

**SIGNALLING PATHWAYS INVOLVED IN INSULIN
CARDIOPROTECTION: ARE THEY COMPARABLE IN
NORMOXIC PERFUSED ISOLATED RAT HEART VS.
ISCHAEMIA/REPERFUSION MODEL?**

By

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DECLARATION

I, the undersigned, hereby declare that the work in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: _____

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OPSOMMING

Inleiding: Dit is welbekend dat toediening van insulien die hart beskerm teen ischemie/reperfusie-beserings, wat lei tot verbeterde hartfunksie. Hierdie effek word wyd ondersoek in modelle van ischemie en reperfusie. Dit is bewys dat 'n verskeidenheid seintransduksie paaie, insluitend PI3-K, PKB/Akt, p70^{S6k} en ERK, betrokke is by hierdie beskermende effek van insulien op die hart. Baie min data is egter beskikbaar rakende die effek van insulien tydens normoksiese toestande. Alhoewel dit bekend is dat insulien 'n inotropiese effek op die normale geperfuseerde hart het, is die presiese sellulêre meganismes wat dit bewerkstellig nog nie nagevors nie. Om dus 'n beter begrip van hierdie meganismes te verkry is dit dus noodsaaklik om die effekte van insulien onder normoksiese perfusie toestande na te vors.

Doelstellings: Om 'n geskikte dosis, waarby insulien sy positiewe inotropiese effek onder normale toestande het, vas te stel, om die moontlike meganismes betrokke by insulien-geïnduseerde verbetering in hartsametreikbaarheid te bestudeer, met spesifieke verwysing na die bloedvoorsiening en koronêre vloei, en om die moontlike betrokkenheid van die PI3-K pad en sy teiken effektore onder normale suurstof-toestande te ondersoek. **Materiaal en metodes:** Geïsoleerde rotharte is geperfuseer deur gebruik te maak van die Langendorff tegniek. Na 'n stabilisasie periode van 10 minute is rotharte blootgestel aan 30 minute perfusie met een van vier oplossings: 'n standaard perfusie oplossing (Krebs-Henseleit buffer met glukose onder spesifieke gaskondisies van 95% O₂, 5% CO₂ – kontrole harte); standaard perfusie oplossing en insulien; standaard perfusie

oplossing met insulien en die stikstofoksied sintase inhibitor L-NAME, of standaard perfusie oplossing, met insulien en die PI3-K inhibitor wortmannin. Met verloop van die perfusie protokol, is ontwikkelde linker ventrikulêre druk (LVDevP), harttempo (HR) en koronêre vloei (CF), sowel as PI3-K en PKB/Akt fosforilasie, gemeet. **Resultate:** Toediening van insulien teen fisiologiese konsentrasies het 'n verbeterde hartfunksie tot gevolg, in vergelyking met harte in die kontrole groep. In teenstelling hiermee het harte wat insulien+L-NAME ontvang het 'n betekenisvolle verlaagde funksie getoon in vergelyking met die kontrole harte en harte wat slegs insulien ontvang het ($p < 0.05$). Harte wat slegs insulien, of insulien+L-NAME ontvang het, het 'n verhoging in gefosforileerde PKB/Akt (Thr308) getoon in vergelyking met kontrole harte. Gefosforileerde PI3-K het ook geneig om hoër te wees in harte wat insulien+L-NAME of insulien+wortmannin ontvang het, as in harte wat slegs insulien ontvang het. **Gevolgtrekking:** Hierdie studie bewys dat fisiologiese konsentrasies van insulien, onder normale suurstof-toestande, 'n positiewe inotropiese effek op hartfunksie uitoefen, soos gesien in die verbeterde LVDevP. Wortmannin-geïnduseerde inhibering van die PI3-K pad het 'n verlaagde PKB/Akt fosforilasie tot gevolg gehad in harte wat insulien+wortmannin ontvang het, terwyl die toediening van L-NAME die voordelige effekte van insulien op hartfunksie onderdruk het. Hierdie resultate dui dus aan dat stikstofoksied 'n rolspeler is in die positiewe inotropiese effek van insulien op hartfunksie tydens normoksiese toestande, aangesien beide inhibitore hierdie effek onderdruk het. Beide inhibitore het ook die betrokkenheid van stikstofoksied en die PI3-K pad by die effek van insulien op harttempo en koronêre vloei onthul.

ABSTRACT

Introduction: It is well documented that insulin offers cardioprotection against the consequences of ischaemia/reperfusion injury. Insulin-induced improvements in cardiac functions are widely investigated in models of ischaemia and reperfusion. It has been shown that many signalling pathways may be involved in the cardioprotection properties of insulin under those conditions. These pathways include PI3-K, PKB/Akt, p70^{S6k}, ERK and many others. However, little data exists on the effects of insulin on the heart under normoxic condition. Some evidence has been presented that insulin has a positive inotropic effect on the normoxic perfused rat heart, but no precise cellular mechanism has been investigated or described in this regard. We believe that an investigation into the effects of insulin on cardiac function and pathways involved under normoxic conditions may help us to better understand the mechanisms of insulin-induced cardioprotection. **Aims:** To determine a suitable dose of insulin at which a positive inotropic response could be detectable under normoxic conditions, to investigate the possible mechanisms involved in insulin-induced increases in contractility with specific reference to the vasculature and the coronary flow and to investigate a possible involvement of PI3-K and its downstream effectors on the insulin effects on cardiac functions under normoxic conditions. **Materials and methods:** Isolated rat hearts were perfused retrogradely using the Langendorff technique. After 10 minutes of stabilization hearts were perfused for 30 minutes either with standard perfusion solution i.e. Krebs-Henseleit buffer + glucose gassed with 95%O₂, 5%CO₂ (control hearts), or with standard perfusion solution plus insulin alone or insulin together with the

nitric oxide synthase inhibitor L-NAME or the PI3-K inhibitor wortmannin. Left ventricular developed pressure (LVDevP), heart rate (HR) and coronary flow (CF) as well as phosphorylated PI3-K and PKB/Akt in heart were measured.

Results: Administration of insulin alone at physiological concentrations showed improved cardiac function compared to hearts in the control group. Hearts that received insulin+L-NAME showed a significant decrease in function compared to the control hearts and the hearts that received insulin alone ($p < 0.05$). Phosphorylated PKB/Akt (Thr³⁰⁸) was increased in hearts that received insulin alone and insulin+L-NAME compared to the control hearts. Phosphorylated PI3-K tended to be higher in hearts where insulin was administered alone compared to the hearts that received insulin+L-NAME or insulin+wortmannin.

Conclusion: This study confirmed that physiological concentrations of insulin exert positive inotropic effects on cardiac function in normoxic perfused rat hearts as seen with the improved LVDevP. Inhibition of PI3-K by wortmannin induced a decrease in phosphorylated PKB/Akt in hearts that received insulin+wortmannin and administration of L-NAME impaired the beneficial effects of insulin on cardiac functions. Therefore these results may indicate that nitric oxide may have a role in the positive effect of insulin on cardiac function in the healthy heart perfused under normoxic conditions. L-NAME as well as wortmannin reversed the positive inotropic effects of insulin. Both inhibitors also unmasked effects of insulin via nitric oxide and PI3-K on heart rate and coronary flow.

RESUME

Introduction: La documentation sur l'insuline et ses propriétés cardioprotectives est vaste. Les améliorations induites par l'insuline des fonctions cardiaques sont largement étudiées sur des modèles ischémie/reperfusion. Il a été démontré que plusieurs voies de signalisation peuvent être impliquées dans les propriétés de cardioprotection de l'insuline dans ces conditions. Ces voies incluent PI3-K, PKB/Akt, p70^{S6k}, ERK et beaucoup d'autres. Cependant, peu de données existent sur les effets de l'insuline sur le coeur dans sous des conditions normales. Des évidences ont été présentée sur le fait que l'insuline a un effet inotropique positif sur le coeur perfusé de rats sous les conditions normales, mais aucun mécanisme cellulaire précis n'a été étudié ou décrit à cet égard. Nous croyons qu'une investigation sur les effets de l'insuline sur la fonction cardiaque sous les conditions normales et les voies impliquées peut nous aider à mieux comprendre les mécanismes de cardioprotection induite par l'insuline. **But :** Déterminer une dose d'insuline appropriée avec laquelle une réponse inotropique positive pourrait être discernable sous des conditions normales, étudier les mécanismes possibles impliqués dans l'accroissement induite par l'insuline de la contractilité avec un regard spécifique sur la vascularisation et l'écoulement coronaire, étudier une l'implication possible de PI3-K et de ses effecteurs sur les effets de l'insuline sur les fonctions cardiaques dans des conditions normales. **Matériels et méthodes :** Des coeurs isolés de rats ont été perfusés de manière rétrograde en utilisant la technique de Langendorff. Après 10 minutes de stabilisation, les coeurs ont été perfusés pendant 30 minutes avec la solution standard de perfusion, c'est-à-dire la solution

tampon de Krebs-Henseleit + glucose aérée avec 95%O₂, 5%CO₂ (coeurs de control), ou avec la solution standard de perfusion plus l'insuline seule ou l'insuline associée à l'inhibiteur de l'oxyde nitrique synthase L-NAME ou à l'inhibiteur de PI3-K wortmannin. Pression développée ventriculaire gauche (LVDevP), fréquence cardiaque (HR) et écoulement coronaire (CF) aussi bien que PI3-K et PKB/Akt phosphoreux des coeur ont été mesurés.

Résultats : L'administration de l'insuline seule aux concentrations physiologiques a montré des fonctions cardiaques améliorées comparées aux coeurs du groupe de control. Les coeurs qui ont reçus l'insuline+L-NAME ont montré un décroissement significatif des fonctions comparés aux coeurs de control et aux coeurs qui ont reçu l'insuline seule ($p < 0.05$). PKB/Akt (Thr308) phosphoreux a augmenté chez les coeurs qui ont reçus l'insuline seule et l'insuline+L-NAME comparés aux coeurs de control. PI3-K phosphoreux a tendance à être plus élevé dans les coeurs où l'insuline seule a été administrée comparé aux coeurs qui ont reçus l'insuline+L-NAME ou l'insuline+wortmannin.

Conclusion : Cette étude a confirmé que les concentrations physiologiques de l'insuline exercent des effets inotropiques positifs sur les fonctions cardiaques des coeurs perfusés normaux de rats comme l'a montré la pression amélioré du ventricule gauche (LVDevP). L'inhibition de PI3-K par le wortmannin a induit une diminution de PKB/Akt phosphoreux dans les coeurs qui ont reçus l'insuline+wortmannin et administration de L-NAME a altéré les effets bénéfiques de l'insuline sur les fonctions cardiaques. Ces résultats peuvent indiquer, par conséquent, que l'oxyde nitrique peut avoir un rôle dans l'effet positif de l'insuline sur les fonctions cardiaques du coeur saint perfusé dans des conditions normales.

L-NAME, aussi bien que le wortmannin, a renversé les effets inotropiques positifs de l'insuline. Les deux inhibiteurs ont également démasqué des effets d'insuline par l'intermédiaire de l'oxyde nitrique et le PI3-K sur l'écoulement coronaire et la fréquence cardiaque.

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CHAPTER 1

INTRODUCTION

It is widely recognized that the heart is the centre of circulation and is the seat of blood circulation within the body. The heart is an organ composed of four chambers, which work closely together. Oxygenated blood from the lungs enters the heart through the left atrium from where it is then pumped into the left ventricle. The filling of the left ventricle occurs during the relaxation phase, known as diastole. The left ventricle empties during its contraction (systole) to facilitate blood circulation in the entire body. Deoxygenated blood from the venous system then re-enters the heart through the right atrium, from where it is pumped into the right ventricle, from where blood is sent back to lungs in order to pick up oxygen and off-load carbon dioxide (Opie, 3rd edition, p8 and 9).

Most of the heart is made up of contractile muscle cells (myocytes) known as *cardiomyocytes*. Myocytes constitute about 75% of the total volume of the myocardium (Brilla et al. 1991); however they are not the most numerous in the organ. The major function of cardiomyocytes is to contract to facilitate the pumping action of the heart. The two main contractile proteins in the heart are the thick myosin filaments and the thin actin filaments that slide over each other during contraction (Opie. 3rd edition, p45). The breakdown of adenosine triphosphate (ATP) is the only source of energy for contraction and other vital functions. The major fuels, in order of importance in the

healthy heart, are free fatty acids, glucose and lactate. These are transformed to acetyl-CoA, which enters the citric acid cycle to produce NADH_2 ($\text{NADH} + \text{H}^+$) (Opie. 3rd edition, p311).

Insulin is a polypeptide hormone that is produced and secreted by the β cells in the pancreas. Insulin exerts a myriad of biological actions, including regulation of glucose uptake and utilisation, protein and lipid synthesis, and gene transcription. It is the primary hormone involved in glucose homeostasis, and an absolute or relative lack of its secretion or action leads to impaired glucose metabolism and diabetes (for review, see Srivastava 1998).

The knowledge of various aspects of insulin action has increased significantly during the past several years, and many signalling steps involved in insulin action have been identified (White and Kahn, 1994). In addition, insulin has been used to improve the efficiency of energy production and recovery of contractile function in the damaged heart (Taegtmeyer & Villalobos 1995).

The biological actions of insulin are initiated by the binding of insulin to its receptor, which is located on the outer surface of the membrane of cells within insulin target tissues.

The insulin receptor (IR) is a hetero-tetrameric glycoprotein composed of two 135-kDa extracellular α -subunits and two 95-kDa transmembrane β -subunits.

The α -subunits possess the insulin binding activity whereas the β -subunits

have an intrinsic protein tyrosine kinase activity. The binding of insulin to the α -subunit of the receptor activates the tyrosine kinase and results in phosphorylation of tyrosine residues of the β -subunit. This phosphorylation subsequently activates several endogenous substrates including insulin receptor substrates (IRS) (Onoda *et al.*, 1989) and related proteins and src-homology 2 (SH2)-domain containing adaptor protein (Shc) (for review see Brownsey, 1997; Srivastava, 1998).

Insulin was first used as a polarising agent for the heart in early 60's by Sodi-Pallares *et al.* in a mixed cocktail of glucose, insulin and potassium for the treatment of acute myocardial infarction (AMI). The use of insulin then shifted to a more metabolic approach since it has the ability to improve the energy production efficiency by increasing glucose uptake and glucose utilisation to improve recovery of contractile function, and to lower the content of free fatty acids (FFA) in the serum (Sidossis *et al.* 1996; Taegtmeyer & Villalobos; 1995).

In mammalian heart and in isolated cardiac muscle preparations, insulin exerts a positive inotropic effect (that is to say it increases the force of contraction of the cardiac muscle). These positive effects on the heart include increases in maximum force generation, improvements in post-ischaemic contractile functions when administered during low flow ischaemia and at reperfusion (Doenst *et al.* 1999, Baines *et al.* 1999; Jonassen *et al.* 2001; Legtenberg *et al.* 2002; Van Rooyen *et al.* 2002; Fischer-Rasokat & Doenst

2003), increases in cardiac output and faster relaxation in both experimental and clinical situations (Reviewed by Aulbach, 1999).

