods, results showed that the phosphorylation and expression of the kinases (PKB, ERKp44/p42, JNKp54/p46 and p38 MAPK) and PTEN were similar in the hearts from both DIO and control rats for each substrate (Fig 14, 15). In view of the above, the effect of low fatty acid as substrate on the phosphorylation and expression of the different proteins at baseline conditions was not evaluated in the two groups.

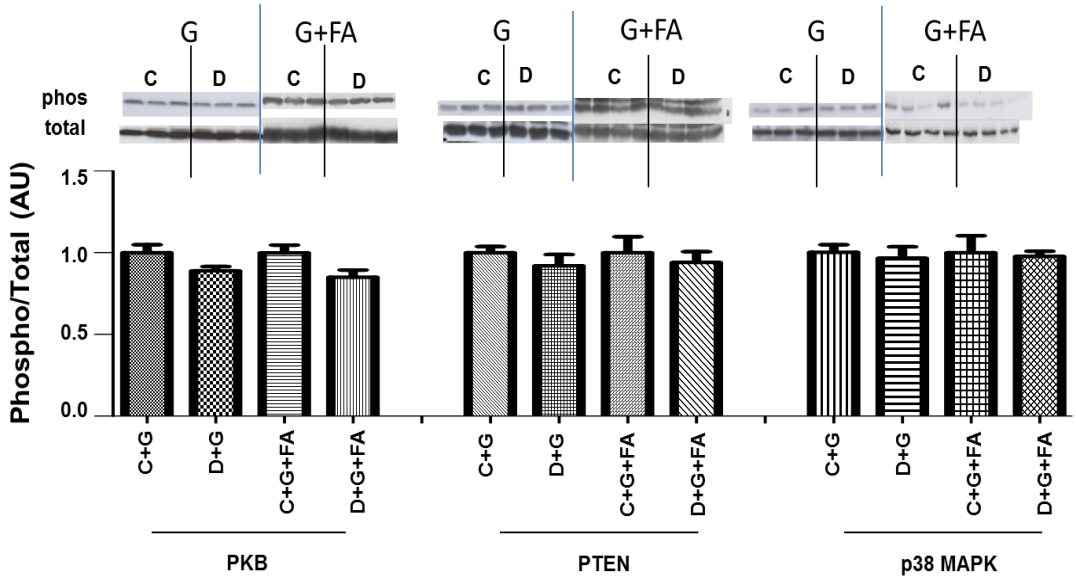


Fig 14. Baseline of PKB, PTEN and p38 MAPK expression and activation patterns in hearts from control and DIO rats. All values were expressed as the ratio between phospho/total arbitrary densitometry units (AU) and were normalized to control hearts as one. Data are means ± SE, n = 4-6 per group. Abbreviations as in protocol I.

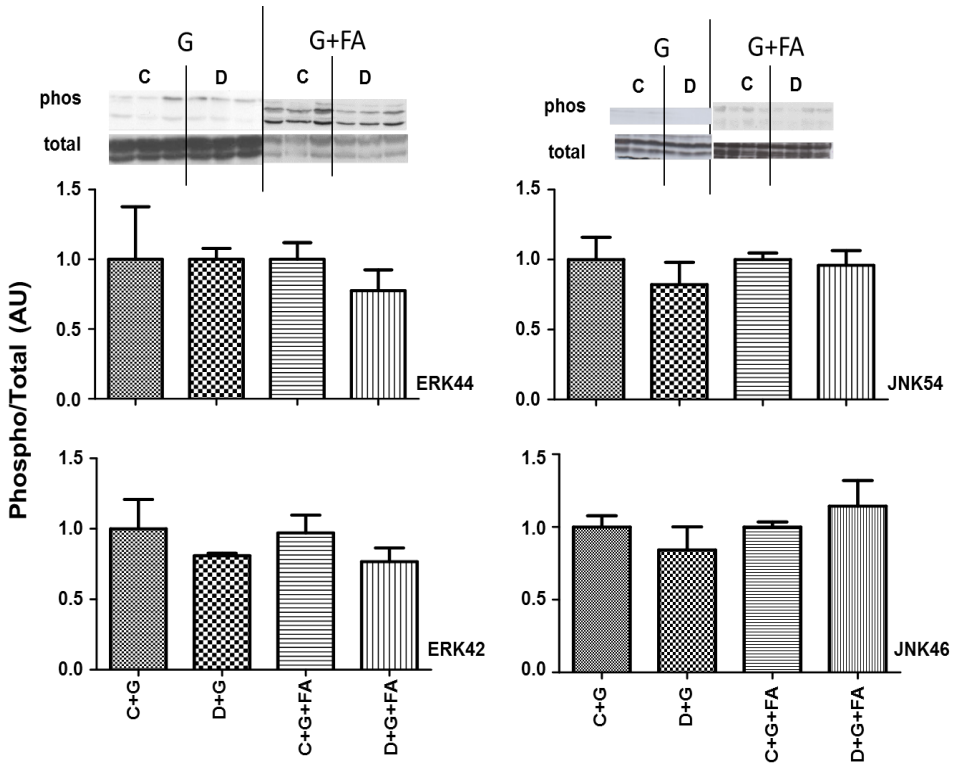


Fig 15. Baseline of ERK and JNK expression and activation patterns in hearts from control and DIO rats. AU: arbitrary units. Data are means ± SE, n = 4-6 per group. Abbreviations as in protocol I.

In summary, regardless of the different substrates in the perfusate, the baseline kinase and PTEN patterns did not differ in the DIO compared to the control groups.

3.2. Myocardial response during reperfusion: Effects of obesity

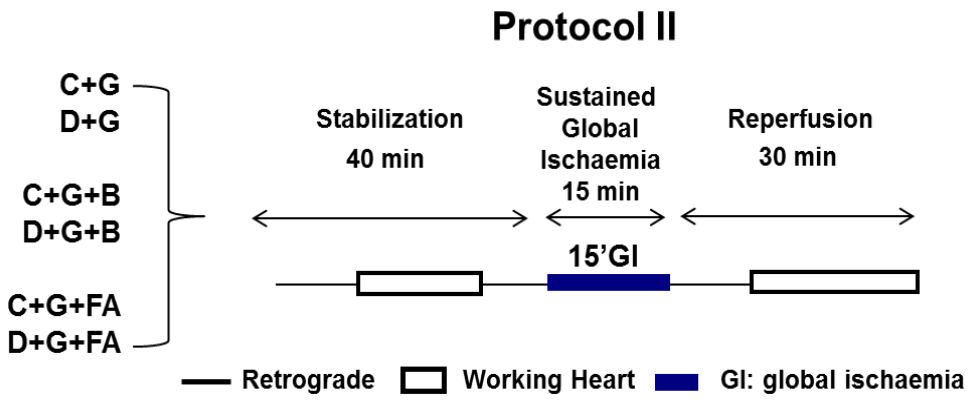
Since exposure of the hearts to 20 min global ischaemia resulted in poor recovery during reperfusion in the working heart model (804), it was decided to use 15 min global ischaemia in the present study.

3.2.1. Effects of obesity on postischaemic functional recovery.

Comparison: postischaemic vs preischaemic function

Substrate: Glucose

When perfused with glucose alone as substrate, exposure of the hearts from both DIO and control rats to 15 min sustained global ischaemia followed by 30 min reperfusion (see protocol II), caused a significant reduction in AO, CO, PSP and TW compared to the values obtained before ischaemia (Table 2). However, postischaemic CF and HR in both groups were not significantly lower (Table 2).



For abbreviations, see Table 1

Table 2

Functional recovery during reperfusion after exposure of the heart to 15 min global ischaemia

		CF	AO	CO	PSP	HR	TW
Pre-ischaemia	C+G	18.5±1.2	46.4±1.7	64.9±2.5	98±2	283±11	14.17±0.64
	D+G	19.1±1.1	43.2±1.3	62.3±2.1	99±1	285±9	13.67±0.47
	C+G+B	18.8±0.8	48.0±1.4	66.8±1.9	101±2	282±8	13.38±0.21
	D+G+B	20.5±1.3	47.3±1.8	67.8±2.5	106±3	264±14	14.23±0.08
	C+G+FA	21.0±1.0	45.8±1.5	66.8±2.3	103±2	254±14	15.31±0.56
	D+G+FA	21.0±1.0	43.3±1.5	64.3±2.3	105±2	248±17	15.40±0.32
Post-ischaemia	C+G	15.5±1.6	21.1±2.9*	36.6±4.0*	89±2*	277±9	7.32±0.90*
	D+G	17.7±0.8	28.4±1.2*	46.1±1.5*	91±1*	272±7	9.38±0.40*
	C+G+B	24.0±1.6&	30.0±2.2&	54.0±3.4&	97±2	268±20	10.64±0.78&
	D+G+B	28.5±1.3&	32.8±2.8&	61.3±2.1&	100±2	261±23	12.55±0.37&
	C+G+FA	27.0±1.0#	35.0±1.8#	62.8±2.0	100±1	265±16	14.14±0.58
	D+G+FA	26.3±1.8#	42.0±1.8	68.3±2.4	104±3	254±15	16.19±0.46

* $p < 0.05$ vs pre-ischaemic C+G or D+G, respectively
& $p < 0.05$ vs pre-ischaemic C+G+B or D+G+B, respectively
# $p < 0.05$ vs pre-ischaemic C+G+FA or D+G+FA, respectively

Data are expressed as means ± SE. n= 5-8 per group.

CF: coronary flow (ml/min) AO: aortic output (ml/min) CO: cardiac output (ml/min)
PSP: peak systolic pressure (mmHg) HR: heart rate (beats/min) TW: total work (mW)

Substrates: Glucose plus low fatty acid

Addition of low fatty acid to the glucose-containing perfusate (see protocol II) was without effect on the reduction of AO, CO and TW in both groups during reperfusion (Table 2). However, in contrast to glucose alone, postischaemic PSP and HR in both groups in the presence of low fatty acid did not differ significantly from their corresponding preischaemic values (Table 2). Interestingly, the combination of glucose and low fatty acid caused significant increases in CF of the hearts from both DIO and control groups during reperfusion compared to the values obtained before ischaemia (Table 2). In summary, the combination of glucose plus low fatty acid in the perfusion, retained the reduction in AO, CO and TW during 30 min reperfusion after 15 min sustained global ischaemia, but improved postischaemic CF to values even higher than preischaemic values in the hearts from both groups. There was no effect on HR.

Substrates: Glucose plus high fatty acid

Addition of a high concentration of fatty acid to the glucose-containing perfusate (see protocol II) had a profound effect on functional recovery during reperfusion after

exposure of the hearts to 15 min sustained global ischaemia.

Comparison of coronary flow values before and after ischaemia, showed that the combination of glucose with a high concentration of fatty acid, caused significant increases of CF in the hearts from both DIO and control rats during reperfusion (Table 2). Surprisingly, the postischaemic AO in hearts from DIO rats did not differ from preischaemic values (Table 2). Furthermore, CO, PSP and TW measured during reperfusion from both DIO and control groups were not lower when compared to the preischaemic values (Table 2). On the contrary, the CO and TW in the hearts from DIO rats were even slightly higher than their preischaemic values (Table 2). Again, the combination of glucose with a high concentration of fatty acid did not affect the HR in both groups (Table 2). In summary, when using the combination of glucose and a high concentration of fatty acid in the perfusion medium, 15 min sustained global ischaemia followed by 30 min reperfusion caused reduction of AO in the control group only, but improved postischaemic CF values to even higher than preischaemic values in the hearts from both DIO and control rats while it was without effects on other parameters.

3.2.2. Comparison of myocardial function during reperfusion between the DIO and control group and effects of substrate composition

In order to further evaluate the data summarized in Table 2, all data obtained during reperfusion were expressed as a percentage of their corresponding pre-ischaemic values to allow comparison between (i) the control and DIO groups, as well as to evaluate (ii) the effect of substrate composition on myocardial recovery during reperfusion in the two groups.

3.2.2.1. Comparison between control and DIO

Substrate: Glucose

In the presence of glucose alone as substrate, comparison of functional recovery in the hearts from the DIO and the control animals, showed that the percentage recovery of AO, CO and TW (when expressed as a percentage of pre-ischaemic values) in the DIO group was significantly higher compared to the control group (% recovery: D+G/C+G, AO 66.4±3.7/45.3±5.9; CO 74.6±3.2/55.9±4.9; TW 69.1±3.4/50.7±4.5, respectively, $p < 0.05$, Fig 16a). Postischaemic CF, PSP and HR were similar in these two groups (Table 2).

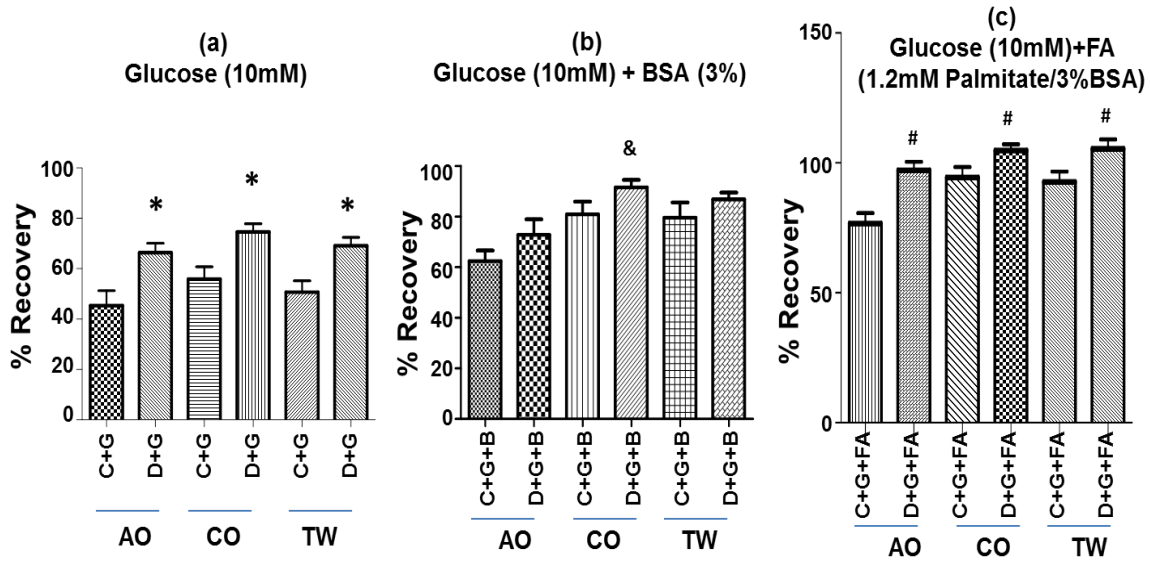


Fig 16. Comparison of % functional recovery during reperfusion of hearts from control and DIO rats exposed to 15 min global ischaemia. Data are expressed as means ± SE.
(a) Glucose (10mM) alone: * p < 0.05 vs C+G;
(b) Glucose (10mM) and BSA (3%): & p < 0.05 vs C+G+B;
(c) Glucose (10mM) and FA (1.2mM Palmitate/3%BSA): # p < 0.05 vs C+G+FA.
n = 5-8 per group. For abbreviations, see protocol I.

AO: aortic output (ml/min) CO: cardiac output (ml/min) TW: total work (mW)

Substrate: Glucose plus low fatty acid

In contrast to glucose as the sole substrate, addition of low fatty acid to the glucose-containing perfusate caused only a significant increase of CO in the DIO group (% recovery: CO, D+G+B/C+G+B 91.7±2.8/81.0±5.0, $p < 0.05$, Fig 16b), while no differences in AO, PSP, HR and TW were observed in the two groups (Table 2).

In summary, in the presence of glucose plus low fatty acid as substrates, hearts from the DIO group presented with an improved CO compared to the control group while this substrate combination had no effect on other parameters during reperfusion.

Substrates: Glucose plus high fatty acid

In the presence of glucose plus 1.2mM palmitate/3%BSA, there were marked increases in AO, CO and TW in the hearts from the DIO group (% recovery D+G+FA/C+G+FA: AO 97.2±3.3/76.6±4.1, CO 104.8±2.5/94.5±3.9, TW 105.4±3.6/92.7±3.9, respectively, $p < 0.05$, Fig 16c). However, postischaemic CF, PSP and HR were similar in these two groups (Table 2). These results therefore exhibited the same pattern observed in hearts perfused with glucose alone as substrate. In summary, in the presence of glucose plus high fatty acid as substrates,

the hearts from the DIO group still showed a significant improvement in AO, CO and TW, compared to those of the control group.

3.2.2.2. Comparison of substrate effects on myocardial function during reperfusion

Glucose plus low fatty acid vs Glucose alone

Addition of low fatty acid to the perfusate significantly increased the postischaemic recovery of CF, CO, PSP and TW in both the DIO and control groups (% recovery control: C+G+B/C+G: CF 124.0±8.0/74.8±8.8, CO 81.0±5.0/55.9±4.9, PSP 96.6±2.1/90.9±0.7, TW 81.4±5.3/50.7±4.5; % recovery DIO: D+G+B/D+G: CF 138.0±9.5/93.4±2.6, CO 91.7±2.9/74.6±3.2, PSP 96.6±2.1/92.5±1.1, TW 86.9±2.7/69.1±3.4, respectively, $p < 0.05$, Figs 17a,b).

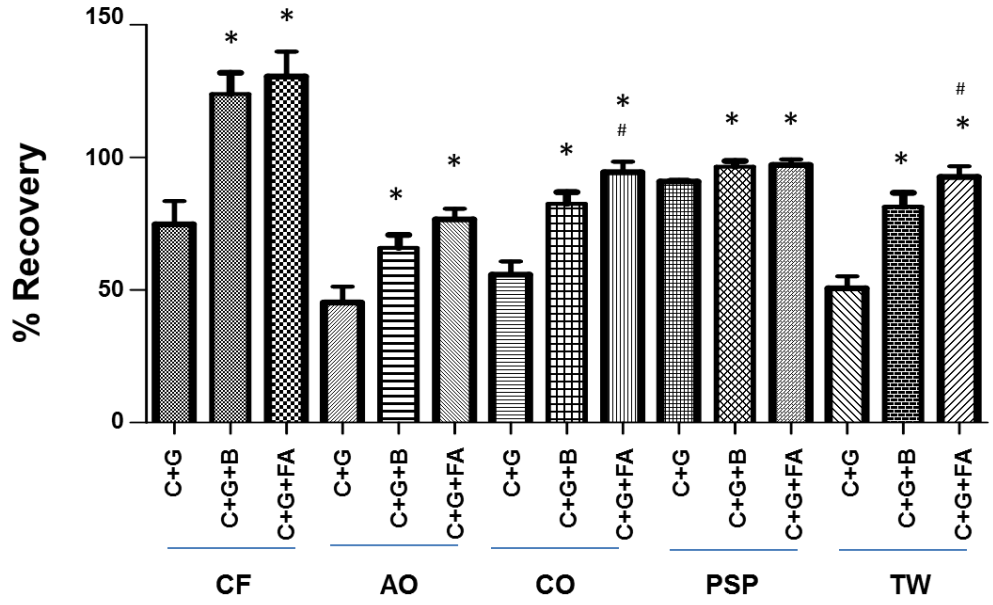


Fig 17a. Effect of substrates on % functional recovery during reperfusion of hearts from control rats exposed to 15 min global ischaemia. Data are expressed as means ± SE. n = 5-8 per group. * p < 0.05 vs C+G, respectively; # p < 0.05 vs C+G+B, respectively. For abbreviations, see protocol I.

CF: coronary flow (ml/min) AO: aortic output (ml/min) CO: cardiac output (ml/min) PSP: peak systolic pressure (mmHg) TW: total work (mW)

However, a marked increase in postischaemic AO was observed in hearts from control animals only but not in the DIO group (% recovery AO: C+G+B/C+G 66.0±4.8/45.3±5.9, $p < 0.05$, Fig 17a).

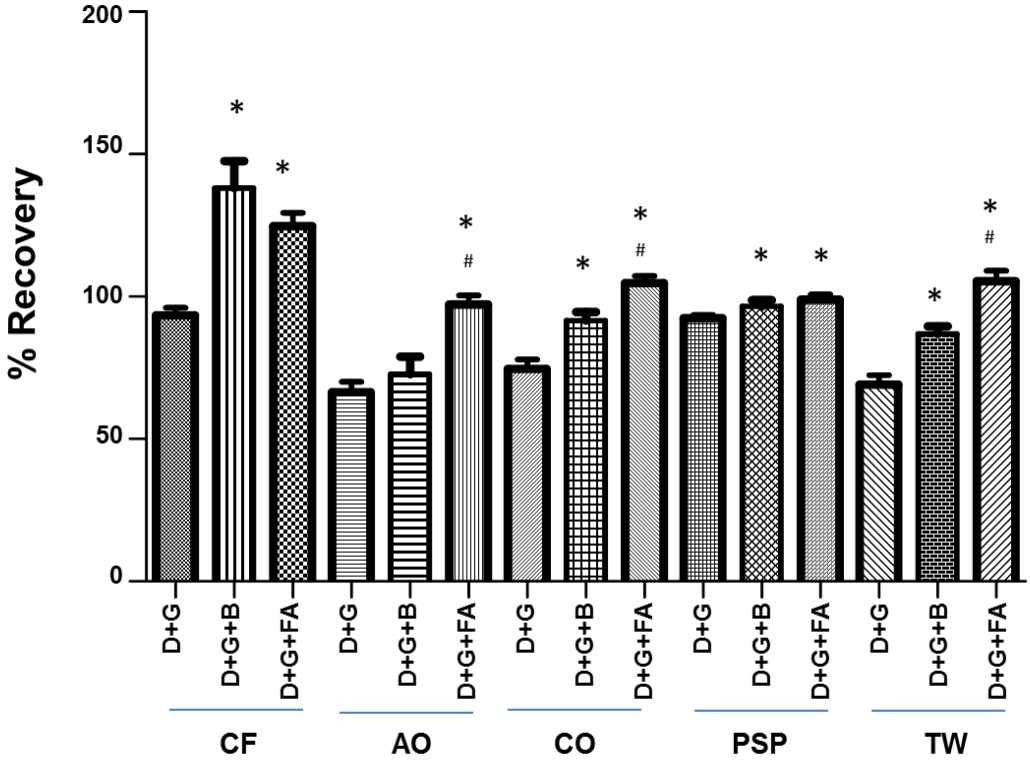


Fig 17b. Effect of substrates on % functional recovery during reperfusion of hearts from DIO rats exposed to 15 min global ischaemia.
Data are expressed as means ± SE. n = 5-8 per group.
*** p < 0.05 vs D+G, respectively; # p < 0.05 vs D+G+B, respectively.**
For abbreviations, see protocol I.
CF: coronary flow (ml/min) AO: aortic output (ml/min) CO: cardiac output (ml/min)
PSP: peak systolic pressure (mmHg) TW: total work (mW)

Glucose plus high fatty acid vs Glucose alone

The combination of glucose and a high concentration of fatty acid resulted in significant increases in all parameters (except HR) of mechanical performance in both the DIO and the control groups when compared to values obtained with glucose as the only substrate (% recovery DIO: D+G+FA/D+G: CF 124.7±4.6/93.4±2.6, AO 97.2±3.3/66.4±3.7, CO 104.8±2.5/74.6±3.2, PSP 99.0±1.8/92.5±1.1, TW 105.4±3.6/69.1±3.4. % Recovery control: C+G+FA/C+G: CF 130.5±9.4/74.8±8.8, AO 76.6±4.1/45.3±5.9, CO 94.5±3.9/55.9±4.9, PSP 97.2±2.1/90.9±0.7, TW 92.7±3.9/50.7±4.5, respectively, $p < 0.05$, Fig 17a,b).

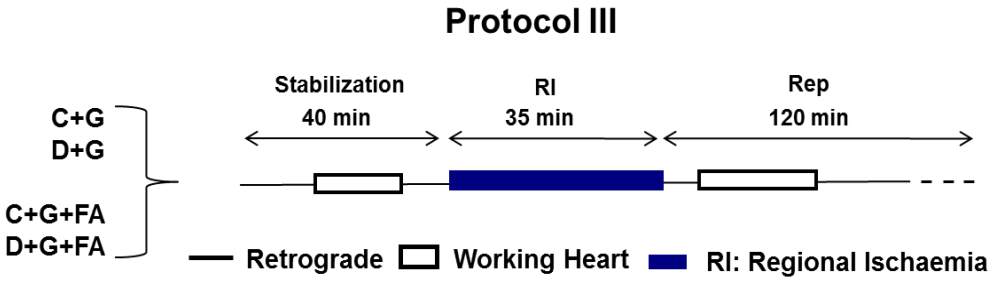
Glucose plus high fatty acid vs Glucose plus low fatty acid

Addition of a high concentration of fatty acid to the glucose-containing perfusate caused a further increase in functional recovery of CO and TW in the hearts from both the DIO and control animals compared to the values obtained when low fatty

acid was present (% recovery DIO: D+G+FA/D+G+B: CO 104.8±2.5/91.7±2.9, TW 105.4±3.6/86.9±2.7. % Recovery control: C+G+FA/C+G+B: CO 94.5±3.9/81.0±5.0, TW 92.7±3.9/81.4±5.3, respectively, $p < 0.05$, Fig 17a,b). Surprisingly, in contrast to the control group, an additional marked increase in postischaemic AO in the DIO group in the presence of high fatty acid in the perfusate was also observed compared to the group when low fatty acid was present (% recovery AO: D+G+FA/D+G+B 97.2±3.3/72.8±6.2, $p < 0.05$, Fig 17a,b). However, postischaemic CF, PSP and HR during reperfusion were similar in the DIO and control groups (Fig 17a,b, Table 2).

3.2.3. Effects of obesity and substrate on infarct size

Infarct size (IS) is expressed as a percentage of the area at risk in rat hearts. For these studies, hearts were subjected to 35 min regional ischaemia, followed by 120 min reperfusion (see protocol III). In this study and all subsequent studies determining infarct size, the area at risk did not differ between the groups. The averaged value was 48.0±1.2%.



Substrate: Glucose

In the presence of glucose alone as substrate, comparison of the infarct sizes in the two groups of hearts, showed that, after 35 min regional ischaemia followed by 120 min reperfusion, infarct sizes of the hearts from DIO rats were significantly smaller than those of the hearts from control rats (% IS: D+G/C+G 29.7±2.8/43.5±2.5, $p < 0.05$, Fig 18).

Substrates: Glucose plus high fatty acid

In the presence of glucose plus a high concentration of fatty acid, there were no differences in infarct size between the DIO and control group (% IS: D+G+FA/C+G+FA 33.0±3.4/36.6±1.4, $p > 0.05$, Fig 18).

It was evident in both DIO and control groups that the addition of high fatty fatty acid to the glucose-containing perfusate had no further effect on infarct size (% IS:

D+G+FA/D+G 33.0±3.4/29.7±2.8; C+G+FA/C+G 36.6±1.4/43.5±2.5, respectively, p > 0.05, Fig 18). In view of the above, the effect of glucose plus low fatty acid as substrates on infarct sizes was not studied.

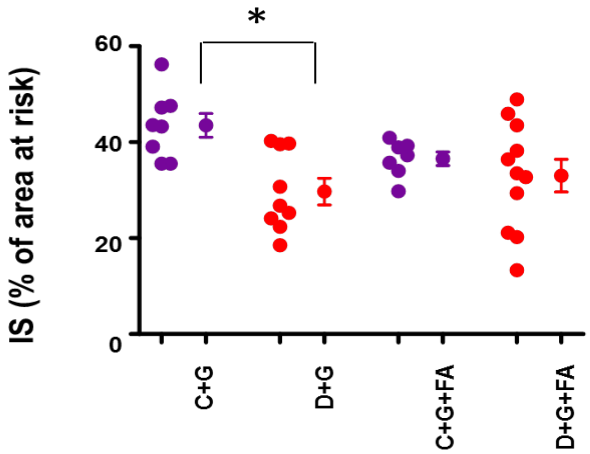


Fig 18. Effect of substrates on infarct size (IS) of the hearts from control and DIO rats subjected to 35 min coronary artery ligation and 120 min reperfusion.
Infarct size was expressed as a percentage of the area at risk. Data are expressed as means ± SE. * p < 0.05 vs C+G.
n = 8 (C+G), 9 (D+G) per group, n = 7 (C+G+FA), 11 (D+G+FA) per group.

Summary

Functional recovery and infarct size during reperfusion from control and DIO group

	Ischaemia / Reperfusion						
	Functional Recovery						Infarct Size
	CF	AO	CO	PSP	HR	TW	
D+G/C+G		↑	↑			↑	↓
D+G+B/C+G+B			↑				
D+G+FA/C+G+FA		↑	↑			↑	
C+G+B/C+G	↑	↑	↑	↑		↑	
C+G+FA/C+G	↑	↑	↑	↑		↑	
C+G+FA/C+G+B			↑			↑	
D+G+B/D+G	↑		↑	↑		↑	
D+G+FA/D+G	↑	↑	↑	↑		↑	
D+G+FA/D+G+B		↑	↑			↑	

3.2.4. Kinase and PTEN expression and activation patterns during reperfusion after ischaemia: effects of obesity and substrate

The optimal time of reperfusion for assessment of kinases and PTEN patterns was described in a previous study (804). Hearts were therefore freeze-clamped at 5, 10 and 30 min reperfusion after 15 min global ischaemia. In all instances (except PTEN), increased kinase phosphorylation indicated activation. In the case of PTEN, increased phosphorylation was indicative of inactivation of the enzyme. At all time points during reperfusion, the total protein expression of all kinases and PTEN was similar in hearts from both DIO and control groups.

3.2.4.1. Comparison between the hearts from DIO and control rats

For comparison purposes, the values obtained in hearts from DIO rats were normalized to those of the control group at each time point.

Substrate: Glucose

When perfused with glucose alone as substrate, at 5 min reperfusion, there was a slight increase in PKBs473 phosphorylation and decreases in PTEN and ERKp44/p42 phosphorylation in the DIO group, but they were not significantly different from the control group (Fig 19a, 20a, 21a).

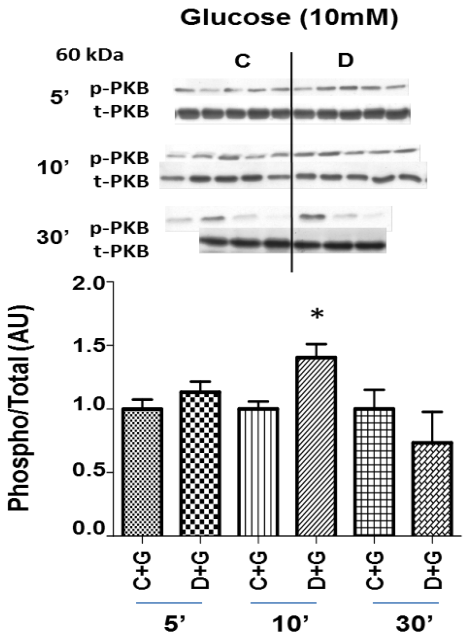


Fig 19a. PKBs473 expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group. All results were expressed as the ratio between phospho/total arbitrary densitometry units (AU) and were normalized to control hearts as one. Data are means ± SE. Substrate: Glucose (10mM) alone. * p < 0.05 vs C+G, n = 3-5 per group.

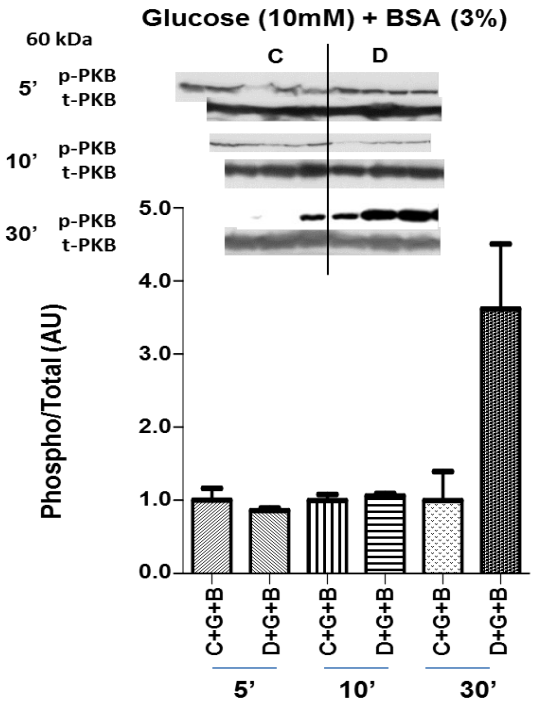


Fig 19b. PKBs473 expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group. AU: arbitrary units.
Data are means ± SE. Substrates: Glucose (10mM) plus BSA (3%).
*** p < 0.05 vs C+G+B, n = 3-5 per group.**

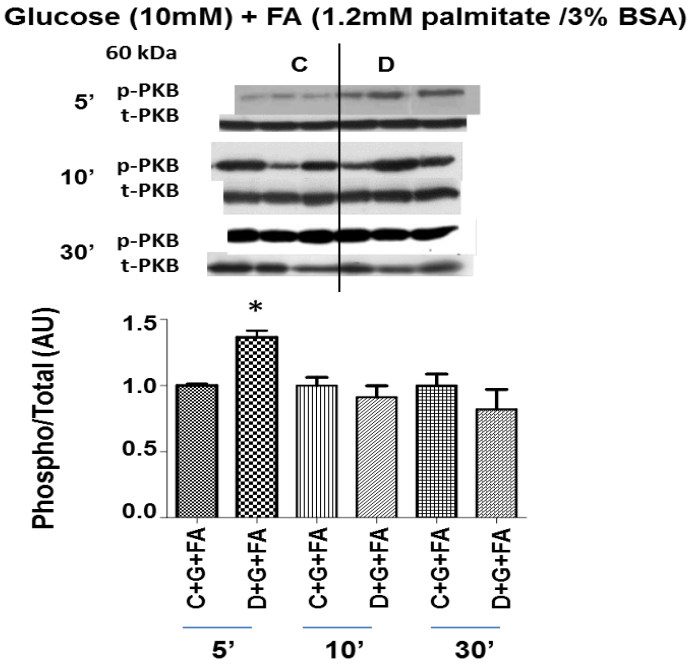


Fig 19c. PKBs473 expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group. AU: arbitrary units.
Data are means ± SE.
Substrates: Glucose (10mM) plus FA (1.2mM Palmitate/3%BSA).
*** p < 0.05 vs C+G+FA, n = 3-5 per group.**

However, hearts from DIO rats exhibited a significant decrease in JNKp54/p46 phosphorylation compared to those of the control group (au D+G/C+G: JNKp54, 0.55±0.04/1.00±0.13, JNKp46, 0.78±0.04/1.00±0.09, $p < 0.05$, Fig 22a). In contrast, no difference in p38 MAPK phosphorylation was observed at 5 min reperfusion between the DIO group and control group (Fig 23a). Interestingly, at 10 min reperfusion, there was a marked increase in PKBs473 phosphorylation in the DIO group compared to the control group (au D+G/C+G: 1.41±0.11/1.00±0.06, $p < 0.05$, Fig 19a), while PTEN phosphorylation did not differ between the groups (Fig 20a). Furthermore, no difference in ERKp44/p42 phosphorylation was observed at this time point (Fig 21a).

In contrast to the decreased JNKp54/p46 phosphorylation observed at 5 min reperfusion, the hearts from DIO rats exhibited markedly increased JNKp54/p46 phosphorylation at 10 min reperfusion compared to those of the control group (au D+G/C+G: JNKp54, 1.80±0.15/1.00±0.13; JNKp46, 1.59±0.11/1.00±0.08, respectively, $p < 0.05$, Fig 22a). However, there was no change of p38 MAPK phosphorylation at 10 min reperfusion in these two groups (Fig 23a).

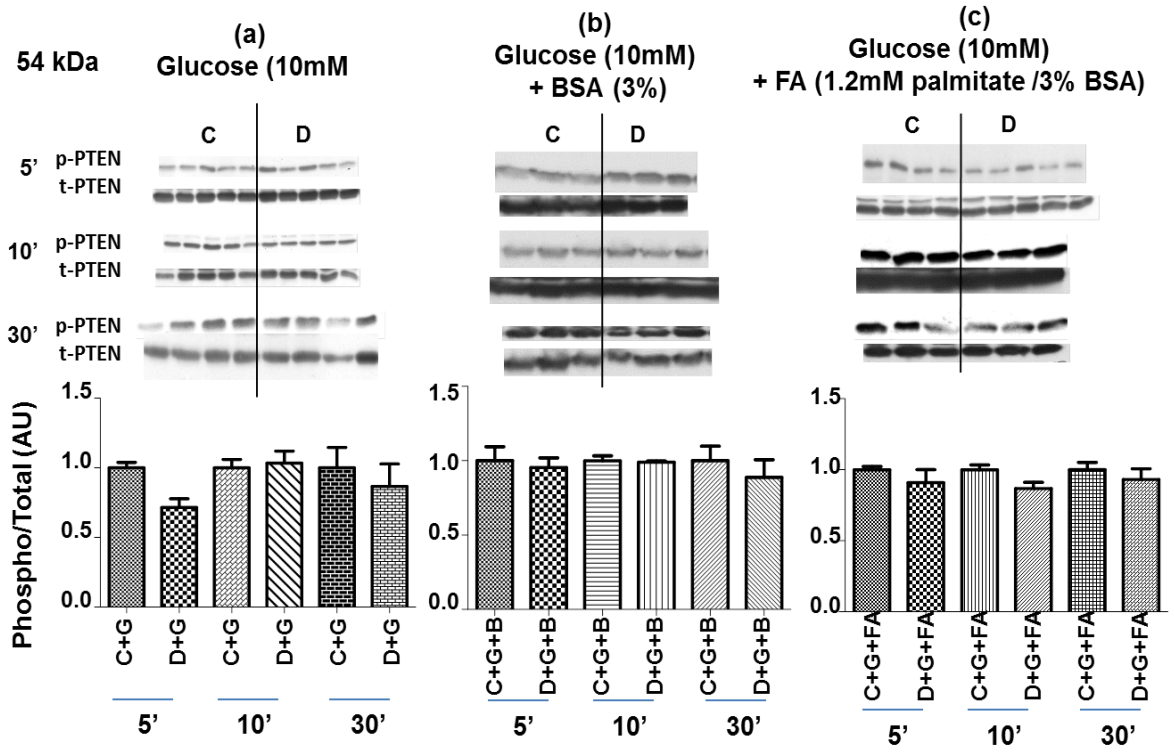


Fig 20. PTEN expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group.
AU: arbitrary units. Substrates: (a) Glucose (10mM) alone; (b) Glucose (10mM) and BSA (3%); (c) Glucose (10mM) and FA (1.2mM Palmitate/3%BSA). n = 3-5 per group.