Among the immediate targets of the insulin receptor, the most extensively studied has been the insulin-receptor substrate-1 (IRS-1). Originally observed as a prominent 185-kDa phosphoprotein, this soluble protein becomes phosphorylated in response to insulin on at least 8 out of 20 potential tyrosine phosphorylation sites. These sites occur within Y-X-X-M motifs which are recognized by several defined proteins which contain SH2 (src-homology 2) domains. IRS-1 therefore appears to act as a nucleation site for the assembly of subsequent downstream signalling proteins. Proteins with SH2 domains which associate with IRS-1 include the adaptor proteins Grb2 and Nck, the protein tyrosine phosphatase SH-PTP2/Syp and p85, the 85-kDa regulatory subunit of PI-3-kinase (See Fig. 1.1, p17).

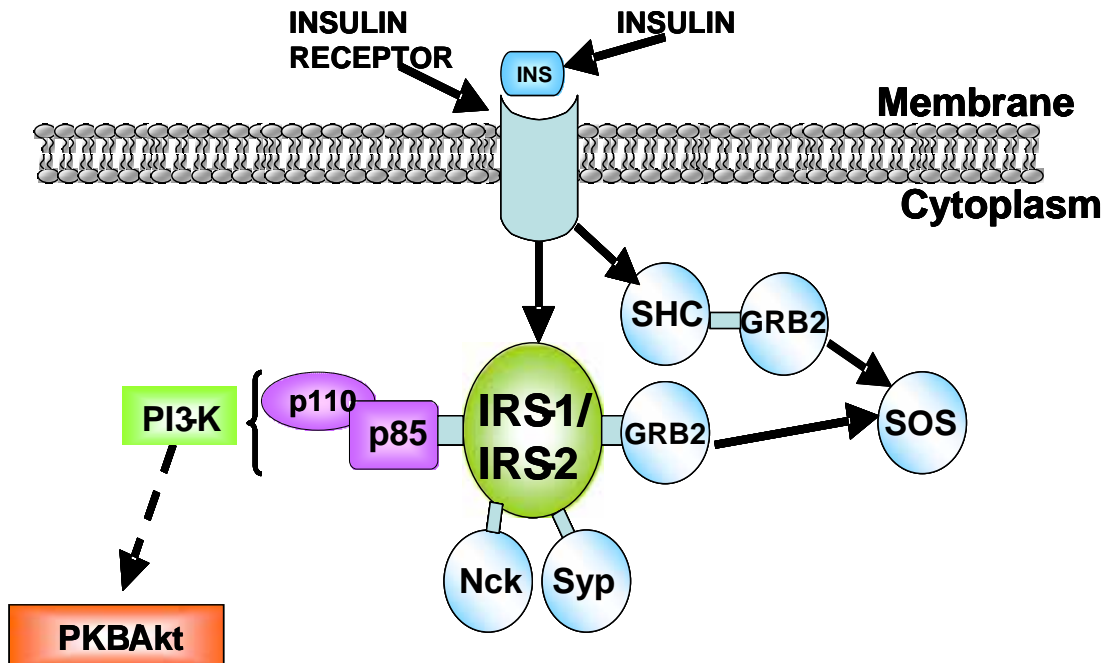


Figure 1.1: Overview of the pathways involved in insulin signalling (Adapted from Shepherd P.R., 1996).

Insulin signalling is mediated by complex multiple cascade pathways characterised by spatial and temporal aspects (Coffer *et al.* 1998; Hue *et al.* 2002). After the binding of insulin to the insulin receptor (IR), the tyrosine kinase activity of IR leads to IR auto-phosphorylation and to the subsequent phosphorylation of insulin receptor substrates (IRS). One of the downstream signalling pathways of IRS involves the activation of phosphatidylinositol 3-kinase (PI3-K) and its recruitment by phosphorylated IRS. PI3-K produces phosphatidylinositol 3,4,5-tris-phosphate [PtdIns(3,4,5)-P₃] and phosphatidylinositol 3,4-bis-phosphate [PtdIns(3,4,-P₂), which bind to the pleckstrin homology (PH) domain of at least two different serine/threonine protein kinases, namely phosphoinositide-dependent protein kinase-1 (PDK-1) and protein kinase B (PKB, also known as Akt) (Coffer *et al.* 1998; Hue *et al.* 2002), (See Fig. 1.2, p19).

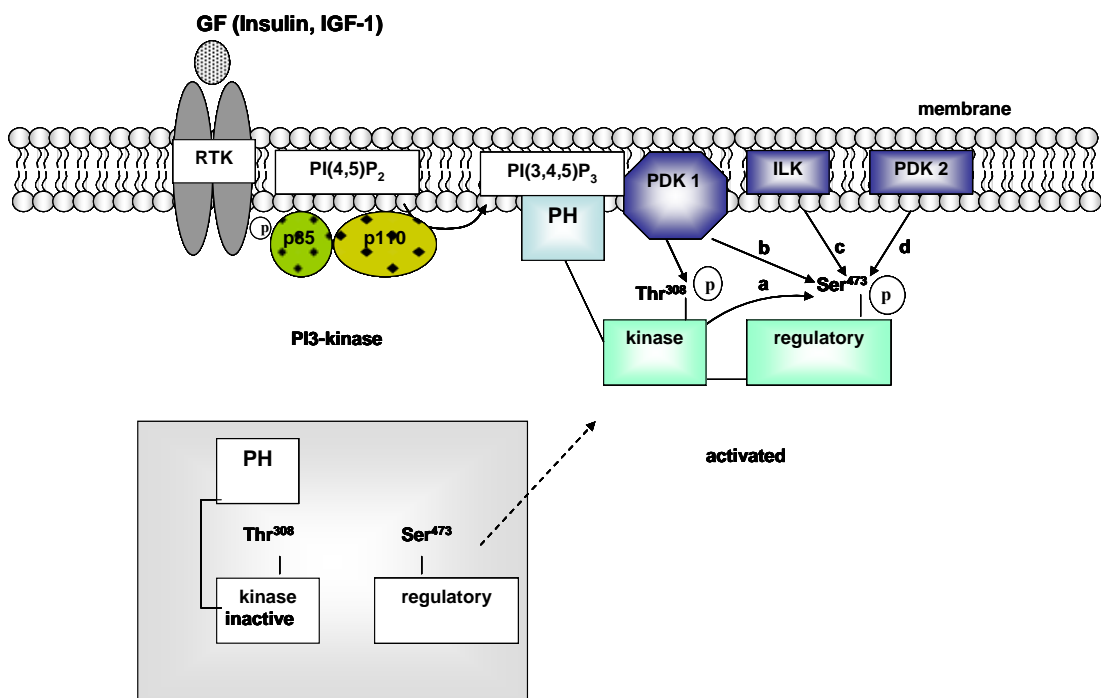


Figure 1.2: Mechanism of activation of PKB/Akt (Adapted from Nicholson and Anderson, 2002).

In un-stimulated cells PKB/Akt is not phosphorylated on Thr³⁰⁸ or Ser⁴⁷³ and resides mainly in the cytosol. Following growth factor (GF) activation of receptor tyrosine kinases (RTKs, or other cell surface receptors, not shown), PI-3K is recruited to the receptor and activated, resulting in the production of PIP₃. This recruits PKB/Akt to the membrane where it is phosphorylated on Thr³⁰⁸ within the catalytic domain by PDK-1 and on Ser⁴⁷³ within the regulatory domain by an ill-defined mechanism, possibly involving (a) auto-phosphorylation, (b) PDK-1, (c) ILK, or (d) an unidentified PDK-2. PKB/Akt is then released from the membrane and translocates to other sub-cellular compartments.

PDK-1 and PDK-2 participate in the phosphorylation and activation of several downstream protein kinases including PKB/Akt and p70^{S6K}. PKB/Akt mediates most short-term effects of insulin which include among others:

- (i) The stimulation of glycogen synthesis by phosphorylation and inactivation of glycogen synthase kinase 3 (GSK-3) (Coffer *et al.*, 1998)
- (ii) Glucose uptake through the recruitment of glucose transporters 4 (GLUT4) to the plasma membrane (Lawrence, 1992);
- (iii) The activation by phosphorylation of phosphodiesterase 3B (PDE 3B, the enzyme responsible for the anti-cyclic adenosine monophosphate (anti-cAMP) effects of insulin) (Wijkander *et al.* 1998);
- (iv) The inhibition of apoptosis by phosphorylation and inactivation of Bad (a pro-apoptotic protein). (Del Peso *et al.*, 1997; Datta *et al.*, 1997). See Fig. 1.3, p21.

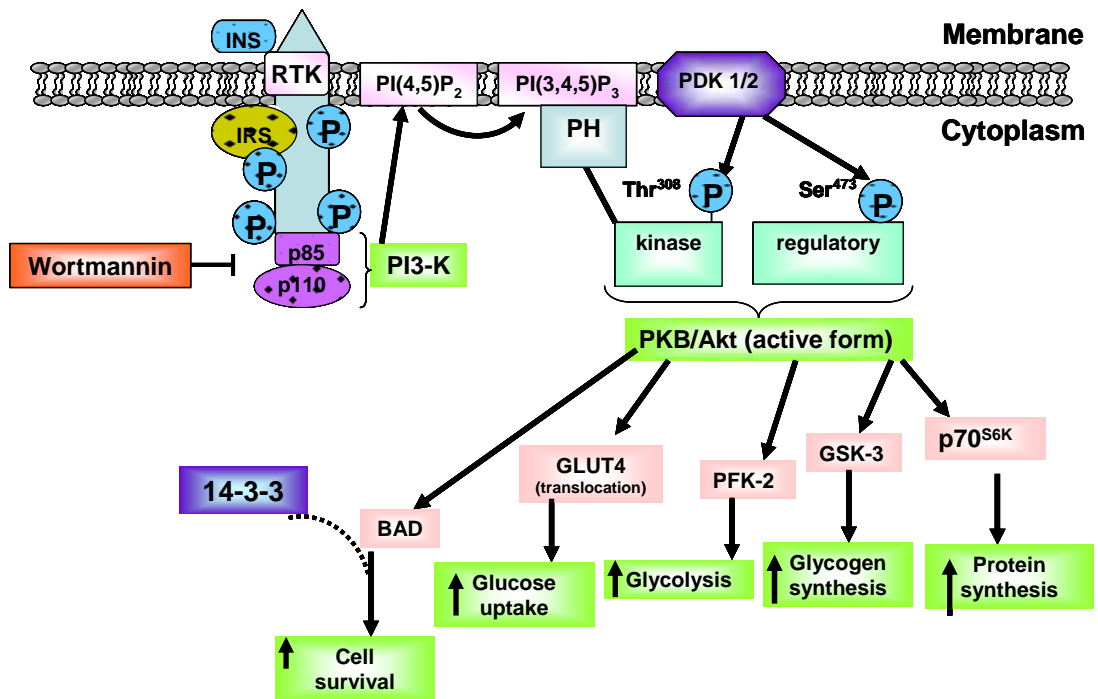


Figure 1.3: Insulin Signalling.

INS = insulin, RTK = receptor tyrosine kinase, IRS = insulin receptor substrate, P = phosphorylation sites, PI3-K = phosphatidylinositol kinase-3, PH = pleckstrin homology, PDK1/2 = phosphatidyl inositol dependent kinase-1/2, PKB/Akt = protein kinase B, GSK-3 = glycogen synthase kinase-3, PFK = phosphofructo kinase-2. GLUT4 = glucose transport, BAD = pro-apoptotic protein, Wortmannin = PI3-K inhibitor.

One of the pathways involved in the insulin cardioprotection is via phosphatidylinositol 3-kinase (PI3-K), which is an heterodimer consisting of a 85 kDa regulatory subunit and a 110 kDa catalytic subunit (Kitamura *et al.* 1998). PI3-K is part of the cascade that activates the serine/threonine kinase Protein kinase B (also known as Akt – PKB/Akt). Most of the different pathways that are activated by Akt are hypothesized to promote cell survival (Matsui *et al.*, 2001)

Studies have shown that insulin, as well as other factors such as ischaemia, stimulate glucose uptake in isolated perfused rat hearts. Glucose is the most used substrate for the hearts perfused in the Langendorff method (for explicit review see Brownsey *et al.*, 1997). Transport of glucose into the myocardial cells is mediated by the glucose transporter (GLUT) isoforms GLUT-4 and GLUT-1. The translocation of GLUTs has been demonstrated in isolated cardiac myocytes (Slot, 1991), in perfused hearts (Watanabe, 1984), and in the heart in vivo (Uphues *et al.*, 1994).

The GLUT-4 isoform is localised mainly on intracellular membrane vesicles in the basal state and translocates to the plasma membrane in response to stimuli (Kolter *et al.* 1992; Egert *et al.* 1999). GLUT 4 is insulin sensitive and is translocated to the sarcolemma where it mediates increased glucose uptake into the myocyte when stimulated by insulin (Stanley *et al.* 1997; King & Opie 1998; Charron & Katz 1998).

Insulin is also involved in the regulation of glycolysis in the heart. Regulation of the glucose transporter GLUT-4 and programmed cell death activity has an important bearing on flux through glycolysis which is generally coordinated with rates of pyruvate oxidation, increasing in response to insulin and to increased cardiac workload during glucose perfusion (reviewed by Brownsey *et al.*, 1997).

It is known that exogenous insulin protects the heart against ischaemia-reperfusion injury of the myocardium (Sodi-Pallares *et al.* 1962; Apstein *et al.* 1983; Oliver & Opie 1994) and there is evidence for clinical efficacy when insulin is given as glucose-insulin-potassium (GIK) (Diaz *et al.* 1998).

Louw (2001) investigated the cardioprotective effects offered by insulin in the ischaemia/reperfusion isolated rat heart model (Louw 2001, Unpublished results, MSc thesis). The study demonstrated and confirmed that insulin administration before, during and after low flow ischaemia, offered cardioprotection that was reflected by improved functional performance during reperfusion and by delayed of time to onset of ischaemic contracture (Louw 2001, Unpublished results, MSc thesis). Insulin has also been shown to reduced the infarct size (Jonassen *et al.* 2000) in the *in vivo* rat heart, and provided better cardiac reperfusion recovery in working heart (Van Rooyen *et al.* 2002).