After 30 min reperfusion, the difference in PKBs473 phosphorylation had disappeared in these two groups (Fig 19a), while no differences of PTEN and ERK

phosphorylation were observed in these two groups (Fig 20a, 21a). At 30 min reperfusion the phosphorylation of both JNKp54/p46 isoforms was significantly reduced in the DIO group compared to the control group (au D+G/C+G: JNKp54, 0.39±0.06/1.00±0.13; JNKp46, 0.55±0.10/1.00±0.15, $p < 0.05$, Fig 22a). No difference in p38 MAPK phosphorylation was observed in these two groups (Fig 23a).

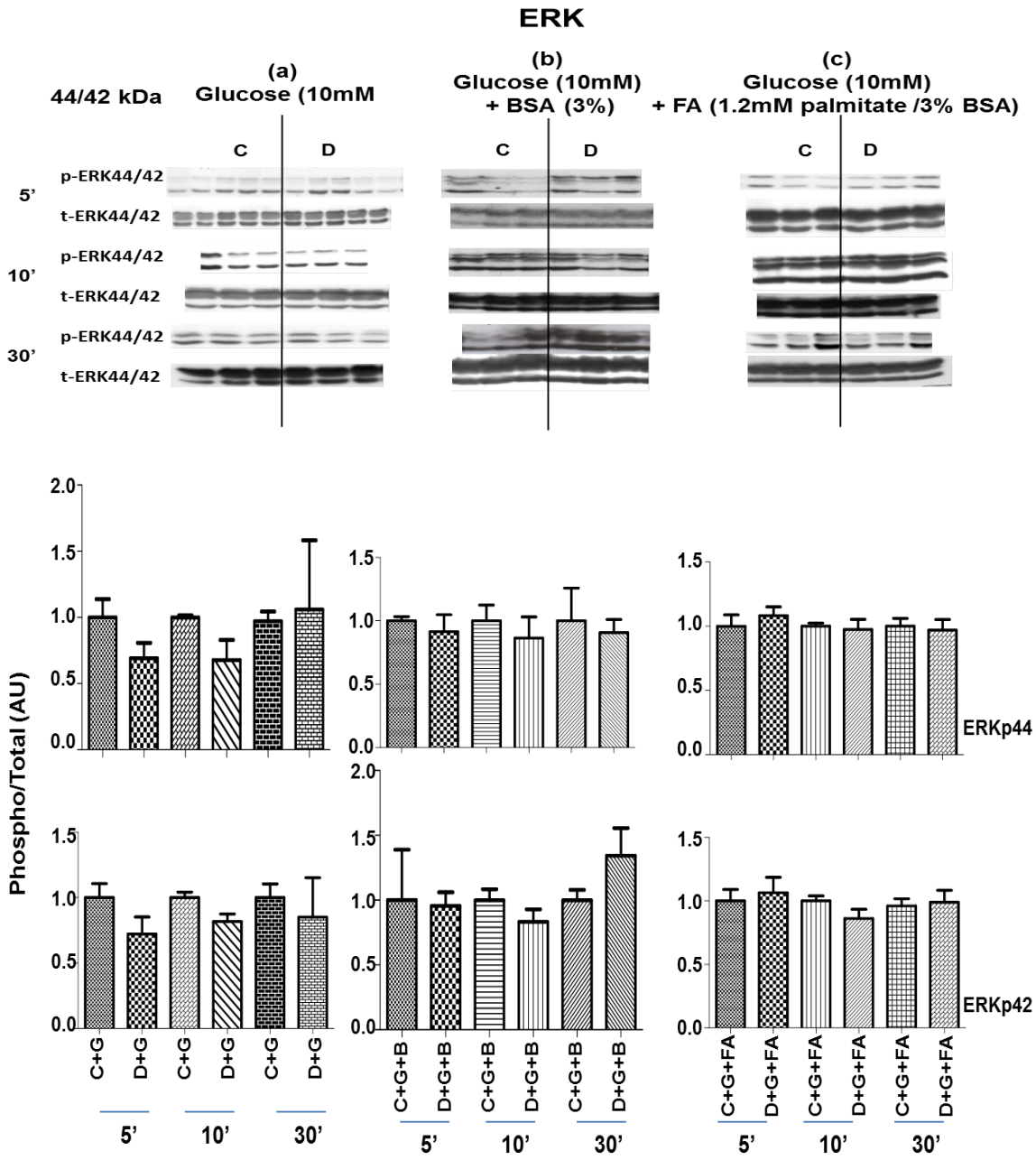


Fig 21. ERK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group. AU: arbitrary units. Substrates: (a) Glucose (10mM) alone; (b) Glucose (10mM) and BSA (3%); (c) Glucose (10mM) and FA (1.2mM Palmitate/3%BSA). n = 3-5 per group.

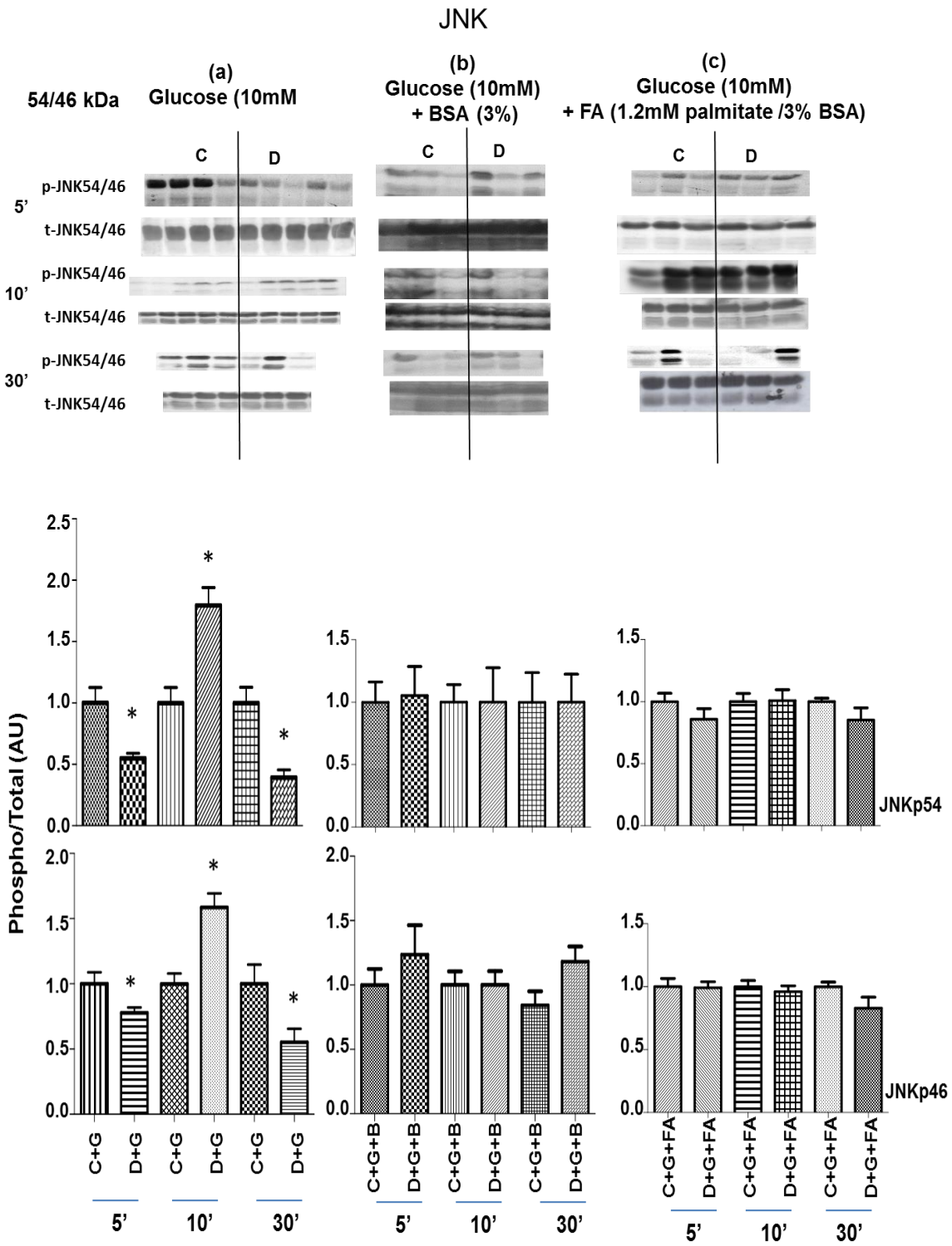


Fig 22. JNK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group. AU: arbitrary units. Substrates: (a) Glucose (10mM) alone: * $p < 0.05$ vs C+G; (b) Glucose (10mM) and BSA (3%); (c) Glucose (10mM) and FA (1.2mM Palmitate/3%BSA). n = 3-5 per group.

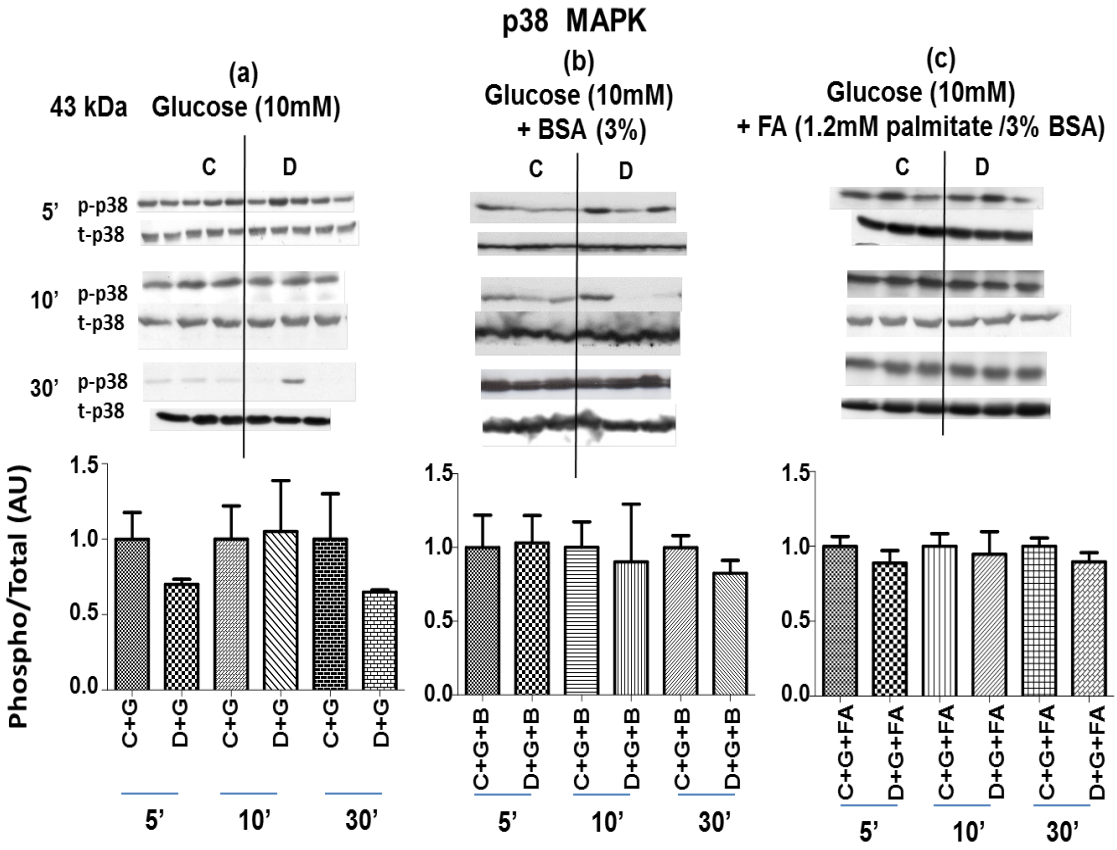


Fig 23. p38 MAPK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group. AU: arbitrary units. Substrates: (a) Glucose (10mM) alone; (b) Glucose (10mM) and BSA (3%); (c) Glucose (10mM) and FA (1.2mM Palmitate/3%BSA). n = 3-5 per group.

Substrates: Glucose plus low fatty acid

When low fatty acid was added to the perfusion medium, the difference in PKBs473 phosphorylation at 10 min reperfusion observed between DIO and control hearts in the presence of glucose alone as substrate, disappeared (Fig 19b). However, at 30 min reperfusion, a significant increase in PKBs473 phosphorylation was observed in the hearts from DIO rats compared to those of the control group (au D+G+B/C+G+B: 3.62±0.89/1.00±0.39, $p < 0.05$, Fig 19b). In contrast, no significant changes were seen in either total or phosphorylated PTEN at 30 min reperfusion in the DIO group (Fig 20b). Similarly, no differences in ERKp44/p42 phosphorylation were observed from 5 min to 30 min reperfusion between the DIO and the control group (Fig 21b). Interestingly, in the presence of glucose with low fatty acid as substrate, the significant differences in JNKp54/p46 phosphorylation from 5 to 30 min reperfusion in the presence of glucose alone disappeared (Fig 22b). The presence of low fatty acid did not change p38 MAPK phosphorylation at all reperfusion time points in these two groups (Fig 23b).

Substrates: Glucose plus high fatty acid

Interestingly, addition of a high concentration of FA to the glucose-containing perfusate caused a significant increase in PKBs473 phosphorylation at 5 min reperfusion in the DIO group compared to those of the control group (au D+G+FA/C+G+FA: 1.37±0.05/1.00±0.02, $p < 0.05$, Fig 19c) with no difference observed at 10 and 30 min reperfusion (Fig 19c). No differences in PTEN, ERKp44/p42, JNKp54/p46 or p38 MAPK phosphorylation were observed at any time point (Fig 20c-23c).

3.2.4.2. Comparison of the effects of different substrates

In contrast to the relatively few significant changes observed when comparing the patterns of kinase and PTEN phosphorylation during reperfusion of hearts from DIO and control groups, the different substrates resulted in markedly different responses.

Glucose plus low fatty acid vs Glucose alone

For comparison purposes, the values obtained in hearts from DIO rats were normalized to those of the control group at each time point.

As described above, when perfused with glucose alone as substrate, there was a marked increase in PKBs473 phosphorylation at 10 min reperfusion in the DIO group compared to the control group (au D+G/C+G: 1.41±0.11/1.00±0.06, $p < 0.05$, Fig 19a). Interestingly, hearts from DIO rats also exhibited a significant decrease in JNKp54/p46 phosphorylation at 5 min reperfusion; but markedly increased JNKp54/p46 phosphorylation at 10 min reperfusion; significantly reduced JNKp54/p46 phosphorylation again at 30 min reperfusion compared to the control group (au D+G/C+G: 5 min JNKp54, 0.55±0.04/1.00±0.13, JNKp46, 0.78±0.04/1.00±0.09, 10 min JNKp54, 1.80±0.15/1.00±0.13; JNKp46, 1.59±0.11/1.00±0.08, 30 min JNKp54, 0.39±0.06/1.00±0.13; JNKp46, 0.55±0.10/1.00±0.15, $p < 0.05$, Fig 22a, respectively). However, no differences in PTEN, ERKp44/p42 and p38MAPK phosphorylation were observed (Fig 20a,21a,23a).

When low fatty acid was added to the perfusion medium, the difference in PKBs473 phosphorylation at 10 min reperfusion observed between DIO and control hearts in the presence of glucose alone as substrate, disappeared (Fig 24a,b). However, at 30 min reperfusion, a significant increase in PKBs473 phosphorylation was observed in the hearts from DIO rats compared to those of the control group.

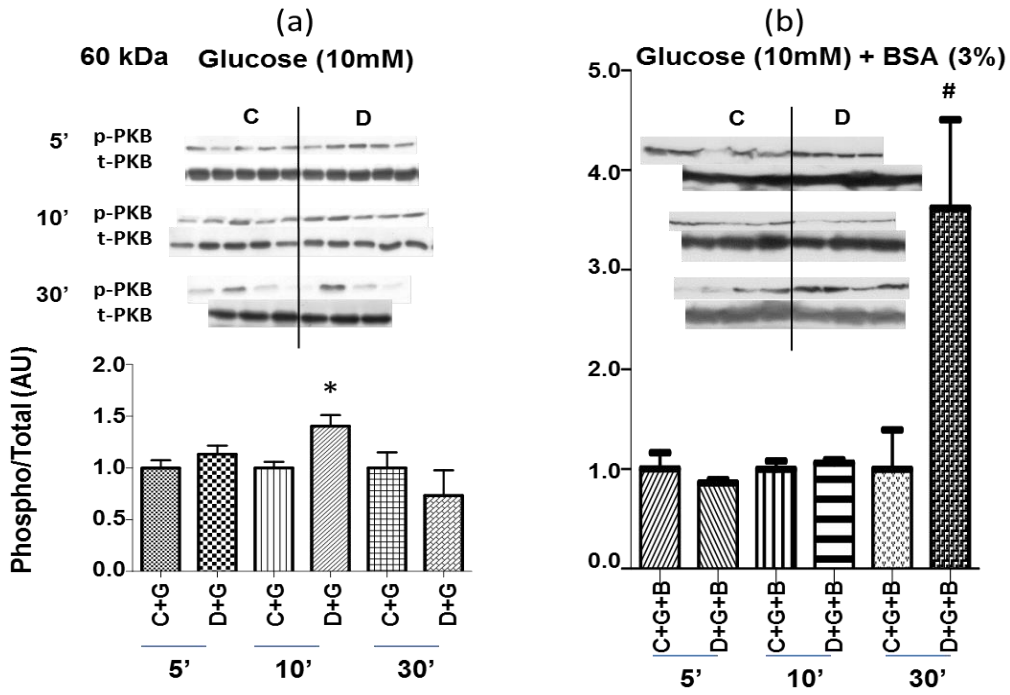


Fig 24. PKBs473 expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus 3%BSA and glucose alone. AU: arbitrary units. Substrates: (a) Glucose (10mM) alone; (b) Glucose (10mM) and BSA (3%): * $p < 0.05$ vs C+G; # $p < 0.05$ vs C+G+B. n = 3-5 per group.

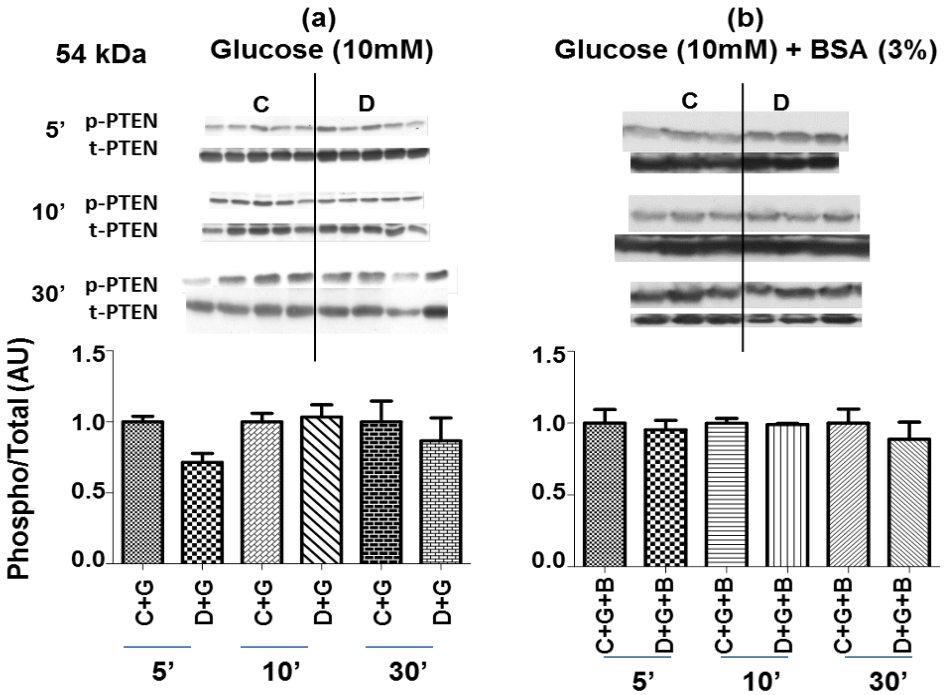


Fig 25. PTEN expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group. AU: arbitrary units. Substrates: (a) Glucose (10mM) alone; (b) Glucose (10mM) and BSA (3%), n = 3-5 per group.

No significant changes were seen in either total or phosphorylated PTEN and ERK from 5 min to 30 min reperfusion in the DIO group (Fig 25,26).

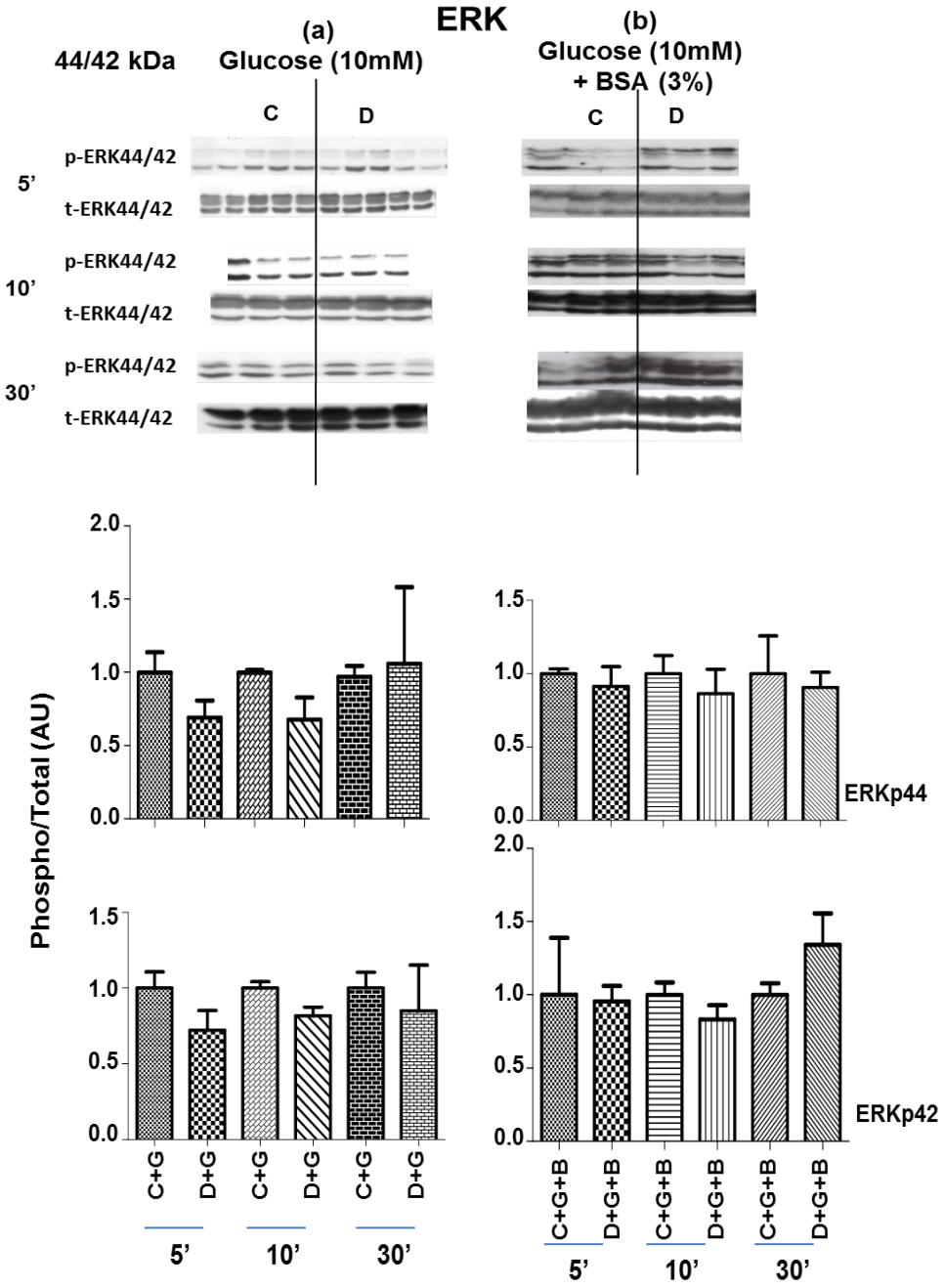


Fig 26. ERK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group. AU: arbitrary units. Substrates: (a) Glucose (10mM) alone; (b) Glucose (10mM) and BSA (3%); n = 3-5 per group.

In the presence of low fatty acid, the significant differences in JNKp54/p46 phosphorylation from 5 to 30 min reperfusion in the presence of glucose alone disappeared (Fig 27a,b).

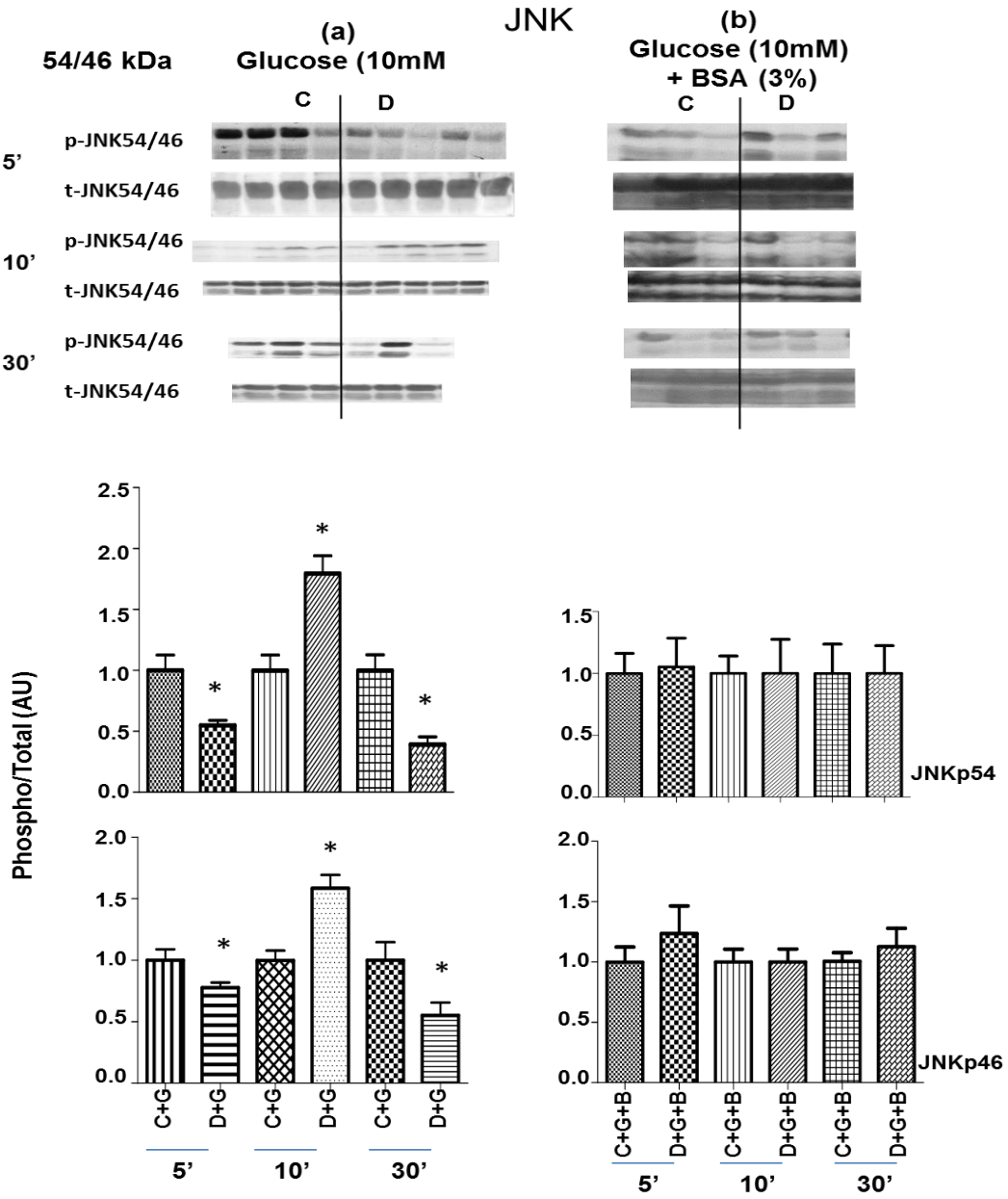


Fig 27. JNK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group. AU: arbitrary units. Substrates: (a) Glucose (10mM) alone; (b) Glucose (10mM) and BSA (3%); * $p < 0.05$ vs C+G; n = 3-5 per group.

Addition of low fatty to the perfusate did not change p38 MAPK phosphorylation at all reperfusion time points in these two groups (Fig 28).

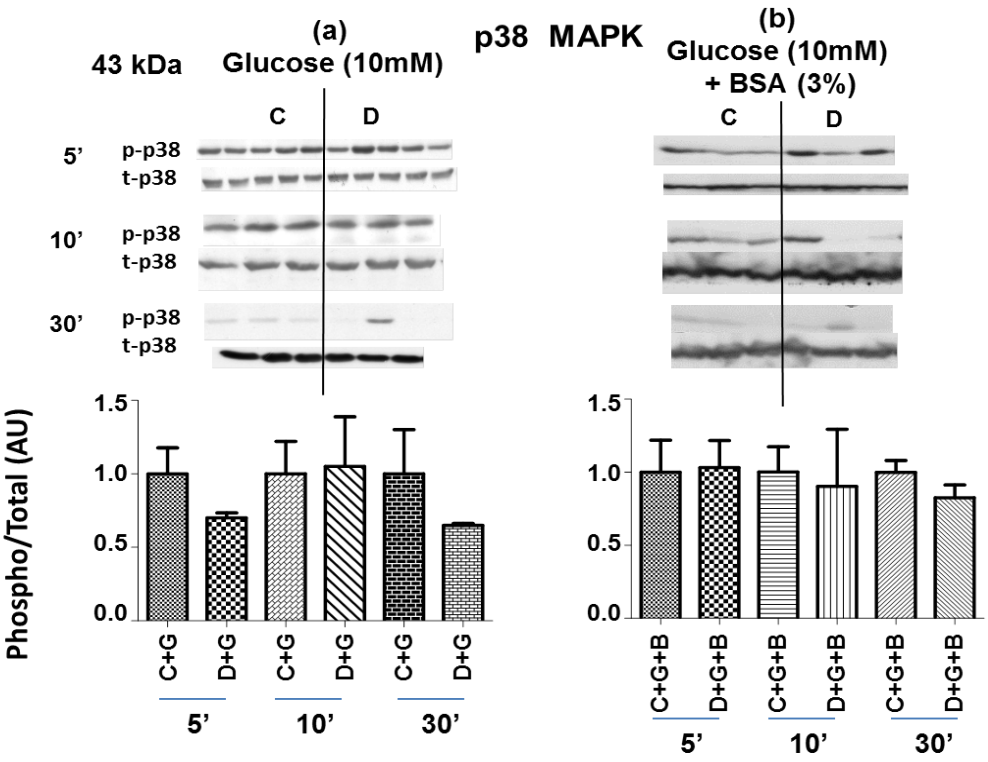


Fig 28. p38 MAPK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: comparison between the hearts from control and DIO group. AU: arbitrary units. Substrates: (a) Glucose (10mM) alone; (b) Glucose (10mM) and BSA (3%); n = 3-5 per group.

Glucose plus high fatty acid vs Glucose alone

For comparison purposes, the values obtained in the presence of glucose plus a high concentration of FA as substrate in hearts from both DIO and control rats were normalized to its group in the presence of glucose alone as substrate.

Control groups

In hearts from control animals, the addition of a high concentration of FA to the perfusion medium caused significant increases in PKBs473 phosphorylation from 5 min till 30 min reperfusion (au C+G+FA/C+G: PKBs473, 5 min 1.30±0.05/1.00±0.01, 10 min 2.48±0.39/1.00±0.09; 30 min 2.45±0.24/1.00±0.17, Fig 29a). In contrast to the increased PKBs473 phosphorylation, no differences of PTEN phosphorylation were observed at all these reperfusion time points (Fig 30a).

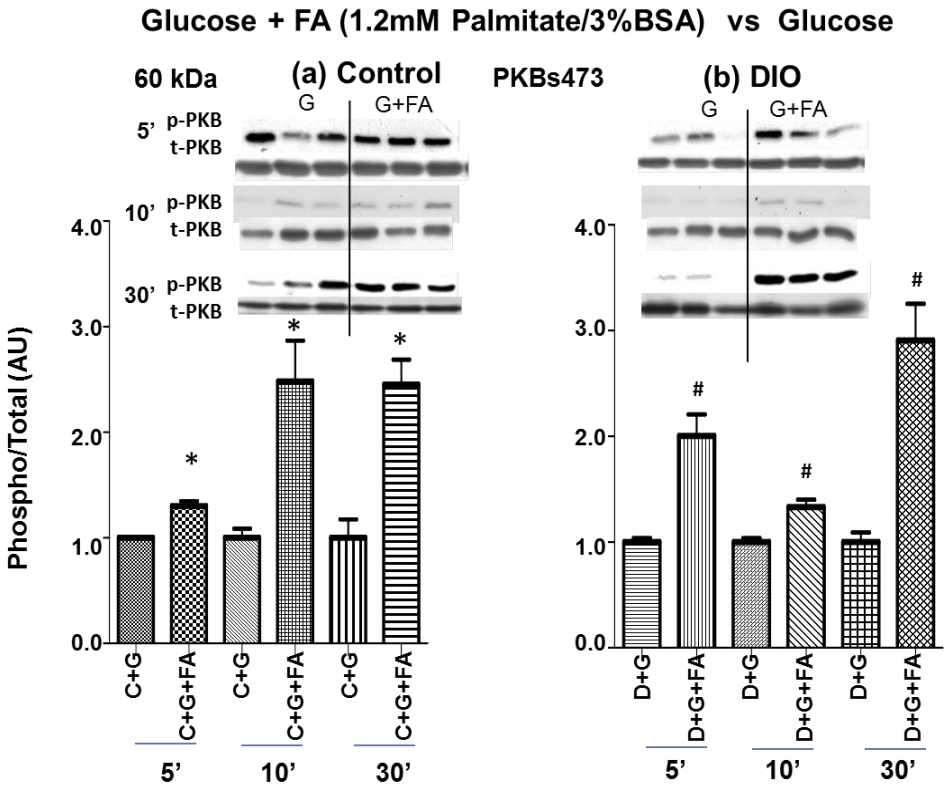


Fig 29. PKBs473 expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus FA (1.2mM Palmitate/3%BSA) and glucose alone. AU: arbitrary units. (a) effect of substrates on the hearts from control rats: * $p < 0.05$ vs C+G, respectively; (b) effect of substrates on hearts from DIO rats: # $p < 0.05$ vs D+G, respectively. n = 3-5 per group.

Surprisingly, this substrate combination resulted in a significant increase in ERKp44/p42 phosphorylation at 10 min reperfusion (au ERKp44, 1.50±0.19/1.00±0.05, ERKp42, 1.16±0.06/1.00±0.04; $p < 0.05$, Fig 31a), although no differences were observed at 5 or 30 min reperfusion. Furthermore, the addition of high concentration of FA to the perfusion medium also markedly increased JNKp54/p46 phosphorylation at 10 min reperfusion, but decreased its phosphorylation at 30 min reperfusion (au C+G+FA/C+G: 10 min JNKp54, 1.85±0.42/1.00±0.09; JNKp46, 1.35±0.10/1.00±0.09; 30 min JNKp54, 0.34±0.03/1.00±0.04, JNKp46, 0.45±0.05/1.00±0.01, $p < 0.05$, Fig 32a). No differences of p38 MAPK at all reperfusion time points were observed (Fig 33a).

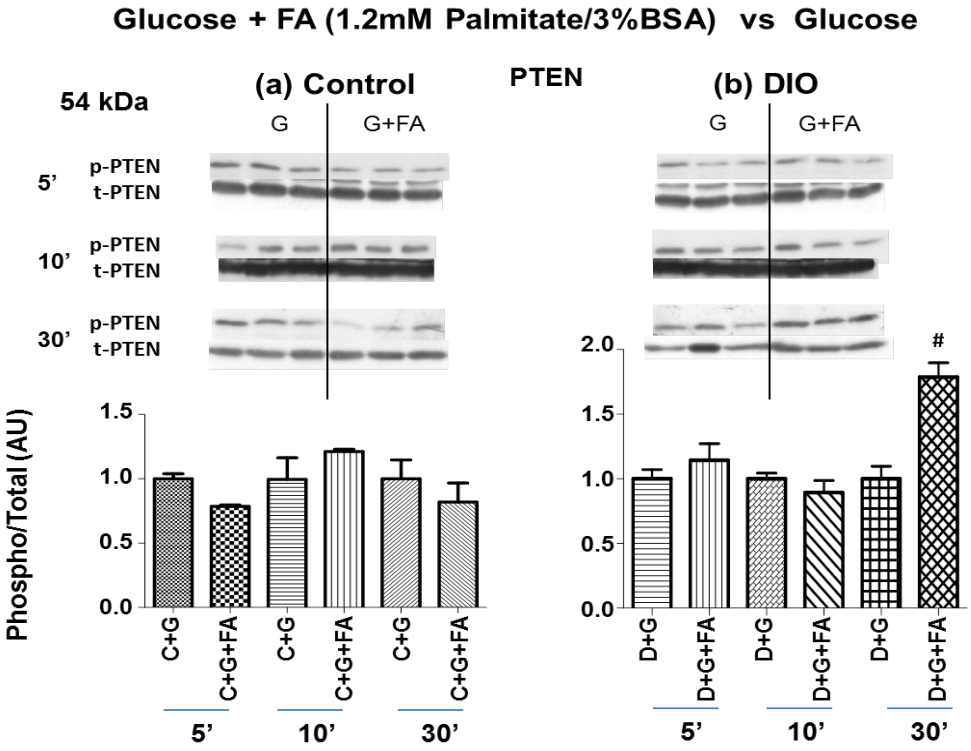


Fig 30. PTEN expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus FA (1.2mM Palmitate/3%BSA) and glucose alone. AU: arbitrary units. (a) effect of substrates on the hearts from control rats; (b) effect of substrates on hearts from DIO rats: # $p < 0.05$ vs D+G. n = 3-5 per group.

DIO groups

In hearts from the DIO group, the combination of glucose with a high concentration of FA as substrate also significantly increased PKBs473 phosphorylation throughout reperfusion (au D+G+FA/D+G: 5 min 2.01±0.20/1.00±0.04, 10 min 1.33±0.07/1.00±0.04, 30 min 2.91±0.35/1.00±0.09, $p < 0.05$, Fig 29b), and interestingly, in contrast to the control groups, it also markedly increased PTEN phosphorylation (decreased PTEN activation) at 30 min reperfusion (au D+G+FA/D+G: 1.79±0.11/1.00±0.10, $p < 0.05$, Fig 30b), although, the difference in PTEN phosphorylation was not observed at 5 and 10 min reperfusion. Addition of high concentration of FA to the perfusate also markedly increased ERKp44/p42 phosphorylation from 5 to 10 min reperfusion (au D+G+FA/ D+G: 5 min ERKp44, 1.54±0.11/1.00±0.11, ERKp42, 1.46±0.13/1.00±0.07; 10 min ERKp44, 1.83±0.19/1.00±0.05, ERKp42, 1.27±0.10/1.00±0.05, $p < 0.05$, respectively, Fig 31b). However, these differences were not observed after 30 min reperfusion (Fig 31b). The differences of JNKp54/p46 phosphorylation observed in the control groups were not apparent in the DIO groups from 5 min to 30 min reperfusion (Fig 32b). Furthermore, there were no changes in p38 MAPK phosphorylation at all reperfusion

times in the DIO groups (Fig 33b).

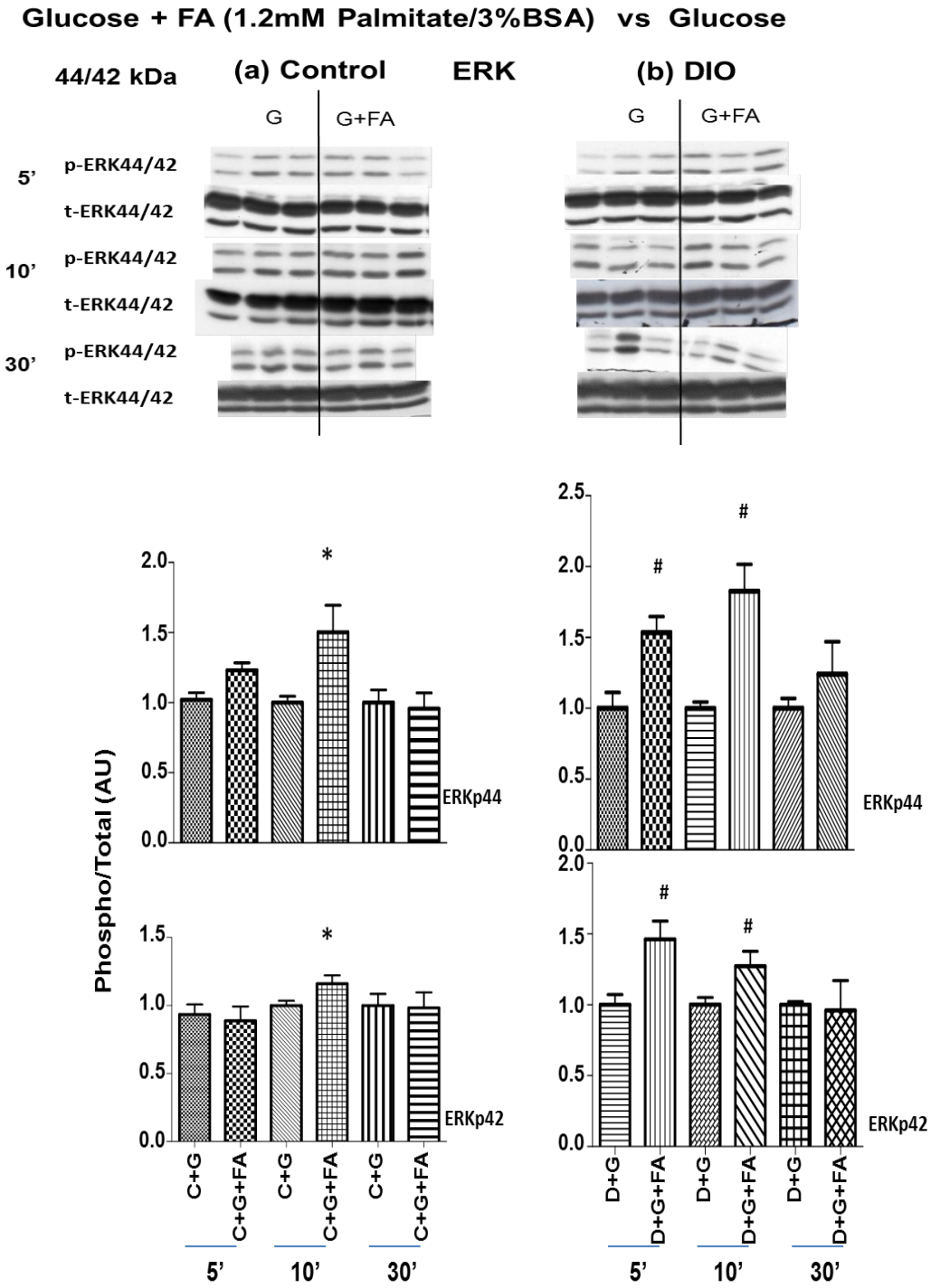


Fig 31. ERK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus FA (1.2mM Palmitate/3%BSA) and glucose alone. AU: arbitrary units. (a) effect of substrates on the hearts from control rats: * p < 0.05 vs C+G, respectively; (b) effect of substrates on hearts from DIO rats: # p < 0.05 vs D+G, respectively. n = 3-5 per group.

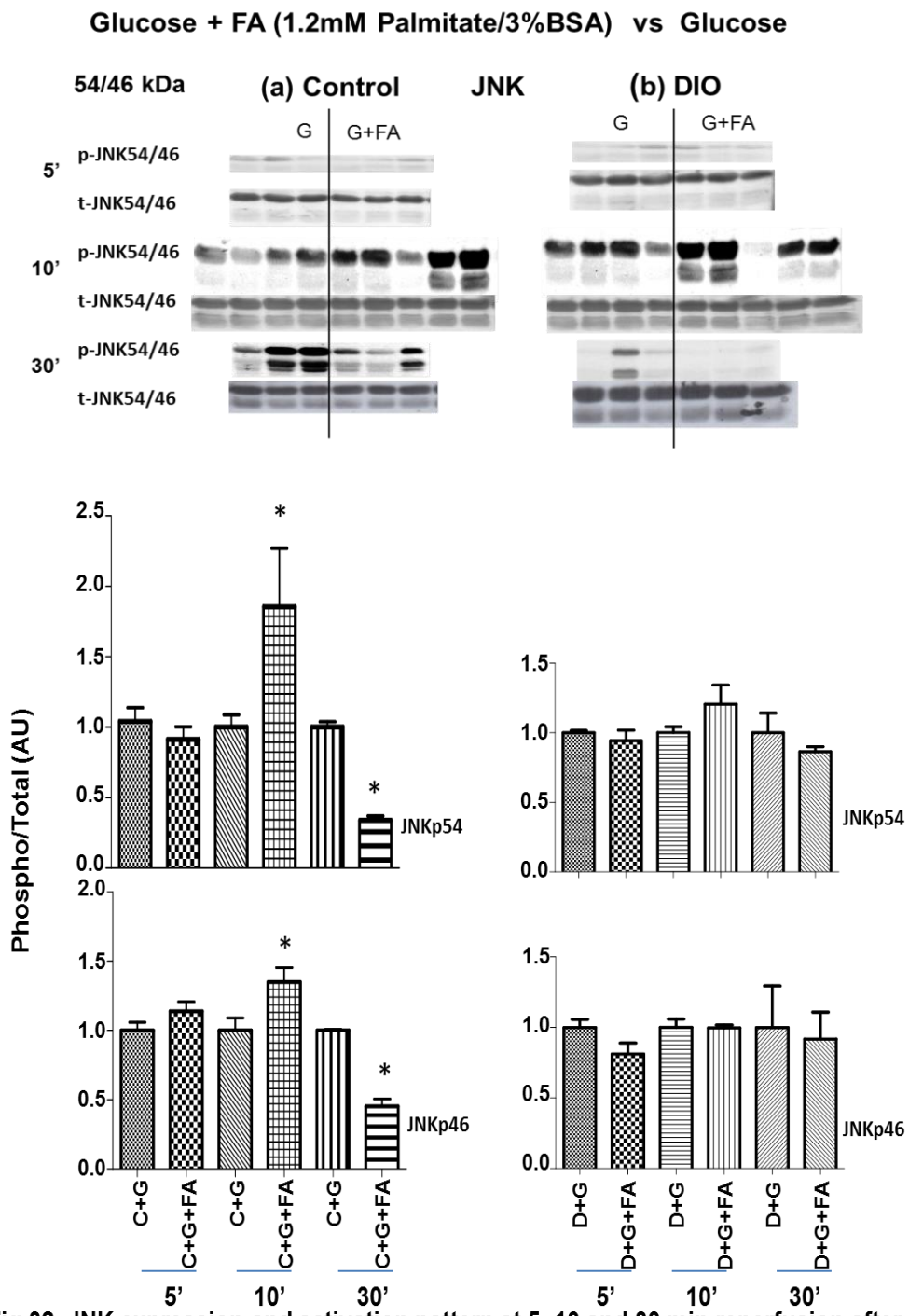


Fig 32. JNK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus FA (1.2mM Palmitate/3%BSA) and glucose alone. AU: arbitrary units.
(a) effect of substrates on the hearts from control rats: * $p < 0.05$ vs C+G, respectively;
(b) effect of substrates on hearts from DIO rats. n = 3-5 per group.

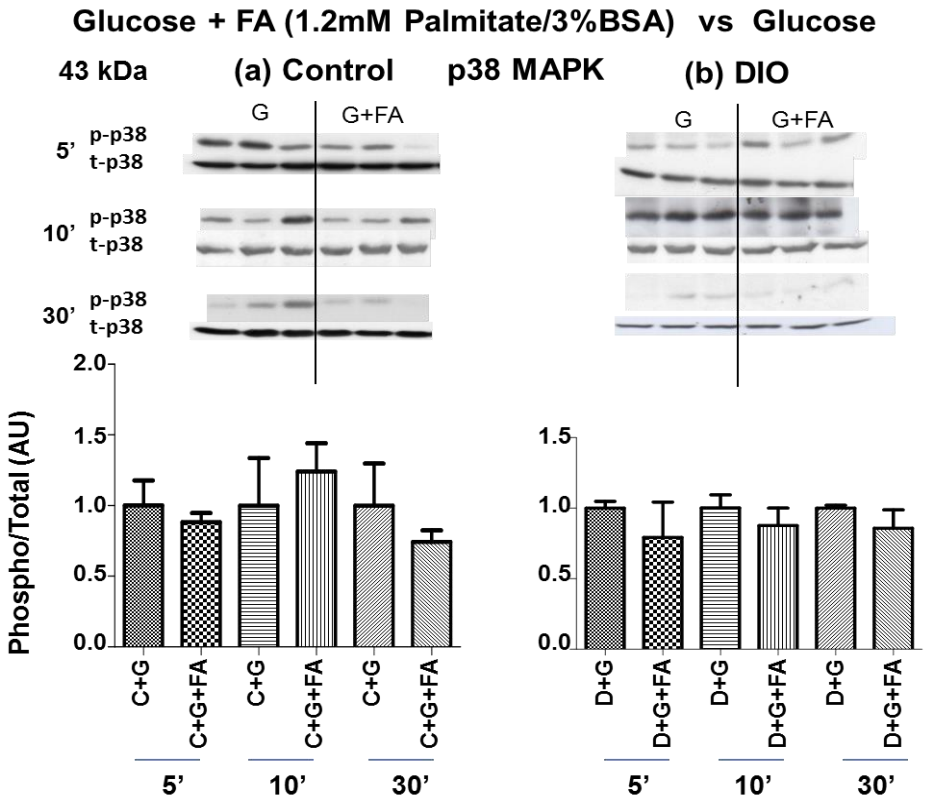


Fig 33. p38 MAPK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus FA (1.2mM Palmitate/3%BSA) and glucose alone. AU: arbitrary units. (a) effect of substrates on the hearts from control rats; (b) effect of substrates on hearts from DIO rats. n = 3-5 per group.

Glucose plus high fatty acid vs Glucose plus low fatty acid

The values obtained in the presence of glucose plus a high concentration of FA as substrate in hearts from both DIO rats and control rats were normalized to its group in the presence of glucose plus low fatty acid as substrate.

Control groups

In the control group, the addition of a high concentration of FA to the perfusate did not change phosphorylation of PKBs473 at 5 and 10 min reperfusion, but significantly increased its phosphorylation at 30 min reperfusion compared to addition of only low fatty acid as substrate (au C+G+FA/C+G+B: 30' 2.28±0.19/1.00±0.18, $p < 0.05$, Fig 34a). In contrast, no differences in PTEN, ERKp44/p42, JNKp54/p46 or p38 MAPK phosphorylation at all reperfusion times were observed in these two groups (Fig 35a-38a).

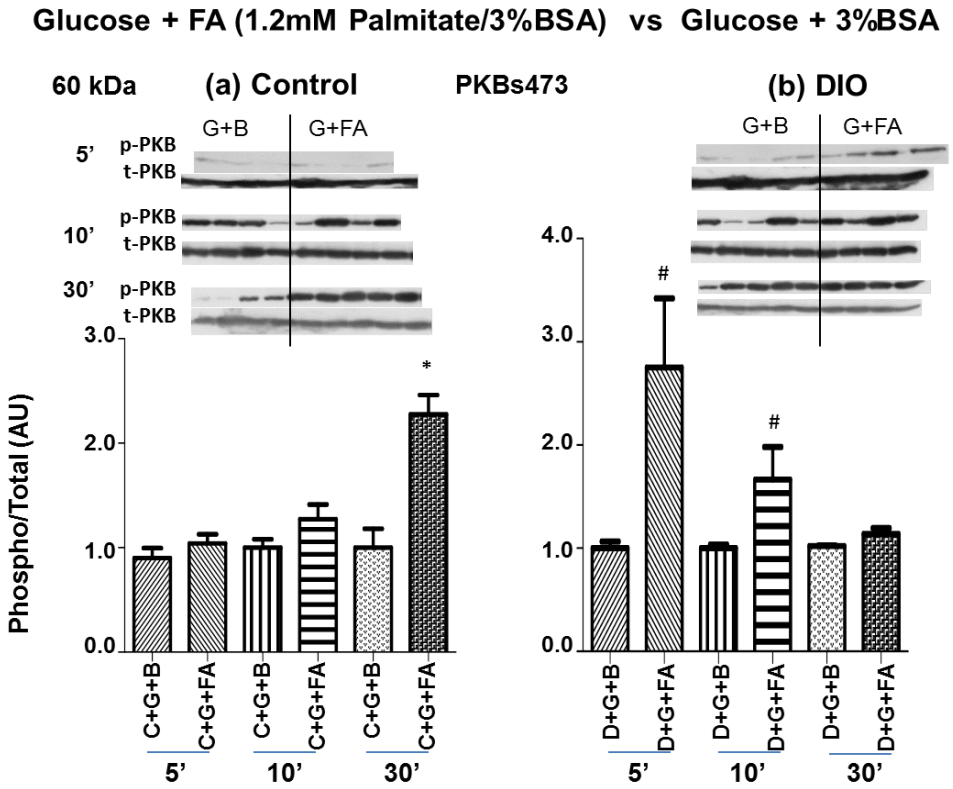


Fig 34. PKBs473 expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus FA (1.2mM Palmitate/3%BSA) and glucose plus 3%BSA. AU: arbitrary units. (a) effect of substrates on the hearts from control rats: * $p < 0.05$ vs C+G+B; (b) effect of substrates on hearts from DIO rats: # $p < 0.05$ vs D+G+B, respectively. n = 3-5 per group.

DIO groups

In contrast to the control group, addition of a high concentration of FA to the glucose-containing perfusate, caused significant higher phosphorylation of PKBs473 at 5 and 10 min reperfusion in hearts from the DIO group compared to those hearts perfused with glucose plus low fatty acid as substrate (au D+G+FA/D+G+B: 5 min 2.75±0.67/1.00±0.06; 10 min 1.67±0.32/1.00±0.03, $p < 0.05$, Fig 34b), but, the difference in PKBs473 phosphorylation disappeared after 30 min reperfusion. Increased PTEN phosphorylation at 5 min reperfusion was also observed (au D+G+FA/D+G+B: 5 min 1.45±0.02/1.00±0.07, $p < 0.05$, Fig 35b), although, the difference was absent at 10 and 30 min reperfusion (Fig 35b).

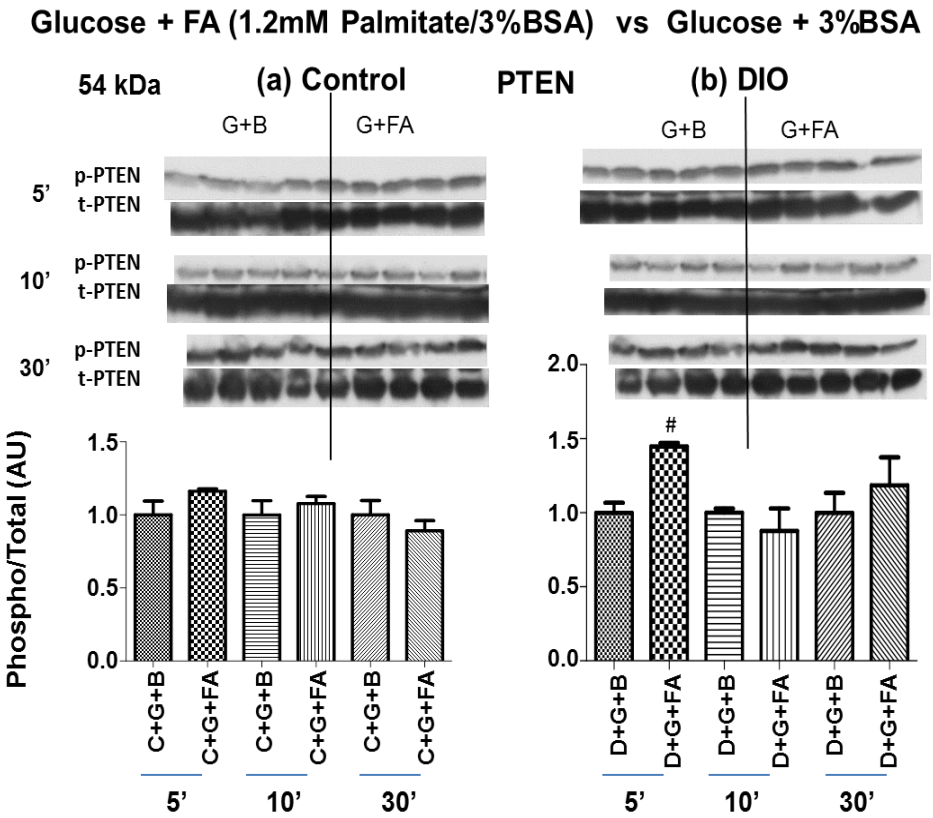


Fig 35. PTEN expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus FA (1.2mM Palmitate/3%BSA) and glucose plus 3%BSA. AU: arbitrary units. (a) effect of substrates on the hearts from control rats; (b) effect of substrates on hearts from DIO rats: # $p < 0.05$ vs D+G+B. n = 3-4 per group.

Surprisingly, in contrast to the absence of changes in ERKp44/p42 phosphorylation during reperfusion observed in the control groups, hearts from the DIO animals showed a significant increase in ERKp44/p42 phosphorylation at 10 min reperfusion compared to those hearts perfused with low fatty acid (au D+G+FA/D+G+B: 10' ERKp44, 1.51±0.12/1.00±0.19; ERKp42, 1.61±0.12/1.00±0.12, $p < 0.05$, Fig 36b). This difference was not observed at 5 and 30 min reperfusion (Fig 36b). As observed in the control groups, there were no differences in JNKp54/p46 (Fig 37b) or p38 MAPK (Figs 38b) phosphorylation during all reperfusion times when comparing the response observed with addition of high FA vs low fatty acid in the perfusate. There were no differences in the expression of proteins between any of the groups

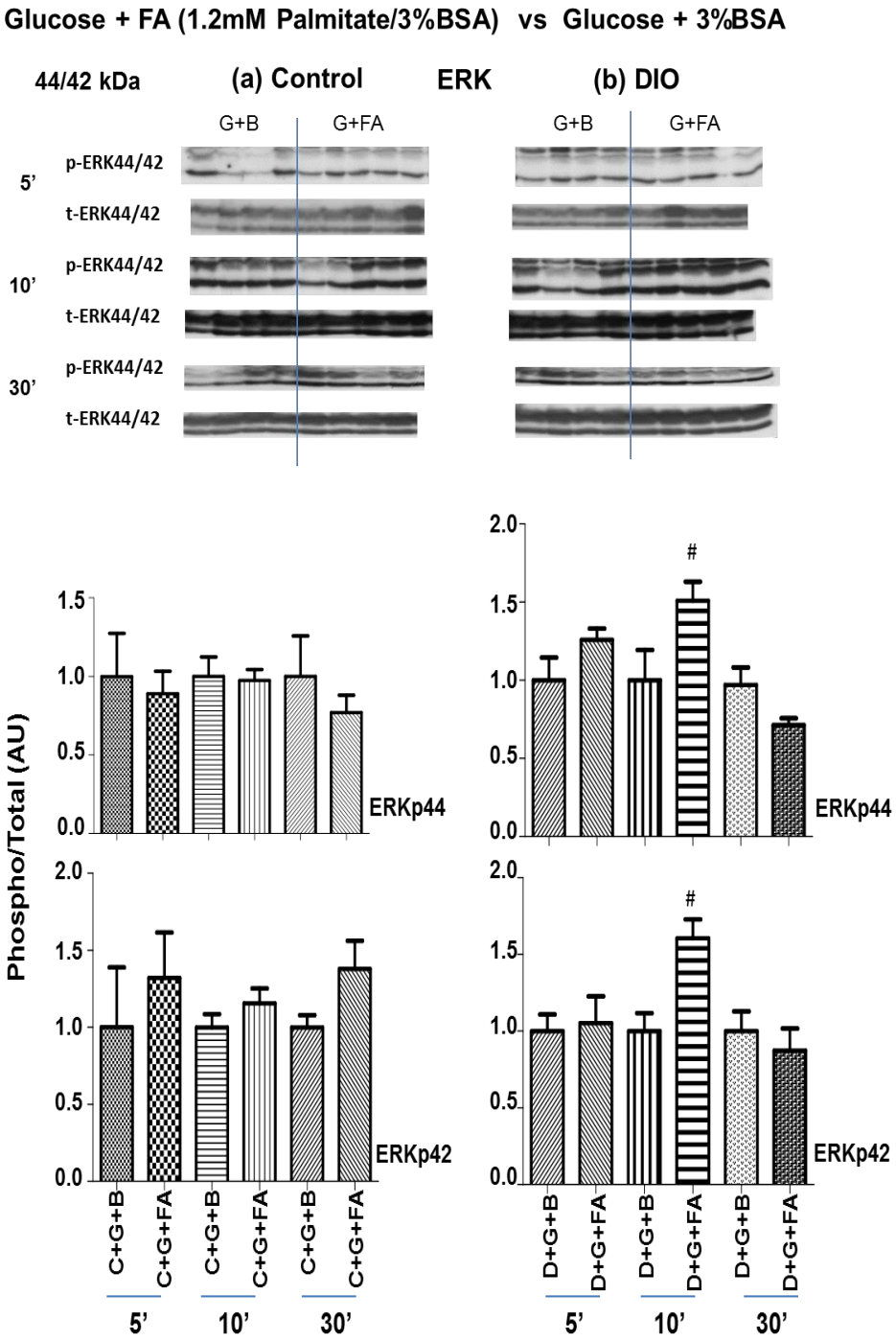


Fig 36. ERK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus FA (1.2mM Palmitate/3%BSA) and glucose plus 3%BSA. AU: arbitrary units.
(a) Effect of substrates on the hearts from control rats; (b) Effect of substrates on hearts from DIO rats; # $p < 0.05$ vs D+G+B, respectively. n = 3-4 per group.

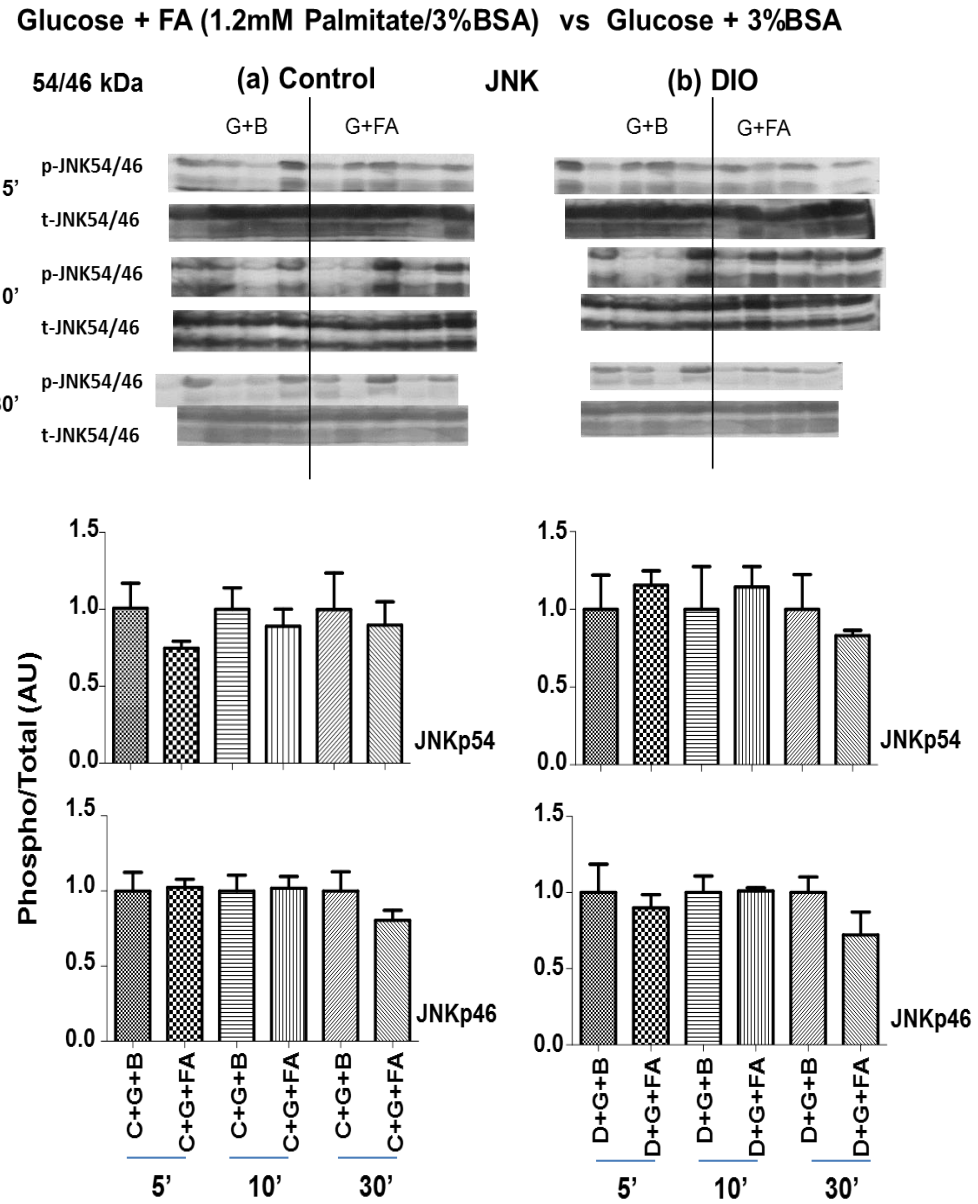


Fig 37. JNK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus FA (1.2mM Palmitate/3%BSA) and glucose plus 3%BSA. AU: arbitrary units.
(a) effect of substrates on the hearts from control rats;
(b) effect of substrates on hearts from DIO rats. n = 3-4 per group.

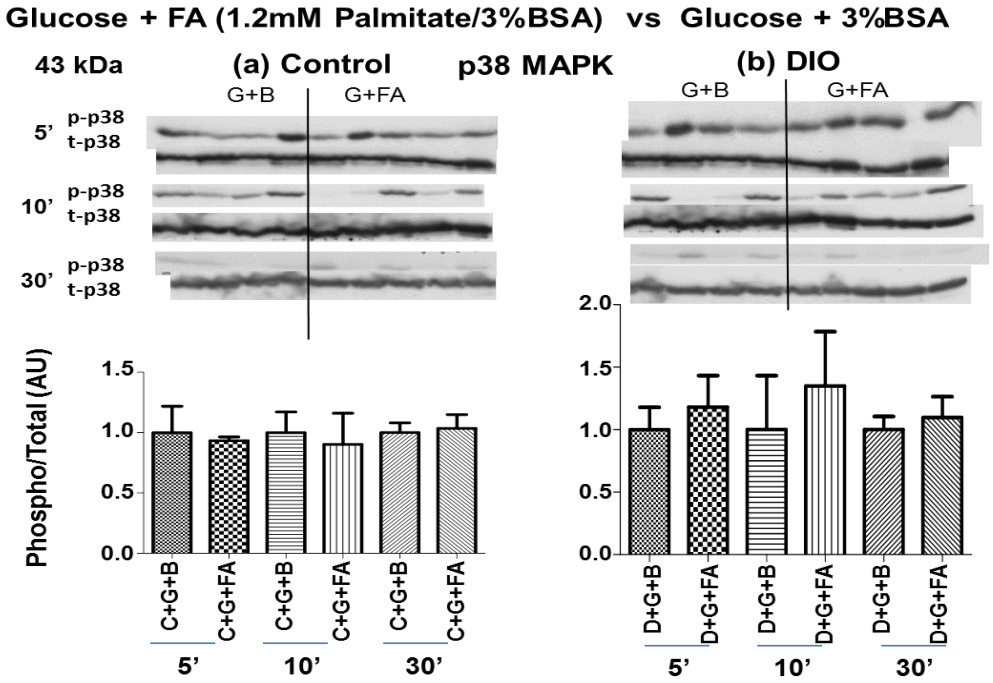


Fig 38. p38 MAPK expression and activation pattern at 5, 10 and 30 min reperfusion after 15 min global ischaemia: effects of substrates, comparison between glucose plus FA (1.2mM Palmitate/3%BSA) and glucose plus 3%BSA. AU: arbitrary units.
(a) effect of substrates on the hearts from control rats;
(b) effect of substrates on hearts from DIO rats. n = 3-4 per group.

Comparison of blots indicated (i) significant differences in the activation patterns of JNK and PKB during reperfusion of control and DIO hearts, particularly with glucose as substrate (ii) addition of a high concentration of fatty acids to the perfusate had a profound effect on the pattern of kinase activation of both control and DIO hearts.

Summary
kinase pattern during reperfusion after 15 min global ischaemia from control and DIO group

	Ischaemia	Reperfusion 5'	Reperfusion 10'	Reperfusion 30'
D+G/C+G		JNK54/46 ↓	PKB, JNK54/46 ↑	JNK54/46 ↓
D+G+B/C+G+B				PKB ↑
D+G+FA/C+G+FA		PKB ↑		
C+G+B/C+G				JNK54/46 ↓
C+G+FA/C+G		PKB ↑	PKB, JNK54/46, ERK44/42 ↑	PKB ↑, JNK54/46 ↓
C+G+FA/C+G+B				PKB ↑
D+G+B/D+G				PKB ↑
D+G+FA/D+G		PKB, ERK44/42 ↑	PKB, ERK44/42 ↑	PKB, PTEN ↑
D+G+FA/D+G+B		PKB ↑	PKB, ERK44/42 ↑	

Chapter IV

Discussion: effects of obesity

The aim of the study was to assess the effects of obesity coupled to insulin resistance and substrate supply on myocardial functional recovery and infarct size, in conjunction with intracellular signalling during reperfusion after ischaemia.

A number of important and novel findings emerged from this study as follows:
(I) In the presence of glucose alone as substrate, the hearts from DIO rats exhibited an improved tolerance to ischaemia/reperfusion (I/R) injury as reflected by an increase in functional recovery and a reduction in infarct size compared with the age-matched controls (Table 2, Figs 16-18). This was associated with early activation of PKB and JNKp54/p46 at 10 min reperfusion, with down regulation of these kinases after 30 min reperfusion (Fig 19a,22a).
(II) Contrary to expectations, the combination of a high concentration of fatty acids and glucose as substrates afforded significantly more protection against I/R injury in hearts from both DIO and control rats, when compared with the respective groups perfused with glucose alone as substrate (Fig 17). Surprisingly, during reperfusion after 15 min ischaemia, the DIO rat hearts recovered not only better than the controls, but the values were actually higher than those obtained before ischaemia (Fig 17). This improved protection in both groups was associated with increased activation of the RISK pathway (Fig 29, 31).

4.1. Models used

Some of the impediments to understanding myocardial metabolism in obesity are: (I) the difficulty in obtaining data from patients, (II) the expense involved and poor characterization of large animal models of obesity, and (III) the limitations of existing rodent models. The majority of studies have been performed in rodents and in smaller numbers in other species such as rabbits or dogs. The most relevant rodent model of pathophysiology in humans is the diet-induced obesity (DIO) rat model, whereby increased caloric intake, results in weight gain, increased fat mass, and insulin resistance (805,806). Transgenic animal models involving targeted gene overexpression or deletion have also been used, such as ob/ob (defective leptin) and

db/db mice (truncated leptin receptor) and Zucker or fa/fa rats (truncated leptin receptor) (434,807-810). The effect of obesity on the heart has been studied in these models using I) in vivo, and II) in vitro (isolated perfused hearts, papillary muscles, or isolated cardiomyocyte) approaches. Comparison of data obtained in these models presents difficulties because of the divergent causes of obesity and/or cardiac pathology and the confounding effects of hypertension and hyperglycaemia in some strains. Furthermore, multiple aspects of these dietary studies must be taken into consideration, such as duration of the dietary intervention, the carbohydrate content and lipid saturation, whether the diets are isocaloric or hypercaloric as well as the animal species studied. For example, high fat diets (HFD) have been used extensively for studies of obesity in vivo, but isocaloric HFDs do not always lead to cardiac dysfunction, insulin resistance or hyperglycaemia (811).

4.1.1. Diet induced obesity (DIO) model

The effects of obesity on substrate selection by the heart have not been extensively investigated (37). To obtain more information regarding these aspects, the rat model of hyperphagia-induced obesity was used in this study.

The rats were fed a high energy diet containing 65% carbohydrate, 19% protein, and 16% fat of which each rat consumed ~30 g per day (570±23 kJ/day). In contrast, a control rat consumed ~20 g of normal rat chow daily (371±18 kJ/day), which contained 60% carbohydrate, 30 % protein and 10% fat. Thus the DIO rats consumed a diet containing more carbohydrate and fat than the controls. However, because of the larger consumption of food by the DIO animals, the actual protein consumption was similar in these two groups. After 16 weeks, the DIO rats showed a significant increase in body weight, associated with elevated serum triglyceride, free fatty acid and insulin concentrations, as well as an increased homeostasis model assessment (HOMA) index, but with normal fasting glucose levels. These changes are common features in most rodent models of obesity (812,813) in which increased serum FA levels are a crucial step in the development of insulin resistance (814,815), often associated with compensatory hyperinsulinemia to maintain euglycemia (816). Although, the causes and consequences of acquired insulin resistance are not completely understood, the model used in the present study has been informative in highlighting certain important role players associated with insulin signalling.

4.1.2. Working heart model

In the normal well-perfused and oxygenated heart, the majority of the energy requirements (50–70%) is met by the oxidation of FA, with the remainder supplied by carbohydrate oxidation (30–50%) (for review, see ref 32). This substrate preference is due to the balance between FA β-oxidation and glucose oxidation via the Randle cycle (26,815) during normoxia. In this study, the working heart model was used, isolated hearts from both DIO and control rats were perfused with glucose alone as substrate as well as with a combination of glucose and FA (palmitate) as substrates. A high concentration of FA (1.2mM palmitate/3%BSA) was used to simulate the elevated in vivo circulating free FA content of these animals (29). It is recognized that this in vitro model has a few shortcomings, for example, the substrates supplied may not accurately reflect the milieu of substrates to which the hearts were exposed to in vivo, as well as the absence of other circulating factors (e.g., hormones). Despite these shortcomings, the isolated working heart model is used by many researchers worldwide and allows characterization of the effects of obesity and substrate combinations on the response of the heart to I/R injury as well as the associated intracellular signalling pathways.

In view of the fact that a reduction in infarct size after coronary artery ligation is not always associated with an improvement in functional recovery during reperfusion due to concomitant stunning, as described previously (804), two models of ischaemia were employed in the present study, namely coronary artery ligation for 35 min for evaluation of infarct size and global ischaemia (15 min) for measurement of functional recovery and evaluation of kinase activation during reperfusion. As motivated in the results section, a 15 min period of global ischaemia was chosen due to the increased susceptibility to ischaemic damage in older rats and the fact that functional recovery after 20min ischaemia is often very low indeed (647, 804). When perfused with glucose (10mM) alone as substrate, there was a significant reduction in AO, CO, PSP and TW during reperfusion after global ischaemia in hearts from both DIO and control rats, indicating that exposure to 15 min global ischaemia was sufficient to elicit ischaemic damage.