AIMS

Insulin-induced improvements in cardiac functions are widely investigated in models of ischaemia and reperfusion. It has been shown that many signalling pathways may be involved in the cardioprotection effect of insulin under those conditions. These pathways include PI3-K, PKB/Akt, p70^{S6K}, ERK and many others. However, little data exists on the effects of insulin on the heart under normoxic condition. We therefore hypothesize that insulin may improve cardiac function under normoxic conditions and we believe that an investigation into the effects of insulin on cardiac function and pathways involved under normoxic conditions may help us to better understand the mechanisms of insulin-induced cardioprotection. Therefore, our study aimed to:

- ❖ Determine a suitable dose of insulin at which a positive inotropic response could be detectable under normoxic conditions. This was achieved by perfusing the hearts with three different concentrations of insulin.
- ❖ Investigate the possible mechanisms involved in insulin-induced increases in contractility with specific reference to the vasculature and the coronary flow. A specific regard was given to nitric oxide.

- ❖ Investigate a possible involvement of PI3-K and its downstream effectors on the insulin effects on cardiac functions under normoxic conditions.

Chapter 2

LITERATURE REVIEW

Background on insulin involvement in cardiac therapy.

The heart uses various substrates, eg. fatty acids and glucose, for the production of high-energy phosphates. During ischaemia, glucose becomes the preferred substrate as glycolysis switches from aerobic to anaerobic conditions. Glucose transport into myocardial cells is mediated by the glucose transporter (GLUT) isoforms GLUT-4 and GLUT-1. GLUT-4 is predominant in the heart and is localised mainly on intracellular membrane vesicles in the basal state and translocates to the plasma membrane in response to stimuli such as insulin, ischaemia, and contraction (Kolter *et al.* 1992; Egert *et al.* 1999).

The effects of insulin on energy production and recovery of contractile function in the heart following ischaemia and reperfusion is well known (Taegtmeyer & Villalobos 1995). Sodi-Pallares *et al.* (1962) were the first to use insulin in a mixed cocktail containing glucose, insulin and potassium (GIK) for the treatment of acute myocardial infarction (AMI) (Sodi-Pallares *et al.* 1962).

This GIK cocktail was first advocated as a polarizing solution (Legtenberg *et al.* 2002; Sundell & Knuuti 2003). Due to the ability of insulin to lower the content of free fatty acids (FFA) in serum via the inhibition of the adipose tissue

triacylglycerol lipase and to enhance glucose utilisation (Sidossis *et al.* 1996) it was argued that it could also be a metabolic treatment.

Braimbridge *et al.* (1969) were the first to clinically use GIK infusion in cardiac surgery and they reported that the cocktail was successful in treating patients with low cardiac output after mitral valve replacement, who were not responding to the treatments available at the time (isoprenaline, digoxin or pacemaking) (Braimbridge *et al.* 1969).

However, during the later part of the 1960's a working group from the British Medical Research Council failed to demonstrate any benefit of GIK in the treatment of myocardial infarction (Medical Research Council Working Party on the Treatment of Myocardial Infarction 1968). This prevented the combination of GIK from becoming a standard treatment for patients with acute myocardial infarction. In a recent review Doenst *et al.* (2003) focused on the analysis of insulin based research, and found that about 81% out of 91 study protocols using insulin or GIK for the heart reported a benefit of the insulin treatment while only 19% failed to prove the benefit of insulin. Doenst *et al.* (2003) mentioned that none of the studies that failed to demonstrate a benefit of insulin reported the occurrence of any life-threatening complications (Doenst *et al.* 2003).

Despite the positive observations, the benefit of insulin had still not been noticed and/or validated by the British Medical Research Council and by some researchers. It is only recently that the concept of GIK has attracted renewed

attention after recent studies that demonstrated a reduction in 1-year mortality for diabetic patients with acute myocardial infarction (Malmberg *et al.* 1995; Fath-Ordoubadi & Beatt 1997).

The renewed interest in GIK as a therapy was also due to results showing a direct positive inotropic effect of insulin on the post-ischaemic heart (Doenst *et al.* 1999) in Sprague-Dawley rat heart, or reduction in infarct size. These findings have been confirmed by other groups that used other experimental models including rabbits and Wistar rats (Baines *et al.* 1999; Jonassen *et al.* 2001).

Studies have shown that GIK reduces the infarct size and improves recovery of mechanical function in rat and dog hearts with myocardial infarction (Ahmed *et al.* 1978, Jonassen *et al.* 2000a). Because of these observations, it was thought that the beneficial effects of GIK in myocardial ischaemia may result from increased myocardial glucose uptake and glycogen content, providing additional stores of substrate for glycolytic energy generation (Maroko *et al.* 1972; Ahmed *et al.* 1978; Jonassen *et al.* 2000; Huisamen *et al.* 2001).

The benefit of GIK has also been attributed to its ability to reduce circulating levels and myocardial uptake of FFA, since increased FFA levels are toxic to the ischaemic myocardium and are associated with increased membrane damage, arrhythmias, and decreased cardiac function *in vivo* (Opie & Owen 1976; Apstein 1998).

Under physiological conditions, insulin controls numerous metabolic processes in various targeted tissues, largely through the receptor-mediated tyrosine phosphorylation of insulin receptor substrates 1 and 2 (IRS-1/2) and possibly other adapter proteins. This results in activation of IRS-1/2 dependent phosphatidylinositol 3-kinase (PI3-K) and its downstream effectors, protein kinase B (PKB, also known as Akt) and atypical protein kinase C (aPKC) (Takata *et al.* 1999, Bandyopadhyay *et al.* 2000). In studies conducted on muscles and adipocytes regarding insulin's action on cells, it appears that both aPKC and Akt control glucose transport, in addition to Akt regulating glucose storage in the form of glycogen (Kohn *et al.* 1996; Bandyopadhyay *et al.* 1997; Takata *et al.* 1999; Hill *et al.* 1999; Bandyopadhyay *et al.* 2000). See Fig. 2.1, p27.

Previous studies on insulin action in the heart.

Insulin is a well known vasodilator and has positive inotropic effects, and hence improves post-ischaemic contractile function of the heart when given during low flow ischaemia and at reperfusion in both experimental and clinical situations (Jonassen *et al.* 2001; Legtenberg *et al.* 2002; Van Rooyen *et al.* 2002; Fischer-Rasokat & Doenst 2003).

Benefit of insulin on contractility

Insulin has been shown by several groups to improve the recovery of contractile function of the heart after a no flow and/or a low flow ischaemia (Ahmed *et al.* 1977, Tomai *et al.* 1999; Jonassen *et al.* 2001; Legtenberg *et al.* 2002; Van Rooyen *et al.* 2002). In her protocol, Louw observed an

increase in the left ventricular developed pressure (LVDevP) in the control hearts that received insulin (Louw 2001, Unpublished results, MSc thesis).

Insulin signalling is an important regulator of substrate metabolism in vertebrates. In the healthy heart, insulin has direct effects on glucose transport (Abel *et al.* 1999; Belke *et al.* 2001), glycolysis (Depre *et al.* 1998), glucose oxidation (Patti & Kahn 1998), glycogen synthesis (Laughlin *et al.* 1992), and protein synthesis (Flaim *et al.* 1983).

Glucose transport

Myocardial glucose metabolism is dependent on the uptake of extracellular glucose, which is regulated by the transmembrane glucose gradient and the activity of glucose transporters GLUT-1 and GLUT-4. In the heart, GLUT-1 is relatively insulin insensitive and is considered to be responsible for basal glucose uptake in the setting of low fasting insulin concentrations and normal conditions. GLUT-4, which is insulin sensitive, is distributed to a greater extent in the intracellular vesicles under normoxic conditions. On stimulation by insulin or ischaemia, GLUT-4 is translocated to the sarcolemma where it mediates increased glucose uptake into the myocyte (Stanley *et al.* 1997; King & Opie 1998; Charron & Katz 1998).

Activation of PKB/Akt by insulin leads to the protection of the myocardium via various processes among which the translocation of glucose transporters to increase glucose uptake (Lawrence, 1992).

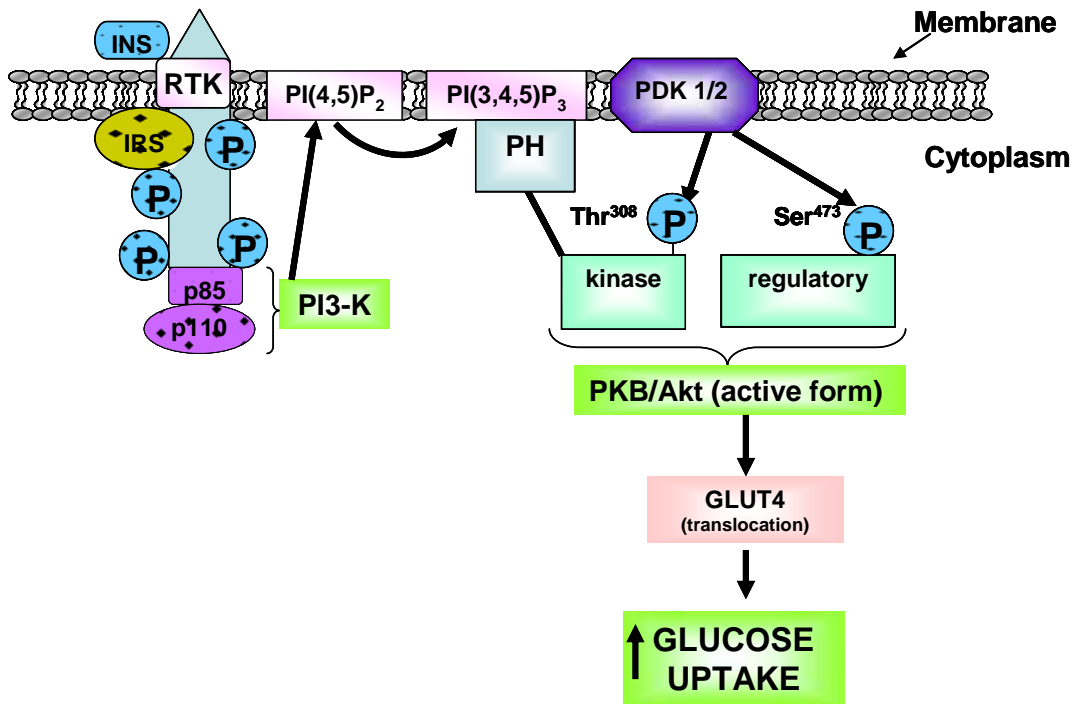


Figure 2.1: Pathways involved in insulin-mediated increase of glucose uptake

The action of insulin on the cell is initiated by the binding of insulin to its receptor which results in the stimulation of the insulin receptor's intrinsic tyrosine kinase activity. This mediates auto-phosphorylation of the receptor and subsequent phosphorylation of substrate proteins on multiple tyrosine residues. The major substrates of the insulin receptor (IR) are the insulin receptor substrate (IRS) proteins IRS-1, IRS-2, IRS-3 and IRS-4, but the most investigated are IRS-1 and IRS-2 (Sun *et al.* 1991; Sun *et al.* 1995; Lavan *et al.* 1997a; Lavan *et al.* 1997b).

Insulin administration before ischaemia

Apstein *et al.* (1983) showed that high glucose and insulin administration during moderate but not severe ischaemia has a protective effect on the heart. This observation was also confirmed by Van Rooyen *et al.* (2002). In their study, Apstein *et al.* (1983) observed that the beneficial effects of the glucose and insulin administration during moderate ischaemia were:

- Improved contractile function during the post-ischaemic recovery period,
- The prevention of contracture during ischaemia,
- The lesser degree of contracture during recovery, and
- The higher level of myocardial adenosine triphosphate (ATP) during the ischaemic period.

Insulin administration at reperfusion

Focusing on the effect of insulin on the heart at reperfusion, Jonassen *et al.* (2000) demonstrated that in rat neonatal cardiomyocytes, administration of insulin at the onset of reoxygenation following a simulated ischaemic insult resulted in a significant reduction both in total myocyte death and in the development of apoptosis compared to models where insulin was administered to the whole heart before or during low flow ischaemia (Jonassen *et al.* 2000a). In the isolated, perfused heart, these cardio-protective effects of insulin seemed to be mediated via the tyrosine kinase and phosphatidylinositol 3-kinase (PI3-K) signalling pathways since the protective effect observed with insulin at reperfusion was completely abolished when the hearts were treated with lavendustin-A (a tyrosine kinase inhibitor, 0.1 $\mu\text{mol/L}$ was used) or with wortmannin (a PI3-K inhibitor, 1 $\mu\text{mol/L}$ was used) (Jonassen *et al.* 2001). This suggested that insulin improves post-ischaemic recovery of function through PI3-K. In addition, insulin increases cardiac contractility and has an anti-apoptotic effect on cardiomyocytes (Jonassen *et al.* 2001; Louw 2001, Unpublished results, MSc thesis).

In vivo, many of the effects of insulin on cardiac metabolism and function are related to the systemic effects of insulin, such as increased peripheral and coronary vasodilatation (Baron 1994), increased sodium and water uptake by the kidneys (DeFronzo 1981), and changes in the delivery of substrates to the heart (Brownsey *et al.* 1997; Jagasia *et al.* 2001). For example, insulin's anti-lipolytic effect will reduce the delivery of FFAs to the heart, which in

conjunction with increased intracellular Malony-coenzyme A levels, reduces fatty acid oxidation rates (Awan & Saggerson 1993).

Effects of insulin on cardiac function

A key physiological action of insulin is the stimulation of glycogen synthesis, involving an increase in the glucose uptake and activation of the enzyme glycogen synthase (GS). Insulin has been demonstrated to cause inactivation of glycogen synthase kinase-3 (GSK-3) in several cell types; and in rabbits *in vivo* (Welsh & Proud 1993; Cross *et al.* 1994; Borthwick *et al.* 1995).

As the 90 kDa ribosomal S6 kinase p90^{rsk} (also called MAPKAP kinase 1 or rsk-2) can phosphorylate and inactivate GSK-3 *in vitro* and as it lies downstream of the insulin-stimulated mitogen activated-protein (MAP) kinase cascade it provides an attractive mechanism whereby insulin could stimulate GS by both activation of protein phosphatase 1 (PP 1) and inactivation of GSK-3 (Sutherland & Cohen 1994; Seger & Krebs 1995).

Some studies have also demonstrated that activation of GS, inactivation of GSK-3 and stimulation of glucose uptake by insulin are blocked by wortmannin (a fungal inhibitor of phosphatidylinositol 3-kinase) placing the relevant pathway(s) downstream of that enzyme (Arcaro & Wymann 1993). One must also consider that the time span where insulin effects on the heart can be observed depends on the dose and model used. Hurel *et al.* (1996) have shown for example that it takes less than 10 min to observe insulin

effects on cultured human myoblasts from the gastrocnemius muscle (skeletal muscle) of healthy subjects (Hurel *et al.* 1996).

Ischaemia and cardiac function

Myocardial ischaemia takes place when there is a reduction of the coronary flow resulting in an inadequate oxygen supply to the tissue. It is a multi-factorial process characterised by several changes like loss of energy, membrane damage, and metabolite accumulation (Opie 1998, p515).