4.2. Baseline in isolated hearts

It is important to note that hearts, when perfused ex vivo have been removed from their in vivo metabolic and neurohormonal environments. Animals with hyperphagia-induced obesity exhibit an altered serum lipid profile (29,30), which in turn, is

expected to alter their substrate utilization: increased FA uptake and metabolism have been reported in obesity and diabetes in human and animals (for reviews, see refs. 18,32,817). These changes in lipid metabolism probably persist after removal and during perfusion of these hearts ex vivo, particularly in the presence of a high concentration of FA in the perfusate. Our results show that, the baseline function in different substrates, as well as PTEN, PKB and MAPK expression and activities were similar in the hearts isolated from DIO and control rats when perfused for 30 min under identical conditions in the working heart mode (Table 1, Fig 13-15). This suggests that, although the rats from the DIO group were insulin resistant, it was not yet sufficient to impair cardiac function and intracellular PKB and MAPK expression and activities of the hearts when perfused under normoxic conditions. The insulin signalings in insulin target tissues from the DIO rats were not evaluated in this study. **These results support the general concept that insulin responsiveness of the heart is relatively intact in insulin resistant and type 2 diabetic animals** (for review, see ref 32).

4.3. Substrate effects on ischaemia/reperfusion injury

The results obtained in this study showed that functional recovery during reperfusion after exposure to 15 min global ischaemia was significantly improved in the hearts from obesity induced insulin resistant rats in the present of different substrates when compared with age matched controls (Table 2). These beneficial effects of obesity were further corroborated by the finding that infarct sizes were also significantly smaller in the hearts from these DIO rats when perfused ex vivo with glucose as the only substrate (Fig 18). Interestingly, the significant improvement in functional recovery observed when hearts were perfused with the combination of glucose and FA as substrates, was not associated with a further reduction in infarct size (Fig 18). These observations confirm those made by Donner and coworkers (817a) after 32 weeks of feeding a similar high carbohydrate diet.

4.3.1. Glucose:

The increased tolerance to I/R injury of the hearts from the DIO group in the presence of glucose alone as substrate, may be attributed to the presence of intramyocardial triacylglycerol (TAG) levels. It is well-established that glucose metabolism accounts for only a small percentage of energy produced during reperfusion, for example, when the hearts from diabetic rats were perfused in the

presence of glucose alone as substrate, glucose oxidation provided only 20% of the total ATP requirements (321,818). It was reported that in rat hearts perfused with glucose as the sole substrate, FA derived from endogenous TAG breakdown contributed 36% to the energy expenditure, and this decreased to 11% when palmitate was added to the perfusate (43). Thus, it is possible that FA derived from endogenous TAG breakdown in the hearts from DIO rats, could contribute to the energy balance during reperfusion associated with improved functional recovery in this scenario. Although not measured in the present study, both human and animal studies have shown that obesity and diabetes increase intramyocardial TAG stores due in part to elevated circulating FA and TAG (32). It was previously demonstrated that the circulating TAG and FA levels were significantly elevated in the DIO rat model used in the present study (29,30) further supporting a possible role for endogenous TAG in the protection observed. The intramyocardial TAG stores and breakdown in hearts from the obese rats during I/R need to be further investigated.

4.3.2. Addition of FA:

Contrary to expectations, addition of FA to the perfusate afforded more protection against I/R injury not only in the DIO group but also in the control group (Fig 16,17). The bovine serum albumin (3%BSA) contributed 0.3 mM to the FA concentration in the buffer and this low concentration of FA in itself appears to be beneficial, since hearts from both the DIO and control groups perfused with glucose and 3% albumin only, also showed an improvement in functional recovery, which was further increased by elevating the FA concentration to 1.2 mM in the perfusate.

Interestingly, the post-ischaemic CF from both the DIO and control groups were significantly higher than pre-ischaemic CF in the presence of FA (Table 2, Fig 16,17), but the post-ischaemic AO were still reduced compared to pre-ischaemic AO except the hearts from DIO group (Table 2, Fig 16,17). The post-ischaemic CO and TW from both the DIO and control groups were lower than pre-ischamic values in the presence of low FA, but they remained same as pre-ischamic values in the presence of high FA (Table 2, Fig 16,17). Furthermore, comparison of myocardial function during reperfusion between the DIO groups and between control groups also showed that the post-ischaemic CF from both the DIO and control groups were significantly higher in the presence of FA than post-ischaemic CF in the presence of glucose alone (Table 2, Fig 16,17). The increased post-ischaemic CF was associated with increased AO, CO, PSP and TW in the presence of FA (Table 2, Fig 16,17). The results

indicated that the hearts from both the DIO and control groups in the presence of FA performed as the positive inotropic effects during reperfution than the hearts in the presence of glucose alone in I/R injury. Coronary vessels carrying 5% to 10% of the cardiac output run over the surface of the heart, giving rise to branches which penetrate the heart muscle and which in turn branch into smaller vessels (microcirculation) that supply the heart's capillary network with blood. This coronary flow is regulated by the heart, changing according to the heart's metabolic needs, and maintained near the minimum level required for the supply of oxygen. The main parameters dictating cardiac oxygen consumption are heart rate (chronotropy), cardiac contractility (inotropy), and left ventricular (LV) wall stress. The mechanisms by which the coronary bed adapts blood flow to the cardiac workload represent one component of coronary autoregulation, that is, the recruitment of the coronary blood flow reserve to match coronary blood flow (O_2 supply) to energy needs (O_2 demand). This is accomplished via metabolic byproducts and adenosine, but it can also be modulated through an integrated regulation of substance release from the endothelium or from the myocardium itself, neural control, myocardial compressive forces, and aortic perfusion pressure. In contrast to the normal heart, where fatty acid and glucose metabolism are tightly regulated, the dynamic relationship between fatty acid β-oxidation and glucose oxidation is perturbed in ischemic and ischemic–reperfused hearts (772-781). These metabolic alterations negatively impact both cardiac efficiency and function. Specifically there is an increased reliance on glycolysis during ischemia and fatty acid β-oxidation during reperfusion following ischemia as sources of ATP production (772-781). In this study, the data showed that in the presence of FA, the hearts from both DIO and control groups increased the post-ischaemic CF, associated with improved cardiac contractility (inotropy). Thus, it is possible that in our study, the FA in the working heart model predisposed the hearts towards FA metabolism, also during reperfusion, which in turn, may play an important role in eliciting the improved response of these hearts to I/R injury. However, the role of post-ischaemic CF in I/R injury has not been evaluated and warrants further investigation.

It is important to note that, although the presence of FA as substrate improved functional recovery in both DIO and control groups, the hearts from DIO rats still exhibited significantly more protection against I/R injury than those of controls (Fig 16,17).

4.4. FA and the obesity paradox:

It is generally accepted that ischaemia causes disturbances in the balance between FA and glucose oxidation and that increased FA β-oxidation as a source of ATP generation, at the expense of glucose oxidation during reperfusion, negatively influences cardiac efficiency, despite the restoration of coronary flow (for reviews, see ref 26,32). Several experimental studies have shown a rapid rise in FA oxidation rate, during reperfusion of the isolated working rat, as well as swine hearts (27,29,32,778,779,819) and the detrimental effects of high concentrations of FA on I/R injury are also well documented in hearts from obese or diabetic rats (28,774,820-822). The rapid recovery of FA β-oxidation in the post-ischaemic myocardium can lead to ROS accumulation which is suggested to be harmful. A high concentration of FA could also abolish the cardioprotective effects of insulin (790). This suggests that high circulating plasma FA, as found in obesity and diabetes, and a further increase during an ischaemic event, may both be involved in increased ischaemic damage (28).

In the present study, however, in the presence of a high concentration of FA, the hearts from both the DIO and the control rats exhibited an increased tolerance to I/R injury than when perfused with glucose alone as substrate. The high concentration of FA should have had a much greater impact on glucose uptake, glycolysis, and glucose oxidation than the more physiologically relevant concentrations of this substrate. The results obtained in this study may therefore support an opposite hypothesis, proposing that excessive, rather than reduced, FA metabolism can be beneficial in certain I/R conditions (823,824), and, under these hemodynamic stress conditions, the negative effect of FA on glucose metabolism may be abrogated (252,825).

In contrast to many studies showing worse outcomes after ischaemia in obese individuals, our results are in agreement with those of several other groups. For example, King and colleagues (826) showed that in the presence of a high concentration of FA (1.2mM palmitate/3%BSA) with glucose (11mM), isolated hearts from streptozotocin-induced diabetic as well as normal control rats, showed improved functional recovery after low-flow I/R, by decreasing ATP depletion. Furthermore, Ito and coworkers (827) recently reported that neonatal rabbit hearts perfused with 2.4mM palmitate and 5.5mM glucose, showed increased tolerance to I/R injury by increasing palmitate oxidation, tricarboxylic acid (TCA) cycle activity, and ATP generation. It is also important to mention that, in the reversibly injured myocardium,

FA oxidation rapidly recovered during reperfusion to replenish the ATP pool, and did not depress recovery of mechanical function in the diabetic rat hearts (28,822,828). However, the parameters in the cycling of FA between long-chain acyl-CoA synthase and mitochondrial and cytosolic thioesterase reactions (807), as well as TAG-FA cycling (285) were not assessed in the present study. Clearly, more studies are required to better understand the contributions of both endogenous and exogenous substrates to energy production in I/R under both normal and insulin resistant conditions.

Thus, it is possible that in our study, the altered serum lipid profile of the DIO rats, predisposed their hearts towards FA metabolism, also during reperfusion, which in turn, may play an important role in eliciting the improved response of these hearts to I/R injury. This is also substantiated by the fact that use of palmitate as substrate, further enhanced cardioprotection.

Although obesity has been implicated as one of the major risk factors for type 2 diabetes mellitus (DM), coronary heart disease (CHD) and hypertension (HTN), several studies from clinical cohorts of patients with established cardiovascular disease (CVD) reported an “obesity paradox” where overweight and obese patients with DM, CHD and HTN, and peripheral arterial disease (PAD), tended to have a more favourable short- and long-term prognosis (6,41). An explanation for these conflicting findings regarding the impact of obesity on I/R injury has not yet been provided.

The obesity paradox has also been observed in a number of animal studies. Hypertensive rats fed an isocaloric HFD compared to a LFD, exhibited a reduction in left ventricular hypertrophy (LVH) and improved contractile function (829,830). Additionally, isocaloric HFD feeding for 8 wks following myocardial infarction-induced heart failure, resulted in increased mitochondrial respiration, despite elevated ceramide levels and modest attenuation of contractile dysfunction (831). Isocaloric HFD feeding for 16 wks, post-MI increased myocardial tissue triglyceride accumulation, but did not alter mitochondrial function and increased cardiac function as assessed by increased fractional shortening (831,832).

Interestingly, sham-operated animals exhibited decreased mitochondrial function in response to the HFD (832). This concept was further substantiated in a study that examined mice following short-term HFD feeding (833). Wright et al. found that decreased glucose utilization and increased FA utilization occurred following as little as 2 wks of HFD feeding and these metabolic changes preceded impaired insulin

signalling, changes in PPAR gene expression, mitochondrial uncoupling, ROS production or myocardial triglyceride accumulation. Thus altered myocardial substrate utilization represents the earliest change that develops in response to an increase in caloric intake and precedes mitochondrial and contractile dysfunction and cardiac steatosis. It has also been suggested that certain molecular changes that occur in response to lipid overload may be deleterious under non-stressed conditions but could be protective in the face of additional pathological insults (811). These variables mediate disparate effects on the development of obesity and their related comorbidities. In a growing number of studies, high-fat feeding has been shown to attenuate some of the defects associated with pressure-overload and ischaemic injury (829-832).

Therefore, both animal-based studies as well as epidemiological studies in humans have suggested the existence of an obesity paradox, but whether or not similar mechanisms account for the potential beneficial effects of high-fat feeding observed in the animal models described above is currently not known.

4.5. Obesity and inflammation:

Obesity has also been shown to be associated with low-grade chronic inflammation and dysregulated cytokine production, contributing to insulin resistance. Particularly interesting is the low-grade chronic inflammation associated with increased circulating TNFα.

TNFα is is known to be involved in the pathogenesis and progression of myocardial ischaemia/reperfusion injury. The formation and release of TNFα lead to binding to its two receptor subtypes to initiate downstream signal transduction cascades. Myocardial TNFα and TNF receptor activation have ambivalent roles in myocardial ischaemia/reperfusion injury. Excessive TNFα expression and subsequent cardiomyocyte TNF receptor type 1 stimulation, induce contractile dysfunction, hypertrophy, fibrosis and cell death (834-836), while lower TNFα concentrations and subsequent cardiomyocyte TNF receptor type 2 stimulation, are thought to be protective (836,837). Apart from its concentration and receptor subtype, the myocardial action of TNFα depends on the duration of exposure and its localization (834). While detrimental during sustained ischaemia, TNFα (838), endotoxin and endotoxin derivatives (839,840) can be utilized as pharmacological pre- or post-conditioning mimetics (840). Classic ischaemic pre-conditioning depresses the I/R induced endogenous increase of TNFα in isolated rat hearts (841,842) and in rabbit

hearts in vivo (843). The reduction in infarct size induced by ischaemic pre-conditioning is lost in TNFα-KO mice (844) suggesting a role for this cytokine in cardioprotection. Classic pre-conditioning is mimicked by TNFα when administered prior to regional or global ischaemia in mice (844) and isolated rat (845,846) and rabbit hearts (847). This protection by exogenous TNFα requires a washout phase before sustained ischaemia, suggesting that TNFα acts as a trigger of pre-conditioning. The magnitude of infarct size reduction largely depends on the dose of TNFα, and only lower doses are protective (846). Use of higher TNFα doses or TNFα without a washout phase before the sustained ischaemia, can even increase infarct size (846).

Since, in the present study, the hearts were perfused for 40 min before 15 min global ischaemia, it is possible that the inflammatory cytokines, such as TNF-α, may be washed out, particularly in hearts from the DIO rats. This may contribute to the increased tolerance to ischaemia/reperfusion (I/R) injury. The activation of the Survivor Activating Factor Enhancement (SAFE) pathway is involved in the activation of TNFα (548,549). The 'RISK-free' pathway also can confer protection in ischaemic pre-conditioning (548-551). The upstream and downstream activators of the SAFE pathway have been poorly studied. Many pharmacological agents capable of mimicking ischaemic pre- or post-conditioning may confer their cardioprotective effect via the SAFE pathway. TNFα also initiates the activation of an alternative cardioprotective pathway; i.e., the janus kinase (JAK)/signal transducer and activator of transcription (STAT3) pathway (848-851). The JAK/STAT3 pathway is suggested to protect via phosphorylation and inactivation of glycogen synthase kinase β, resulting in inhibition of mitochondrial permeability transition pore (MPTP) opening (for review, see 852-855). However, the contribution of TNFα to the improved tolerance to I/R in obesity has not been evaluated and warrants further investigation.

4.6. Intracellular signalling

To gain more insight into the events occurring in the hearts of the control and obese rats when exposed to ischaemia/reperfusion and the role of the substrates used, it was decided to evaluate intracellular signalling events during reperfusion with special emphasis on the activation patterns of a number of kinases as well as the phosphatase PTEN.

4.6.1. Control vs DIO:

The improved cardioprotection in the hearts from DIO rats was observed in three different substrates. However, significant differences were observed between the intracellular signalling events in the hearts from control and obese rats. In general, increased activation of PKB was present in all DIO hearts when compared to controls, although the pattern differed depending on the substrate used. For example, increased activation of this kinase occurred at 5 min of reperfusion with glucose, albumin and high concentration of fatty acid, at 10 min with glucose alone as substrate and at 30 min with glucose plus albumin (representative of low fatty acid) (Fig 19a,b,c). The other significant changes in intracellular signalling patterns were observed with glucose only as substrate. For example, the hearts from the DIO group exhibited less JNKp54/p46 activation at 5 min, but increased activation at 10 min and interestingly reduced activation again at 30 min reperfusion (Fig 22a). However, changes in JNK patterns in hearts from DIO rats disappeared in the presence of FA. Regardless of the substrate used, no significant differences were seen in ERKp44/p42 activation during reperfusion (Fig 21).

4.6.2. Substrate effects:

Interestingly, the substrates present had major effects on the pattern of kinase activation during reperfusion of the heart itself. The combination of glucose with a high concentration of FA had a profound effect on both the PKB and ERK pathways in hearts from both DIO and control groups when compared with use of glucose as only substrate: both DIO and control groups maintained activation of PKB for 30 min, while ERKp44/p42 activation disappeared at this time (Fig 29,31). In contrast, in the presence of glucose plus albumin (i.e. low concentration of FA), the increased activation of PKB was only seen in the DIO group at 30 min reperfusion, but not in the control group (Fig 19b), in addition, both groups did not show changes in ERK signalling (Fig 21b). **These results suggest that both the PKB and ERK pathways were involved in the protection against the I/R injury in the presence of a high concentration of FA, however, in the presence of a low FA concentration, the PKB pathway, but not the ERK pathway was affected in the hearts from the DIO animals. These observations support a major role for PKB activation during reperfusion, with ERK being only important in the presence of high FA concentrations.**

In contrast to the control group, in the presence of glucose plus a high concentration

of FA, PTEN phosphorylation and thus inactivation, was also increased at 30 min reperfusion, coinciding with up-regulation of PKB at this time-point in the hearts from DIO rats (Fig 30b). It is well established that active PTEN acts to downregulate the PI3K/PKB pathway (19,558). Inhibition of PTEN during 30 min reperfusion is consistent with up-regulation of PKB in hearts from DIO rats and, is also in agreement with the role of PTEN in I/R injury (20,559). This observation suggests an important role for PTEN in the response of hearts from obese animals to I/R injury and in the acute setting of I/R (20,21,559).

It is worth mentioning that PTEN may play a significant role in the regulation of the size and contractile function in cardiomyocytes (564,566) as well as in the regulation of the L-type calcium currents (565). PTEN was also reported to be involved in ischaemic pre-conditioning in the rat heart (20). However, these aspects fell beyond the limits of the present study.

Thus, the results obtained in the present study suggest that PKB activation is always associated with cardioprotection against I/R injury: the best protection was observed in the hearts from DIO rats perfused with the combination of glucose and fatty acids which was associated with prolonged PKB activation throughout the reperfusion period. ERKp44/p42 activation under these conditions was significant but transient (Fig 29,31).

It is well known that in the myocardium, the activation of the pro-survival kinase signalling cascades, PI3-K/PKB and Ras/ERK, the so-called RISK pathway, during early reperfusion, is associated with a reduction in infarct size and improvement of postischaemic mechanical function, as was reported in procedures such as ischaemic pre- or post-conditioning or the administration of pharmacological agents both in vitro and in vivo (for reviews, see refs. 9,11).

The mechanism through which the recruitment of these pro-survival kinase pathways mediates cellular protection is not certain, but may in part be attributed to their ability to phosphorylate and modulate a diverse array of pro-and anti-apoptotic proteins.

There is abundant evidence that the PKB and ERK pathways exert transcriptional, translational, and post-translational protective effects through phosphorylation of diverse target molecules such as the Bcl-2 family proteins, and GSK-3β (9,11,163,375,856), which ensures that mitochondrial integrity is preserved during exposure short- and long-term stress. It has become apparent in recent years that the Bcl-2 family of proteins and the mitochondrial permeability transition pore (mPTP) are important regulators of the mitochondrial death pathway that is activated by

stress in cardiomyocytes (9,11,857). The direct inhibitory effects of the RISK pathway on apoptotic Bcl-2 family proteins and opening of the mPTP are critical for protection of cardiomyocytes against I/R injury (9,11,857). However, it is currently unclear whether phosphorylation and inhibition of GSK-3β by PKB is the only mechanism whereby inhibition of the mPTP confers cardioprotection (856).

PKB activation is also involved in increased glucose uptake by enhancing sarcolemmal Glut-4 expression in I/R (535). In addition, the PKB/mTOR/p70S6K complex is protective by promoting the post-ischaemic synthesis of contractile proteins (375). It is well documented that PKB overexpression in cardiac myocytes is associated with enhanced Ca^{2+} influx through L-type Ca^{2+} channels and increased Ca^{2+} release from sarcoplasmic reticulum leading to increased cytoplasmic Ca^{2+} (858,859). Whether these aspects contribute to the enhanced tolerance to I/R damage in hearts from obese rats remains to be determined.

In the present study, although ERKp44/p42 activation seems to be less outspoken than PKB, it may promote survival of cardiomyocytes by interacting with other signalling pathways, for example, IL-10 mediated ERK1/2 activation was shown to inhibit TNFα induced apoptotic signalling by blocking inhibitor-Kappa-B kinase (IKK) phosphorylation and subsequent NF-kB activation (544). ERK1/2 has been found to suppress gap junction permeability in response to mitoKATP channel opening during I/R, thus reducing myocardial damage (546). Interestingly, ERK1/2 has also been shown to compensate for loss of PKB activity in the post-infarcted myocardium and promote cardioprotection in response to erythropoietin (538).

Obesity and associated insulin resistance are characterized by decreased glucose uptake, altered lipid metabolism and impairment in PI3-K/PKB-dependent signalling in both metabolic and vascular insulin target tissues (5,6). However, these aspects were not investigated in the current study. Obesity has also been shown to be associated with low-grade chronic inflammation and dysregulated cytokine production, contributing to insulin resistance by activation of JNK, IKK, and others as negative feedback mechanisms in the regulation of insulin action via serine phosphorylation of IRS-1, which down-regulates the IRS/PI3-K/PKB pathway (10,17,18). However, as mentioned before, in this study, pre-ischaemic function (regardless of substrate) as well as basal PTEN, PI3-K/PKB, JNK and MAPK expression and activity did not differ between the control and DIO groups.

The role played by the JNK in ischaemic injury is much less clear. This is partly due to the fact that potent and selective inhibitors of the JNK have only very recently been

developed and have not been used widely in the study of I/R injury. It is however well established that JNK is activated during reperfusion only but not during the ischaemic period (583,585). However, conflicting evidence exists regarding the effects of JNK activation in I/R injury. For example, it has been reported that the JNK regulates proapoptotic death signalling events during I/R (for review, see ref 15), while genetic or pharmacologic inhibition of JNK were shown to be cardioprotective by a number of studies (14,15,606). In contrast, it has also been suggested that JNK was capable of transducing antiapoptotic signals and mediate survival in the postischaemic cardiomyocyte (613,618), but the mechanisms of these pro-survival effects were much less clear than the mechanisms promoting cell death (15). A novel antiapoptotic role for JNK was recently reported by Shao and colleagues who suggested that JNK phosphorylation was prerequisite for the full activation of PKB in the survival of postischaemic cardiomyocytes (13). Thus, the effect of JNK on myocardial cell survival in the setting of I/R needs to be further investigated. These effects of JNK are complex and likely to depend on localization, timing, substrates and insulin resistance as well as the isoform activated. As discussed above, in the presence of glucose alone as substrate, the JNK activation after 10 min reperfusion was associated with cardioprotection in hearts from obese rats (Fig 22). Interestingly, when high fatty acids were added to the perfusate, JNK activation were still remained high at early reperfusion and it even was significantly increased in the hearts from control group (Fig 32). This indicated that the addition of high concentrations of FA to the perfusate did not abolish the activation of JNK observed in hearts from the DIO rats.

4.7. Other factors

There are of course several other factors or kinases that have been implicated in I/R injury through their effects on apoptotic cell death, such as PKA, Rho kinase and JAK-STAT pathways. These factors and their signalling pathways have not been evaluated in the present study and should be investigated in future studies.

The elevated circulating fatty acid concentrations in the DIO rats could have served as endogenous ligands for the PPAR/PGC-1 signalling pathway (32,860,861). This may have changed transcriptional genes involved in fatty acid β-oxidation and lipogenesis in these hearts. The cardioprotective effects of PPARα agonists (fibrates, GW7467) have been shown to be associated with an increase in fatty acid β-oxidation during reperfusion (862-864). The rapid activation of AMPK during reperfusion (780) may contribute to the increased fatty acid β-oxidation during

reperfusion and residual oxidative ATP generation (463,769,865). It is therefore possible that the PPAR/PGC-1 signalling pathway together with the AMPK-ACC-MCD axis may result in a greater contribution of fatty acid β-oxidation to oxidative ATP production in the hearts from DIO rats, thereby contributing to the improved cardioprotection seen in these hearts compared to controls.

It is of interest that the reduction in infarct size (substrate glucose) observed in hearts from DIO animals, was associated with an improvement in functional recovery. This improvement was also seen in the presence of low (glucose + albumin) or high (1.5 mM) circulating FA concentrations. These results therefore argue that it is possible that the cardioprotective effects seen in the DIO hearts was effective to override the stunning normally associated with reperfusion (866). It is also possible that FA per se stimulates functional recovery, since infarct sizes remained unchanged when the perfusate contained glucose with FA.

The beneficial effects of high circulating FA obtained in an experimental setting, as in this study, may, in part, explain the findings obtained in clinical studies assessing the impact of obesity on outcomes following myocardial infarction and reperfusion. The controversial results reported by others may be related to several potential factors: (i) differences in the severity of the insulin resistant state (other studies used normal or type 2 diabetic rat hearts (867), (ii) the severity of the ischemic insult in which ischemic contracture is frequently associated with increased severity of ischemic injury (44,868); (iii) differences between perfusion models (working heart vs. retrograde) and (iv) species differences, for example, isolated working rat hearts oxidize fatty acids in the perfusate at significantly greater rates but oxidize glucose and lactate at lower rates than their mouse counterparts (243,780,869,870,871) and fatty acid-induced inhibition of glucose oxidation was reported to be much more potent in the rat (43) than in the mouse (790).

The current results indicated that the obesity and associated insulin resistance as well as a high circulating concentration of fatty acid, did cause intrinsic changes in the myocardium in ischaemia and reperfution, resulting in an increased tolerance to I/R injury and these beneficial effects on function were associated with activation of the PI3K/PKB and MAPK pathways during the onset of reperfusion after 15 min global ischaemia. In addition, we showed that the PI3K/PKB and MAPK pathway phosphorylation status was substrate dependent. Thus these results clearly indicated that obesity and the presence of a high concentration of fatty acids during I/R were not inherently detrimental but whether these observations reflected a reduced degree

of myocardial stunning, or decreased apoptosis in the myocardium from insulin resistant animals in I/R, requires further study. The exact mechanism(s) involved in these beneficial actions of obesity and high concentration of fatty acid on the heart also still remain to be established. However, as suggested by the present study, further studies on the impact of fatty acids on myocardial injury during ischaemia and reperfusion should take precedence before considering the potential benefits of obesity and high concentrations of fatty acid for the hearts.

In summary, the results obtained in this study clearly indicate that obesity and the presence of high concentrations of fatty acids during I/R per se are not detrimental.

The finding that obese insulin resistance and fatty acids modulate the activation of the PI3-K/PKB and MAPK pathways during I/R may have relevance to obesity in humans.

Chapter V

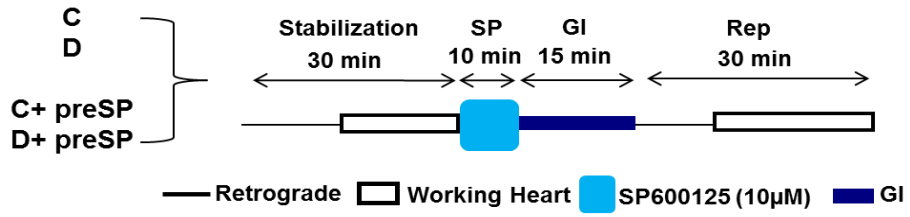
Results: Effects of JNK inhibition on myocardial response to ischaemia/reperfusion injury in control and DIO rats

The results described in Chapter III show that hearts from obese, insulin resistant rats are more resistant to I/R injury than their age-matched control counterparts, regardless of the substrate used in the ex vivo perfusion experiments. Marked time-dependent changes in JNK activation were observed during reperfusion of hearts from these obese rats, particularly when glucose was present as the only substrate (Fig 22a,b.c). Interestingly, when high fatty acids were added to the perfusate, JNK activation were still remained high at early reperfusion and it even was significantly increased in the hearts from control group (Fig 32). **It is worth mentioning that after I/R injury, activation of JNK during early reperfusion were observed in all hearts from control and DIO rats in the present of all three different substrates.**

In this section of the study the significance of JNK activation during reperfusion was investigated on the outcome of I/R in hearts from control and obese rats by using a specific JNK inhibitor, SP600125. To assess the role of JNK during both phases of the experimental protocol, the drug was administered either before induction of ischaemia (pretreatment) or during the first minutes of reperfusion (posttreatment).
In the pretreatment protocol, the JNK inhibitor, SP600125 (SP:10uM) was administered for 10 min only without wash out before induction of 15 min global ischaemia (see Protocol IV,V). In the post-tretreatment protocol, SP (10uM) was administered for 10 min immediately during reperfusion after 15 min global ischaemia (see Protocol VI,VII). In view of the results obtained before, the effects of the inhibitor on parameters during reperfusion after 15 min ischaemia were studied in hearts perfused with glucose alone or glucose plus a high concentration of fatty acid as substrates. Hearts perfused with glucose plus low fatty acids were not included in this study.

Protocol IV
Functional recovery
preSP

Substrates: Glucose (10mM)

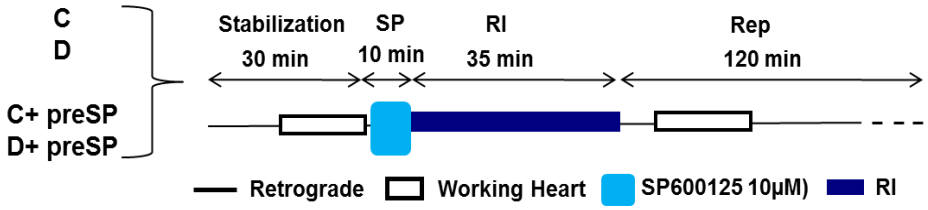


C+ preSP: the hearts from control rats pretreated with SP600125 (10mM)
D+ preSP: the hearts from DIO rats pretreated with SP600125 (10mM)

Abbreviations see Protocol I, II

Protocol V
Infarct Size
preSP

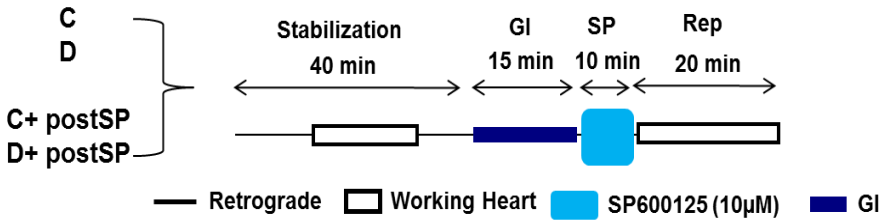
Substrates: Glucose (10mM)



Abbreviations see Protocol I, II, III

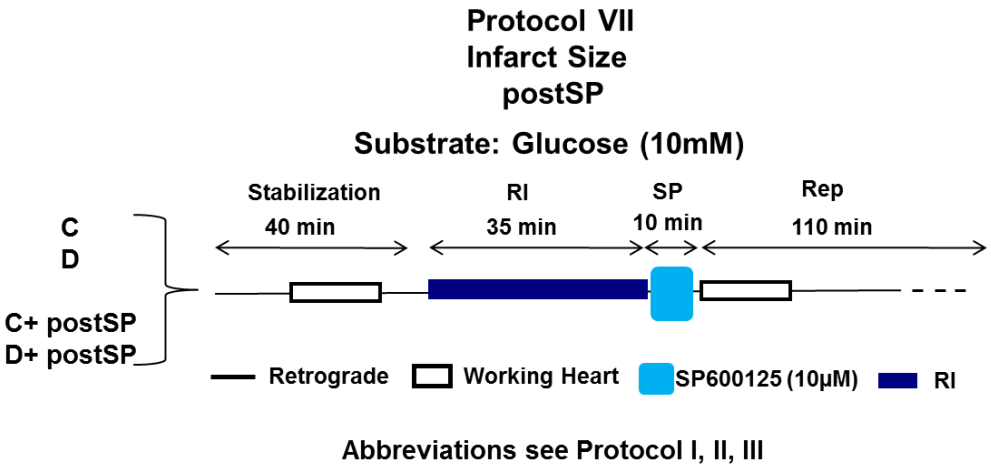
Protocol VI
Functional recovery
postSP

Substrates: Glucose (10mM)



C+ postSP: the hearts from control rats treated with SP600125 (10mM) during reperfusion after ischaemia
D+ postSP: the hearts from DIO rats treated with SP600125 (10mM) during reperfusion after ischaemia

Abbreviations see Protocol I, II



5.1. Effects of SP600125 (10uM) on the JNK transcription factor, c-Jun.

c-Jun, is a target of downstream transcription factor of JNK. To evaluate the inhibitory effect of SP600125 on the JNK pathway in our study, we first examined the effect of the drug on serine 63 phosphorylation of c-Jun, employing Western blotting.

c-Jun, is a downstream transcription factor of JNK. For this study hearts were also subjected to 15min global ischaemia, followed by 30 min reperfusion. Based on a previous review (876), it was decided to use SP600125 at a concentration of 10microM.

Substrate: glucose

The results obtained showed that when perfused with glucose alone at a concentration of 10 mM as substrate in the **absence** of SP, there was no significant change in c-Jun phosphorylation in the DIO group compared to those of the control group from 5 to 30 min reperfusion (Fig 39, 40).

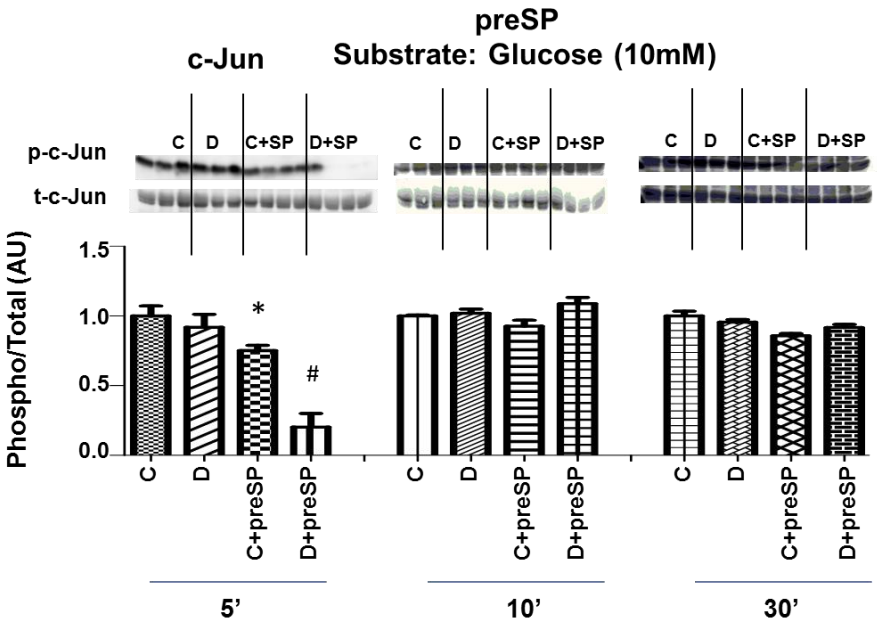


Fig 39. Effects of JNK inhibitor SP on c-Jun expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group pretreated with SP or without treatment. All values were expressed as the ratio between phospho/total arbitrary densitometry units (AU) and were normalized to control hearts as one. Substrate: Glucose (10mM). * p < 0.05 vs C, # p < 0.05 vs C, respectively. n = 3-4 per group.

With preSP, in the presence of glucose alone as substrate, c-Jun phosphorylation was inhibited at 5 min reperfusion in the hearts from both control and DIO rats (au C+preSP/C: 0.75±0.04/1.00±0.07, D+preSP/D: 0.20±0.10/0.92±0.09, $p < 0.05$, respectively, Fig 39). In both pretreated groups, c-Jun phosphorylation was significantly lower in hearts from DIO rats (au D+preSP/ C+preSP: 0.20±0.10/0.75±0.04, $p < 0.05$, Fig 39). However, this inhibition was not sustained through 10 and 30 min reperfusion.

With postSP, in the presence of glucose alone as substrate, the same pattern was observed as in pretreatment after 5 min reperfusion in control and DIO hearts (au C+postSP/C: 0.44±0.03/1.00±0.04, D+preSP/D: 0.38±0.03/0.84±0.02, $p < 0.05$, respectively, Fig 40). Although the reduced c-Jun phosphorylation at 10 min reperfusion was not remained in the DIO groups, but a marked inhibition of c-Jun at 30 min reperfusion was still observed in the hearts from DIO rats (au D+postSP/D: 0.40±0.05/1.02±0.08, $p < 0.05$, Fig 40).

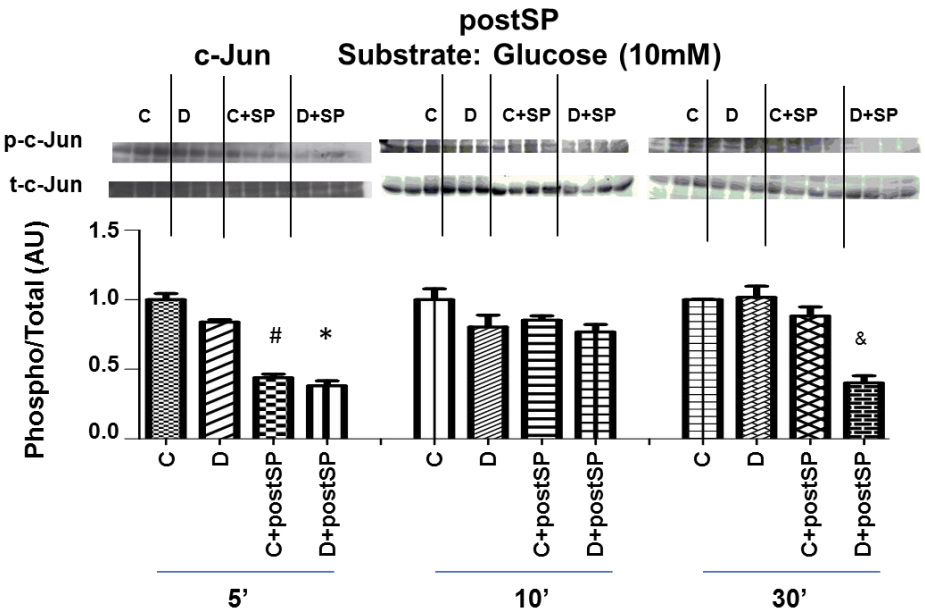


Fig 40. Effects of JNK inhibitor on c-Jun expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group posttreated with SP or without treatment. AU: arbitrary units. Substrate: Glucose (10mM). * p < 0.05 vs C, # p < 0.05 vs C, & p < 0.05 vs D, respectively. n = 3-4 per group.

Substrate: glucose with high fatty acid

The results obtained showed that when perfused with glucose plus a high concentration of fatty acid as substrates in the **absence** of SP, there were no differences in c-Jun phosphorylation between the DIO and control groups at all reperfusion time points ($p > 0.05$, Fig 41,42).

PreSP, in the presence of glucose with a high concentration of fatty acid, inhibited c-Jun phosphorylation at 5 min reperfusion in the hearts from both control and DIO rats (au C+preSP/C: 0.59±0.03/1.00±0.06, D+preSP/D: 0.77±0.03/1.08±0.14, $p < 0.05$, respectively, Fig 41). However, this inhibition was not sustained through 10 and 30 min reperfusion (Fig 41).

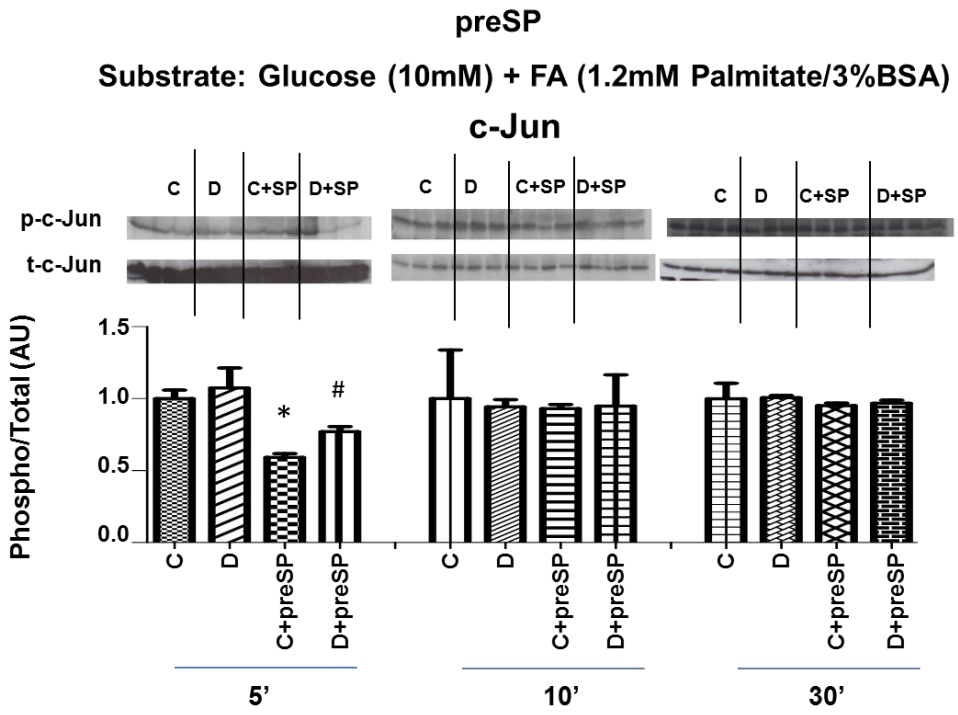


Fig 41. Effects of JNK inhibitor SP on c-Jun expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group pretreated with SP or without treatment. AU: arbitrary units.
Substrate: Glucose (10mM)+ FA (1.2mM Palmitate/3%BSA). * p < 0.05 vs C,
n = 3-4 per group.

PostSP in the presence of glucose with a high concentration of fatty acid as substrates, inhibited c-Jun phosphorylation at 5 min reperfusion in the hearts from both control and DIO rats (au C+postSP/C: 0.81±0.08/1.00±0.06; D+postSP/D: 0.52±0.10/1.08±0.14, $p < 0.05$, respectively, Fig 42). Surprisingly, in the hearts from DIO rats, this inhibition was still observed at 10 min reperfusion (au D+postSP/D: 0.75±0.03/1.06±0.11, $p < 0.05$, Fig 42).

In conclusion, our results showed that SP600125 at a concentration of 10 uM administrated either before ischaemia or during early reperfusion after ischaemia causes a significant reduction in c-Jun phosphorylation during early reperfusion regardless of the substrate. c-Jun, is a target of downstream transcription factor of JNK. Selective inhibitors of JNK have only very recently been developed and have not been used widely in the study of I/R injury. SP600125 (anthrax [1,9-cd]pyrazole-6 (2H)-one), is a small-molecule, cell-permeable, selective and reversible ATP-competitive JNK inhibitor. Based on a literature review and our results, we decided to use SP600125 at a concentration of 10 uM in our experiments.

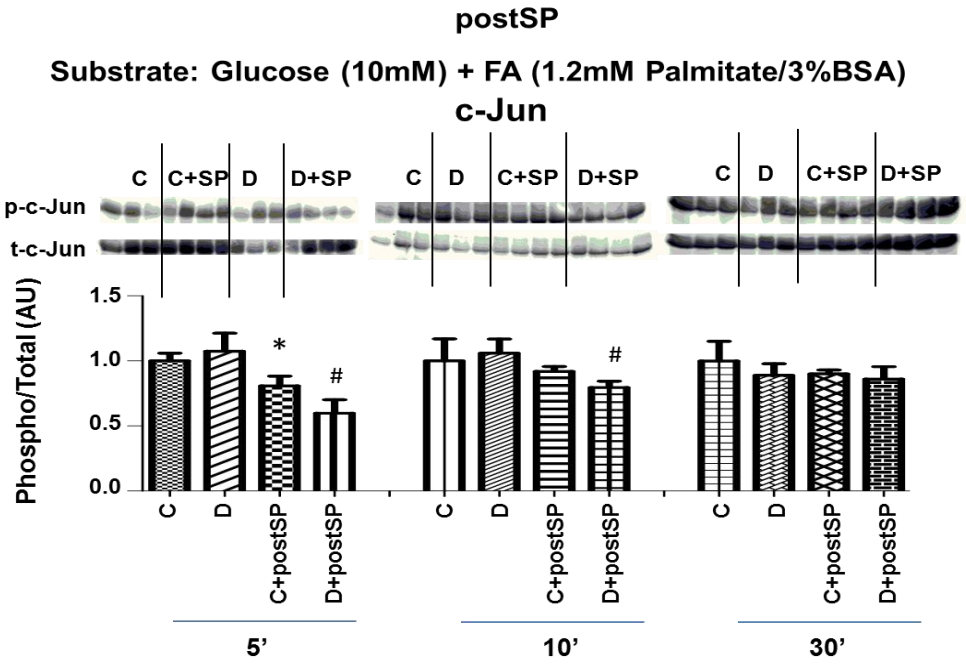


Fig 42. Effects of JNK inhibitor on c-Jun expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group posttreated with SP or without treatment. AU: arbitrary units. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA). * p < 0.05 vs C, # p < 0.05 vs D, respectively. n = 3-4 per group.

5.2. Effects of SP600125 on I/R injury

Substrate: glucose

5.2.1. Pretreatment with SP600125

5.2.1.1. Functional parameters from both DIO and control groups

Comparison of postischaemic vs preischaemic function

As mentioned before, in the presence of glucose alone as substrate without SP treatment, exposure of the hearts from both DIO and control rats to 15 min sustained global ischaemia followed by 30 min reperfusion (see protocol II), caused a significant reduction in AO, CO and TW compared to the values obtained before ischaemia, but was without effect on the PSP and HR in both groups (table 3). Pretreatment of control hearts with SP had no effect on thr reduction in mechanical recovery during reperfusion, while a significant further reduction in AO, CO and TW was observed in the DIO hearts when pretreated with SP

Table 3

Effects of JNK inhibitor SP600125 (SP) pretreatment on functional recovery

Substrates: Glucose (10mM)

		CF	AO	CO	PSP	HR	TW
Pre-ischaemia	C	17.1±0.8	45.0±1.2	62.1±1.6	98±2	284±7	13.40±0.47
	D	19.3±0.8	43.0±1.0	62.3±1.5	97±1	273±8	13.42±0.38
	C+preSP	17.0±0.7	45.0±0.7	62.0±0.6	96±1	269±17	13.19±0.20
	D+preSP	19.5±2.3	45.7±1.4	65.2±3.3	95±1	276±12	13.18±0.42
Post-ischaemia	C	15.0±1.1	22.2±2.4*	37.1±3.1*	88±2	280±7	7.53±0.72*
	D	18.1±0.7	28.1±1.4*	46.3±1.5*	91±1	262±7	9.34±0.34*
	C+preSP	15.0±1.7	21.7±5.4#	36.7±6.9#	88±2	241±21	7.03±1.32#
	D+preSP	15.3±1.3&	18.0±5.4#&	33.3±5.0#&	84±3	215±45	6.13±1.14#&

* p < 0.05 vs pre-ischaemic C or D, respectively
# p < 0.05 vs pre-ischaemic C+preSP or D+preSP, respectively
& p < 0.05 vs post-ischaemic D, respectively

Data are expressed as means ± SE. n= 5-6 per group

Abbreviations see Protocol I

CF: coronary flow (ml/min) AO: aortic output (ml/min) CO: cardiac output (ml/min)
PSP: peak systolic pressure (mmHg) HR: heart rate (beats/min) TW: total work (mW)

Comparison of percentage recovery: DIO vs control

As described in Chapter II, mechanical performance during reperfusion was also expressed as a percentage of the values obtained during perfusion before induction of ischaemia. As previously observed (Fig 16a) in the presence of glucose alone as substrate, the hearts from DIO group recovered better during reperfusion after 15 min sustained global ischaemia (as indicated by the improvement of AO, CO and TW) than those of the controls.

When the hearts were pretreated with SP before ischaemia, the significant differences in AO, CO and TW during reperfusion between the DIO and control group disappeared (% recovery D+preSP/C+preSP: AO: 40.1±12.0/48.8±12.2 , CO: 52.5±8.7/59.2±11.0; TW: 46.7±8.5/53.3±9.9, $p > 0.05$, Fig 43). Postischaemic parameters of CF, PSP and HR were similar in the DIO and control group with or without pretreatment with SP (Fig 43).

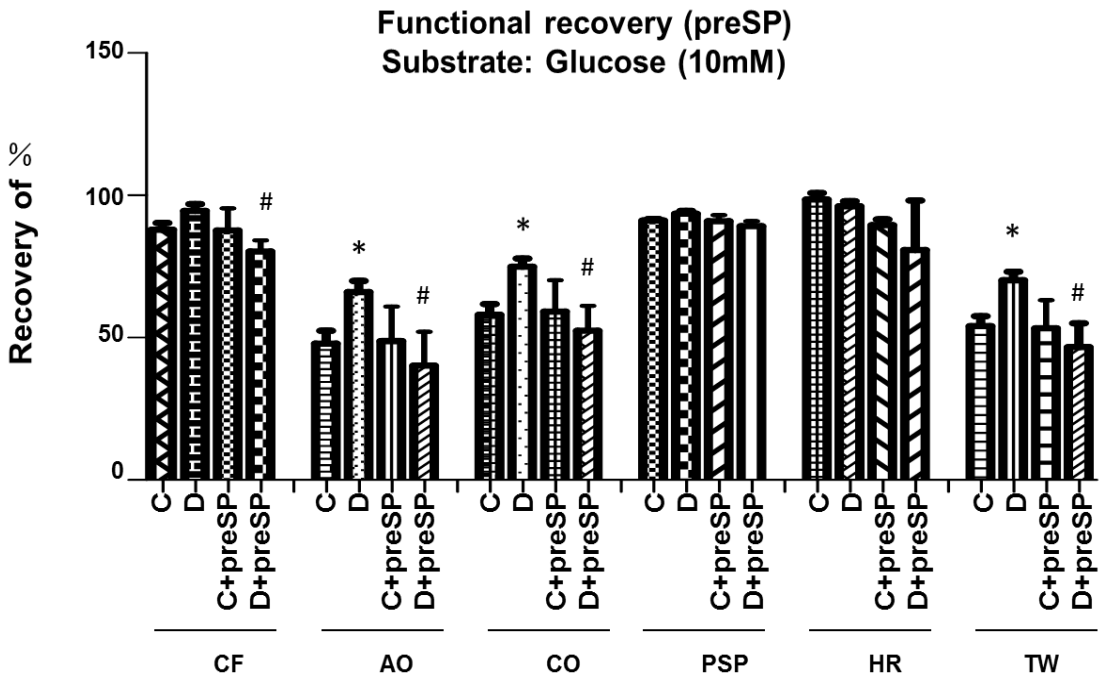


Fig 43. Comparison of % functional recovery during reperfusion of hearts pretreated with SP (preSP) before exposed to 15 min global ischaemia from control and DIO rats. Substrate: Glucose (10mM). Data are expressed as means ± SE. * p < 0.05 vs C, # p < 0.05 vs D, respectively. n= 5-6 per group.

5.2.1.2. Effect of SP600125 pretreatment on infarct size

As described before, in the presence of glucose alone as substrate, comparison of the infarct sizes in the two groups of hearts, showed that, after 35 min regional ischaemia followed by 120 min reperfusion, infarct sizes of the hearts from DIO rats were significantly smaller than those of the hearts from control rats (Fig 44).

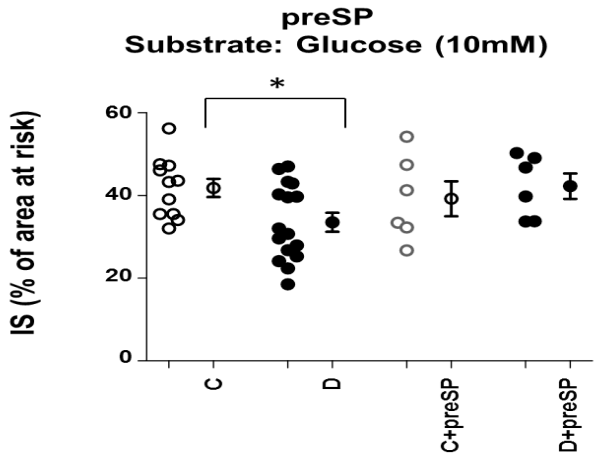
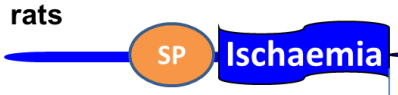


Fig 44. Comparison of % infarct sizes of hearts pretreated with SP (preSP) before exposed to 35 min regional ischaemia from control and DIO rats. Substrate: Glucose (10mM). Data are expressed as means ± SE.* p < 0.05 vs C. n = 11-14 (C, D) per group, n= 6 (C+preSP, D+preSP) per group.

When the hearts were pretreated with SP, the difference of infarct size between the DIO and control group disappeared (% IS: D+preSP/C+preSP: 38.5±3.1/39.2±4.2, $p > 0.05$, Fig 44). Furthermore, within the same group, pretreatment with SP had no effect on infarct size (C+preSP/C: 39.2±4.2/41.8±2.2, D+preSP/D: 38.5±3.1/33.5±2.3, $p > 0.05$, respectively, Fig 44).

Summary

Functional recovery and infarct size during reperfusion of hearts pretreated with SP (preSP) before exposed to global or regional ischaemia from control and DIO rats



	% of functional recovery						Infarct Size
	CF	AO	CO	PSP	HR	TW	
D/C	↑	↑	↑	≈	≈	↑	↓
D+preSP/C+preSP	≈	≈	≈	≈	≈	≈	≈
C+preSP/C	≈	≈	≈	≈	≈	≈	≈
D+preSP/D	↓	↓	↓	≈	≈	↓	≈

Substrate: Glucose (10mM)

5.2.1.3. Effects of pretreatment with SP600125 on kinase and PTEN expression and activation patterns during reperfusion

Comparison: DIO vs control group

At 5 min reperfusion, there was no difference in PKBs473 phosphorylation between the DIO and the control group (Fig 45) with or without the JNK inhibitor, but hearts from the DIO group pretreated with SP exhibited significantly higher PTEN phosphorylation (au D+preSP/C+preSP: 1.41±0.09/0.77±0.09, $p < 0.05$, Fig 46). At this time point, no difference in ERK phosphorylation was observed in these two groups (Fig 47). DIO hearts pretreated with SP presented with significantly reduced JNKp54 phosphorylation (au D+preSP/C+preSP: 0.53±0.05/0.82±0.06, $p < 0.05$, Fig 48). No difference in p38 MAPK phosphorylation at 5 min reperfusion was observed in these two groups (Fig 49).

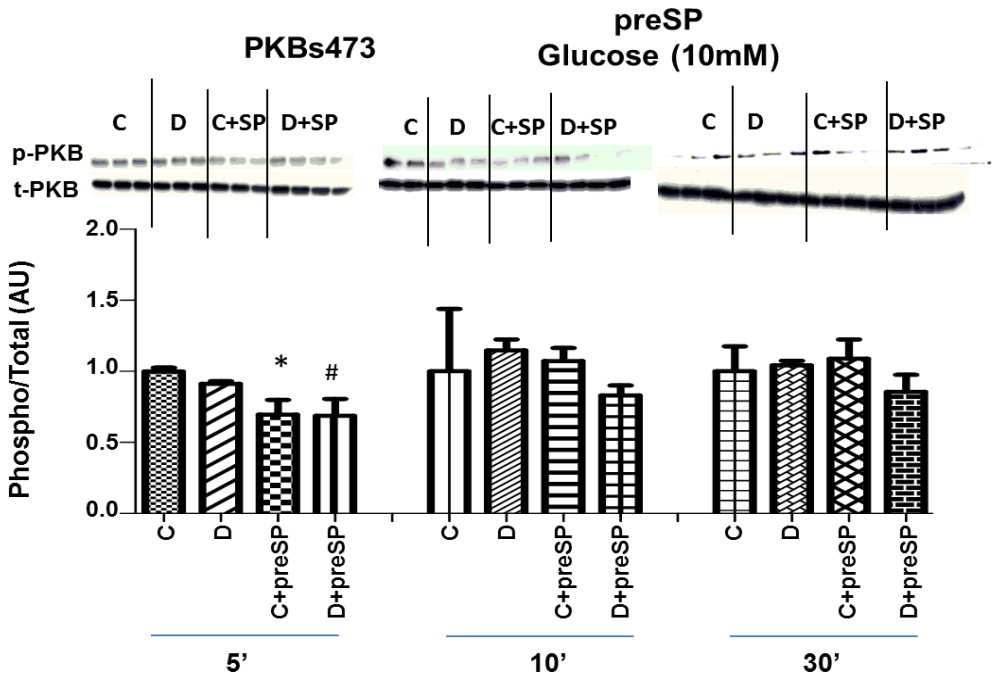


Fig 45. Effect of preSP on PKBs473 expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. All values were expressed as the ratio between phospho/total arbitrary densitometry units (AU) and were normalized to control hearts as one. Substrate: Glucose (10mM). * $p < 0.05$ vs C; # $p < 0.05$ vs D, respectively. n = 3-4 per group.

At 10 min reperfusion, the differences in PTEN and JNKp54 phosphorylation disappeared, and ERKp44/p42 phosphorylation at this time point still remained the same between the treated groups (Fig 47). Interestingly, at 30 min reperfusion, no difference in PKBs473 phosphorylation was observed, but an increased PTEN phosphorylation appeared again in the DIO group (au D+preSP/C+preSP: 1.66±0.22/ 0.82±0.04, $p < 0.05$, Fig 46). After 30 min reperfusion, no differences in ERKp44/p42, JNKp54/p46 or p38 MAPK phosphorylation were observed between the DIO and control groups (Figs 47- 49). The pretreatment of the hearts with SP had no effects on total expression of the proteins at all reperfusion time points.

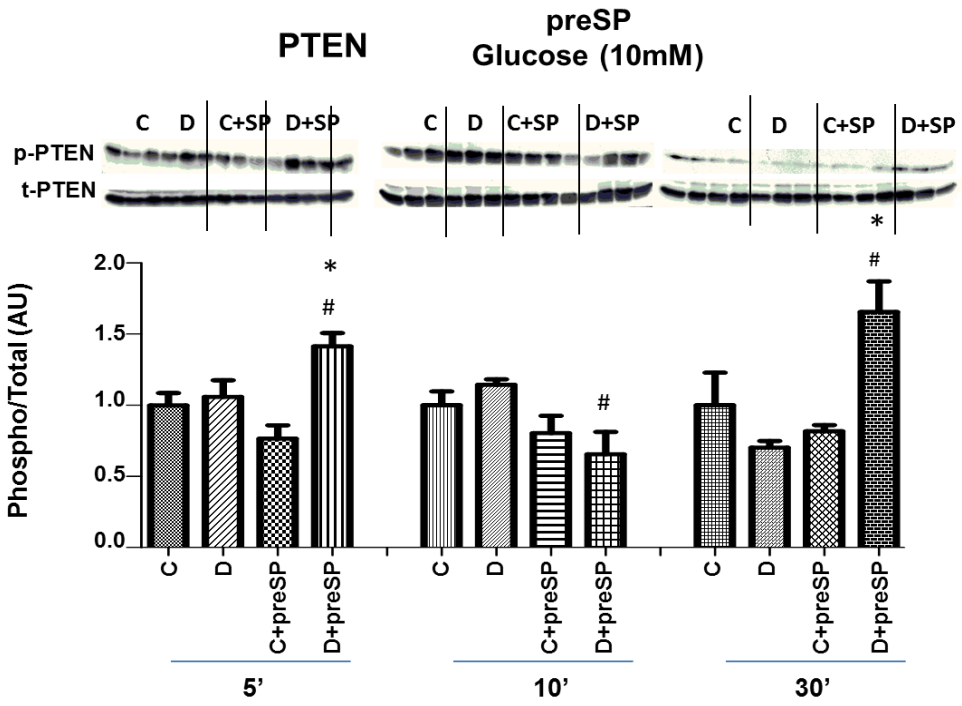


Fig 46. Effect of preSP on PTEN expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrate: Glucose (10mM).
*** p < 0.05 vs C+preSP; # p < 0.05 vs D, respectively. n = 3-4 per group.**

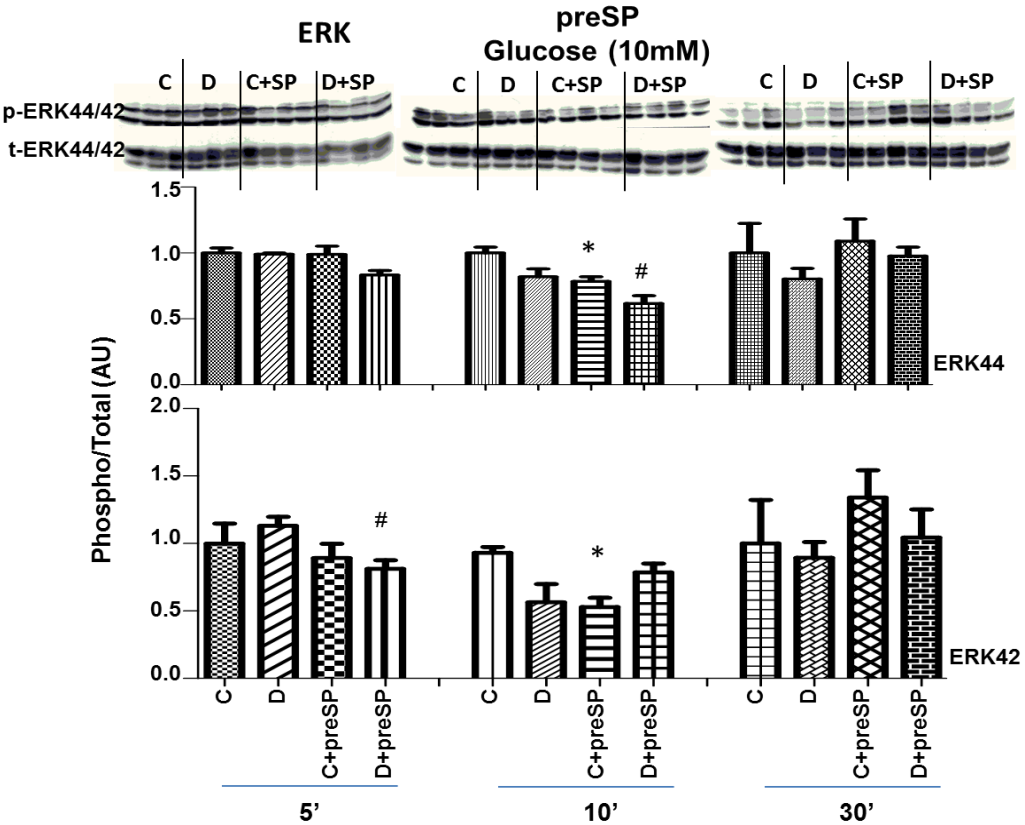


Fig 47. Effect of preSP on ERK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group.
AU: arbitrary units. Substrate: Glucose (10mM).
*** p < 0.05 vs C; # p < 0.05 vs D; & p < 0.05 vs C+preSP, respectively. n = 3-4 per group.**

Effects of SP600125 on hearts from control rats

PreSP significantly decreased the phosphorylation of PKBs473 and JNKp46 at 5 min reperfusion compared to the untreated control group (au C+preSP/C: PKBs473: 0.70±0.10/1.00±0.03, JNKp46: 0.60±0.07/1.00±0.05, $p < 0.05$, Figs 45,48). Neither ERK nor p38 MAPK phosphorylation were affected (Figs 47,49).

At 10 min reperfusion, the decreased phosphorylation of PKBs473 and JNKp46 disappeared (Fig 48), but JNKp54 phosphorylation as well as ERKp44/p42 phosphorylation were significantly reduced (au C+preSP/C: JNKp54: 0.65±0.14/1.00±0.12, ERKp44: 0.78±0.04/1.00±0.04, ERKp42: 0.53±0.07/1.00±0.06, $p < 0.05$, Fig 47,48), with no changes in p38 MAPK phosphorylation.

However, after 30 min reperfusion, pretreatment with SP was without effect on all kinases and PTEN phosphorylation (Figs 45-49).

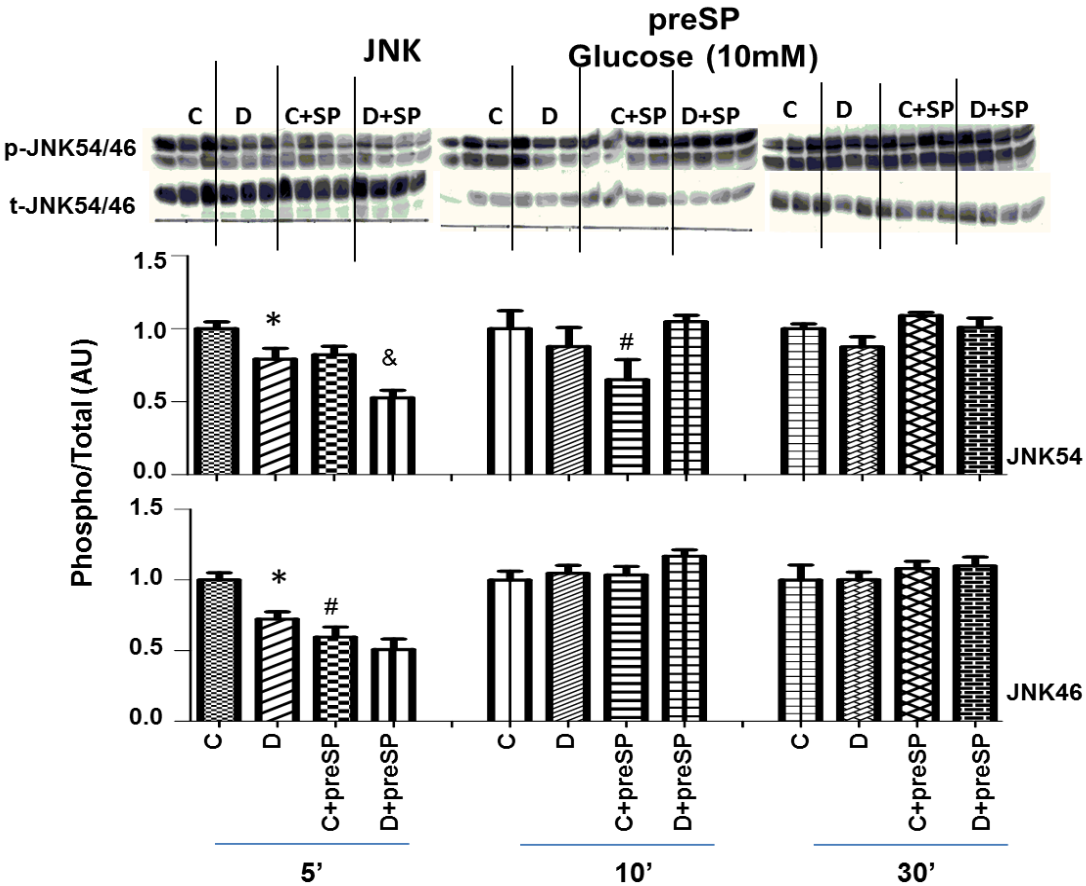


Fig 48. Effect of preSP on JNK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrate: Glucose (10mM).
***# $p < 0.05$ vs C, respectively; & $p < 0.05$ vs C+preSP. n = 3-4 per group.**

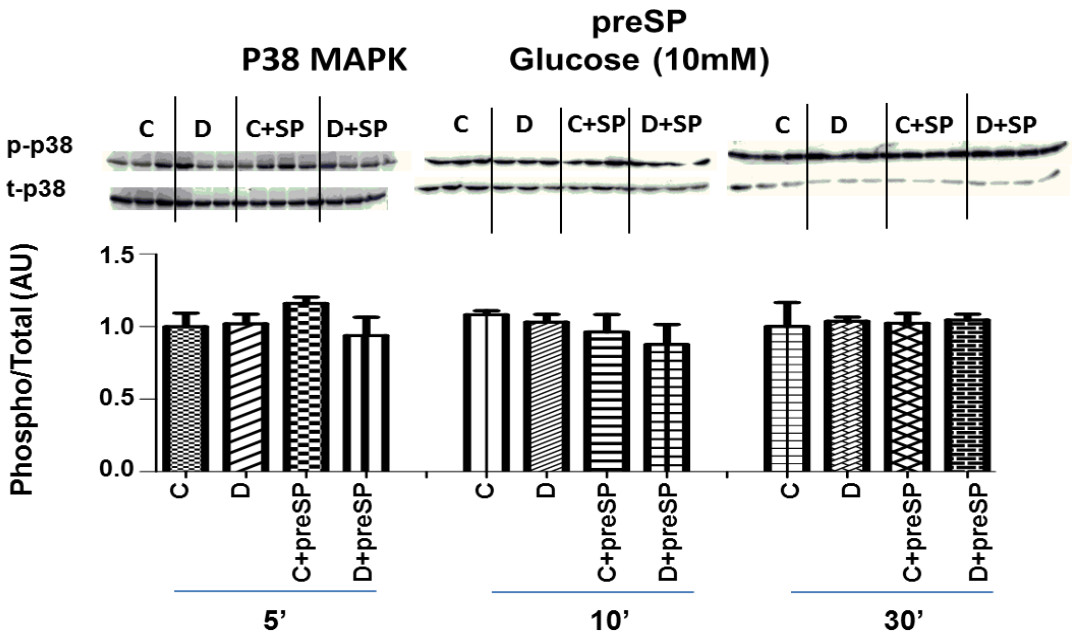


Fig 49. Effect of preSP on p38 MAPK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrate: Glucose (10mM). n = 3-4 per group.

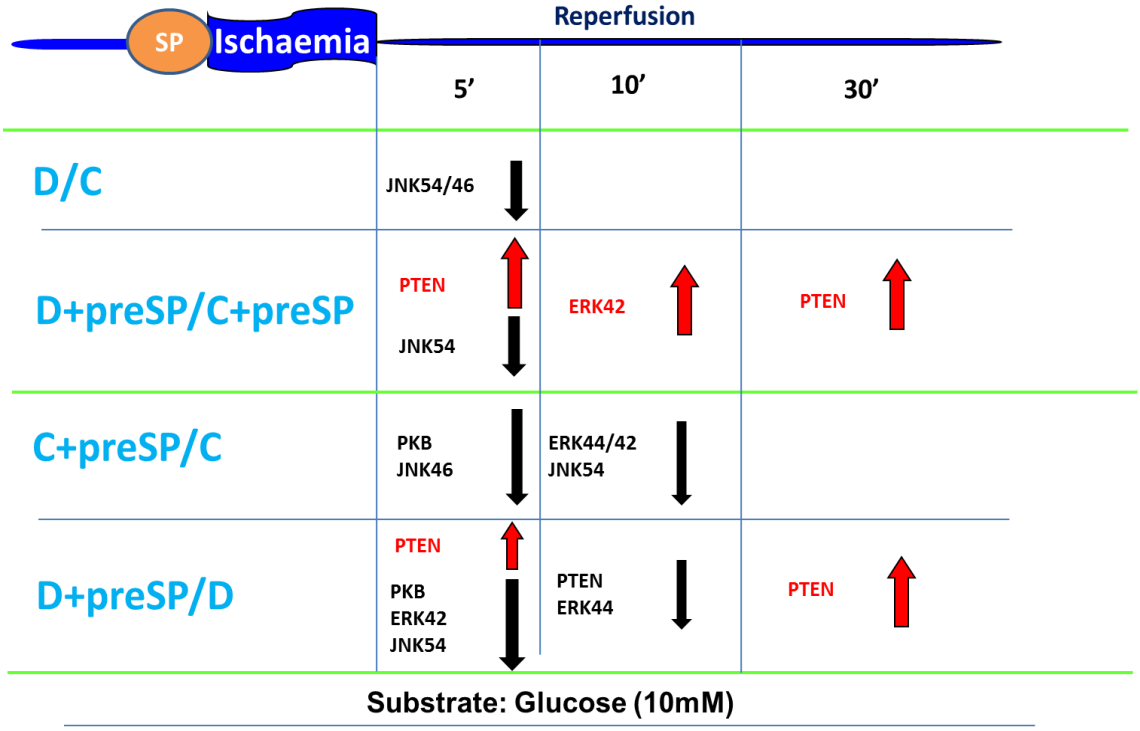
Effects of SP600125 on hearts from DIO rats

PreSP significantly reduced the phosphorylation of PKBs473 and ERKp42 at 5 min reperfusion compared to the untreated DIO group (au D+preSP/D, PKBs473: 0.69±0.12/0.91±0.02, ERKp42: 0.81±0.06/1.13±0.07, $p < 0.05$, respectively, Figs 45,47), but no change in ERKp44 phosphorylation was observed at this time point (Fig 47). PTEN phosphorylation was increased compared to the untreated DIO group (au D+preSP/D, 1.41±0.09/1.06±0.12, $p < 0.05$, Fig 46) while JNKp54/p46 phosphorylation was not decreased by SP pretreatment (Fig 48).

At 10 min reperfusion, the decrease in PKBs473 phosphorylation disappeared, and in contrast to 5 min reperfusion, the phosphorylation of ERKp44, but not ERKp42 was significantly reduced (au D+preSP/D: ERKp44: 0.62±0.06/0.82±0.06, $p < 0.05$, Fig 47). Interestingly, at 10 min reperfusion, in contrast to 5 min reperfusion, PTEN phosphorylation was markedly reduced compared to the untreated DIO group (au D+preSP/D: 0.66±0.16/1.14±0.04, $p < 0.05$, Fig 46) with no changes observed in either JNK or p38 MAPK phosphorylation. At 30 min reperfusion, PTEN phosphorylation was increased again (au 1.66±0.22/0.70±0.05, $p< 0.05$, Fig 46) with no noticeable changes in PKBs473, ERK or JNK (Figs 45-49).

Summary

Protein phosphorylation during reperfusion of hearts pretreated with SP (preSP) before exposed to global ischaemia from control and DIO rats



5.2.2. Posttreatment with SP600125

Substrate: glucose

5.2.2.1. Functional parameters from both DIO and control groups

Comparison: postischaemic vs preischaemic function

As previously observed, the results obtained showed that 15 min sustained global ischaemia followed by 30 min reperfusion caused a significant reduction in AO, CO and TW in the hearts from both DIO and control rats, but was without effect on the CF, PSP and HR in both groups (table 4).

Posttreatment with the JNK inhibitor did not affect this reduction in AO, CO and TW but, in addition, also resulted in lower CF and HR in hearts from control rats (table 4). In contrast, posttreatment with SP caused a significant reduction in AO, CO and TW during reperfusion of hearts from DIO rats, when compared to their untreated counterparts.

Table 4

Effects of JNK inhibitor SP600125 (SP) treated during reperfusion (postSP) on functional recovery

Substrates: Glucose (10mM)

		CF	AO	CO	PSP	HR	TW
Pre-ischaemia	C	17.1±0.8	45.0±1.2	62.1±1.6	98±2	284±7	13.40±0.47
	D	19.3±0.8	43.0±1.0	62.3±1.5	97±1	273±8	13.42±0.38
	C+postSP	17.4±1.1	48.8±3.0	66.2±4.0	89±3	275±10	13.31±0.81
	D+postSP	17.1±1.2	45.2±2.0	62.3±2.9	93±1	245±13	12.71±0.71
Post-ischaemia	C	15.0±1.1	22.2±2.4*	37.1±3.1*	88±2	280±7	7.53±0.72*
	D	18.1±0.7	28.1±1.4*&	46.3±1.5*&	91±1	262±7	9.34±0.34 *&
	C+postSP	12.9±1.0#	15.0±5.8#	27.9±5.6#	80±3	240±7#	5.04±1.18#
	D+postSP	15.3±1.7	20.0±3.8#$	35.3±4.7#$	86±3	239±36	6.76±0.82#$

* p < 0.05 vs pre-ischaemic C or D, respectively
\# p < 0.05 vs pre-ischaemic C+postSP or D+postSP, respectively
& p < 0.05 vs post-ischaemic C, respectively
$ p < 0.05 vs post-ischaemic D, respectively

Data are expressed as means ± SE. n= 5-6 per group.