Reperfusion of the ischaemic heart improves cardiac metabolism by increasing contractile activity and the washout of harmful products (Neely & Grotyohann 1984). Reperfusion also provides the heart with oxygen and substrate. Despite its beneficial effects on the ischaemic heart, reperfusion might also induce cell injury by worsening the injury sustained during ischaemia (Tennant & Wiggers 1935). These injuries which may include arrhythmias, fibrillation and myocardial stunning, may have a deleterious impact on cardiac function at reperfusion (Bolli 1991; Reimer *et al.* 1993).

Signalling Pathways involved in insulin cardioprotection

Insulin signalling is mediated by complex multiple cascade pathways characterised by spatial and temporal aspects. Previous work on tissues and cells showed that insulin signalling is initiated by its binding to the insulin receptor (IR) (Coffer *et al.* 1998; Hue *et al.* 2002). This activates the tyrosine kinase activity of IR leading to IR auto-phosphorylation and to the subsequent phosphorylation of insulin receptor substrates (IRS). One of the

downstream signalling pathways of IRS involves the activation of phosphatidylinositol 3-kinase (PI3-K) and its recruitment by phosphorylated IRS. Phosphorylation of PI3-K produces phosphatidylinositol 3,4,5-tris-phosphate [PtdIns(3,4,5)-P₃] and phosphatidylinositol 3,4-bis-phosphate [PtdIns(3,4)-P₂], which bind to the pleckstrin homology (PH) domain of at least two different serine/threonine protein kinases, namely phosphoinositide-dependent protein kinase-1 (PDK-1) and protein kinase B (PKB, also known as Akt) (Coffer *et al.* 1998; Hue *et al.* 2002).

PDK-1 participates together with PDK-2 in the phosphorylation and activation of several downstream protein kinases including Akt and p70^{S6K}. Akt mediates most short-term effects of insulin which include among others:

- (i) The stimulation of glycogen synthesis by phosphorylation and inactivation of glycogen synthase kinase 3 (GSK-3) (Coffer *et al.*, 1998);
- (ii) Glucose uptake through the recruitment of glucose transporters 4 (GLUT4) to the plasma membrane (Lawrence, 1992);
- (iii) The activation by phosphorylation of phosphodiesterase 3B (PDE 3B, the enzyme responsible for the anti-cyclic adenosine mono phosphate (anti-cAMP) effects of insulin) (Wijkander *et al.*, 1998);
- (iv) The inhibition of apoptosis by phosphorylation and inactivation of Bad (a pro-apoptotic protein) (Del Peso *et al.*, 1997; Datta *et al.*, 1997).

The stimulation of heart glycolysis by insulin is due to the concomitant recruitment of GLUT4 and an increase in fructose-2,6-P₂ concentration which itself results from phosphofructo kinase-2 (PFK-2) activation. Insulin has an important role in normal vascular function. In healthy subjects, insulin increases not only blood flow but also blood volume in skeletal muscle, classifying insulin as a true vasodilatory hormone (Raitakari *et al.* 1995; Mather *et al.* 2001; Hue *et al.* 2002).

The protein kinases

The signalling pathways that determine myocardial susceptibility to injury during ischaemia and reperfusion include protein phosphorylation and those pathways that may regulate myocyte loss through programmed cell death (Jonassen *et al.* 2001).

Protein kinase B/Akt

Protein kinase B, also known as serine-threonine kinase Akt, (PKB/Akt) is a member of the second messenger-dependent family of serine/threonine kinases that have been implicated in the signalling pathways downstream of the growth factor receptor tyrosine kinases (Meier *et al.* 1997; Nicholson & Anderson 2002). PKB/Akt activation is induced by several growth factors including nerve growth factor (NGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor such as β fibroblast growth factor (β FGF) and heparin binding growth factor (HBGF), insulin and insulin-like growth factor-1 (IGF-1) (Franke *et al.* 1997; Downward 1998).

PKB/Akt is a downstream effector of phosphatidylinositol-3 kinase (PI3-K). It was originally cloned as the human homologue of (Stool *et al.*, 1977) *v-akt*, a retrovirus associated (Akt8) oncogene. Akt is a multifunctional signalling intermediate in the regulation of apoptosis, cell cycle progression, and energy metabolism. Three mammalian isoforms of Akt – Akt1 (PKB α), Akt2 (PKB β) and Akt3 (PKB γ) exist. Akt is expressed ubiquitously with elevated expression in brain, heart and lungs (Johnson 1988; Jones *et al.* 1991; Brazil & Hemmings 2001; Scheid & Woodgett 2001)

All three isoforms of PKB/Akt share three domain structures consisting of an N-terminal pleckstrin homology (PH) domain, followed by a kinase domain related to protein kinases A and C (containing Thr³⁰⁸ in Akt1), and a C-terminal regulatory domain (containing Ser⁴⁷³ in Akt1) (Franke *et al.* 1997; Scheid & Woodgett 2001). PH domains of Akt have high-affinity recognition of phosphoinositide head groups.

Activation of PI3-K leads to phosphorylation of membrane phosphatidylinositol-4,5-bis-phosphate [PtdIns(4,5)-P₂], generating phosphatidylinositol-3,4,5-tris-phosphate [PtdIns(3,4,5)P₃] and phosphatidylinositol-3,4,-bisphosphate [PtdIns(3,4)P₂].

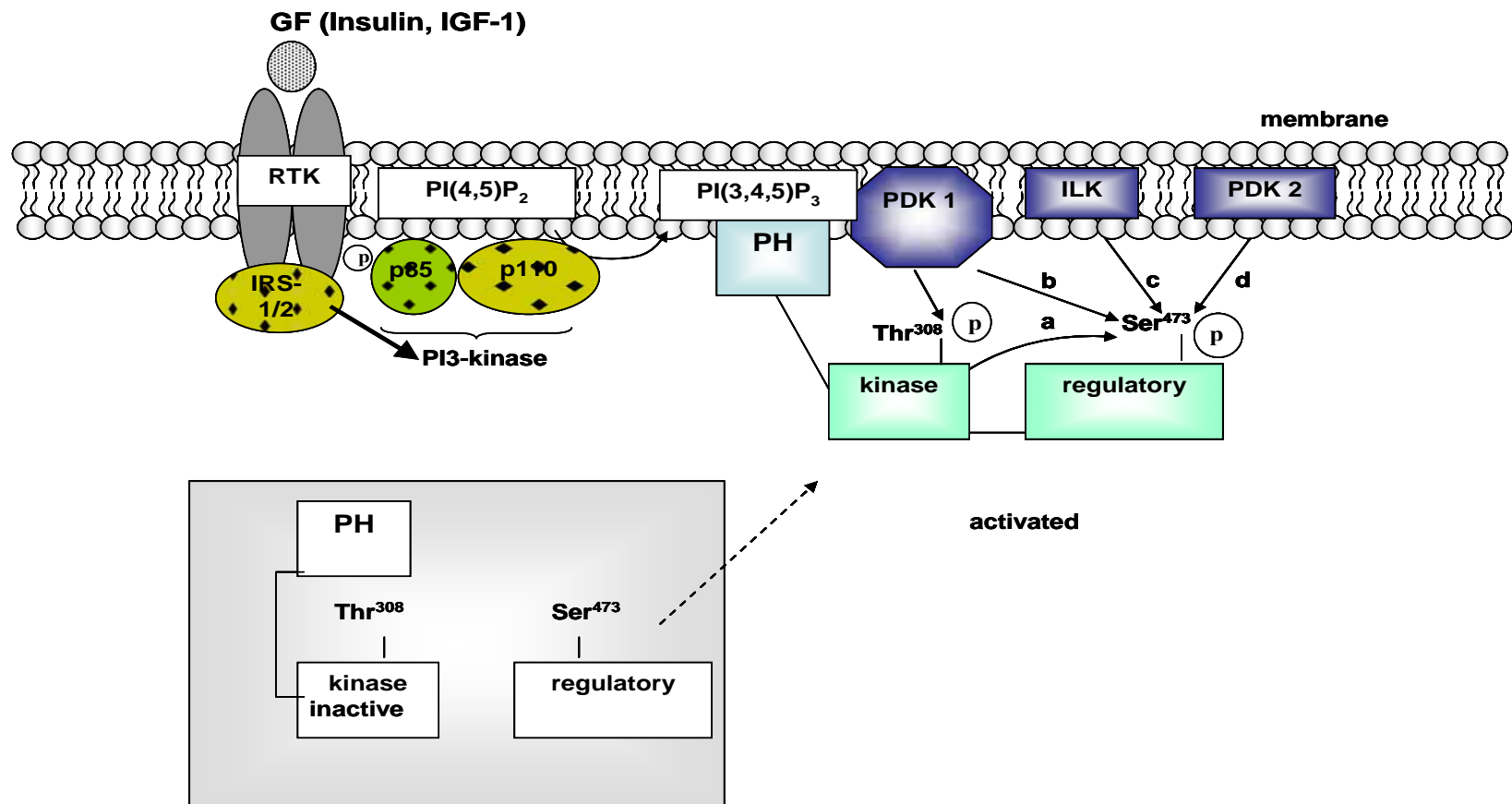


Figure 2.2: Mechanism of activation of PKB/Akt.

In unstimulated cells PKB/Akt is not phosphorylated on Thr³⁰⁸ or Ser⁴⁷³ and resides mainly in the cytosol. Following growth factor (GF), insulin and insulin-like growth factor-1 (IGF-1) activation of receptor tyrosine kinases (RTKs, or other cell surface receptors, not shown), PI-3K is recruited to the receptor and activated, resulting in the production of PIP₃. This recruits PKB/Akt to the membrane where it is phosphorylated on Thr³⁰⁸ within the catalytic domain by PDK-1 and on Ser⁴⁷³ within the regulatory domain by an ill-defined mechanism, possibly involving (a) auto-phosphorylation, (b) PDK-1, (c) ILK, or (d) an unidentified PDK-2. PKB/Akt is then released from the membrane and translocates to other sub-cellular compartments (Adaptation from Nicholson and Anderson, 2002).

Although phosphorylation of Thr³⁰⁸ partially activates Akt (Alessi *et al.* 1996), phosphorylation of the Ser⁴⁷³ in a C-terminal regulatory domain is required in order to induce full activation of Akt (Andjelkovic *et al.* 1999; Scheid *et al.* 2002). Many stimuli have been identified that are capable of activating Akt in different organs and tissues. These stimuli include insulin, IGF-1, ischaemia/reperfusion (hypoxia), pressure overload and β -adrenergic receptor agonist for example in the prevention of apoptosis in rat neonatal cardiomyocytes (Matsui *et al.* 1999; Aikawa *et al.* 2000; Chesley *et al.* 2000) and in ischaemia/reperfusion injury (Yamashita *et al.* 2001; Gao *et al.* 2002).

In ischaemia/reperfusion models, many downstream targets of PKB/Akt exist but the most relevant to cardioprotection and widely investigated are those implicated in anti-apoptotic effects, cell growth, cardiac function, and protein synthesis.

Protein kinase B/Akt and apoptosis

In cardiomyocytes, insulin activates PI3-K and PKB/Akt. Activation of PI3-K appears largely necessary for the anti-apoptotic effects of IGF-I (Matsui *et al.* 1999). In many recent studies, PKB/Akt has been shown to positively mediate cell survival in skeletal muscle, neurons, endothelial and epithelial cells (Dudek *et al.* 1997; Fujio *et al.* 1999; Edwards *et al.* 2002; Wang *et al.* 2002; Suhara *et al.* 2003).

Activation of PKB/Akt *in vivo* reduces cardiomyocyte apoptosis and infarct size after transient ischaemia. Studies have shown that activation of PKB/Akt leads to phosphorylation of the pro-apoptotic Bcl-family protein Bad, that is involved in the anti-apoptotic signalling in many tissues (Aikawa *et al.* 2000; Matsui *et al.* 2001; Negoro *et al.* 2001). PKB/Akt has also been reported to mediate phosphorylation and inhibition of caspase-9 in cell models (Cardone *et al.* 1998).

Some studies have shown that activation of PKB/Akt not only reduces cardiomyocyte death in a model of *in vivo* ischaemia-reperfusion but also substantially improves regional and overall cardiac function. The functional improvement observed appeared out of proportion to the survival benefit in both *in vitro* and *in vivo* models. Following that observation, Matsui *et al.* (2001) investigated whether PKB/Akt activation had more direct effects on cardiomyocyte function after transient hypoxia. They found that activation of PKB/Akt in cardiomyocytes *in vitro* dramatically protected cardiomyocytes from hypoxia-induced dysfunction.

They assessed cardiomyocyte function by measuring the cellular contraction and Ca^{2+} handling and found that inhibition of endogenous PKB/Akt substantially accelerated the hypoxia-induced decline in cardiomyocyte function. Their results demonstrated that acute activation of PKB/Akt

mediates meaningful rescue *in vivo* and that this reflects the combined benefits of inhibition of apoptosis and preservation of function (Matsui *et al.* 2001). These findings imply that there is a convergence of pathways controlling survival and function in cardiomyocytes.

Phosphatidylinositol-3 kinase

Phosphoinositide 3-kinases (PI3-Ks) are a unique family of enzymes that contain both lipid and protein kinase activity (Dhand *et al.* 1994). This family of protein and lipid kinases is involved in multiple biological processes including cell proliferation and survival, cytoskeletal remodelling and membrane trafficking (Cantley 2002).

PI3-K is a heterodimeric protein consisting of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (Kitamura *et al.* 1998). PI3-K is implicated in various metabolic effects of insulin. Recent studies have shown that insulin improves post-ischaemic recovery of function through PI3-K in isolated rat heart and that PI3-K is implicated in the regulation of PKB/Akt since inhibition of PI3-K by wortmannin affects PKB/Akt (Franke *et al.* 1995; Kohn *et al.* 1995; Louw 2001; Van Rooyen *et al.* 2002).

Based on their biochemical properties, these enzymes are divided into three classes. At least three members (α , β and γ) of class I PI3-Ks are expressed

in the mammalian heart (Prasad *et al.* 2003). While PI3-K α and β (class IA PI3-Ks) associate with p85-like regulatory proteins docking to phosphorylated tyrosines in YXXM motives, PI3-K γ (the unique class IB PI3-K) binds directly or in association with the p110 adaptor to the $\beta\gamma$ subunits of G proteins and is thus activated by G-protein-coupled receptors (GPCRs) (Vanhaesebroeck *et al.* 2001).

Table 1: Regulation of cardiac function upon modulation of PI3-K and its immediate effectors (Modification from Prasad *et al.* 2003).

	Molecule modulated	Effect	Reference:
<i>In vivo</i> studies	Constitutively active PKB/Akt	Increased heart size and contractility	Shioi <i>et al.</i> 2002, Condorelli <i>et al.</i> 2002
	PI3-K γ knockout	Enhanced contractility, PLB phosphorylation, and cAMP generation	Crackower <i>et al.</i> 2002.
	Conditional PTEN knockout	Spontaneous hypertrophy and reduced contractility	Crackower <i>et al.</i> 2002.
<i>In vitro</i> studies	Inhibition of PI3-K with inhibitors	Increased PLB phosphorylation and myocyte contractility	Jo <i>et al.</i> 2002.
	Disruption of PI3-K– β -ARK interaction	Attenuation in β -AR internalization	Naga Prasad <i>et al.</i> 2002.