Abbreviations see Protocol I, Table 1

CF: coronary flow (ml/min) AO: aortic output (ml/min) CO: cardiac output (ml/min)
PSP: peak systolic pressure (mmHg) HR: heart rate (beats/min) TW: total work (mW)

Comparison of percentage recovery: DIO vs control

As described before, the hearts from the DIO group recovered better during reperfusion after 15 min sustained global ischaemia (as indicated by the improvement of AO, CO and TW) than those of the controls (% recovery D/C, AO: 66.1±3.8/48.0±4.5, CO: 74.9±2.9/58.1±3.7, TW: 70.2±3.1/54.0±3.5, $p < 0.05$, Fig 50). When the hearts were posttreated with SP for 10 min after ischaemia, the differences in AO, CO and TW during reperfusion between the DIO and control group disappeared (% recovery D+postSP/C+postSP, AO: 49.0±7.6/38.4±11.7, CO: 60.2±6.2/48.8±9.4, TW: 56.5±5.4/42.9±8.8, $p > 0.05$, Fig 50). The CF, PSP and HR did not differ between the DIO and control group (Fig 50).

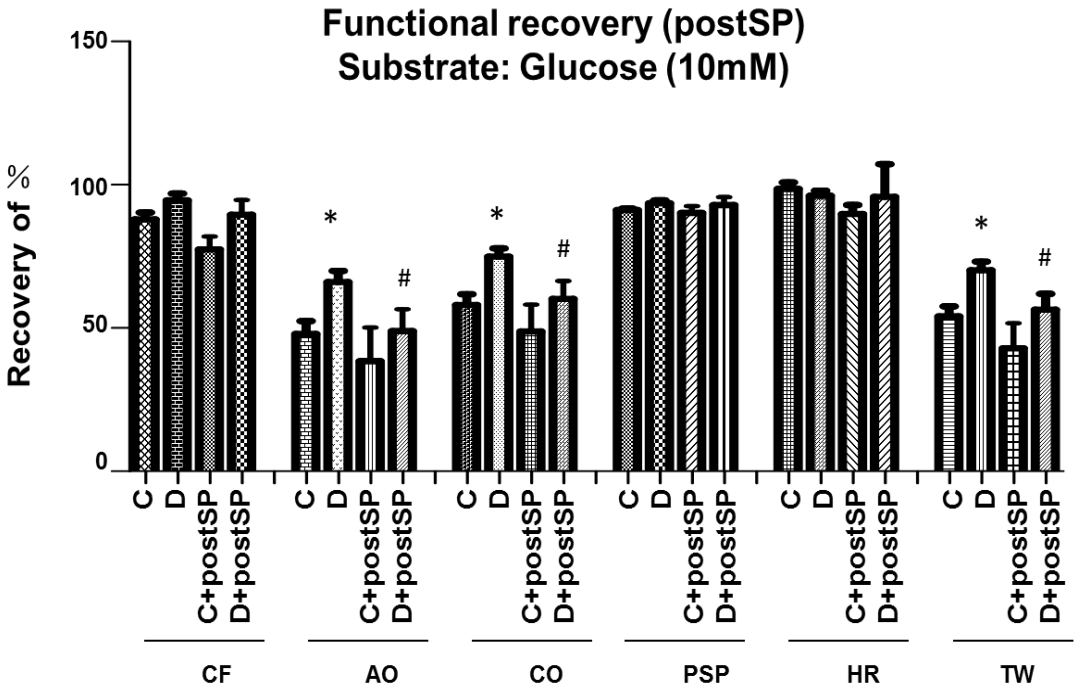
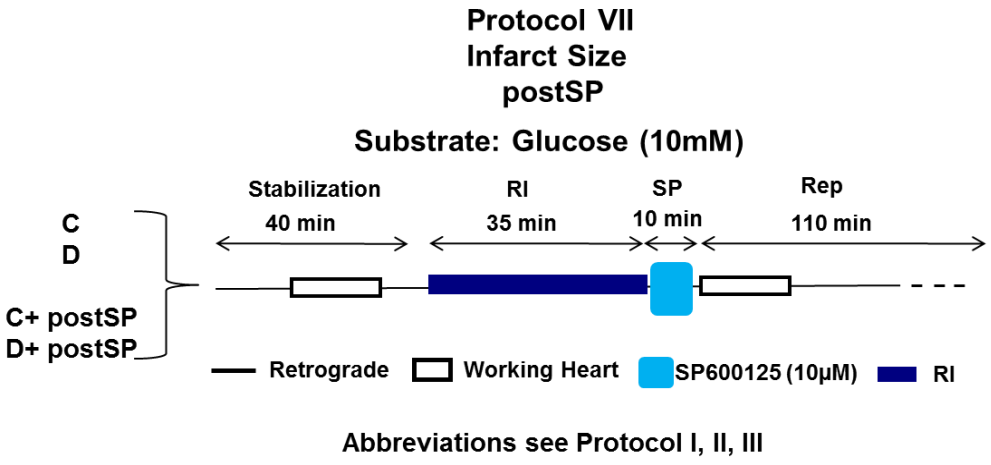


Fig 50. Comparison of % functional recovery during reperfusion of hearts treated with SP (postSP) after exposed to 15 min global ischaemia from control and DIO rats. Substrate: Glucose (10mM). Data are expressed as means ± SE.
*** p < 0.05 vs C, respectively; # p < 0.05 vs D, respectively. n= 5-6 per group.**

5.2.2.2. Effects of SP600125 posttreatment on infarct size

The inhibitor was administered according to protocol VII.



Abbreviations see Protocol I, II, III

As described before in the presence of glucose alone as substrate, infarct sizes of the hearts from DIO rats were significantly smaller than those of hearts from control rats (% IS: D/C 34.5±2.2/41.8±2.2, $p < 0.05$, Fig 51).

PostSP abolished this decrease in infarct size (% IS: D+postSP/C+postSP:

40.1±2.4/38.2±3.5, p > 0.05, Fig 51) with hearts from the DIO animals now presenting with significantly large infarct size (% IS D+postSP/D: 40.1±2.4/34.5±2.2, p < 0.05, Fig 51). In contrast, postSP had no effect on infarct sizes of hearts from control rats (% IS C+postSP/C: 38.2±3.5/41.8±2.2, p > 0.05, Fig 51).

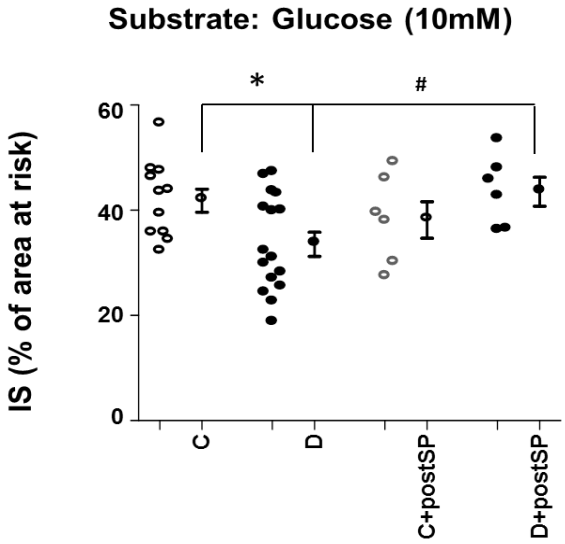


Fig 51. Comparison of % infarct sizes of hearts treated with SP (postSP) after exposed to 35 min regional ischaemia from control and DIO rats. Substrate: Glucose (10mM). Data are expressed as means ± SE.
*** p < 0.05 vs C; # p < 0.05 vs D. n = 11 (C), 16 (D) per group, n = 6 (C+postSP, D+postSP) per group.**

Summary

Functional recovery and infarct size of hearts treated with SP during reperfusion (postSP) after exposed to global or regional ischaemia from control and DIO rats

	% of functional recovery						Infarct Size
	CF	AO	CO	PSP	HR	TW	
D/C	≈	↑	↑	≈	≈	↑	↓
D+post/C+postSP	≈	≈	≈	≈	≈	≈	≈
C+post/C	≈	≈	≈	≈	≈	≈	≈
D+post/D	≈	↓	↓	≈	≈	↓	↑

Substrate: Glucose (10mM)

5.2.2.3. Effects of SP600125 posttreatment on kinase and PTEN expression and activation patterns during reperfusion

Comparison: DIO vs control group

PostSP, at 5 min reperfusion, did not change PKBs473 phosphorylation in the hearts from control group (Fig 52), but caused a stimulation in the DIO hearts. PTEN phosphorylation, on the other hand, was significantly increased by PostSP in both groups (Fig 53). A marked reduction in ERKp44/p42 was observed in hearts from DIO group (au D+postSP/C+postSP ERKp44: 0.49±0.07/0.87±0.07, ERKp42: 0.55±0.03/0.75±0.06, respectively, $p < 0.05$, Fig 54). At 5 min reperfusion, no differences in JNK phosphorylation were observed in both control and DIO groups (Figs 55). However at this time point a marked activation of MAPK phosphorylation was seen in both groups (Fig 56).

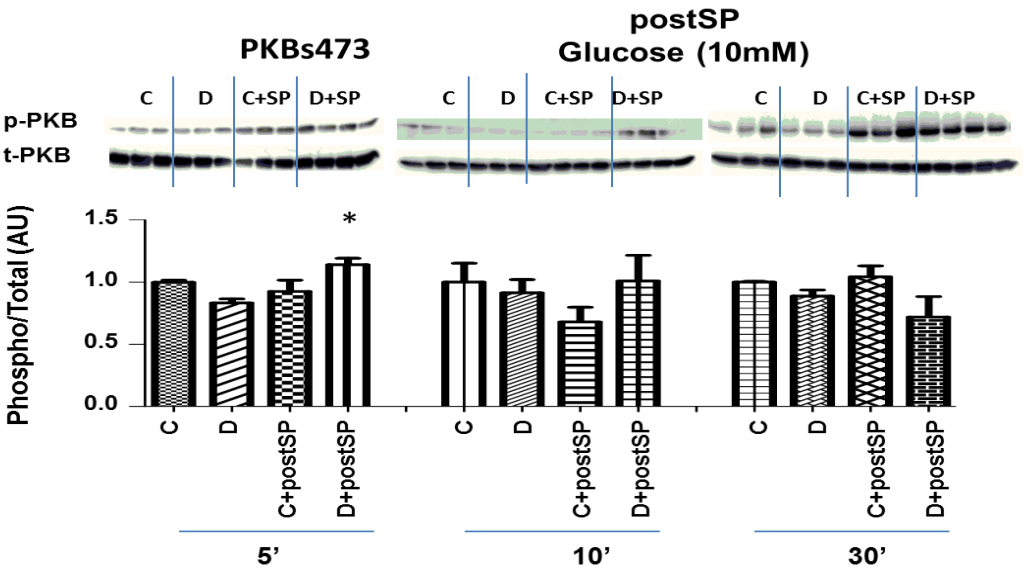


Fig 52. Effect of postSP on PKBs473 expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. All values were expressed as the ratio between phospho/total arbitrary densitometry units (AU) and were normalized to control hearts as one. Substrate: Glucose (10mM). * $p < 0.05$ vs D. n = 3-4 per group.

At 10 minutes reperfusion, although PKBs473, PTEN and ERKp44/p42 phosphorylation did not change in the hearts from both groups (Fig 52-54), the hearts posttreated with SP from DIO group exhibited significantly higher JNKp54 phosphorylation (au 0.71±0.11/0.38±0.07, $p < 0.05$, Fig 55). Furthermore, no difference in p38 MAPK phosphorylation was observed at 10 min reperfusion (Fig 56). Interestingly, at 30 min reperfusion, a marked decrease of JNKp46 phosphorylation was observed in the hearts from the DIO rats (au 0.74±0.05/1.31±0.21, $p < 0.05$, Fig 55), but no differences in the phosphorylation of the other kinases were observed between the DIO and control groups.

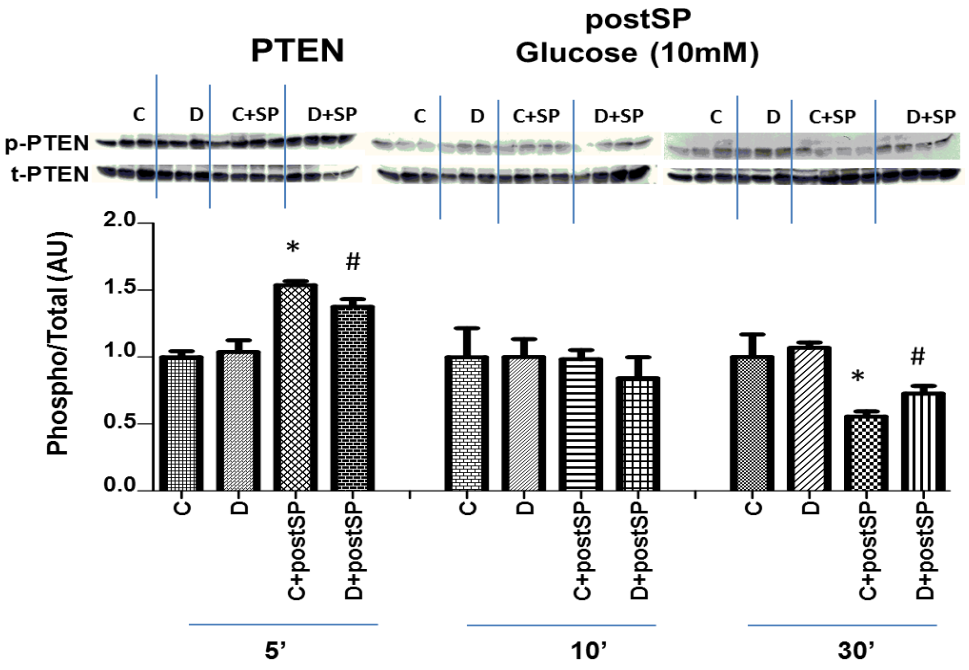


Fig 53. Effect of postSP on PTEN expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrate: Glucose (10mM).
*** p < 0.05 vs C; # p < 0.05 vs D. n = 3-4 per group.**

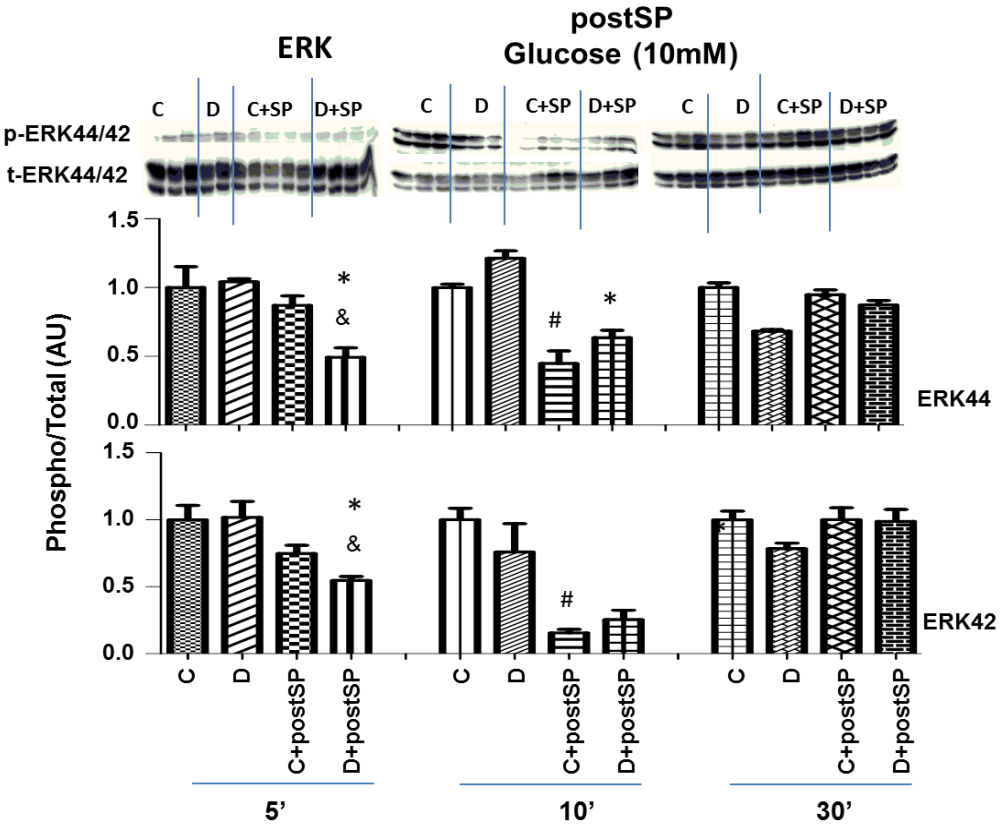


Fig 54. Effect of postSP on ERK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrate: Glucose (10mM). * p < 0.05 vs D; & p < 0.05 vs C+postSP, # p < 0.05 vs C, respectively. n = 3-4 per group.

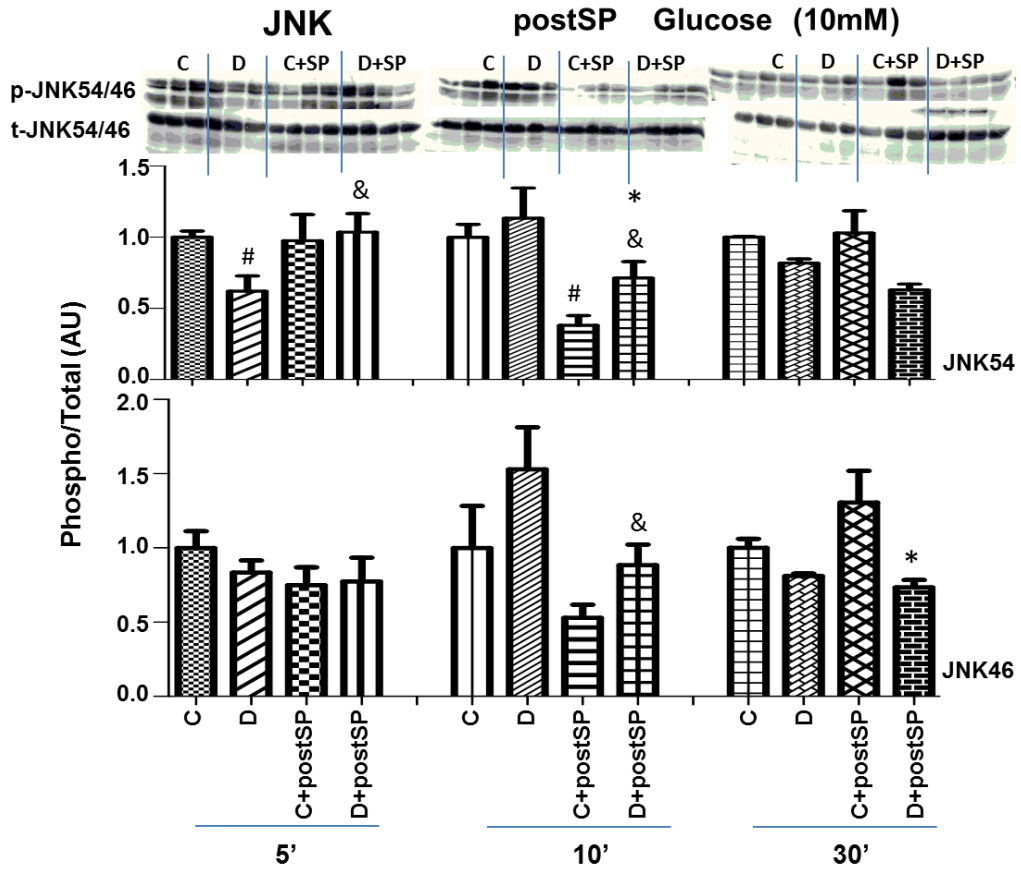


Fig 55. Effect of postSP on JNK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrate: Glucose (10mM). # p < 0.05 vs C; * p < 0.05 vs C+postSP; & p < 0.05 vs D, respectively. n = 3-4 per group.

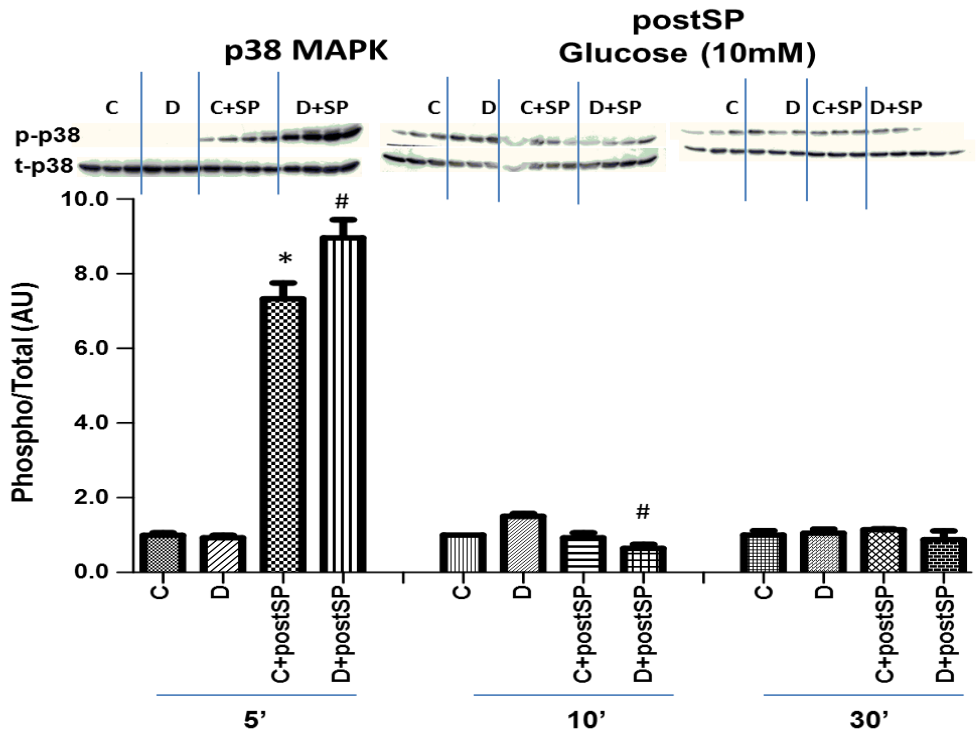


Fig 56. Effect of postSP on p38 MAPK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrate: Glucose (10mM).
*** p < 0.05 vs C; # p < 0.05 vs D. n = 3-4 per group.**

Effects of SP600125 posttreatment on hearts from control animals

Posttreatment with SP did not change PKBs473 phosphorylation from 5 to 30 min reperfusion, but it significantly increased PTEN phosphorylation at 5 min reperfusion compared to those of untreated hearts (au C+postSP/C, 1.54±0.03/1.00±0.04, $p < 0.05$, Fig 53). There were no differences in ERKp44/42 and JNKp54/46 phosphorylation at 5 min reperfusion in the control groups (Figs 54,55). Surprisingly, the hearts posttreated with SP markedly increased p38 MAPK phosphorylation at this time point (au C+postSP/C, 7.33±0.42/1.00±0.06, $p < 0.05$, Fig 56).

However, at 10 min reperfusion, the difference in PTEN phosphorylation disappeared but marked decreases in ERKp44/p42 and JNKp54 phosphorylation were observed with postSP (au C+postSP/C, ERKp44: 0.45±0.09/1.00±0.02; ERKp42: 0.17±0.03/1.00±0.09, JNKp54: 0.38±0.07/1.00±0.09, $p < 0.05$, respectively, Figs 54,55). However, the difference in p38 MAPK phosphorylation disappeared at this time point.

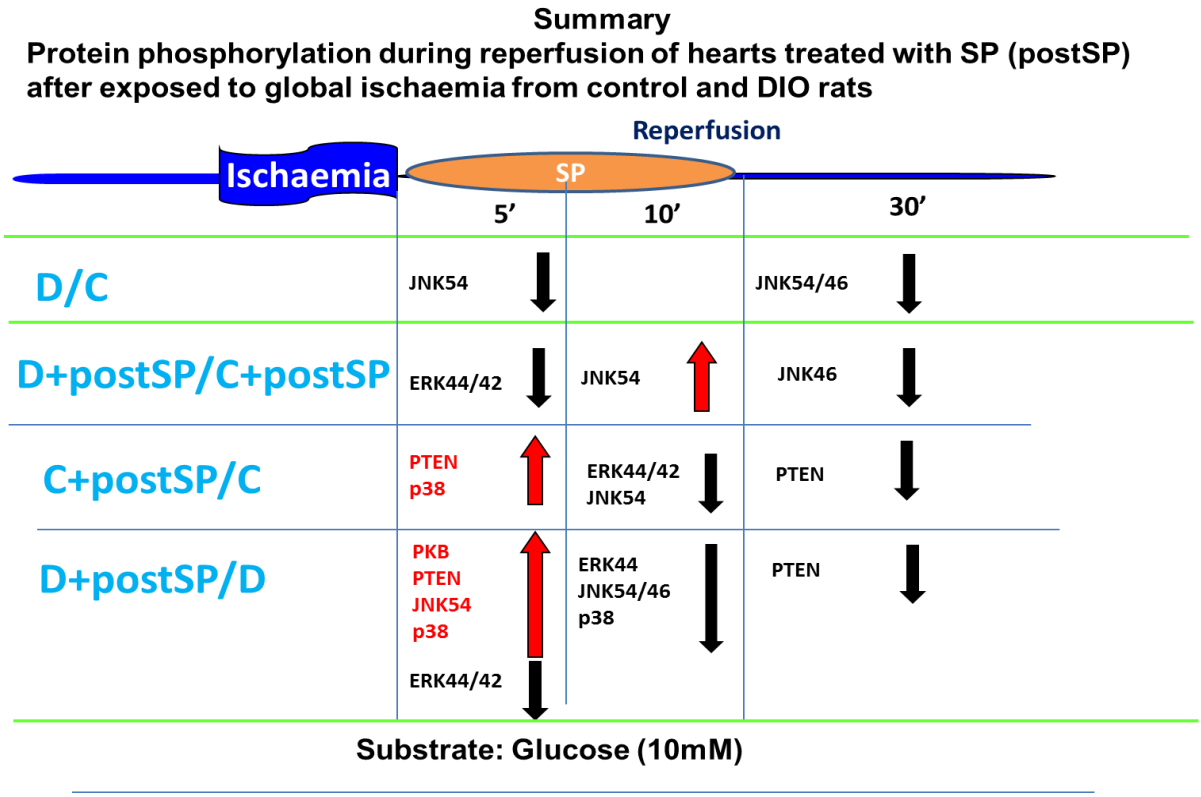
After 30 min reperfusion, posttreatment with SP caused a significant reduction in PTEN phosphorylation (au C+postSP/C: 0.56±0.04/1.00±0.17, $p < 0.05$, Fig 53), but no differences in the phosphorylation of other kinases (Figs 54-56).

Effects of SP600125 posttreatment on hearts from DIO animals

Posttreatment of hearts with SP not only resulted in a significantly increased phosphorylation of PKBs473 and PTEN (au D+postSP/D, PKBs473: 1.14±0.05/0.83±0.03, PTEN: 1.38±0.06/1.04±0.09, respectively, $p < 0.05$, Figs 52,53), but also a significantly reduced ERKp44/p42 phosphorylation at 5 min reperfusion compared to the untreated DIO group (au D+postSP/D, ERKp44: 0.49±0.07/1.04±0.02, ERKp42: 0.55±0.03/1.02±0.12, respectively, $p < 0.05$, Fig 54). Furthermore, JNKp54 and p38 MAPK phosphorylation was markedly increased (au D+postSP/D, JNKp54: 1.04±0.13/0.62±0.11, p38 MAPK: 8.97±0.48/0.93±0.07, respectively, $p < 0.05$, Figs 55,56).

Interestingly, at 10 min reperfusion, although the differences in PKBs473 and PTEN phosphorylation disappeared, ERK44/42 phosphorylation was still significant lower (au D+postSP/D, ERKp44: 0.64±0.05/1.21±0.05, ERKp42: 0.25±0.07/0.76±0.21, $p < 0.05$, Fig 54). Furthermore, in contrast to 5 min reperfusion, the phosphorylation of JNKp54/p46 and p38 MAPK was markedly reduced (au D+postSP/D, JNKp54: 0.71±0.11/1.13±0.21, JNKp46: 0.88±0.14/1.53±0.28, p38 MAPK: 0.64±0.11/1.51±0.07, $p < 0.05$, respectively, Figs 55,56).

At 30 min reperfusion, postSP did not change PKBs473 phosphorylation, but PTEN phosphorylation was markedly reduced, in contrast to the elevated values obtained at 5 min reperfusion, (au D+postSP/D, 0.73±0.06/1.07±0.04, $p < 0.05$, Fig 53). No differences in the phosphorylation of other kinases were observed in the DIO groups (Figs 54-56).



5.3. Effects of SP600125

Substrate: glucose plus fatty acid

5.3.1. Effects of pretreatment with SP600125

5.3.1.1. Functional parameters from both DIO and control groups

Comparison: postischaemic vs preischaemic function

The results obtained showed that in the presence of glucose and a high concentration of fatty acid without SP treatment, exposure to 15 min sustained global ischaemia followed by 30 min reperfusion caused an increase in CF in the hearts from DIO rats during reperfusion compared to the preischaemic values (postischaemic/preischaemic D: 24.1±2.0/19.2±0.7, $p < 0.05$, Table 5). Although CF of hearts from control rats was also increased, the difference was not significant (Table 5). Surprisingly, in contrast to the reduction in AO and TW observed in the

control group, the postischaemic AO and TW in hearts from DIO rats did not differ from preischaemic values (Table 5). Furthermore, the CO, PSP and HR measured during reperfusion from both DIO and control groups were similar to the values obtained before ischaemia (Table 5).

Table 5

Effects of JNK inhibitor SP600125 (SP) pretreatment on functional recovery
Substrates: Glucose (10mM) + FA (1.2mM palmitate/3%BSA)

		CF	AO	CO	PSP	HR	TW
Pre-ischaemia	C	18.5±2.3	45.0±1.5	63.5±3.4	103±2	251±15	14.75±0.63
	D	19.2±0.7	44.9±1.7	64.1±2.3	102±1	276±12	14.58±0.74
	C+preSP	18.0±1.6	46.8±2.2	64.8±3.6	103±3	277±9	14.71±0.95
	D+preSP	18.8±1.0	46.5±2.4	65.3±3.4	99±2	261±8	14.29±0.90
Post-ischaemia	C	23.5±2.4	29.5±3.9*	53.6±6.3	98±1	289±7	11.81±1.60*
	D	24.1±2.0*	39.8±2.1	63.9±3.5	100±2	281±11	14.76±1.01
	C+preSP	15.3±0.5#&	26.5±4.3#	41.8±4.7#	96±4	247±4#&	8.59±1.05#&
	D+preSP	17.6±1.0&	23.6±4.3# &	41.2±4.3#&	88±3#&	245±9&	8.18±1.04#&

* $p < 0.05$ vs pre-ischaemic C or D, respectively
\# $p < 0.05$ vs pre-ischaemic C+preSP or D+preSP, respectively
& $p < 0.05$ vs post-ischaemic C or D, respectively

Data are expressed as means ± SE. n= 5-6 per group.

Abbreviations see Protocol I

CF: coronary flow (ml/min) AO: aortic output (ml/min) CO: cardiac output (ml/min)
PSP: peak systolic pressure (mmHg) HR: heart rate (beats/min) TW: total work (mW)

PreSP caused not only a reduction in AO, CO and TW in both DIO and control groups, but also decreased CF and HR in the controls when compared to the values obtained before ischaemia (Table 5). In contrast to the response of the control animals, PSP in hearts pretreated with SP from the DIO group was also markedly lower compared to the preischaemic values (Table 5). In summary, pretreatment with SP caused a reduction in AO, CO and TW in both DIO and control groups.

Comparison of percentage recovery: DIO vs control

Similar to what was found without SP treatment, the hearts from DIO animals recovered better during reperfusion after 15 min sustained global ischaemia, indicated by the improvement in AO and TW (AO: 39.8±2.1/29.5±3.9, TW:

14.76±1.01/11.81±1.60, p < 0.05, respectively, Table 5, Fig 57). With preSP, the differences in AO and TW between DIO and control hearts disappeared (Fig 57). In addition, postischaemic recovery of CF, CO, PSP and HR was similar in the two groups when pretreated with SP. (Fig 57).

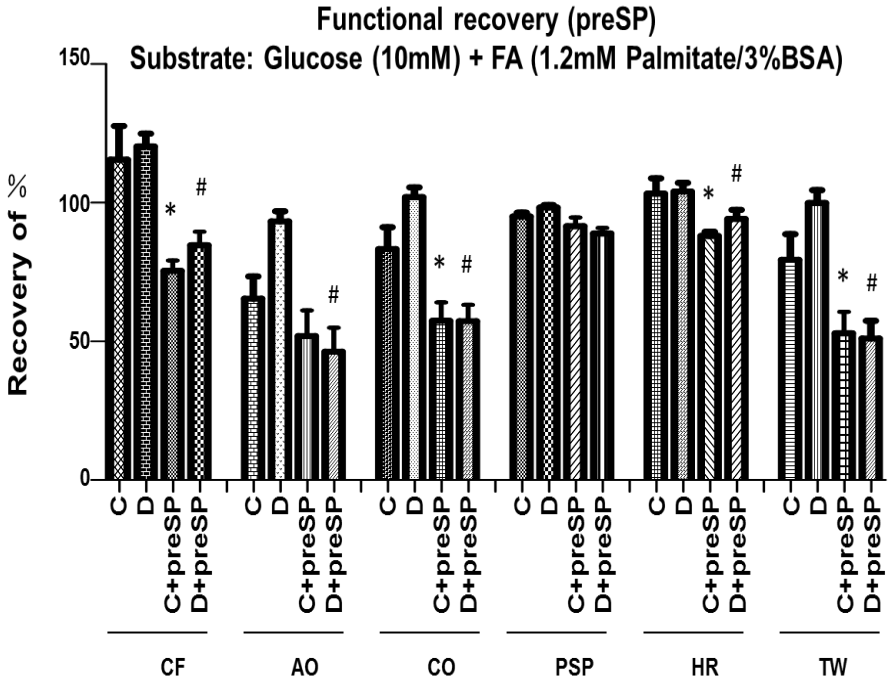


Fig 57. Comparison of % functional recovery during reperfusion of hearts pretreated with SP (preSP) before exposed to 15 min global ischaemia from control and DIO rats. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA). Data are expressed as means ± SE. * p < 0.05 vs C, respectively; # p < 0.05 vs D, respectively. n= 5-6 per group.

Comparison of percentage recovery: effects of SP600125

Control group

In control hearts, pretreatment with SP significantly reduced CF, HR and TW compared to its untreated counterparts (% recovery: CF 75.5±3.8/115.6±12.1, HR 88.1±1.5/103.3±5.6, TW 53.0±7.7/85.9±7.6, respectively, p < 0.05, Fig 57). However, AO and CO were not significantly different from its group without SP pretreatment (Fig 57).

DIO group

In the DIO group, preSP significantly reduced all postischaemic functional recovery parameters during reperfusion compared to untreated hearts (% recovery, CF: 84.7±4.8/120.4±4.6; AO: 46.3±8.6/93.4±3.6; CO: 57.3±5.9/102.1±3.5; PSP: 88.9±2.0/98.3±0.9; TW: 51.1±6.3/100.0±4.6; respectively, p < 0.05, Fig 57).

5.3.1.2. Effect of SP600125 pretreatment on infarct size

As described before in the presence of glucose with a high concentration of fatty acid as substrates, infarct sizes of the hearts from DIO rats were similar to that of the hearts from control rats (% IS: D/C, 31.4±3.3/ 34.4±1.8, p > 0.05, Fig 58).

PreSP did not affect infarct size in any of the hearts when compared to the untreated groups (% IS, C+preSP/C: 33.0±2.7/34.4±1.8, D+preSP/D: 28.4±1.2/31.4±3.3, p > 0.05, respectively, Fig 58).

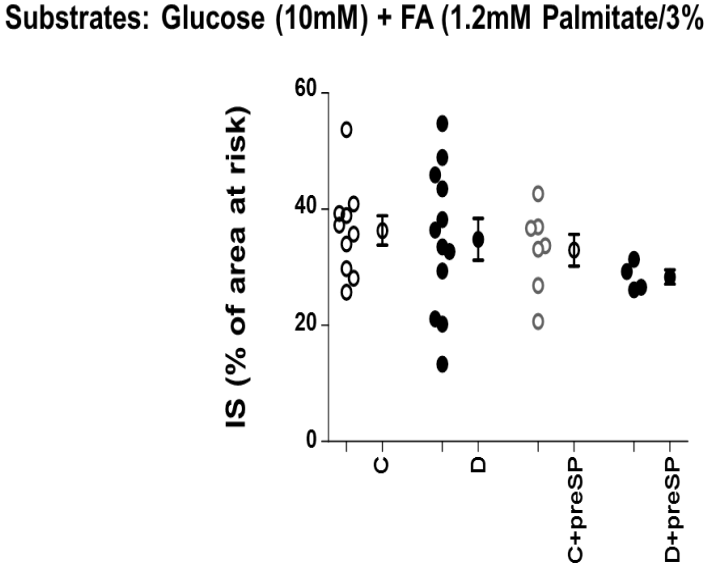


Fig 58. Comparison of % infarct sizes of hearts pretreated with SP (preSP) before exposed to 35 min regional ischaemia from control and DIO rats. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA). Data are expressed as means ± SE. n = 10 (C), 12 (D) per group, n = 7 (C+preSP, D+preSP) per group.

Summary

Functional recovery and infarct size during reperfusion of hearts pretreated with SP (preSP) before exposed to global or regional ischaemia from control and DIO rats

	% of functional recovery						Infarct Size
	CF	AO	CO	PSP	HR	TW	
D/C	≈	↑	≈	≈	≈	↑	≈
D+preSP/C+preSP	≈	≈	≈	≈	≈	≈	≈
C+preSP/C	↓	≈	↓	≈	↓	↓	≈
D+preSP/D	↓	↓	↓		↓	↓	≈

Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA)

5.3.1.3. Effects of SP600125 pretreatment on the kinase and PTEN expression and activation patterns during reperfusion

Comparison: DIO vs control groups

The DIO hearts pretreated with JNK inhibitor exhibited significantly lower PKBs473 phosphorylation at 5 min reperfusion compared with the control pretreated group (au D+preSP/C+preSP: 0.76±0.08/1.08±0.05; $p < 0.05$, Fig 59), but the differences were not observed after 10 min reperfusion (Fig 59). At 10 min of reperfusion, PTEN phosphorylation was significantl;y higher in the DIO group (Fig 60). At this time point,, hearts from the DIO group also exhibited significantly higher ERKp44/p42 phosphorylation compared with the control group (au D+preSP/C+preSP: ERKp44, 1.03±0.03 / 0.75±0.11; ERKp42, 0.94±0.04/0.72±0.10; $p < 0.05$, Fig 61).