β -ARK, β -adrenergic receptor kinase; cAMP, cyclic adenosine monophosphate; PI3-K, phosphatidylinositol 3-kinase; PKB/Akt, protein kinase B (also known as Akt); PLB, phospholamban; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

PI3-K and cardiac contractility

Stimulation of β -adrenergic receptors (β -AR) leads to dissociation of heterotrimeric G protein into $G\alpha$ and $G\beta\gamma$ subunits, allowing $G\alpha$ to activate adenylyl cyclase (AC) and increase intracellular cyclic adenosine monophosphate (cAMP) levels. Increased cAMP activates protein kinase A (PKA), which phosphorylates troponin I, the L-type Ca^{2+} -channel, and phospholamban (PLB), leading to enhanced contractility. This is represented by the classic paradigm of contractility regulated by the adrenergic agonists. (See Fig. 2.3, p45).

A study by Jo *et al.* (2002) has shown potentiating effects in the contractile and relaxant responses of adult ventricular rat myocytes to adrenergic stimulation in the presence of the selective PI3-K inhibitors LY294002 and wortmannin. The potentiating effects of PI3-K inhibitors in the myocytes were not accompanied by an increase in β -AR-induced cAMP levels, but were associated with increased PLB phosphorylation.

This study suggests that PI3-K plays a role in regulating contractility of cardiomyocytes, possibly by either directly or indirectly modulating PLB phosphorylation. Furthermore, the potentiating effects of PI3-K inhibitors on contractile and relaxation events of myocytes were completely abolished upon inhibition of $G\beta_i$ signalling by pertussis toxin, suggesting the involvement

of $G\beta_i$ -mediated signalling in the regulation of PI3-K activity (Jo *et al.* 2002; Prasad *et al.* 2003).

PI3-K and β -adrenergic signalling

➤ Receptor function

Adrenergic and muscarinic cholinergic receptor systems are important for the heart because they function in homeostatic regulation of the cardiovascular system. At least nine subtypes of adrenergic receptors (ARs) have been cloned, and of them, beta-ARs (β -ARs) are the most predominant subtypes present in the heart.

β -ARs are further divided into three isoforms designated β_1 -, β_2 -, and β_3 -ARs. The β -AR signal system is one of the most powerful regulators of cardiac function, mediated by the effects of sympathetic transmitters. Failing human hearts are exposed to increased circulating levels of catecholamines, with consequent marked abnormalities in the β -AR signalling system (Rockman *et al.* 2002).

Chronic increase in catecholamines leads to both β -AR desensitisation and down regulation (diminished receptor number) that is, in part, attributed to increased levels of myocardial beta-adrenergic receptor kinase – 1 (β -ARK1)

(Koch *et al.* 2000). β -ARK1 activation and its association with the plasma membrane are facilitated by binding to the liberated $G_{\beta\gamma}$ subunits of the heterotrimeric G protein (Pitcher *et al.* 1998). Activated β -ARK1 phosphorylates the β -AR, allowing for β arrestin to bind and interdict further coupling. The phosphorylated receptor binds β arrestin, leading to the recruitment of adaptor protein (AP)-2 and clathrin, which forms the clathrin-coated endocytic vesicles and promotes receptor internalisation (Perry & Lefkowitz 2002; Claing *et al.* 2002). In one of their previous studies, Naga Prasad *et al.* (2002) showed an important link between activation of PI3-K and GPCRs function specifically in the process of internalisation.

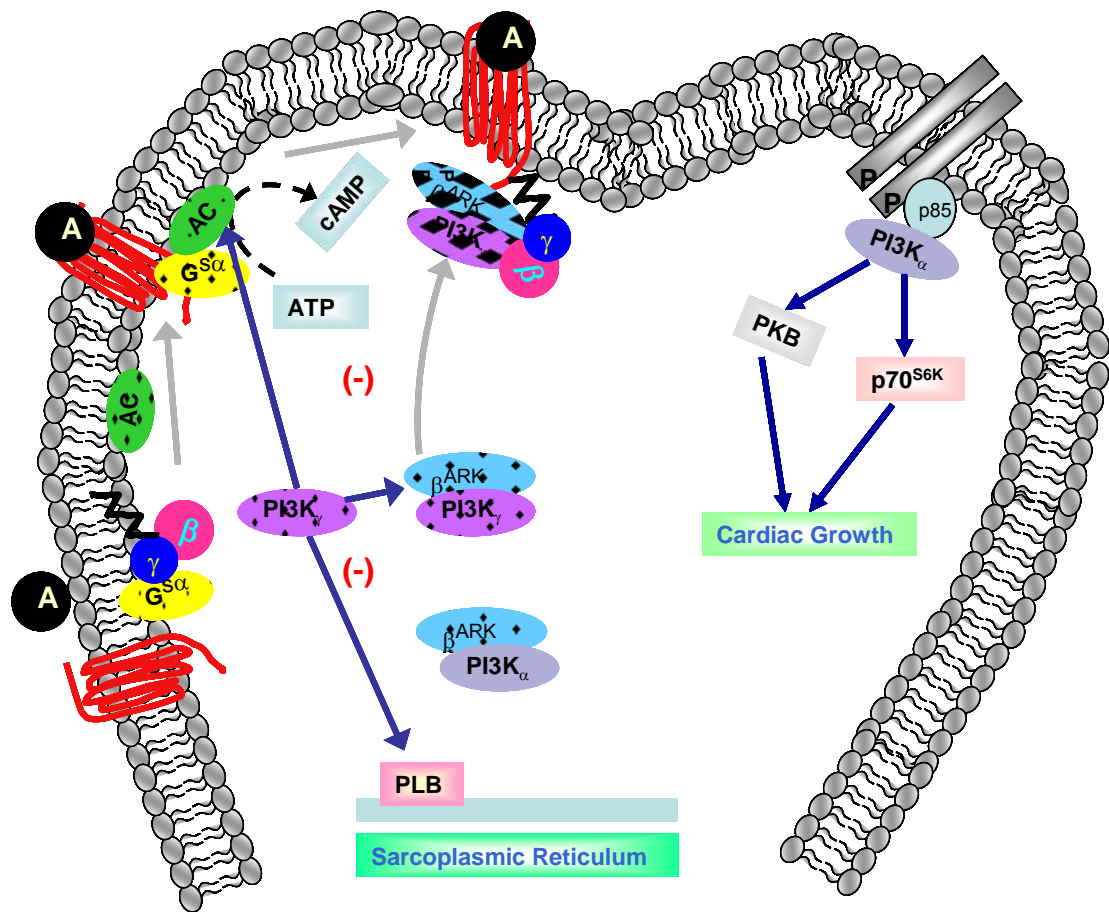


Figure 2.3: Potential regulation of molecules by PI3-K.

Agonist binding to β -ARs leads to the dissociation of heterotrimeric G proteins into G_{α} and $G_{\beta\gamma}$ subunits. G_{α} activates AC generating cAMP, which increases the level of cardiac contractility. Absence of PI3-K _{γ} leads to enhanced contractility and cAMP generation, suggesting that PI3-K negatively (-) regulates AC.

The release of $G_{\beta\gamma}$ subunits activates and translocates β -ARK1 to the agonist-occupied receptor complex. The interaction of β -ARK1 and PI3-K to form a cytosolic complex is independent of the PI3-K isoform, because the binding motif is conserved in all PI3-K isoforms. β -ARK1 will complex with either PI3-K _{α} or PI3-K _{γ} , depending on the availability of the isoform in a given tissue, the predominant isoforms in heart tissues being PI3-K _{α} and γ , with very little expression of PI3-K _{β} isoform.

Upon agonist stimulation, β -ARK1 mediates translocation of PI3-K to the receptor complex. At the receptor complex, PI3-K generates D-3 phosphoinositides that regulate receptor internalization through the recruitment of essential adaptor proteins. Pharmacologic inhibition of PI3-K shows enhanced PLB phosphorylation, suggesting a negative regulation of PLB by PI3-K. Activation of growth factor receptors leads to activation of PI3-K _{α} , PKB/Akt, and p70S6K, which together modulate the increase in cardiomyocyte size (Reproduced from Naga Prasad *et al.*, 2003).

➤ **Evidence that some isoforms of PI3-K gamma may play an anti-inotropic role**

The molecular details underlying the mechanism of action of PI3-K γ in cardiac cells remain to be defined. However, PI3-K γ -deficient cardiomyocytes showed basal alterations in the signalling events classically involved in the control of contractility. This process, known as modulation of cardiac inotropism, involves the activation by GPCRs of heterotrimeric G-proteins, the induction of cAMP synthesis and the subsequent activation of PKA.

PKA, in turn, phosphorylates L-type Ca²⁺ channels, enhancing the transmembrane Ca²⁺ current that activates troponin C and the contractile machinery. At the same time, PKA inhibits PLB, leading to sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) activity and the re-uptake of Ca²⁺ into the intracellular stores (Rockman *et al.* 2002). An increased concentration of cAMP both under basal conditions and after stimulation of β -ARs was shown in isolated mutant cardiomyocytes, where an increased contractility (and relaxation) was detected in PI3-K γ -null hearts. β -ARs are the primary players in the positive modulation of cardiac inotropism (Alloatti *et al.* 2003). A possible cause of the increased concentration of cAMP after β -adrenergic stimulation could be that the lack of PI3-K γ promoted an abnormal elevation in the number of active β -ARs. PI3-K γ appears to be involved in β -AR internalisation (Rockman *et al.* 2002).

In vitro studies showed that the phosphoinositide kinase homology domain of PI3-K γ interacts with β -AR kinase 1, forming a cytosolic complex that is translocated to the plasma membrane upon β -AR stimulation. This event causes the activation of PI3-K γ , which then facilitates the recruitment of the clathrin adaptor AP-2 to the desensitized receptor (Naga Prasad *et al.* 2000; Naga Prasad *et al.* 2002). This process, however, appears to play a minor role *in vivo*.

The mammalian β -ARs comprise at least three different isoforms. Although the β 1-AR isoform couples only to G $_{\alpha s}$ -containing G-proteins and is the major isoform responsible for the enhancement of the intracellular cAMP concentration, the β 2-AR isoform can bind both G $_{\alpha s}$ and G $_{\alpha i}$ G-proteins (Rockman *et al.* 2002). In addition, in the mouse heart, β 2-AR causes localised increases in the cAMP concentration and does not induce a positive inotropic response, thus suggesting that the receptor is physically restrained in its interactions with the activating downstream signalling machinery (Xiao *et al.* 1999). In PI3-K γ -deficient cardiomyocytes, in contrast with the possible confinement of the receptor, specific stimulation of β 2-ARs caused a strong global enhancement of cyclic adenosine monophosphate (cAMP) production, indicating that PI3-K γ activity was required to maintain the β 2-AR in its uncoupled state.

In agreement with this view, treatment of cardiomyocytes with PI3-K inhibitors such as wortmannin has been shown to enhance both β 2-AR-dependent L-type Ca^{2+} flux and cell shortening (Koss & Kranias 1996). These findings thus imply that PI3-K products might be needed to compartmentalise specific receptor-mediated signalling events, and that, in this specific case, they might prevent β -ARs from activating $G_{\alpha s}$.

Although this hypothesis is promising, several aspects of PI3-K γ function in the modulation of cAMP metabolism are still far from being elucidated. For example, the finding that PI3-K γ -null cardiomyocytes show increased basal levels of cAMP and enhanced levels of contractility even in the absence of β -adrenergic stimulation requires comprehensive investigation.

Cyclic nucleotides

Regulation of cardiac contractility occurs primarily by either an increase in the concentration of activating Ca^{2+} ions or an alteration in the response of the contractile proteins to a given Ca^{2+} concentration (McClellan *et al.* 1993).

Cyclic guanosine monophosphate (cGMP) is the nitric oxide (NO) second messenger and it has been shown that cGMP decreases relative myofilament response to calcium (Ca^{2+}) and therefore enhances myocardial relaxation and reduces diastolic tone. Several studies have demonstrated a dissociation

between cGMP concentrations and contractile state, because low concentration of acetylcholine induced a negative inotropic effect even in the absence of any change in cGMP concentration (Vila-Petroff *et al.* 1999).

Cyclic adenosine monophosphate (cAMP) is the second messenger hormone of β -adrenergic signalling in the heart and it mediates the inotropic effects of β -adrenergic agonists in cardiac myocytes (Belhassen *et al.* 1996). It is generated from adenosine trisphosphate (ATP) by the action of adenylyl cyclase (AC).

Most β -ARs, like the β 1 receptor, are coupled to $G\alpha_s$, which increases heart rate and contractility through the activation of AC and the production of cAMP. The increased intracellular concentration of cAMP activates protein kinase A (PKA), which phosphorylates troponin I, the L-type Ca^{2+} -channel and PLB. These processes can be opposed by the anti-adrenergic effect of the M2 muscarinic receptor, which couples to $G\alpha_i$ and thus promotes a decrease of intracellular cAMP.

Nitric oxide

Nitric oxide (NO) is a free radical gas which plays a role in signal transduction in diverse processes. NO is synthesised from L-arginine, in a NADPH-dependent reaction, by nitric oxide synthase (NOS). NOS has many

isozymes: The neuronal and endothelial NOS that are calcium dependent enzymes; the smooth muscle NOS and macrophages NOS that are activated by various cytokines and are calcium-independent, the brain NOS that is stimulated by the activation of ionotropic glutamate receptors such as N-methyl-D-aspartate (NMDA) receptors and non NMDA receptors or metabotropic glutamate receptors (Garthwaite *et al.* 1988; Moncada *et al.* 1989; Bredt & Snyder 1989; Ignarro 1990; Garthwaite 1991; Bredt & Snyder 1992; Okada 1992; Yamada & Nabeshima 1997a; Yamada & Nabeshima 1997b; Zou *et al.* 1998).

NO was identified in the 1970s as an endothelial vasorelaxing factor and as such, is an important indirect regulator of cardiac function, for example through its modulation of coronary reserve. Recent research on cardiac NO has focused on its direct modulation of myocardial contractility (Massion *et al.* 2003).

Nitric oxide synthase expression

In normal heart extracts, the neuronal isoform of nitric oxide synthase (nNOS) is thought to be localised in the sarcoplasmic reticulum, as suggested by its co-immunoprecipitation with the cardiac ryanodine receptor (RyR2), whereas nNOS- α is expressed in mitochondria (Elfering *et al.* 2002). In addition, both adrenergic and cholinergic nervous fibres express the nNOS, and eNOS is

richly represented in endothelial and endocardial cells. Finally, the calcium-independent NOS (inducible nitric oxide synthase, or iNOS, encoded by NOS2) is induced in cardiomyocytes and inflammatory cells infiltrating the myocardium in response to inflammatory cytokines under stress conditions (ischaemia) (Jung *et al.* 2000).

Nitric oxide and cardiac function

Nitric oxide (NO) has been implicated as a mediator of many cellular processes, including endothelium-dependent relaxation of blood vessels, chemical communication between peripheral nerves and smooth muscles and inhibition of platelet aggregation. The major quantity of the endogenous physiological NO in the heart is produced by the coronary endothelium, but NO can also be produced within the cardiac myocytes themselves by the constitutive NO synthase NOS-3. NO influences many major facets of cardiac myocyte function, including signal transduction, Ca²⁺ cycling, and mitochondrial respiration (Vila-Petroff *et al.* 1999).

Spatial confinement of NOS isoforms plays a central role in regulating cardiac contractile function. Recent studies have shown that nNOS (the neuronal isoform of NOS) influences the frequency-mediated rise in cardiac contractility and calcium cycling in a manner previously thought to be specific to eNOS. The intracellular mechanisms accounting for the inotropic effects of NO are

diverse (Balligand & Cannon 1997; Balligand 1999). With exogenous NO, several of these intracellular mechanisms were found to operate in a concentration-dependent bimodal fashion, resulting in inotropically inverse effects at higher NO exposure (Brunner *et al.* 2001). These effects are mediated principally through cGMP-related mechanisms, specifically via the reduction of Ca²⁺ influx through L-type Ca²⁺ channels – either through activation of cGMP-dependent phosphodiesterase or cGMP-dependent protein kinase (PKG) (Belhassen *et al.* 1996; Vila-Petroff *et al.* 1999).

In recent research, one group found that nNOS knockout (nNOS^{-/-}) mice have an attenuated positive force-frequency response in conjunction with impaired increase in SR calcium load (Khan *et al.* 2003), consistent with a facilitatory effect of nNOS on SERCA activity. In contrast, another group found that there was an increased SR calcium load in myocytes from nNOS^{-/-} mice that was attributed to a compensatory increase in trans-sarcolemmal calcium entry in the hearts (Sears *et al.* 2003).

In normal hearts, the tonic release of NO directly influences several components of cardiac function. Acute inhibition of NOS decreases contractile force in isolated rat hearts, induces regional wall thickening in pigs, left ventricular diameter shortening and stroke work in dogs (Puybasset *et al.*

1996; Kojda *et al.* 1997; Heusch *et al.* 2000; Cotton *et al.* 2001; Recchia *et al.* 2002).

Nitric oxide regulation of diastolic and systolic cardiac properties in normal mammalian heart

Non-specific inhibition of NOS or genetic deletion of eNOS and iNOS has little effect on the basal contractile function in the mammalian heart and in isolated cardiomyocytes (Massion *et al.* 2003). The neuronal NOS is a possible exception since its acute inhibition or chronic genetic ablation resulted in an increase in L-type calcium current and amplitude of contractile shortening in isolated mouse cardiomyocytes (at low catecholamine concentrations), as well as contractility *in vivo* (Ashley *et al.* 2002). Inhibition or genetic ablation of nNOS resulted in a slowing of calcium transient decay and myocyte re-lengthening in one study (Sears *et al.* 2003), and in an unaffected basal relaxation *in vivo* in another (Khan *et al.* 2003); however, the higher heart rate of nNOS^{-/-} mice may confound the interpretation of their tau values, i.e., had they been corrected for heart rate, tau values of nNOS^{-/-} may have unveiled an impaired relaxation compared with wild type (WT), compatible with the first study. These results can represent a possible effect of nNOS on calcium re-uptake into the sarcoplasmic reticulum (SR).

In isolated hearts, NOS inhibition elevates left ventricular end-diastolic pressure (LVEDP) and decreases the preload-recruitable rise in cardiac output (Prendergast *et al.* 1997). Endothelial NOS (eNOS) activation appears to play a major role in insulin-mediated mitigation of cardiomyocyte apoptosis as well as infarct size in a rat model of ischaemia-reperfusion injury (Gao *et al.* 2002). NO plays a key role in cardioprotective effects of acute corticosteroid therapy during ischaemia-reperfusion injury (Hafezi-Moghadam *et al.* 2002). This study has also shown that the PI3-K/Akt signalling pathway tightly regulates activation of eNOS in ischaemia-reperfusion injury

Nitric oxide regulation of diastolic and systolic cardiac properties in diseased and in normal mammalian heart.

In the basal state, the effect of NO is bimodal, with a positive inotropic effect at low concentrations of NO exposure but a negative one at higher concentrations. Several studies also found no effect at all of either exogenous or endogenous NO. It is difficult to define the meaning of low or high concentrations of NO since there is no standardised protocol set when it comes to the concentrations of different reagents used for experiments. The lack of standardisation may account for the differences observed between studies.

Although iNOS and nNOS are upregulated with cardiac disease, non-specific NOS inhibitors have little effect on basal function and force–frequency relationships in heart failure (Massion *et al.* 2003). This is perhaps explained by the biodegradation of the NO produced, e.g. by myoglobin, as exemplified in the mild phenotype of iNOS overexpressors; however, the fact that myoglobin content is decreased in several cardiomyopathies suggests that additional factors are at play to account for the relative insensitivity of the failing myocardium in the face of upregulated NOS. eNOS down regulation may impair the Frank–Starling adaptation of the terminally failing heart, both through defective stretch-dependent contractile reserve and altered relaxation dependent on paracrine eNOS signalling (Wunderlich *et al.* 2003).

Altered NOS expression/regulation in the diseased heart participates in cardiac dysfunction. During cardiac diseases, the relative abundance of each NOS may dramatically change, with upregulation of nNOS and iNOS, but down regulation of eNOS (Massion *et al.* 2003). Concurrent changes in the abundance/interaction of allosteric modulators (caveolin-3, hsp90), availability of cofactors, concentration of endogenous inhibitors, or biochemical uncoupling further affect all NOS activity.

The NOS compartmentalisation is also disrupted as a consequence of cell ultrastructure remodelling such as loss of T-tubular structure in diseased

cardiomyocytes. The nature and intensity of stimuli affecting each NOS are profoundly modified in failing hearts, with e.g. increased circulating catecholamines, relative changes in beta-adrenergic isoform expression (e.g. upregulation of beta 3-adrenoceptors) (Moniotte *et al.* 2001).

Beta-adrenergic response.

In normal mammalian heart, the β -adrenergic inotropic effect can be modulated in a bimodal fashion, depending not only on the concentration of NO but also of catecholamines (Massion *et al.* 2003). The response to β -adrenergic stimulation therefore depends on the concentration of NO and catecholamines. Ji *et al.* (1999), in one of their studies, found that at a fixed concentration of NO, cardiac response to β -adrenergic stimulation was increased at low catecholamine levels but decreased at high levels (Ji *et al.* 1999).

In contrast, a NO-dependent attenuation of the beta-adrenergic response is observed in isolated cardiomyocytes, whole heart or *in vivo* animal preparations and in human cardiac diseases. Originally, this was attributed to iNOS in cardiomyocytes (as well as infiltrating inflammatory cells) (Balligand *et al.* 1993) and was confirmed in lipopolysaccharide-induced (LPS) rat ventricular myocytes over-expressing iNOS and in patients with heart failure and iNOS over-expression (Ziolo *et al.* 2001; Ziolo *et al.* 2003).

The mammalian heart is characterised by the expression of all three isoforms of NOS in various cell types of the myocardium, including cardiac myocytes themselves. Their sub-cellular compartmentalisation ensures specific NO signalling to co-localised effectors in response to physical (e.g. stretch) or receptor-mediated stimuli. eNOS and nNOS cooperatively sustain normal excitation contraction coupling and contribute to the early and late phases of the Frank–Starling mechanism of the heart. In addition, they attenuate the β_1/β_2 -adrenergic increase in inotropy and chronotropy, and reinforce the (pre- and post-synaptic) vagal control of cardiac contraction, thereby preventing excessive stimulation by catecholamines.

In the ischaemic and failing myocardium, eNOS coupled to overexpressed β_3 -adrenoceptors further contributes to the attenuation of the inotropic effect of catecholamines, as does iNOS. nNOS expression also increases in the aging and ischaemic heart, but its role remains to be defined (Ziolo & Bers 2003)

In addition, Gao *et al.* (2002) showed that eNOS is a substrate for PKB/Akt and that phosphorylation of eNOS by PKB/Akt results in a calcium-independent NO production. In their study, treatment of hearts with insulin resulted in an increased eNOS phosphorylation and an increased production of NO. When the hearts were treated with wortmannin in the presence of insulin, they observed that eNOS phosphorylation was completely blocked

and the increase of NO production induced by insulin was abolished. When they treated the hearts with L-NAME, there was no effect on eNOS phosphorylation but NO production was reduced in insulin treated hearts. These results led them to the conclusion that, treatment of myocardial tissue in the ischaemia/reperfusion model with insulin activates eNOS and increases NO production through the PI3-K – PKB/Akt pathway.

From the available literature it can be observed that insulin-induced improvements in cardiac functions are widely investigated in models of ischaemia and reperfusion. However, little data exists on the effects of insulin on the heart under normoxic condition. We therefore hypothesize that insulin may improve cardiac function under normoxic conditions and we believe that an investigation into the effects of insulin on cardiac function and pathways involved under normoxic conditions may help us to better understand the mechanisms of insulin-induced cardioprotection. Therefore, in the present study, we will first determine a suitable dose of insulin at which a positive inotropic response could be detectable under normoxic conditions. This will be achieved by perfusing the hearts with three different concentrations of insulin. We will then investigate the possible mechanisms involved in insulin-induced increases in contractility with specific reference to the vasculature and the coronary flow and we will end the present work with a preliminary protocol on the investigation of a possible involvement of PI3-K and its

downstream effectors on the insulin effects on cardiac functions under normoxic conditions.

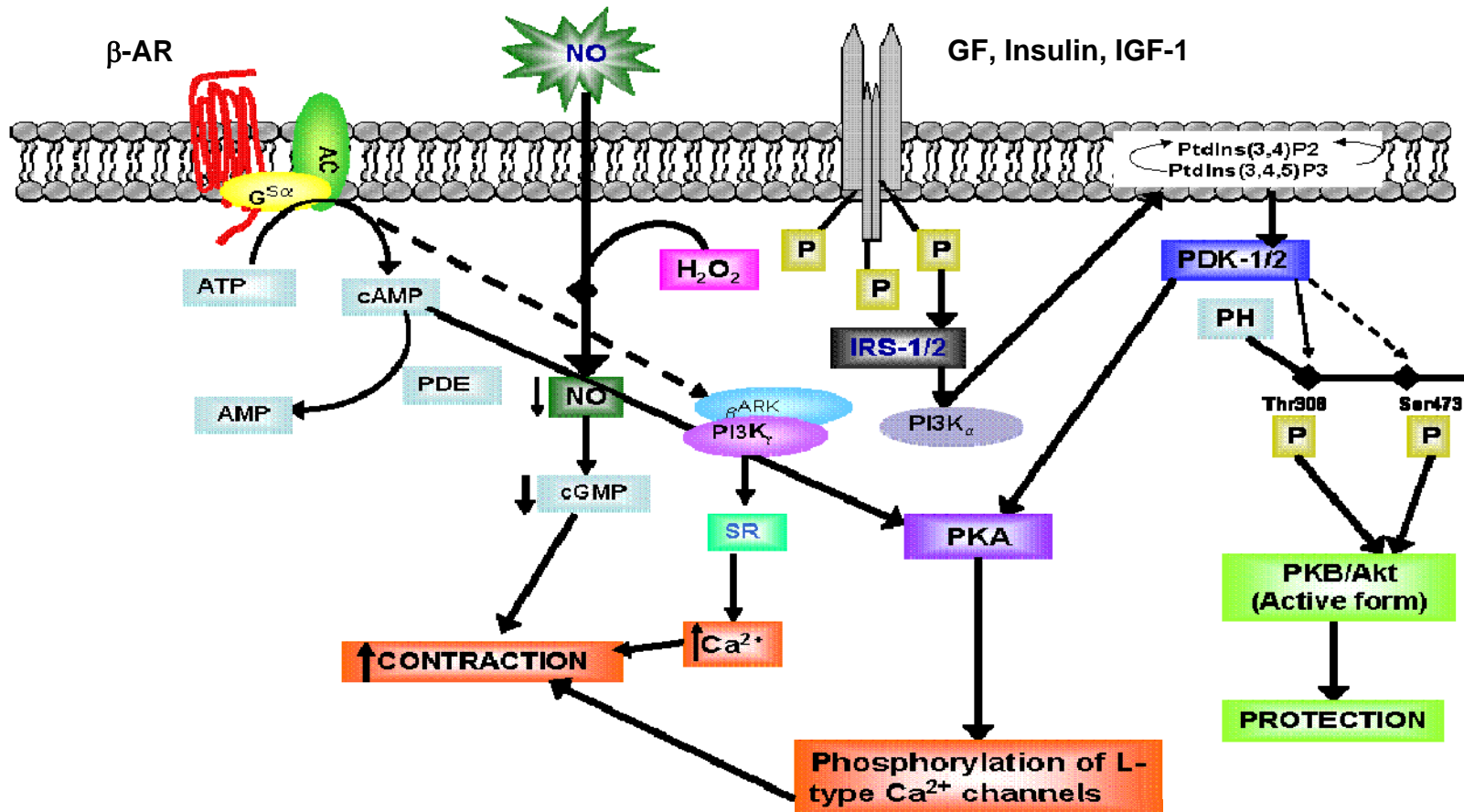


Figure 2.4: Proposed summary of PI3-K, PKB/Akt and NO signalling involved in the increased contractility in the heart. β -AR receptor stimulation produces a positive inotropic effect through activation of AC leading to increases in intracellular cyclic AMP levels. Subsequent activation of cAMP-dependent protein kinase (PKA) leads to downstream phosphorylation of target proteins, including the L-type calcium channel to increase trans-sarcolemmal calcium influx and sarcoplasmic reticulum calcium uptake.

CHAPTER 3

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Male Wistar rats (280–340g) were supplied by the Department of Physiological Sciences Animal Unit at the University of Stellenbosch. The rats were allowed free access to food (standard rat chow) and water. They were maintained in the animal house at a constant temperature of 21°C and were exposed to a twelve-twelve hour light-dark cycle.