However, no differences in JNK and p38 MAPK phosphorylation were seen between the groups at all reperfusion time points (Figs 62,63).

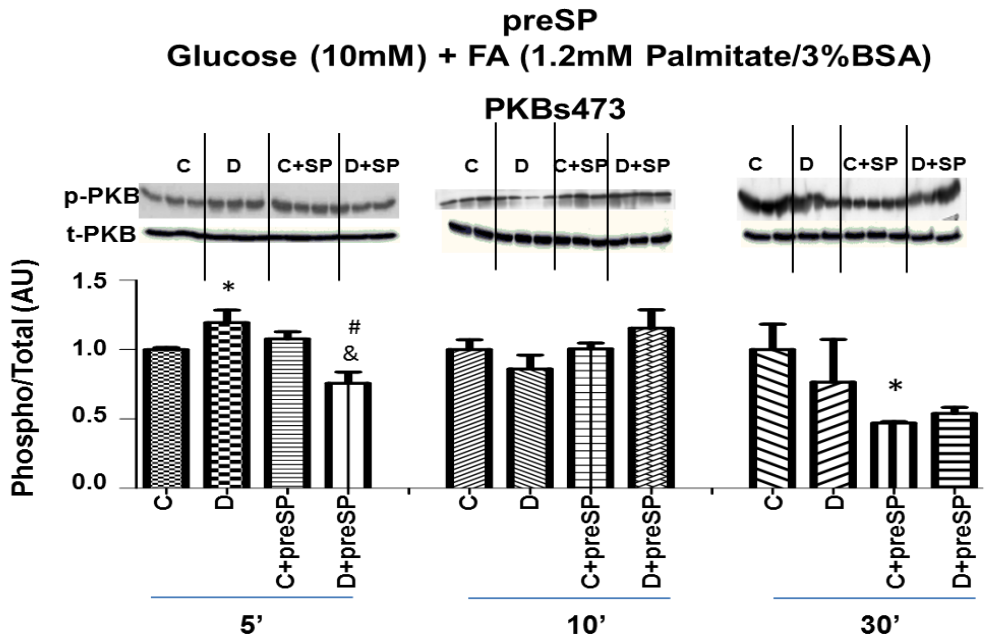


Fig 59. Effect of preSP on PKBs473 expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. All values were expressed as the ratio between phospho/total arbitrary densitometry units (AU) and were normalized to control hearts as one. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA).
*** p < 0.05 vs C; # p < 0.05 vs D, & p < 0.05 vs C+preSP, respectively.**
n = 3-4 per group.

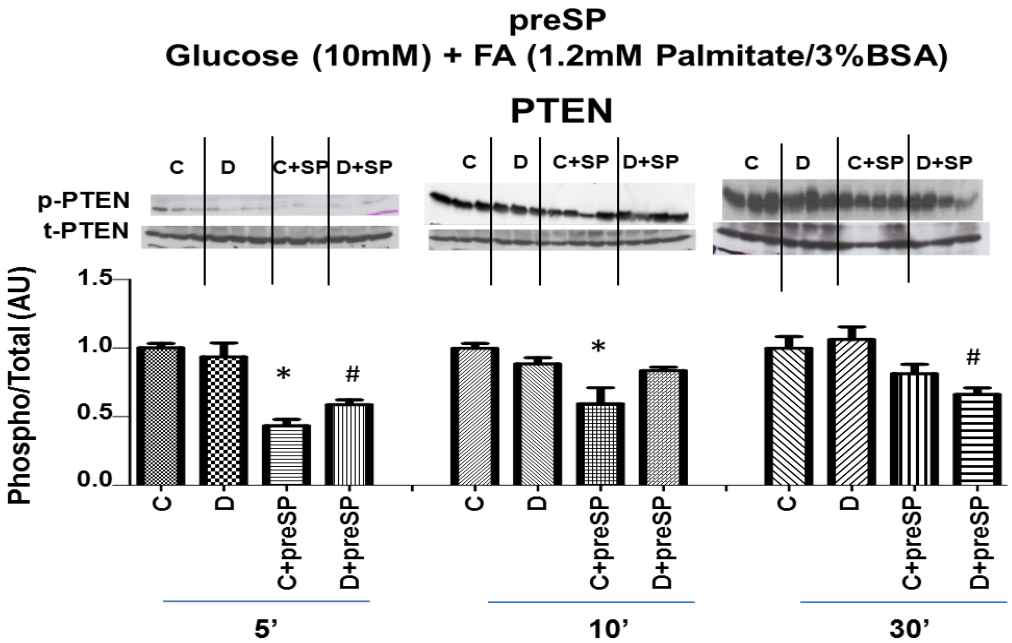


Fig 60. Effect of preSP on PTEN expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA). * p < 0.05 vs C; # p < 0.05 vs C+preSP, respectively. n = 3-4 per group.

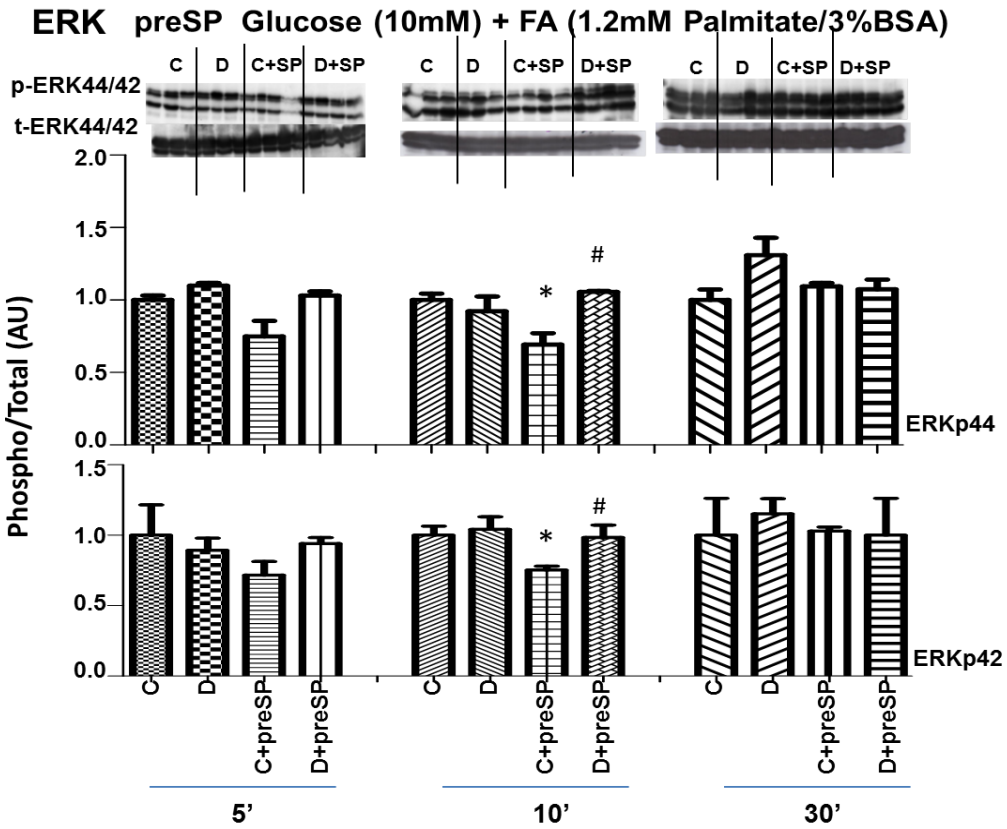


Fig 61. Effect of preSP on ERK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group.
AU: arbitrary units. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA).
*** p < 0.05 vs C; # p < 0.05 vs C+preSP, respectively. n = 3-4 per group.**

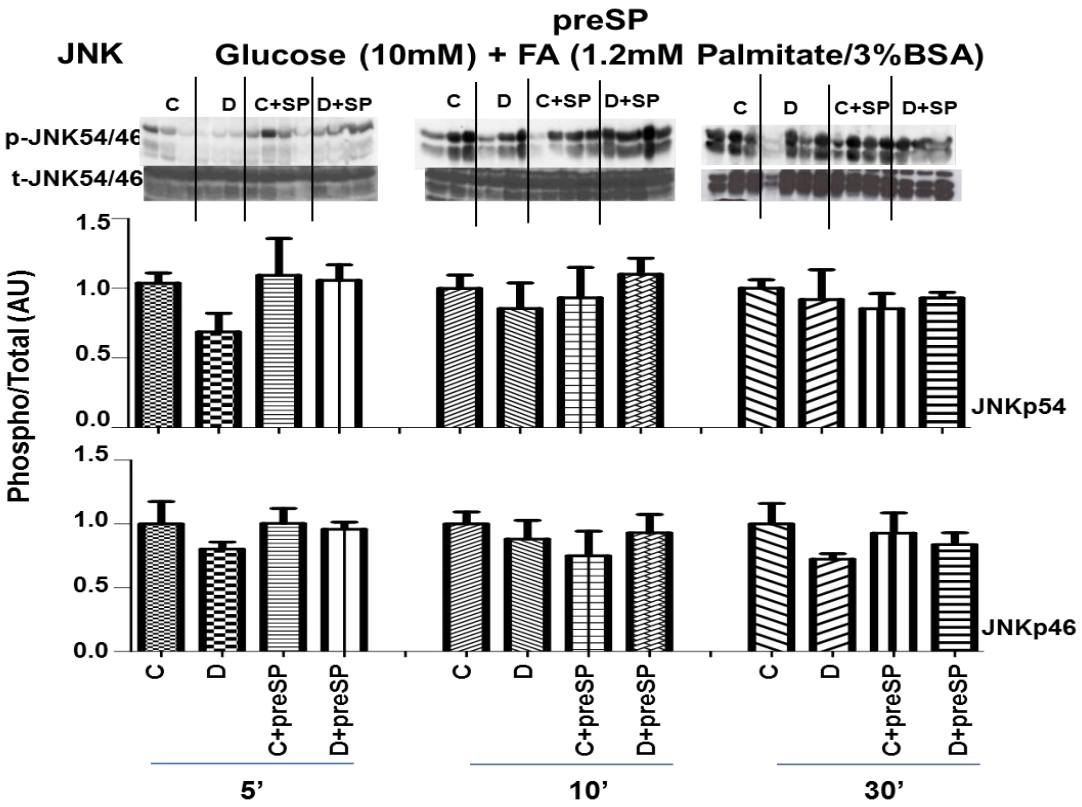


Fig 62. Effect of preSP on JNK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrates: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA). n = 3-4 per group.

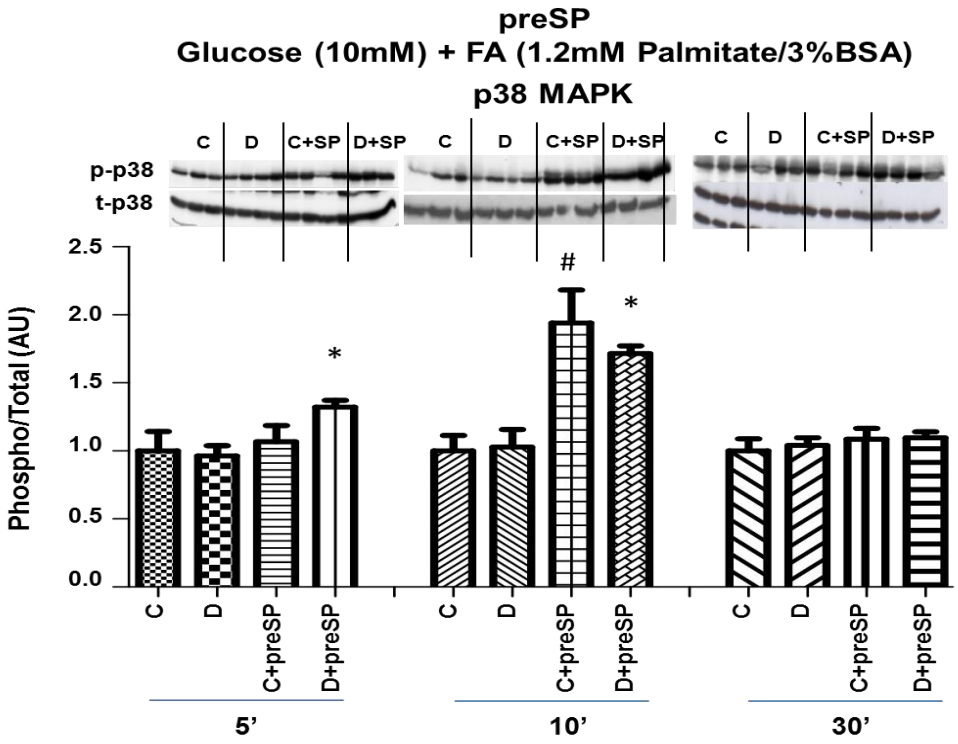


Fig 63. Effect of preSP on p38 MAPK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrates: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA). * $p < 0.05$ vs D; # $p < 0.05$ vs C, respectively. n = 3-4 per group.

Effects of SP600125 on hearts from control rats

In control hearts, pretreatment with SP did not change PKBs473 phosphorylation at 5 and 10 min reperfusion (Fig 59), but it significantly decreased this parameter at 30 min reperfusion compared to those of untreated controls (au C+preSP/C: 0.47±0.01/1.00±0.18, $p < 0.05$, Fig 59).

There were marked decreases in PTEN phosphorylation from 5 to 10 min reperfusion in the pretreated hearts compared to its corresponding untreated group (au C+preSP/C: 5 min 0.43±0.05/1.00±0.03; 10 min 0.59±0.12/1.00±0.03, $p < 0.05$, respectively, Fig 60). Furthermore, preSP markedly reduced ERKp44/42 phosphorylation and increased p38 MAPK phosphorylation at 10 min reperfusion (au C+preSP/C: ERKp44, 0.69±0.08/1.00±0.04; ERKp42, 0.75±0.03/1.00±0.06; p38 MAPK, 1.94±0.24/1.00±0.11, $p < 0.05$, respectively, Figs 61,63). With JNK, PreSP did not change its phosphorylation at all reperfusion times (Fig 62).

However, after 30 min reperfusion, no differences were observed in the phosphorylation of any of the proteins (Figs 61-63).

Effects of SP600125 on hearts from DIO rats

PreSP in the DIO group markedly reduced PKBs473 phosphorylation at 5 min reperfusion compared to those of untreated DIO hearts (au D+preSP/D: 0.76±0.08/1.19±0.09, $p < 0.05$, Fig 59), but the difference was no longer observed after 10 min reperfusion (Fig 59).

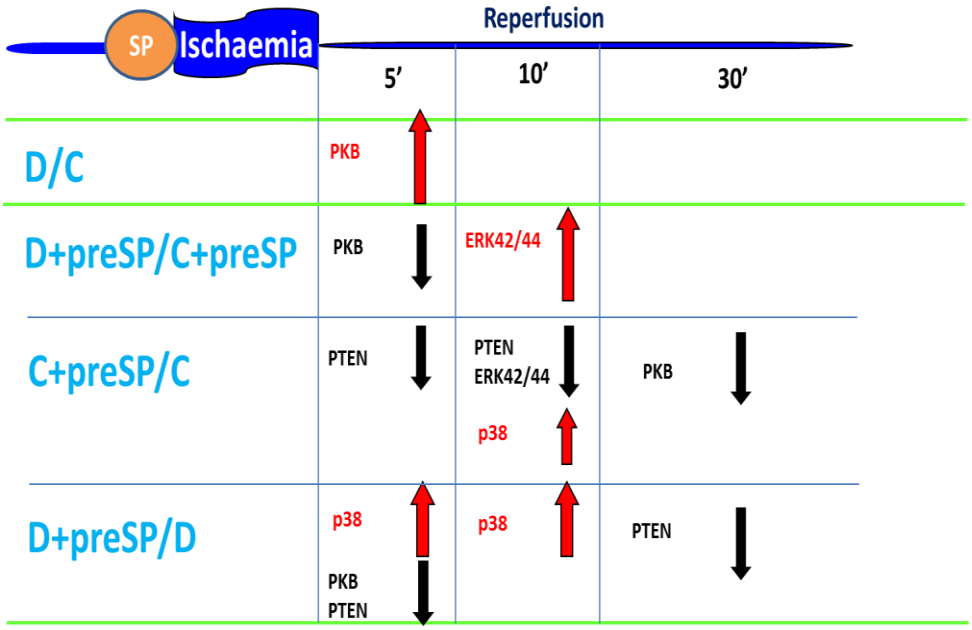
Similar to controls, the phosphorylation of PTEN was markedly decreased at 5 and 30 min reperfusion, but unchanged at 10 min reperfusion (au D+preSP/D: 5 min 0.59±0.03/0.94±0.10, 30 min 0.66±0.05/1.06±0.09, $p < 0.05$, Fig 60).

Furthermore, hearts pretreated with SP showed no effect on ERKp44/p42 and JNKp54/p46 phosphorylation at all reperfusion times (Fig 61).

The phosphorylation of p38 MAPK was significantly increased from 5 to 10 min reperfusion compared to its untreated group (au D+preSP/D: 5 min 1.32±0.05/ 0.96±0.07; 10 min 1.72±0.06/1.03±0.13, respectively, $p < 0.05$, Fig 63), but this difference disappeared at 30 min reperfusion (Fig 63).

Summary

Protein phosphorylation during reperfusion of hearts pretreated with SP (preSP) before exposed to global ischaemia from control and DIO rats



Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA)

5.3.2. Effects of posttreatment with SP600125

5.3.2.1. Functional parameters from both DIO and control groups

Comparison: postischaemic vs preischaemic function

The results obtained showed that without SP treatment, the 15 min sustained global ischaemia followed by 30 min reperfusion caused a significant reduction in AO in hearts from both DIO and control animals compared to the preischaemic values (Table 6), but did not affect CF, CO, PSP, HR and TW (Table 6).

Posttreatment with SP caused a significant reduction in AO, CO, PSP and TW during reperfusion when compared to the values obtained before ischaemia in both groups as well as compared with values obtained during reperfusion of untreated hearts in both groups (Table 6). Interestingly, CF in hearts pretreated with SP from control animals (but not DIO) was also markedly lower compared to the preischaemic values (postischaemic/preischaemic: 10.6±1.2/16.1±0.7, $p < 0.05$, Table 6). In summary, posttreatment with SP caused a reduction in AO, CO, PSP and TW during reperfusion in both groups but only lowered CF in the control group.

Table 6

Effects of JNK inhibitor SP600125 (SP) treated during reperfusion on functional recovery

Substrates: Glucose (10mM) + FA (1.2mM palmitate/3%BSA)

		CF	AO	CO	PSP	HR	TW
Pre-ischaemia	C	15.0±1.8	41.0±1.7	56.0±2.5	103±2	250±11	14.10±0.71
	D	18.5±2.8	40.7±0.7	59.2±3.4	100±3	261±18	13.88±1.10
	C+postSP	16.1±0.7	40.0±0.8	56.1±0.8	102±4	256±13	12.92±0.50
	D+postSP	16.7±0.7	38.0±2.0	54.7±1.8	100±1	243±5	12.15±0.63
Post-ischaemia	C	20.5±4.5	23.8±6.4*	44.3±10.9	98±2	258±13	11.19±1.71
	D	21.0±4.5	33.7±2.0*	54.7±6.3	95±3	276±6	12.54±1.63
	C+postSP	10.6±1.2#&	3.9±1.3#&	14.6±0.9#&	87±3#	264±14	2.82±0.17# &
	D+postSP	15.3±2.7$	8.7±3.5#&	24.0±2.3#$&	81±6#	236±11	4.83±0.54#$&

* p < 0.05 vs pre-ischaemic C or D, respectively
# p < 0.05 vs pre-ischaemic C+postSP or D+postSP, respectively
$ p < 0.05 vs post-ischaemic C+postSP, respectively
& p < 0.05 vs post-ischaemic C, or D, respectively

Data are expressed as means ± SE. n= 5-6 per group.

Abbreviations see Protocol I

CF: coronary flow (ml/min) AO: aortic output (ml/min) CO: cardiac output (ml/min)
PSP: peak systolic pressure (mmHg) HR: heart rate (beats/min) TW: total work (mW)

Comparison of percentage recovery: DIO vs control

When mechanical recovery during reperfusion was expressed as a percentage of the preischaemic values, hearts from DIO group showed a significantly better performance indicated by the improvement of CF, CO and TW than those of controls (% recovery, D+postSP/C+postSP, CF: 92.0±15.0/65.8±7.0, CO: 43.7±3.4/26.0±1.5, TW: 39.7±3.8/23.0±2.1, $p < 0.05$, respectively, Fig 64). No differences were seen in AO, PSP and HR (Fig 64).

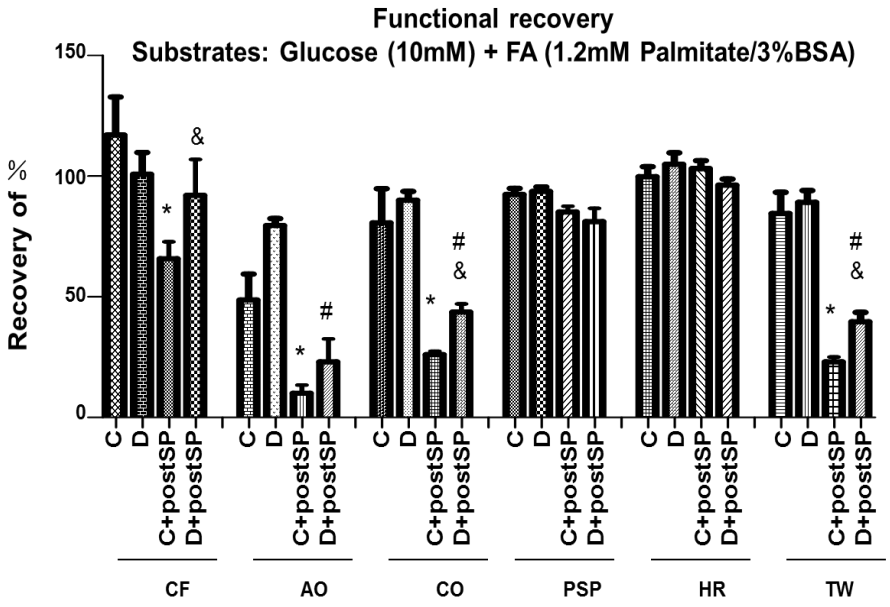


Fig 64. Comparison of % functional recovery during reperfusion of hearts treated with SP (postSP) after exposed to 15 min global ischaemia from control and DIO rats. Substrates: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA). Data are expressed as means ± SE. * p < 0.05 vs C, respectively; # p < 0.05 vs D, respectively; & p < 0.05 vs C+postSP, respectively. n= 5-6 per group.

5.3.2.2. Effect of SP600125 posttreatment on infarct size

As described before in the presence of glucose with a high concentration of fatty acid as substrates, infarct sizes of the hearts from DIO rats were similar to those of the hearts from control rats (% IS: D/C, 33.0±3.4/ 33.6±1.8, p > 0.05, Fig 65).

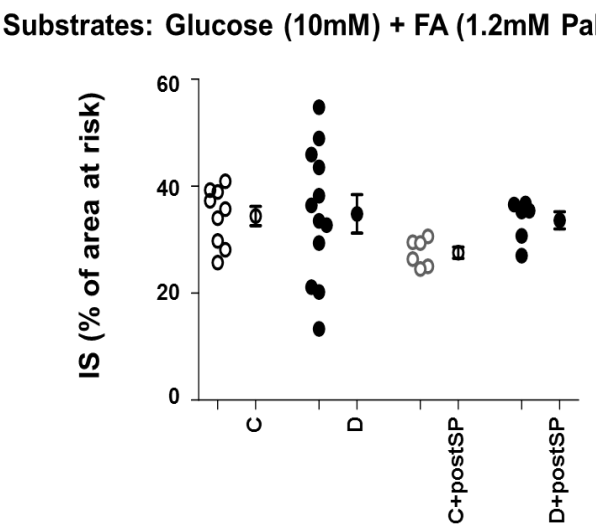


Fig 65. Comparison of % infarct sizes of hearts treated with SP (postSP) after exposed to 35 min regional ischaemia from control and DIO rats. Substrates: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA). Data are expressed as means ± SE. n = 10 (C), 12 (D) per group, n = 6 (C+postSP, D+postSP) per group.

When the hearts from both DIO and control animals were posttreated with SP, infarct size was not affected in either the control or the DIO groups (% IS:

D+postSP/C+postSP: 33.0±1.8/28.2±1.1; D+postSP/D: 33.0±1.8/33.0±3.4; C+postSP/C: 28.2±1.1/33.6±1.8, p > 0.05, respectively, Fig 65).

Summary

Functional recovery and infarct size of hearts posttreated with SP during reperfusion (postSP) after exposed to global or regional ischaemia from control and DIO rats

	% of functional recovery						Infarct Size
	CF	AO	CO	PSP	HR	TW	
D/C	≈	↑	≈	≈	≈	≈	≈
D+postSP/C+postSP	↑	≈	↑	≈	≈	↑	≈
C+postSP/C	↓	↓	↓	≈	≈	↓	≈
D+postSP/D	≈	↓	↓	≈	≈	↓	≈

Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA)

5.3.2.3. Effects of SP600125 posttreatment on the kinase and PTEN expression and activation patterns during reperfusion

Comparison: DIO vs control groups

PostSP had no effects on PKBs473 phosphorylation from 5 to 30 min reperfusion in both groups (Fig 66), however these hearts exhibited significantly higher PTEN and ERKp44/p42 phosphorylation at 5 min reperfusion compared to those of untreated controls (au D+postSP/C+postSP: PTEN, 2.36±0.23/1.09±0.20; ERKp44, 2.40±0.45/1.35±0.23; ERKp42, 2.32±0.58/1.15±0.15; respectively, p < 0.05, Figs 67,68). No differences in JNKp54/p46 phosphorylation from 5 to 30 min reperfusion were observed in both groups (Fig 69), but a markedly increased p38 MAPK phosphorylation at 5 min reperfusion of DIO hearts was seen (au 1.22±0.07/0.75±0.11, p < 0.05, Fig 70).

At 10 min reperfusion, no differences were found in the response of any of these proteins (Figs 66-70) while the only difference found after 30 min reperfusion, was elevated phosphorylation of PTEN (au D+postSP/C+postSP: 1.23±0.07/0.91±0.04, p < 0.05, Fig 67).

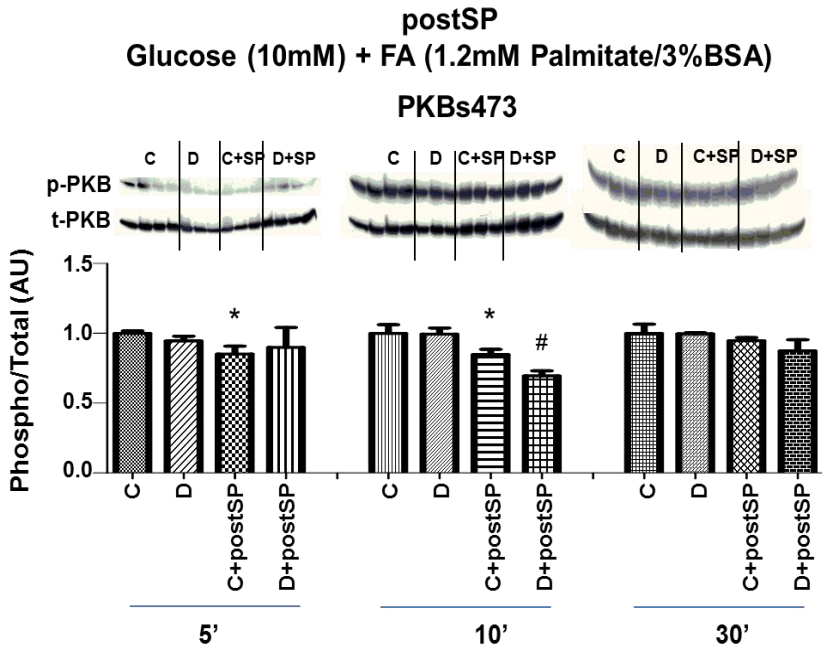


Fig 66. Effect of postSP on PKBs473 expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. All values were expressed as the ratio between phospho/total arbitrary densitometry units (AU) and were normalized to control hearts as one. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA).
*** p < 0.05 vs C; # p < 0.05 vs D, respectively.**
n = 3-4 per group.

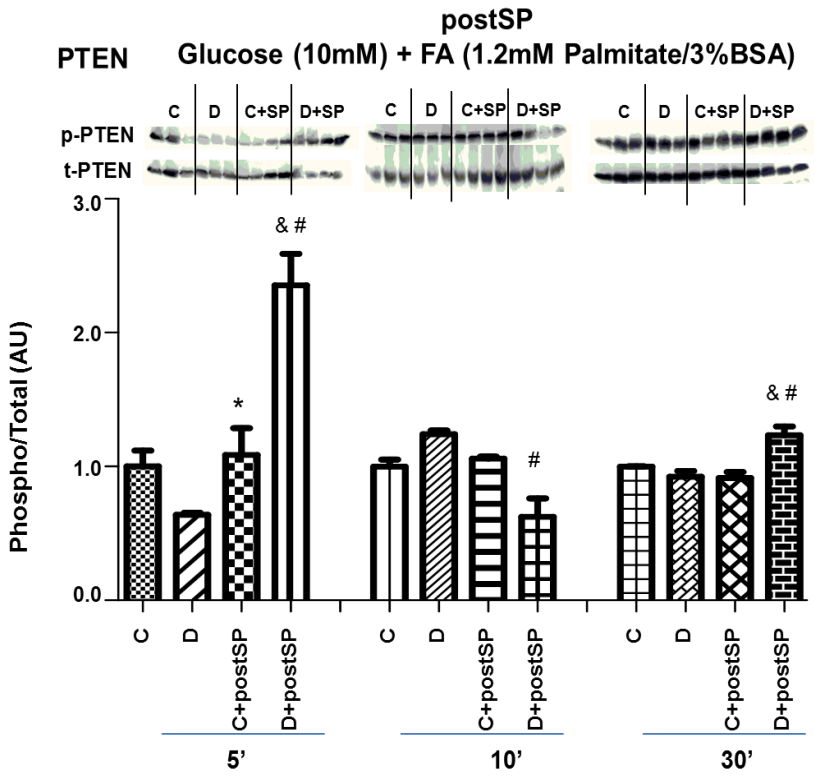


Fig 67. Effect of postSP on PTEN expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group.
AU: arbitrary units. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA).
*** p < 0.05 vs C; # p < 0.05 vs D; & p < 0.05 vs C+postSP, respectively. n = 3-4 per group.**

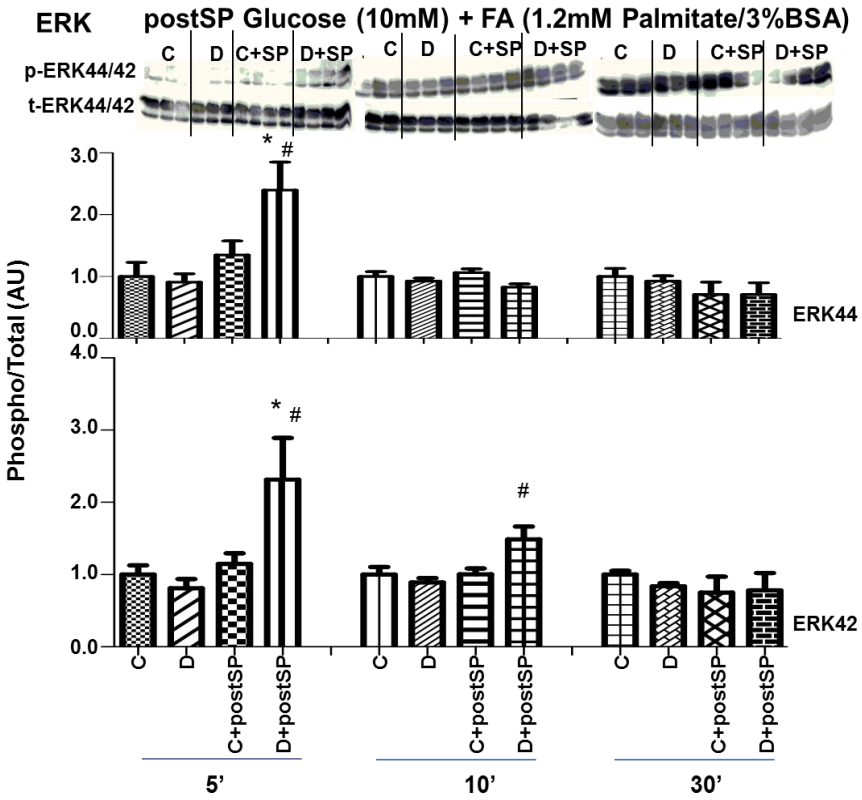


Fig 68. Effect of postSP on ERK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group.
AU: arbitrary units. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA).
*** p < 0.05 vs C+postSP; # p < 0.05 vs D, respectively. n = 3-4 per group.**

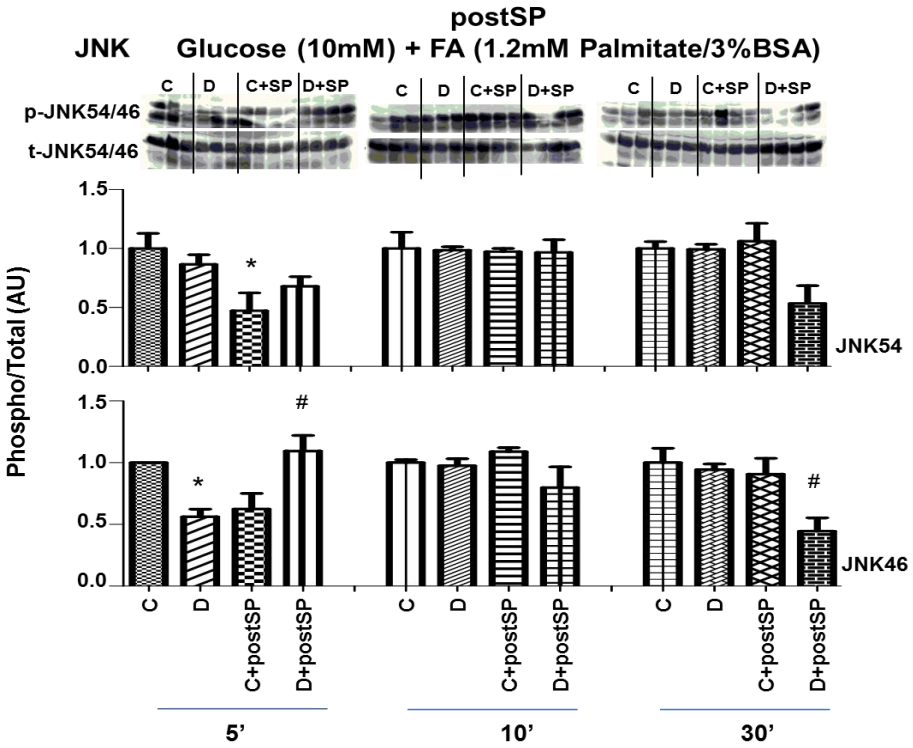


Fig 69. Effect of postSP on JNK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group.
AU: arbitrary units. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA).
*** p < 0.05 vs C; # p < 0.05 vs D, respectively. n = 3-4 per group.**

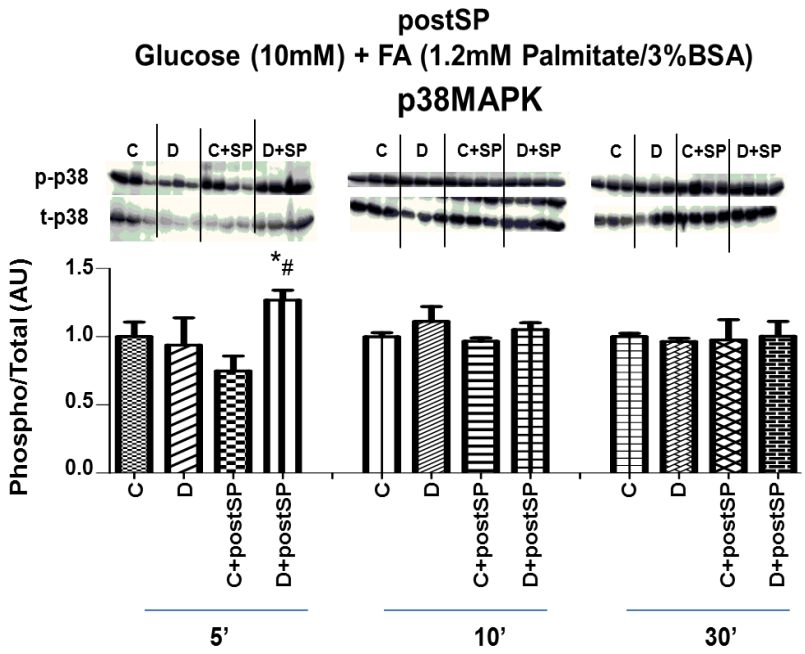


Fig 70. Effect of postSP on p38 MAPK expression and activation pattern during reperfusion after 15 min global ischaemia: comparison between control and DIO group. AU: arbitrary units. Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA). * p < 0.05 vs D; # p < 0.05 vs C+postSP, respectively. n = 3-4 per group.

Effects of SP600125 posttreatment on hearts from control animals

PostSP did not cause any significant changes in the phosphorylation status of the proteins (Figs 66-68,70) with the exception of a significant reduction in JNKp54 phosphorylation at the 5 min reperfusion time point compared to hearts from the untreated group (Fig 69).

Effects of SP600125 posttreatment on hearts from DIO animals

The hearts from the DIO animals postSP resulted in a decrease in PKBs473 phosphorylation at 10 min reperfusion compared to those of untreated hearts (au D+postSP/D: 0.70±0.04/0.99±0.04, $p < 0.05$), however, no differences were observed at 5 or 30 min reperfusion (Fig 66).