DRUGS AND CHEMICALS

Drugs and chemicals used were obtained from the following sources:

Amersham Bioscience:

Anti-rabbit IgG, horseradish peroxidase-labelled secondary antibody, Hyperfilm, Enhanced chemiluminescence detection kit (RPN 2103)

Cell Signalling Technology:

Anti-Akt antibody, anti-PI3K-p110 α antibody, anti-phospho-Akt-Thr (308) antibody, anti-phospho-Akt-Ser (473) antibody

Eli Lilly SA (Fegersheim, France):

Humulin N insulin

Millipore:

PVDF membrane

Sigma Chemicals Company (South Africa):

Bovine Serum Albumin, Bromophenol blue, Gel loading buffer, Lysis buffer,

N-nitro-L-Arginine methyl ester (L-NAME), Wortmannin,

All other reagents were of analytical grade, and were supplied by Merck, Fluka and Sigma Chemical Company (South Africa)

PHYSIOLOGICAL SALT SOLUTION

Krebs-Henseleit (KH) solution containing in mM, 119 NaCl, 25 NaHCO₃, 1.2 KH₂PO₄, 4.7 KCl, 0.59 MgSO₄-7H₂O, 1.25 CaCl₂-2H₂O and 5 mM of glucose and gassed with 95% O₂ and 5% CO₂ at pH 7.4 was used.

INHIBITORS

L-NAME was freshly made before each perfusion and was dissolved in KH solution for a 10 μ M concentration and aliquots were kept at 4°C until used.

Wortmannin was dissolved in DMSO and aliquots of 2mM were stored at -40°C until used.

All other compounds were dissolved in sterile saline unless otherwise stated.

ETHICAL CLEARANCE

Ethical clearance for this study was granted by Sub-committee C of the University of Stellenbosch.

MEASURED VARIABLES

In order to compare the function of the hearts in the different groups, heart rate (HR, bpm), systolic pressure (SP, mmHg), diastolic pressure (DP, mmHg) and the temperature (°C) were measured every 5 minutes from the starting of the experiment until the end of experiment. Coronary flow (CF, ml/min) was measured, by manually collecting the volume of heart's effluent during one minute time period, 5 minutes after starting the experiment and every 5 min until the end of perfusion. The left ventricular developed pressure (LVDevP) was calculated as the difference between the SP and the DP.

EXCLUSION CRITERIA

Rats with body mass outside of the range of 280 – 340 g were not used for the experiments. Hearts were excluded from the study if the following were not met during the stabilisation period:

- coronary flow of 10 – 16 ml/min
- heart rate of 210 – 350 beats per minute
- left ventricular developed pressure of 70 – 150 mmHg

PERFUSION PROTOCOL: ISOLATED PERFUSED RAT HEART

Male Wistar rats (280–340 g) were anaesthetised with thiopentone sodium (120 mg/kg ip). Following cervical dislocation the chest was opened and the heart excised. The hearts were arrested in ice cold KH buffer (4°C). Within 60 seconds of excising the heart the aorta was cannulated with a stainless steel cannula and perfused with KH on a Langendorff perfusion system. Hearts were retrogradely perfused at a constant pressure of 100 cm H₂O. The left atrium and the non-cardiac tissues were removed from the hearts. Temperature was maintained at a constant 37°C by means of a circulating water bath and a water-jacketed glass reservoir. The HR, the DP and SP were recorded via a pressure transducer and data acquisition system (ADInstrument Powerlab 200) connected to the heart *via* a water filled balloon inserted into the left ventricle. The contraction force of the heart against the balloon caused water displacement giving us an indication of pressure. Coronary flow was obtained manually by collecting heart effluent for one minute with a graduated measurement cylinder. The myocardial temperature was monitored with a thermocouple temperature probe inserted into the right coronary sinus.

PRELIMINARY STUDY - INSULIN EFFECT ON CARDIAC FUNCTION

The initial protocol was designed to investigate a suitable dose of insulin at which an effect of insulin on heart function during normal perfusion could be detected. Three doses of insulin, ranging from the physiological to the pathophysiological were used. The three different concentrations of insulin were:

0.3 mIU/mL (Ins₃), 0.6 mIU/mL (Ins₆) and 1.0 mIU/mL (Ins₁₀). Hearts were perfused with KH buffer during the stabilisation period and with the different concentrations of insulin for the remainder of the experimental protocol, except for the control group that received only KH throughout the experiment. All the hearts were allowed 10 minutes stabilisation time before experimental protocol began.

The groups were divided as follows:

Group 1a: Control; KH-buffer (Gluc₅)

Group 1b: KH-buffer + insulin 0.3 mIU/mL (Ins₃)

Group 1c: KH-buffer + insulin 0.6 mIU/mL (Ins₆)

Group 1d: KH-buffer + insulin 1.0 mIU/mL (Ins₁₀)

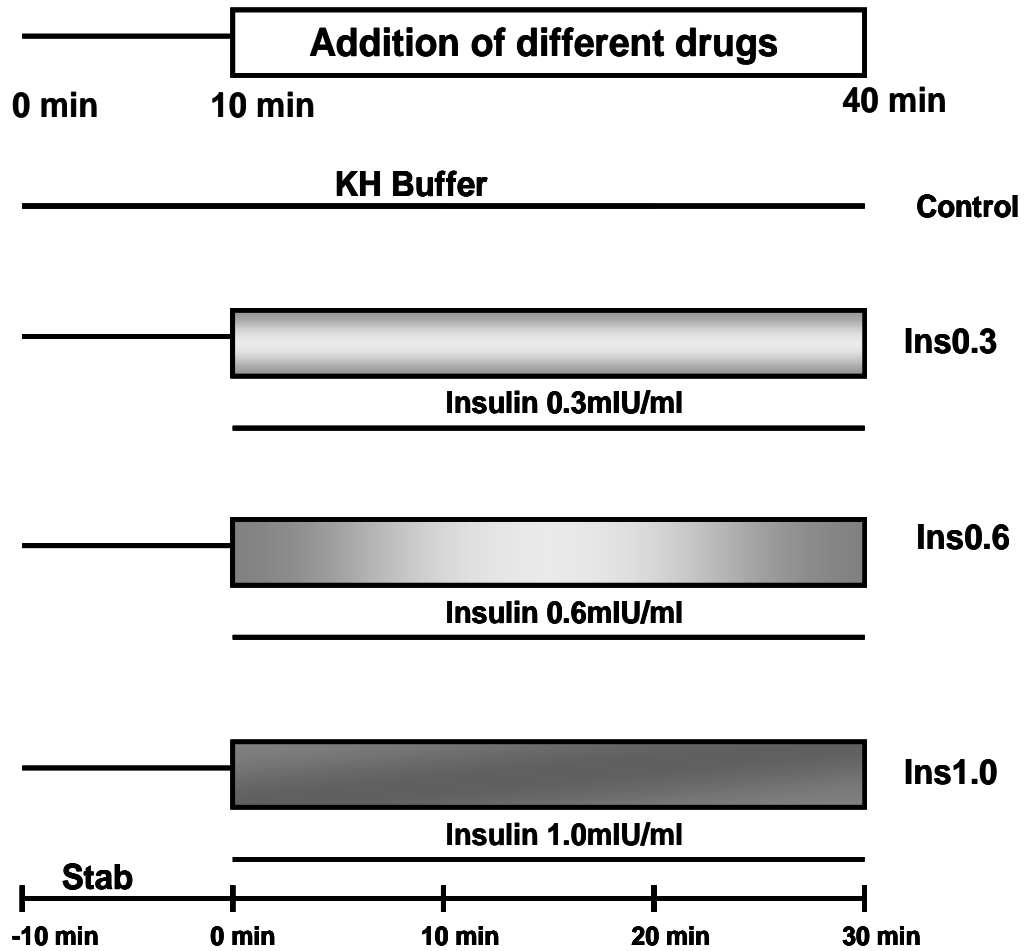


Figure 3.1: Perfusion protocol for insulin dose response

INHIBITOR STUDIES

To investigate the signalling pathways involved in the inotropic effect of insulin on the heart, inhibitors of two specific pathways were added to KH-buffer in the presence of insulin. The first, L-NAME, an inhibitor of nitric oxide synthase (NOS), was used to investigate the involvement of nitric oxide (NO). Secondly, Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3-K), was used for the investigation of the role of PI3-K in the responses seen.

For the purpose of further biochemical analysis, hearts were freeze-clamped at 10, 20, and 30 minutes of experimental time as well as at the end of the stabilisation period with Wollenberger tongs. The Wollenberger tongs were pre-cooled in liquid nitrogen. The freeze-clamped hearts were then stored at -80 °C until analysed.

ROLE OF NITRIC OXIDE IN THE RESPONSES TO INSULIN

This protocol was designed to investigate the involvement of nitric oxide in the effects of insulin on heart, by inhibiting nitric oxide synthase (NOS). L-NAME, a non specific inhibitor of NOS, was used in conjunction with insulin in KH-buffer. As for the insulin protocol, hearts in this group were perfused with Krebs-Henseleit buffer during the stabilisation period and with insulin alone or insulin and L-NAME. The control group received only KH throughout the experiment. The dose of 0.3 mUI/mL of insulin was chosen from the previous protocol and was used for all subsequent protocols where insulin was involved. In the present

protocol, we used KH + insulin throughout the experimental time after the 10 minutes stabilisation. In the last group, L-NAME was administered for 10 minutes in association with insulin immediately after the stabilisation time. After L-NAME administration, hearts received insulin until the end of the experiment. The perfusion protocol for perfusion with L-NAME is represented in figure 3.2.

The groups were divided as follows:

Group 2a: Control: KH-buffer

Group 2b: KH-buffer + insulin 0.3 mIU/mL

Group 2c: KH-buffer + insulin 0.3 mIU/mL + L-NAME 10 μ M

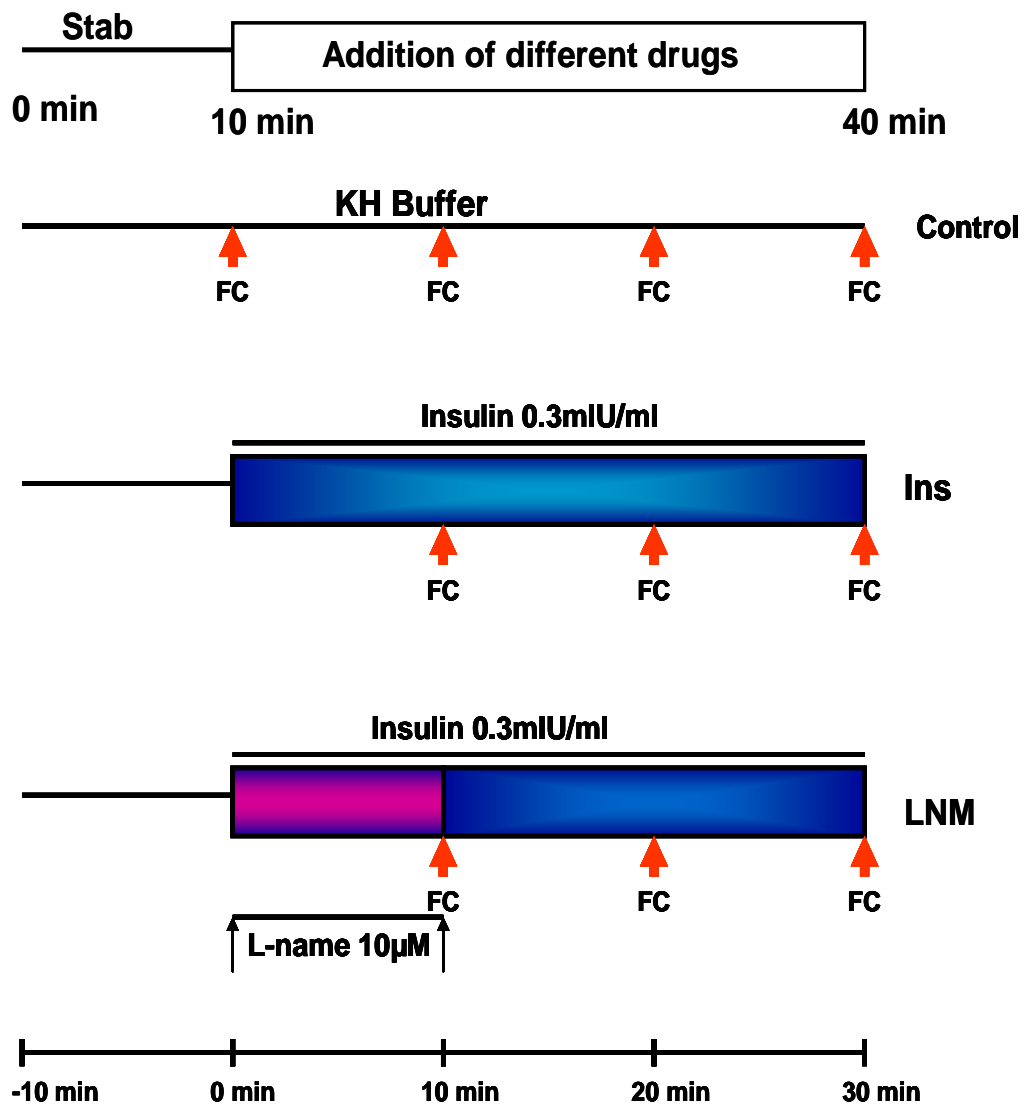


Figure 3.2: Perfusion protocol with L-NAME

ROLE OF PI3-K IN THE RESPONSES TO INSULIN

The setting up of the present protocol aimed to investigate the role of PI3-K in the inotropic effects of insulin in normal hearts. Hearts in this group were perfused with Krebs-Henseleit buffer during the stabilisation period and with insulin alone or insulin and wortmannin. The control group received only KH throughout the experiment. We used KH + insulin throughout the experimental time after the 10 minutes stabilisation. Wortmannin was administered in conjunction with insulin throughout the experiment after the stabilisation period for the last group of the present protocol.

The perfusion protocol for perfusion with wortmannin is represented in figure 3.3.