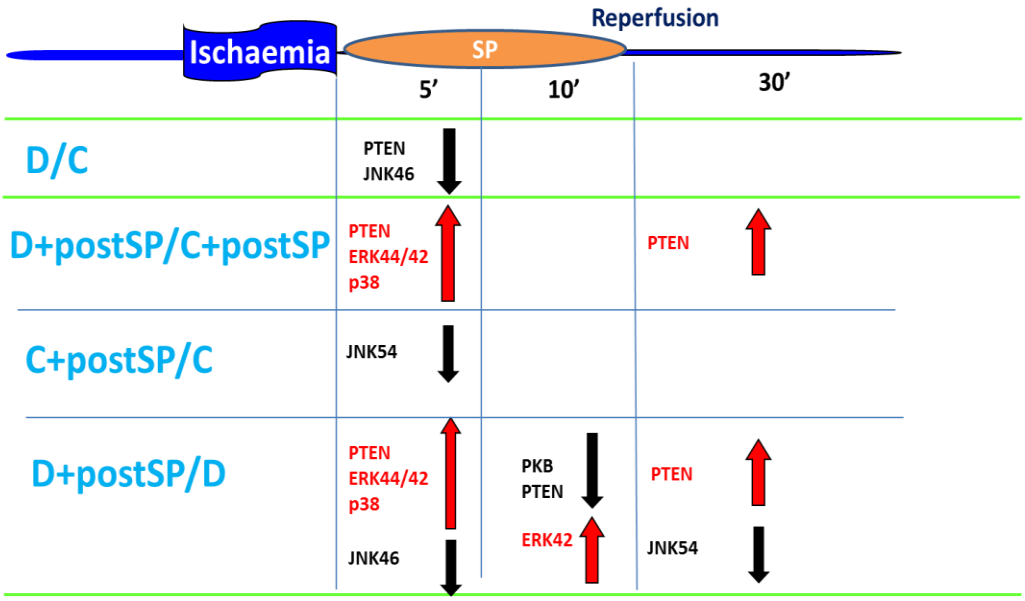
Interestingly, in contrast to the control group, there were marked differences in PTEN phosphorylation during reperfusion in the DIO groups: postSP caused a marked increase in PTEN phosphorylation at 5 min, a reduction at 10 min and a second increase again at 30 min reperfusion compared to the untreated DIO group (au D+postSP/D: 5 min 2.36±0.23/0.64±0.02; 10 min 0.62±0.14/1.24±0.03, 30 min 1.23±0.07/0.92±0.04, $p < 0.05$, respectively, Fig 67).

Furthermore, the DIO hearts posttreated with SP exhibited also markedly increased ERKp44/p42 phosphorylation at 5 min reperfusion but, interestingly, only ERKp42

phosphorylation was still higher at 10 min reperfusion compared to the untreated DIO group (au D+postSP/D: 5 min ERKp44: 2.63±0.40/0.91±0.14; ERKp42: 3.27±1.04/0.81±0.13, 10 min ERKp42: 1.49±0.18/0.89±0.06, $p < 0.05$, respectively, Fig 68). In contrast to the control group, hearts from the DIO animals posttreated with SP presented with significantly increased JNKp46 phosphorylation at 5 min, but reduced JNKp46 phosphorylation at 30 min compared to the untreated DIO group (au D+postSP/D 5 min JNKp46: 1.10±0.13/0.56±0.06, 30 min JNKp46: 0.53±0.15/0.99±0.04, $p < 0.05$, respectively, Fig 69). In addition, postSP also markedly increased p38 MAPK phosphorylation at 5 min reperfusion (au D+postSP/D: 1.22±0.07/0.94±0.20; $p < 0.05$, Fig 70), while having no effect after 10 min reperfusion (Fig 70).

Summary

Protein phosphorylation during reperfusion of hearts treated with SP (postSP) after exposed to global ischaemia from control and DIO rats



Substrate: Glucose (10mM) + FA (1.2mM Palmitate/3%BSA)

Chapter VI

Discussion: Effects of JNK inhibition on myocardial response to ischaemia/reperfusion injury in control and DIO hearts:

6.1. Effects of SP600125 on functional recovery in ischaemia/reperfusion injury

The results obtained in chapter III showed that hearts from DIO rats exhibited an increased tolerance to ischaemia/reperfusion (I/R) injury as reflected by an increase in functional recovery, as well as a reduction in infarct size, when compared with age-matched controls.This was observed with both glucose and glucose plus a high concentration of FA as substrates in the perfusate. Our data also indicated that the pattern of JNK activation during reperfusion not only differed between hearts from control and obese rats, but was also affected by the period of reperfusion and substrate present in the perfusate.

With glucose as substrate in ex vivo perfused hearts, obesity resulted in marked fluctuation in the activation pattern of JNK, with activation at 10 min, a reduction at 5 and 30 min reperfusion. These changes coincided with activation of PKB at 10min. Interestingly, addition of fatty acid to the perfusate, abolished these fluctuations in JNK activity while having a profound stimulatory effect on the activation of PKB in hearts from DIO animals when compared to controls. Furthermore, the presence of fatty acid in the perfusate resulted in higher levels of ERK activation, particularly in hearts from the obese animals.

Obesity has been shown to be associated with low-grade chronic inflammation and dysregulated cytokine production, contributing to insulin resistance by activation of amongst others, JNK and IKK, as negative feedback mechanisms in the regulation of insulin action via serine phosphorylation of IRS-1 which down-regulates the IRS/PI3-K/PKB pathway (10,17,18). In fact, JNK has been increasingly recognized as playing an important role in insulin resistance and suppression of this pathway has been shown to improve insulin resistance and glucose tolerance (for review see 871a). However, as far as we know, evaluation of the role of JNK in hearts from insulin resistant rats, has not yet been performed.

In view of (i) the reported overexpression of JNK in states of obesity and insulin resistance (297,336,351,379,382-384) and (ii) the possibility that JNK activation is a prerequisite for PKB activation during reperfusion after ischaemia (13) and (iii) the

lack of knowledge and conflicting published evidence with regards to the importance of JNK activity during reperfusion after ischaemia, we used a specific inhibitor of JNK in an effort to shed more light on the problem. Selective inhibitors of JNK have only very recently been developed and have not been used widely in the study of I/R injury. In view of the results described in chapters III, we hypothesize that activation of the JNK pathway may provide cellular protection in I/R injury, and that acute inhibition of JNK will be detrimental to the heart, especially in conditions of insulin resistance, and thus possibly exacerbate ischaemic injury.

The results obtained in Chapter V suggest that inhibition of JNK does have a profound effect on mechanical performance (but not infarct size) during reperfusion in hearts from both control and obese, insulin resistant rats. Main observations were (i) the effects were substrate dependent: with glucose as substrate, SP pre- as well as posttreatment reduced mechanical recovery in the DIO hearts only. However, with glucose plus high fatty acids as substrates, pre- and posttreatment with the inhibitor significantly reduced mechanical recovery in both groups; (ii) with glucose as substrate, the effects of SP pretreatment on DIO hearts were associated with a reduction in JNK and PTEN activation during early reperfusion. SP posttreatment of hearts perfused with glucose plus high fatty acids was not accompanied by a significant change in JNK phosphorylation, but a very significant activation of p38MAPK occurred within 5min of reperfusion.

Inhibition of JNK

To elucidate the roles of JNK in insulin resistance and I/R injury, we used SP600125, a specific inhibitor of JNK (872,872a) in our experiments. SP600125 has been widely used as a JNK inhibitor; although it is not specific for any JNK isoform. This; may be advantageous in maximizing its pharmacological effect, and inhibition of the isoforms of JNK may prevent possible isoform compensation during the course of ischaemic injury. A recent study showed that SP600125 inhibited several other kinases in vitro, including p70 S6 kinase, AMP-dependent protein kinase and cyclin-dependent protein kinase 2/cyclin A (872a,873). The phosphorylation state of these kinases was not determined in this study. It is therefore still possible that SP600125 exhibited the observed effects through the inhibition of other kinases (874).

However, IC50 values calculated for JNK1, JNK2 and JNK3 were 40, 40 and 90 nM; respectively (872,872a), but it is highly selective and has a 300-fold selectivity over the related MAPKs, ERK and p38 (875).

Based on a literature review, we decided to use SP600125 at a concentration of 10 μM in our experiments (876). To demonstrate the inhibitory effect of SP600125 on the JNK pathway in our study, we first examined the effect of the drug on serine 63 phosphorylation of c-Jun, a downstream target of JNK, employing Western blotting. It was reported that SP600125 inhibits Ang II induced c-Jun phosphorylation in HMCs with an IC50 of 5 to 10 μM, which is similar to the IC50 value detected in Jurkat T cells (872). In that same report, partial inhibition of other MAPK pathways was observed only when SP600125 was used at concentrations greater than 25 μM (872).

Our results showed that SP600125 at a concentration of 10 μM administrated either before ischaemia or during early reperfusion after ischaemia significantly inhibited the JNK pathway (Figs 39,40) and all subsequent studies were done using SP at this concentration.

The usually high endogenous levels of (mammalian cells) ATP may effectively reduce the efficacy of an ATP-competitive inhibitor. This has been observed with SP600125, where competition with high intracellular concentrations of ATP has been one of the reasons used to explain an increase in IC50 for JNK inhibition from 0.2 uM to 5–10 uM in vitro (872).

JNK inhibition: effect on mechanical recovery

As mentioned above, inhibition of JNK activation had a profound effect on mechanical recovery during reperfusion: with glucose alone as substrate, pre-as well posttreatment with SP600125, caused a significant further reduction in AO, CO and TW during reperfusion of hearts from DIO rats only, while in the case of the controls, it had no further detrimental effects (Tables 3,4)

However, with addition of fatty acid to the perfusate, both pretreatment and posttreatment with SP600125 resulted in a further reduction in AO, CO, PSP and TW during reperfusion in both the DIO and control groups (Tables 5,6). Inhibition of JNK also negated the smaller infarct development observed in DIO animals when perfused with glucose as substrate since inhibition of JNK at the onset of reperfusion, for the first time, resulted in larger infarct development in hearts from these animals (see Fig 51).

It was worth mentioning that, in the presence of SP600125, the patterns of reduced post-ischaemic CF was different in the DIO and control groups. Comparison of myocardial function during reperfusion between the DIO groups and between the controls showed that in the DIO group, only the pretreatment with SP not the posttreatment with SP significantly decreased post-ischaemic CF compared to the DIO group without treatment regardless of the substrates (Table 3-6). Interestingly, in the control group, only in the presence of high FA, both pre and posttreatment with SP significantly reduced post-ischaemic CF compared to the control group without treatment (Table 3-6). These reduced post-ischaemic CF were associated with decreased AO, CO and TW (Table 3-6). Interestingly, in the DIO group, although the posttreatment with SP did not decreased post-ischaemic CF, it significantly reduced post-ischaemic AO, CO and TW compared to the DIO group without treatment regardless of the substrates (Table 3-6). These results further indicated that the JNK inhibition in I/R performed as negative inotrophic effects during reperfusion on the hearts from control group depend on the substrate (only in the presence of high FA), but on the hearts from DIO group independent on the treatment period of SP and substrates. The inotrophic effects of SP on the hearts without I/R injury have not been evaluated in this study and warrant further investigation.

In summary, we demonstrated that a single dose of SP600125 administered either before ischaemia or during reperfusion after ischaemia enhanced myocardial I/R injury, particularly in the case of hearts from DIO rats. These results suggest that activation of the JNK pathway may be one of the mechanisms contributing to cardioprotection against ischaemia in obesity.

Although not evaluated in the present study, the JNK pathway appears to play an important role in myocardial energy metabolism in I/R injury as the hearts from control animals were not affected to the same extent as hearts from the DIO animals by inhibition of JNK. This suggests a role for limited endogenous TAG breakdown for energy metabolism in I/R injury. On the other hand, the hearts from the DIO group showed a significant reduction in functional recovery when treated with the JNK inhibitor, suggesting that the JNK pathway plays an important role in FA metabolism during I/R.

Contrary to our results, the inhibition of JNK signalling has been demonstrated to be

protective against I/R by limiting apoptosis in endothelial cells (877).

A study by Khandoudi et al. (878) has also suggested that inhibition of the JNK pathway is associated with the improved postischaemic hemodynamics observed with Rosiglitazone (RGZ: a peroxisome proliferator-activated receptor (PPAR)-γ agonist), infusion ex vivo. The inhibition of JNK signalling by RGZ in this case may contribute to the improved cardiac function. In human pancreatic islets, JNK inhibition via SP600125 was also protective as it has been shown to preserve whole-islet mass (879).

However, our data clearly demonstrates that the JNK pathway may play an important role in maintaining myocardial function in I/R, particularly in insulin resistant conditions. The substrate present in ex vivo experimentation may also determine the outcome since recovery of hearts from control animals was also affected by JNK inhibition in the presence of glucose plus as high fatty acid as substrates. It would be useful to identify the roles of the different JNK isoforms in I/R injury, given their differing specificity for downstream transcription factors (616,880) as well as in stress-induced activation (11).

In this study, we only investigated the role of the JNK inhibitor (SP600125) at 10 uM concentration on hearts in ischaemia/reperfusion, not included different concentrations of SP in I/R injury, and numerous in vitro and in vivo studies have shown that JNK is activated during reperfusion after ischaemia (575-579), the role of SP on inotrophic effects on the normal hearts in physiological condition without I/R injury was not evalauated in this study.

6.2. Effects of SP600125 on intracellular signalling in ischaemia/reperfusion injury.

In addition to the effects on c-jun, pre- as well as posttreatment with SP600125 had profound effects on the activation state of the PKB, ERK and p38MAPK pathways as well as on the phosphatase PTEN, in both groups during reperfusion.

It is evident that inhibition of JNK before the onset of ischaemia, abolished the activation of PKB during the first 5 min of reperfusion regardless of the substrate used. The second noticeable change is that the phosphatase PTEN is strongly phosphorylated with glucose as substrate, especially in hearts from the DIO rats. This should lead to inhibition of the phosphatase and a stronger potential to activate PKB.

However, in the presence of inhibition of JNK, this is not seen as stated above. With addition of fatty acids to the perfusate, the phosphorylation of PTEN is inhibited accompanied by further downregulation of PKB activation until 30min reperfusion (See summary, p159). Thirdly, administration of the JNK inhibitor at the onset of reperfusion, resulted in high levels of activation of p38, especially in the DIO hearts perfused with glucose plus fatty acid. Activation of p38 MAPK during reperfusion is well-known to be associated with a reduction in functional recovery and increased infarct size during reperfusion after ischaemia as well as increased apoptosis (881). This may account for the marked inhibition of functional recovery observed after SP posttreatment (Table 5,6)

It is well-established that activation of ERK and PKB during early reperfusion (the so-called RISK pathway) is associated with improved recovery after I/R. The SP-induced reduction in PKB activation during early reperfusion of hearts perfused with glucose plus palmitate may also contribute to the marked reduction in functional recovery seen in such hearts.

It has recently been suggested that part of JNK's cardioprotective effect is due to reactivation of PKB by JNK (13). This particular study showed that activation of JNK is essential for PKB phosphorylation at the onset of reperfusion: activation of JNK phosphorylates PKB on Thr450, demonstrating that JNK activation is a prerequisite for full PKB activation by phosphorylation at Thr308 and Ser473.

In the current study, activation of PKB coincides with cardioprotection but the fluctuations observed in the activity of ERK are variable and did not always correlate with improved functional recovery or smaller infarct size development. Apart from a marked reduction in ERK activation by SP pretreatment in the presence of glucose as substrate, ERK seems to be least affected by the interventions used in this study. For example, in hearts perfused with either glucose or glucose plus palmitate, ERK activation was similar at several reperfusion time points in hearts from DIO and control animals despite relatively large differences in functional recovery or infarct size. It should be noticed that the improved functional recovery of obese hearts in the presence of fatty acid as substrate did not coincide with smaller infarct sizes (Fig 16c, 18). Furthermore, with inhibition of JNK before ischaemia, ERK activation was lower in DIO hearts during reperfusion in the presence of glucose but higher in the presence of glucose and fatty acid. Under these conditions, the hearts from DIO

animals had poor functional recovery and this was the one condition where the infarct development tended to be larger in hearts from the DIO animals. However, in the current study we did not inhibit ERK either before or after ischaemia to determine the significance of its activation.

In summary, the results obtained demonstrated that the JNK-specific inhibitor SP600125 administered either before or directly after myocardial ischaemia, resulted in inhibition of PKB activation, in the presence of marked activation of the p38MAPK pathway especially during the critical first 5 min of reperfusion.Thus, inhibition of the JNK pathway by SP600125 exacerbated I/R injury, particularly in hearts from DIO rats. These results indicate that the concept of the elevated expression of JNK in obesity being only associated with negative effects, e.g. induction of insulin resistance via serine phosphorylation of IRS-1 or the induction of apoptosis, should be reconsidered. In the absence of JNK activation, the myocardium is significantly more susceptible to ischaemic damage (882), particularly in hearts from obese insulin resistant rats.

Based on the significant reduction in functional recovery during reperfusion induced by inhibition of JNK, the results described in this chapter suggest that activation of JNK both prior to and during early reperfusion, is required for mechanical recovery during reperfusion. Thus the data presented here, solidify the hypothesis that JNK signaling during early reperfusion, may be an important contributor to the improved recovery during reperfusion, particularly in hearts from obese animals. However, despite the marked SP-induced reduction in mechanical function during reperfusion, infarct size remained unchanged and was not enlarged as would be expected.

JNK signalling: conflicting reports

Despite the convincing data obtained in the study thus far, the many contradictory findings with regard to the importance of JNK in I/R injury, need to be recognized.

Although a critical role for the JNK signalling pathway in post-ischaemia cell survival, necrosis, and apoptosis has been demonstrated (579,883,884), conflicting evidence exists regarding the significance of JNK activation in I/R injury (see literature survey 1.4.4.1). For example, it has been reported that the JNK regulates proapoptotic death signalling events during I/R (for review, see ref 15), while genetic or pharmacologic inhibition of JNK was shown to be cardioprotective in a number of studies (14,15,606). In contrast, it has also been suggested that JNK is capable of transducing

antiapoptotic signals and mediates survival in the postischaemic cardiomyocyte (613,615,616,618), but the mechanisms of these pro-survival effects were much less clear than the mechanisms promoting cell death (14,15,606, 613-618).

Contradictory results have also been obtained with SP600125. Direct protective effects of the drug during I/R have been observed in many tissues, including lung, kidney, liver, brain, and heart (579,885-889) and have been attributed to reducing apoptosis and death in a Fas ligand-initiated extrinsic pathway (890). JNK activation can either phosphorylate Bcl-2 proteins that regulate mitochondrial-mediated apoptosis, or, alternatively, translocate to mitochondria where it can directly trigger mitochondrial permeabilization (891). In addition, SP600125 protected cardiac myocytes from cell death following beta-adrenergic stimulation (600).

Evidence has also been presented that JNK-1 is required to protect the heart against lethal reperfusion injury following brief but not extended ischaemia. JNK-1 inactivation decreases the thresholds of ROS/calcium that are required to open the mPTP after brief ischaemia such that signals that would normally initiate reversible stunning are sufficient to open the mPTP and cause infarction. Conversely, when ischaemia is prolonged, JNK-1 inactivation increases the ROS/calcium thresholds required for mPTP opening, thus conferring protection (616).

The phosphorylation of PKB-Thr-308, PKB-Thr-450, and GSK3-S9 was all reduced when JNK-1 was inhibited during brief ischaemia/reperfusion, but these same targets were more highly phosphorylated when JNK-1 was inhibited during extended ischaemia/reperfusion (616).

In the in vitro cardiac myocyte hypoxia reoxygenation model, activated JNK-1 was protective and inhibition of JNK-1 decreased PKB phosphorylation and total PKB activity when glucose and ATP were sustained during hypoxia but injurious when glucose and ATP were depleted (613). In contrast, Hreniuk et al. (737) showed that cardiac myocytes exposed to hypoxia reoxygenation in the absence of glucose activated JNK-1 and increased cell death whereas JNK-1 inhibition had the opposite effect.

Detrimental effects of SP600125 have also been noted in the heart. SP600125 significantly enhanced the activation of the proapoptotic protease, caspase-3, and

increased the numbers of apoptotic cardiac myocytes in culture in response to their energy depletion following exposure to potassium cyanide and 2-deoxy-D-glucose (619). Similarly, chronic SP600125 treatment in vivo in the cardiomyopathic hamster model of heart failure increased the number of apoptotic (TUNEL-positive) myocytes and the area of interstitial fibrosis (894).

In summary, conflicting evidence exists regarding the significance of JNK activation in I/R injury and contradictory results have been obtained with SP600125. The wide variety in experimental models and protocols may have contributed to the confusion and should be kept in mind when evaluating the role of JNK in the heart. Clearly, in view of the many contradictory reports, the role of JNK in I/R injury needs to be carefully re-evaluated.

JNK and the RISK pathway

As discussed before, it is well known that in the myocardium the activation of the pro-survival kinase signalling cascades, PI3-K/PKB and Ras/ERK, the so-called RISK pathway, during early reperfusion is associated with a reduction in infarct size and improvement of postischaemic mechanical function, as was reported in interventions such as ischaemic pre- or post-conditioning or the administration of pharmacological agents both in vitro and in vivo (for reviews, see ref 9,11). There is abundant evidence that the PKB and ERK pathways exert transcriptional, translational, and post-translational protective effects through phosphorylation of diverse target molecules such as the Bcl-2 family proteins and GSK-3β (9,11,520,517,531,448,895), which ensure that mitochondrial integrity is preserved against short- and long-term stress. It has become apparent in recent years that Bcl-2 family proteins and the permeability transition pore are important regulators of the mitochondrial death pathway that is activated by stress in cardiomyocytes (9,11).

These results again highlight the importance of the PKB survival pathway in protecting the heart against brief as well as extended ischaemia. The results obtained in the present study confirm a role for PKB activation in the improved functional recovery of hearts from obese rats, while that of ERK remains doubtful.

The role of p38 MAPK activation in I/R injury is controversial, it can be both protective as well as detrimental, and recent evidence suggests that the mechanism of p38 MAPK activation may differ according to the experimental conditions. Many reports showed that p38 MAPK activation during myocardial ischaemia enhances lethal

injury (160,642-644) and inhibition of its activation protects against it (642,645,646). Studies from our laboratory (Lochner, Marais et al), demonstrated the detrimental effect of p38 MAPK activation in ischaemic preconditioning and β-adrenergic preconditioning (647). The generally accepted view is that IPC transiently activates p38 MAPK during the pre-conditioning phase (648-651), and reduces the p38 MAPK activation occurring during the sustained ischemic phase (647). For more details of p38 MAPK isoforms in I/R refer to the literature survey (Chapter 1.4.4.2). As mentioned previously, the marked rapid activation of p38MAPK induced by posttreatment with SP, may be important in the reduction in functional recovery.

As discussed before, activation of PTEN is a downregulator of the PI3K/PKB pathway (19,558). It is worth mentioning that PTEN may play a significant role in the regulation of the contractile function in cardiomyocytes as well as in the regulation of the L-type calcium currents (564-566). Our data showed that the inhibition or activation of PTEN occurred in hearts either pretreated or posttreated with SP during different reperfusion times. These observations were however not always consistent with phosphorylation of PKB in hearts from both DIO and control rats.

Summary

Despite the many controversies surrounding the role of JNK in I/R injury and the apparent shortcomings in using SP600125 as inhibitor, our results suggest that JNK may be an important role player in the response of the heart to ischaemia/reperfusion injury, particularly in obesity.

Chapter VII

Conclusion

Obesity protects against ischaemia/reperfusion injury?

Our study demonstrates that, in contrast to other studies where obesity associated with increased plasma fatty acids levels were reported to be detrimental to I/R damage, dietary-induced obesity increase the tolerance of the ex vivo myocardium to I/R injury. In addition, it was also found that a high concentration of palmitate as substrate was not detrimental to hearts of normal rats during I/R, suggesting that fatty acids may indeed have salutary effects on cardiac function. The finding that fatty acids are in fact beneficial to the ischaemic/reperfused heart is in contrast to the generally accepted view that they are indeed bad for the ischaemic heart, especially during reperfusion. Although the exact mechanism whereby fatty acids exert their beneficial effects, is still unknown, this study has shown that this protection was associated with early activation of the PKB and JNK pathways during reperfusion.

A possible limitation in the present experimental approach is that only two exogenous substrates (glucose and palmitate) were examined in the perfusate, thereby neglecting the role of pyruvate, lactate, and ketone bodies and insulin. In addition, lipids are heavily implicated in development of insulin resistance in skeletal muscle (896). This seems to be linked to an imbalance between lipid supply and lipid oxidation, the latter being related to decreased mitochondrial oxidative capacity in states of insulin resistance. A detailed study of myocardial oxidative and lipid metabolism in hearts from obese insulin resistant rats is required. This is currently in progress in our laboratory. Our understanding of the effect of obesity on cardiac function and metabolism is greatly limited by the paucity of human data, particularly relating to the effects of comorbidities (insulin resistance, diabetes, hypertension, hyperlipidemia) in this population.

The present study also stressed the role of substrates in the outcome of ischaemia/reperfusion injury. This was evident not only in the extent to which functional recovery occurred, but also in the pattern of intracellular signaling observed and the effect of reperfusion time. It does seem, however, that activation of PKB during early reperfusion plays a pivotal role, regardless of the substrate present

in the perfusate of the ex vivo perfused heart. ERK activation seems to be less important.

Obesity paradox

Overwhelming evidence supports the importance of obesity in the pathogenesis and progression of CV disease. On the other hand, an increasing number of studies have shown that overweight and obese patients with established CV diseases seem to survive better than leaner patients–the so-called “obesity paradox”. The underlying explanation for these results is unknown, although many potential explanations exist.

The results obtained in this study lends support to the concept of the obesity paradox. It is known that obese insulin resistance is associated with a reduction in insulin-stimulated glycogen synthesis, which is in turn, a consequence of reduced glucose transport. Insulin resistance also leads to enhanced FA production, which inhibits insulin signaling (10,304,308,320). Although it is generally accepted that an increase in myocardial reliance on FA in obesity has detrimental consequences in the heart, the results obtained in this study suggest the opposite. Metabolic dysregulation in obesity is accompanied by adaptive as well as maladaptive responses of the heart. Insulin resistance may be adaptive when it is protecting the heart from excess fuel uptake or maladaptive when it is associated with ROS formation and activation of signalling pathways of programmed cell death. Our observations support the notion that obesity-induced insulin resistance gave rise to a number of adaptive responses. A major question is whether or not insulin resistance affects myocardial metabolism in our model of diet-induced obesity. Clearly further detailed analyses of the myocardial metabolic processes in obesity are required.

Role of JNK

In the present study pharmacological manipulation of JNK by administration of the selective JNK inhibitor, SP, during myocardial ischaemia and reperfusion suggested an important role for JNK in the outcome of I/R injury. The detrimental effect of SP following ischaemic exposure, was associated with inactivation of PKB suggesting that cross-talk between JNK and PKB pathways in the post-ischemic myocardium may be a major contributing factor to the outcome of I/R injury. These results also suggest that JNK and its downstream signalling pathways may be critical in

mediating protection in I/R. The JNK-mediated activation of pro-survival signals appears to dominate over JNK-mediated activation of pro-death signals in this particular model.

Increasing numbers of new JNK small molecular inhibitors have been identified with good potency, selectivity, and bioavailability. It will be of great interest to see if these inhibitors will shed more light on the actions of this kinase. It is clear, however, that further investigations are essential before a connection between bench observations and the bedside can be achieved.

Clinical implications

Our results are potentially of clinical significance, and may suggest a new therapeutic strategy for treating insulin resistance associated with obesity and heart disease. Our findings suggest that interventions targeting JNK may have some important therapeutic implications in the treatment of I/R injury. Whether this is a viable approach in insulin resistance remains to be determined.

However, as literature has abundantly demonstrated, the complexity of the signalling transduction network makes it impossible and imprudent to label any particular molecule as definitively “bad” or “good.” Using genetic approaches to achieve complete inactivation (knockout) or activation (knockin) of signalling pathways, although very powerful, have major limitations in uncovering their intricate roles in the dynamic process of stress response.

Potential further studies

The present study focused on two time intervals, namely 15 min global ischaemia , and 35 min regional ischaemia which represent reversible and irreversible cell damage respectively. Two substrate combinations were used namely glucose alone and glucose in combination with either low or high fatty acid concentrations. In order to evaluate the role of the fatty acids per se, hearts will have to be perfused with fatty acids alone, subjected to the same experimental protocols as in the present study and using the same endpoints. In view of the apoptotic actions of JNK, markers of apoptosis will also be included in this study.

The further studies will focus on the relationship between the 14-3-3 proteins, PKB, JNK etc and apoptosis in hearts from control and obese rats perfused with glucose and fatty acids.

JNK activation is probably associated with mitochondrial pro-apoptotic factors (584-587). PKB activation protects against apoptosis also through the Bcl-2 family proteins after a wide variety of stimuli including the withdrawal of growth factors, UV irradiation, matrix detachment, cell cycle disturbance, DNA damage, and treatment of cells with anti-Fas antibody (reviewed in 716-719).

14-3-3 proteins are a family consisting of highly conserved acidic proteins, with molecular weights of 25-30 kD, that are expressed in all eukaryotic cells. It is composed of at least seven mammalian isoforms (β, γ, ε, η, σ, τ and ζ) (896a,b).

14-3-3 acts as an adaptor or "chaperone molecule", which is able to move freely from cytoplasm to nucleus and vise-versa (897).

14-3-3 proteins play important roles in the decision between cell death and survival through the cell cycle, regulating their response to DNA damage, and controlling many of the signalling pathways following internal injury or external cytokine-mediated cues (898,899).

The role of 14-3-3 in apoptosis has been well documented and indicated to mediate an essential anti-apoptotic signal by binding to members of the Bcl-2 family, Bcl-2-associated death promoter (BAD) and Bcl-2-associated X protein (BAX), thereby inhibiting their proapoptotic activities (902-904).

It would therefore seem as if there is a close working relationship between 14-3-3 proteins and JNK, PKB/Akt, in cell death or survival in the pre-diabetic heart. The immunoprecipitates will be analysed for the presence of BAX, BAD, JNK & PKB/Akt associated with 14-3-3. If possible, a mouse strain with cardiac specific expression of a dominant negative 14-3-3 will be obtained to investigate the importance of this protein family in the regulation of apoptosis in the pre-diabetic heart.

Specific Acknowledgements

I owe exceptional thanks for Mrs Sonia Genade as the lab technician performed partial perfution in Chapter V for pre and posttreatment of JNK inhibitor and Dr Amanda Genis performed partial Westing blots in Chapter V for pre and posttreatment of JNK inhibitor.

ADDENDUM:

LYSIS BUFFER FOR WESTERN BLOTTING:

	Stock	10 ml
20 mM Tris-HCl 1 mM EGTA	200 mM	1 ml
1 mM EDTA	100 mM	100 µl
150 mM NaCl	1 M	1.5 ml
1 mM β-glycerophosphate		0.002g
2.5 mM tetra-Na-Pirophosphate		0.01g
1 mM Na_3VO_4 Weekly (0.018g/10ml)	10 mM	1 ml
*** 50 µg/ml PMSF**	100 mM	30 µl
10 µg/ml Leupeptin		10 µl
10 µg/ml Aprotinin		10 µl
1% Triton X-100	10%	1 ml

BRADFORD REAGENT:

The Bradford reagent is prepared as follows and kept as stock in a fridge:

Dissolve 500mg Coomassie Brilliant Blue in 250ml 95% Ethanol

Add 500ml phosphoric acid and stir

Make up to 1L with dH_2O

The working solution is prepared as a 1:5 dilution (i.e. 10ml Bradford stock + 40ml of dH_2O) and filtered through a double layer of filter paper.

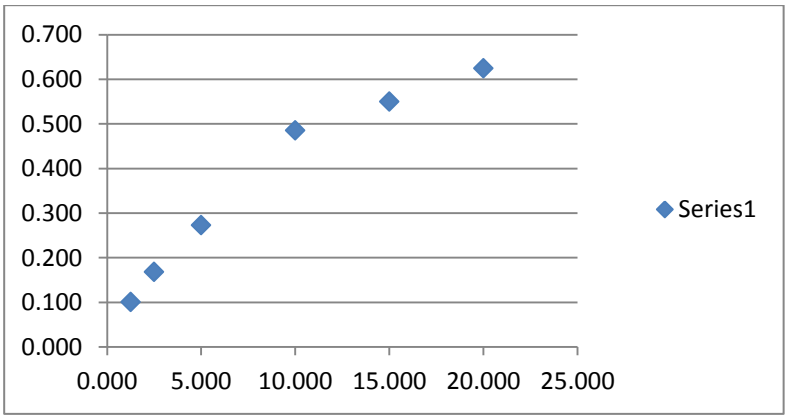
METHOD:

- A BSA stock solution of known concentration (determined by reading the optical density at 280nm and calculating the concentration from the E^{280} value of albumin = 1.51 is prepared.
- A standard curve containing protein concentrations ranging from 2.5ug to 25ug in 100uL is generated by dilution with distilled water.

- Lysates are diluted 1:10 with distilled water to dilute all detergents that may interfere with the assay, e.g. Triton X-100.
- A volume (e.g. 10uL) of the diluted samples is then further diluted with distilled water to 100uL.
- 900uL of Bradford reagent is then added to each standard and sample, including a blank of distilled water, and mixed by vortexing.

All samples are left for 15min for full colour development before reading the OD at 595nm.

An example of a standard curve:



As can be seen from the standard curve, this reaction reaches a saturation in colour development with increasing concentrations. All samples are therefore diluted to fall on the linear portion of the standard curve. The protein concentrations of the samples are then calculated from this standard curve.

LAEMMLI SAMPLE BUFFER PREPARATION:

Final concentration:

62.5 mM Tris-HCl (pH 6.8)

4% SDS

10% Glycerol

0.03% Bromophenol Blue

5% β-mercaptoethanol

Preparation of a 3X sample buffer stock:

First solution:

0.5 M Tris + 0.4% SDS

- Dissolve 9.09g Tris in dH_2O
- Add 6ml of the 10% stock SDS
- pH the solution with HCl to 6.8 and make up to a final volume of 150ml with dH_2O

Second solution:

- Weigh off 60g of glycerol
- Add 99.9ml from the solution made up in step 1 and add to the glycerol in the beaker.
- Add 26.4g of SDS
- **dissolve thoroughly**, the SDS must be fully dissolved before the Bromophenol Blue is added as you cannot see whether the SDS is dissolved or not afterwards.
- Lastly, add 0.225g of Bromophenol Blue
- **dissolve thoroughly**

Lysates containing equal amounts of protein are prepared by diluting all samples with a volume of lysis buffer in order to also obtain the equal protein in an equal volume. The diluted samples are then further diluted with Laemmli buffer in a ratio of 2:1, boiled for 5min and stored at -80^{o}C.

WESTERN BLOT RUNNING BUFFER

10 x Running Buffer

Tris: 60.6g

Glycine: 288g

SDS: 20g

Make up to 2L with distilled water

Store at 4ºC and dilute 10 times with distilled water at room temperature for use.

WESTERN BLOT TRANSFER BUFFER

10 x Transfer Buffer

Tris: 6.06g

Glycine: 28.83g

Methanol: 400ml (20%)

Dissolve Tris, glycine and methanol in dH2O and make up to 2L

No need to set pH

Store at 4°C

TRIS-BUFFERED SALINE (TBS)

10 x TBS stock

Tris: 48.4g

NaCl: 160g

Dissolve in dH2O

Set pH with HCl to 7.6

Make up to 2litres and store at 4°C

TBST

Add 1ml of Tween-20 to 100ml of TBS stock solution and make up to 1L with distilled water.

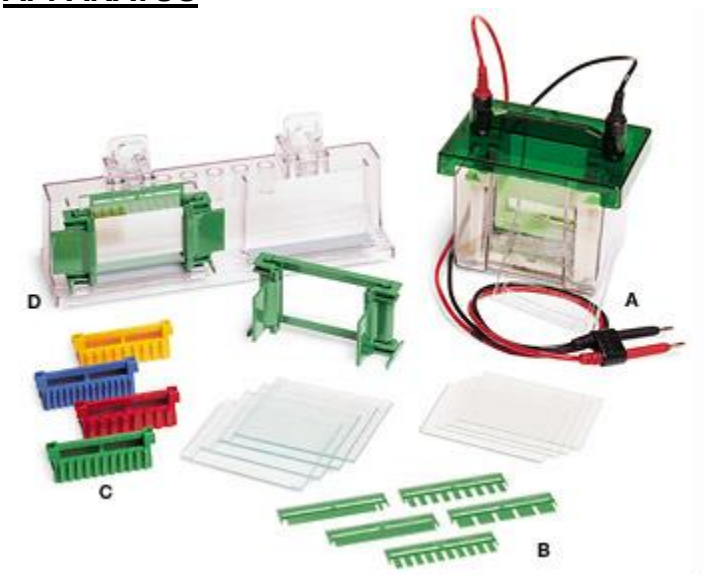
BLOCKING SOLUTION

Dissolve 5g of fat free milk powder in 100ml of TBST

GEL COMPOSITION

Reagent	**Stock**	**7.5% Gel**	**10% Gel**	**12% Gel**	**Stack (4%)**
dH_2O	distilled	5.525 ml	4.9 ml	3.35 ml	3.05 ml
Tris	1.5 M (gel) 0.5 M (stack)	2.50 ml (1.5M)	2.50 ml (1.5M)	2.50 ml (1.5M)	1.25 ml (0.5M)
SDS	10%	100 µl	100 µl	100 µl	50 µl
Acrylamide	40%	1.875 ml	2.50 ml	3.0 ml	0.5 ml
APS (0.1g in 1ml dH_2O) – prepare fresh stock weekly)	10%	50 µl	50 µl	50 µl	50 µl
TEMED	99%	20 µl	20 µl	20 µl	10 µl

APPARATUS



A - gel electrophoresis system tank

B - plastic combs for setting the wells

C - sample loading guide

D - side-by-side casting stand with casting frames and glass plates (consisting of a 0.75mm spacer plate and short plate).

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