The groups were divided as follows:

Group 3a: Control: KH-buffer

Group 3b: KH-buffer + insulin 0.3 mIU/mL

Group 3c: KH-buffer + insulin 0.3 mIU/mL + wortmannin 100 nM

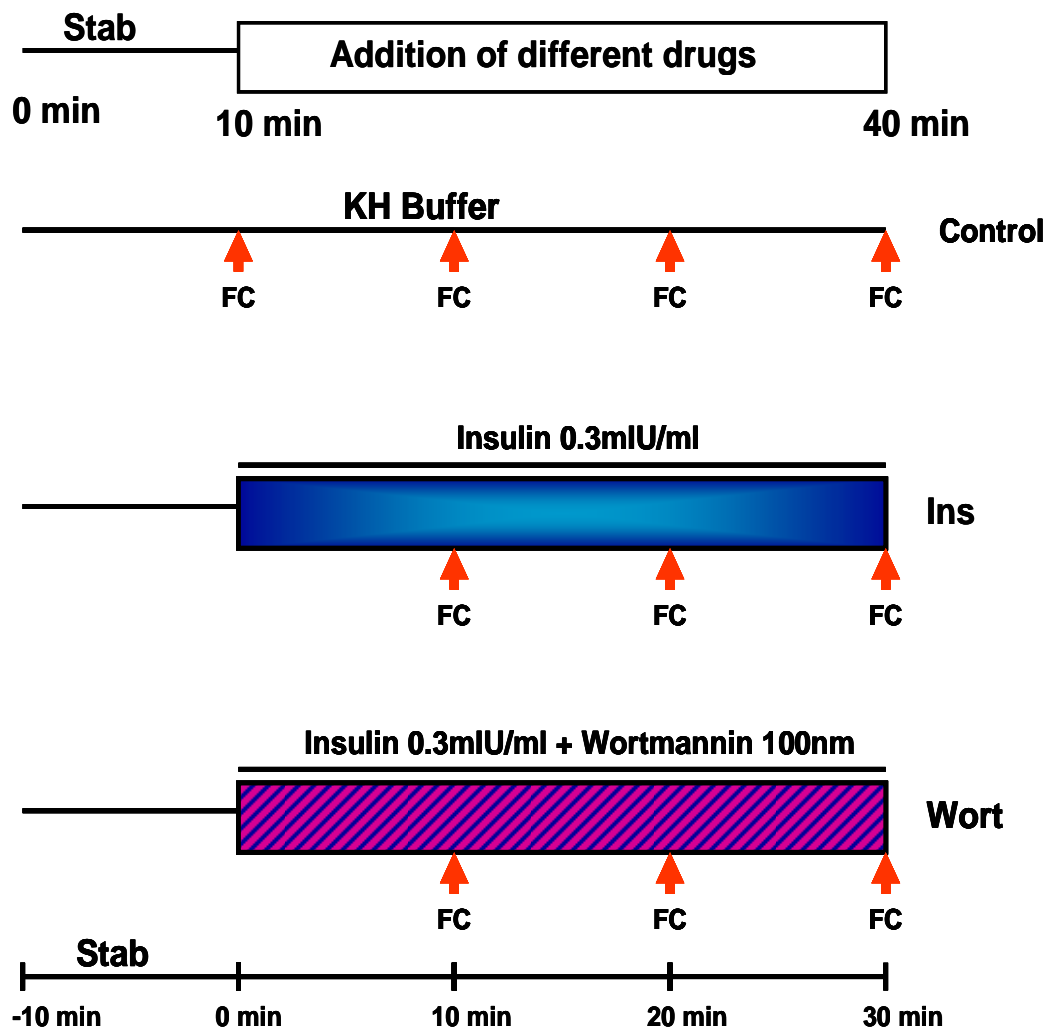


Figure 3.3: Perfusion protocol with wortmannin

MEASUREMENT OF PKB/Akt PHOSPHORYLATION AND PI3-K

The freeze-clamped heart tissue samples stored at -80°C were used following freeze-drying for 24h. The freeze dried tissue was reduced to powder and homogenised with a 1:1 w/v lysis buffer for 10 seconds. The homogenates were then centrifuged (2500 xg, 10 min, 4°C) to remove particulate matter. The protein concentration in the supernatants was determined by the Bradford dye-binding assay using bovine serum albumin (BSA) as a standard (Bradford, 1976).

The lysates were diluted in Laemmli sample buffer, boiled for 5 min. 40 μg protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel, on a standard Mini-PROTEAN 3 system (Bio-Rad). The separated proteins were transferred to a polyvinylidenedifluoride (PVDF) membrane. Following transfer, the membranes were stained with Ponceau S Red (reversible stain) to visualise the proteins, and to assess the quality and quantity of the transfer. Following scanning and removal of the Ponceau-S Red, non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline containing 0.1% tween20 (0.1% TBS-T). The amounts of total protein as well as phosphorylation of PKB/Akt were visualized using appropriate antibodies. Membranes were probed with either an anti-Akt antibody (for total Akt), anti-phospho-Akt (Thr 308) antibody, anti-phospho-Akt (Ser 473) antibody or an anti-PI3K-p110 antibody. The membranes were washed with TBS-T and the immobilised primary antibodies were conjugated with a diluted horseradish peroxidase conjugated secondary antibody. After washing thoroughly with TBS-T,

the membranes were incubated in an enhanced chemmiluminescent detection reagent (ECL Plus™) for 5 mins and were quickly exposed to an autoradiography film. Films were subsequently scanned and the bands analysed using densitometry (UN-SCAN-IT, Silkscience). Protein were expressed in arbitrary units and all proteins were normalized to control (the value of 1 was taken as the total protein content of the control).

STATISTICAL ANALYSIS

Results are reported as the mean \pm standard error of the mean. Data were analysed by ANOVA with a Bonferroni post hoc test. The condition of $P < 0.05$ was considered to be statistically significant.

CHAPTER 4

RESULTS

FUNCTIONAL DATA - INSULIN DOSE RESPONSE

EFFECTS OF INSULIN CARDIAC FUNCTION

For the investigation of a suitable dose of insulin at which an effect on cardiac function could be detected, three different concentrations of insulin were used, namely: 0.3 mIU/mL; 0.6 mIU/mL; 1.0 mIU/mL.

HEART RATE (FIGURE 4.1.A)

At the end of the stabilisation period the heart rate (HR) for the control group (n = 14) was 308.7 ± 9.9 beats per minutes (bpm); 295.6 ± 9.5 bpm for the group treated with insulin at the concentration of 0.3 mIU/mL (Ins_{0.3}) (n = 12), 295.0 ± 14.7 bpm for the group treated with insulin at the concentration of 0.6 mIU/mL (Ins_{0.6}) and 293.2 ± 8.7 bpm for the group treated with insulin at the concentration of 1.0 mIU/mL (Ins_{1.0}) (n = 7) (See Fig. 4.1.a, p75). There was no significant difference between the four groups.

After 30 min of perfusion with either KH + glucose (Ctrl), or with KH + glucose + insulin at different concentrations (Ins_{0.3}, Ins_{0.6} and Ins_{1.0}), the HR values were as follows: 282.1 ± 11.6 bpm for the control group, 264.0 ± 7.8 bpm for Ins_{0.3}, 267.0

± 13.2 bpm for $\text{Ins}_{0.6}$ and 290.2 ± 8.8 bpm for $\text{Ins}_{1.0}$, no significant statistical difference was observed (See Fig. 4.1.a, p79).

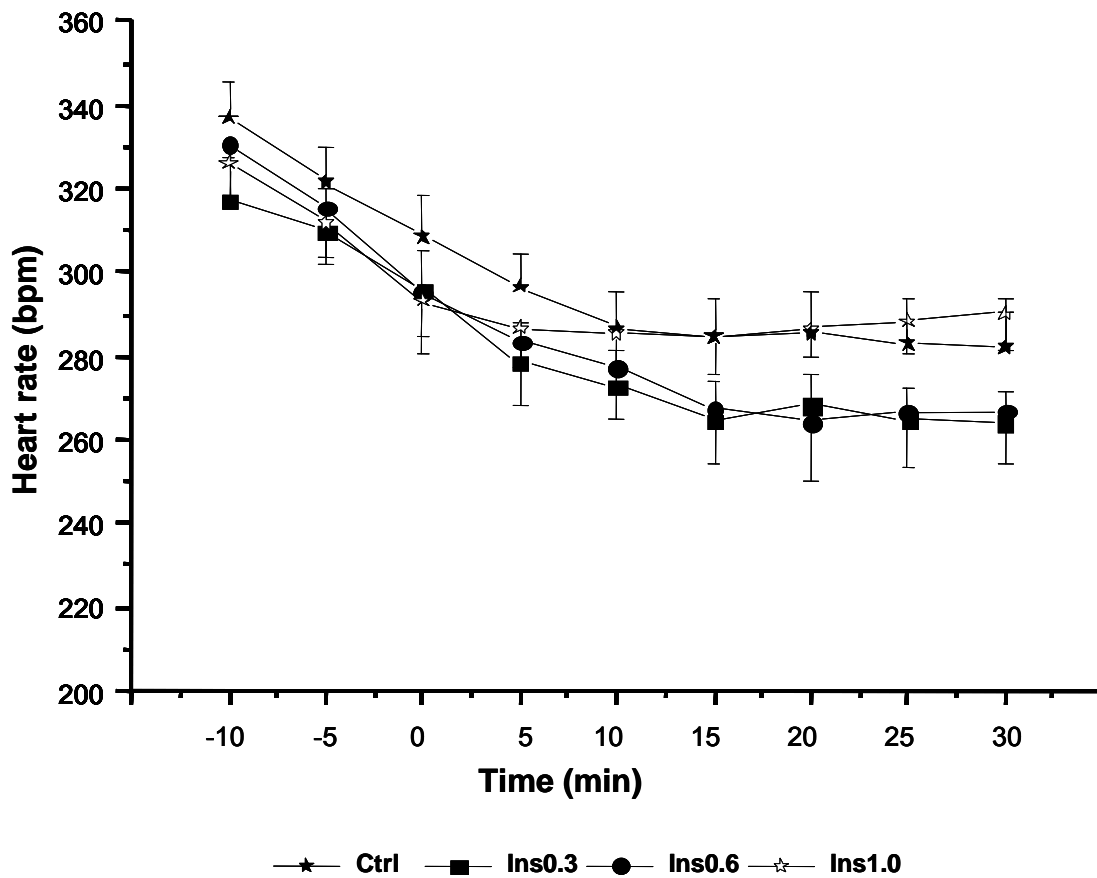


Figure 4.1.a: Effect of three doses of insulin on heart rate.

Heart rate of hearts perfused with KH solution containing either 5 mM glucose (Control group, n = 14) or with 5 mM glucose and insulin at three different concentrations (0.3 mIU/mL (Ins_{0.3}) n = 12, 0.6 mIU/mL (Ins_{0.6}) n = 8, and 1.0 mIU/mL (Ins_{1.0}) n = 7).

LEFT VENTRICULAR DEVELOPED PRESSURE (FIGURE 4.1.B)

Left ventricular developed pressure (LVDevP) of the isolated perfused hearts at the end of the stabilisation time was 78.0 ± 3.8 mmHg for the control group (n = 14), 71.08 ± 2.5 mmHg for Ins_{0.3} (n = 12), 80.40 ± 4.3 mmHg for Ins_{0.6} (n = 8) and 74.91 ± 2.9 mmHg for Ins_{1.0} (n = 7). Thirty minutes after the experiment, the LVDevP was 79.7 ± 3.3 mmHg for the control group, 95.66 ± 4.1 mmHg for the Ins_{0.3} group, 90.32 ± 5.6 mmHg for the Ins_{0.6} group, and 92.38 ± 6.2 mmHg for the Ins_{1.0} group. Hearts perfused with Ins_{0.3} showed a linear increase from the start of the perfusion until 15 min of perfusion. Then the increase slowed down until the end of the experiment. No significant differences were detected within the groups where insulin was administered. Significant difference was observed between the control group and the insulin group at the concentration of 0.3 mIU/mL from the 25th min until the end, $p < 0.05$ (See Fig. 4.1.b, p80).

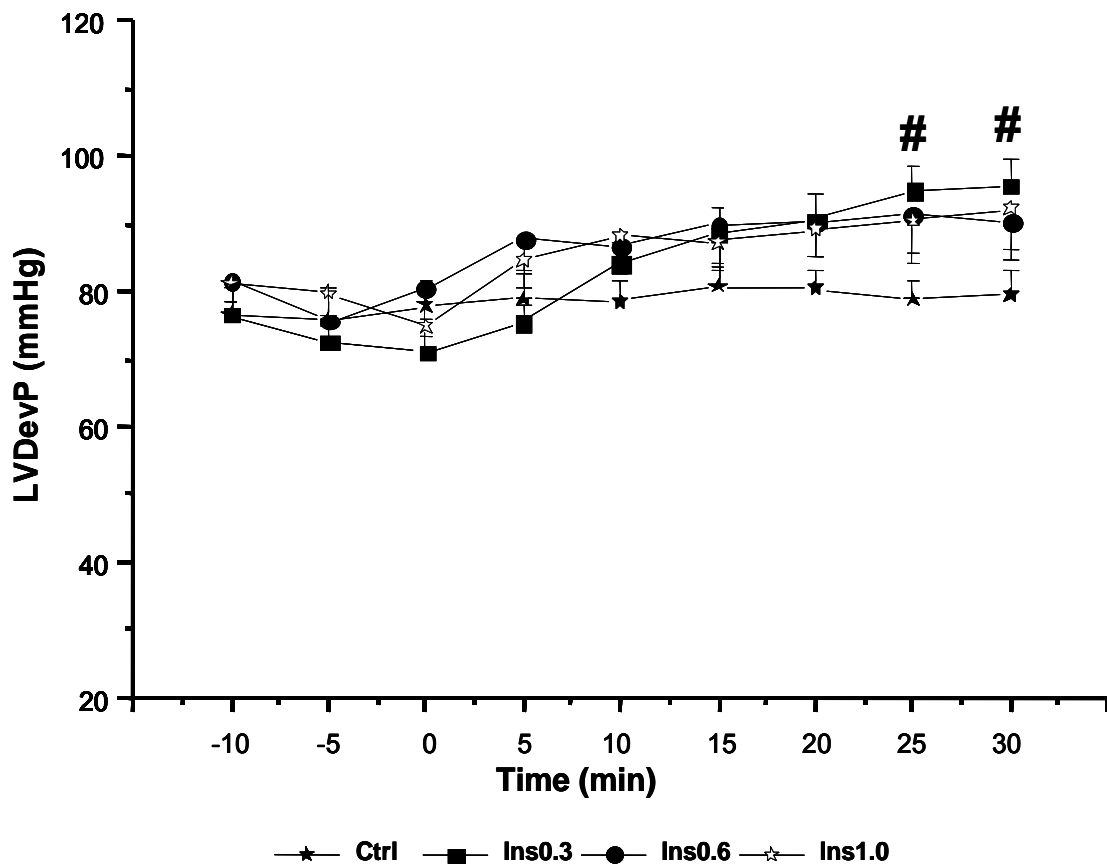


Figure 4.1.b: Effect of three doses of insulin on LVDevP

LVDevP of hearts perfused with KH solution containing either 5 mM glucose (Control group, n = 14) or with 5 mM glucose and insulin at three different concentrations (0.3 mIU/mL (Ins_{0.3}) n = 12, 0.6 mIU/mL (Ins_{0.6}) n = 8, and 1.0 mIU/mL (Ins_{1.0}) n = 7), # p < 0.05

CORONARY FLOW (FIGURE 4.1.C)

The coronary flow (CF) of the group that received the high concentration of insulin seemed to be slightly higher than all other groups, but there was no statistically significant difference between all groups. At the end of the stabilisation period the coronary flow was 13.3 ± 0.4 ml/min for the control group (n = 14), 13.46 ± 0.6 ml/min for the Ins_{0.3} group (n = 12), 15.3 ± 0.9 ml/min for the Ins_{0.6} group (n = 8), and 14.54 ± 0.7 for Ins_{1.0} group (n = 7). At the end of the experiment, the CF values were 11.9 ± 0.5 ml/min, 12.93 ± 0.5 ml/min, 13.4 ± 0.6 ml/min, and 13.71 ± 0.8 ml/min for the control group, the Ins_{0.3} group, the Ins_{0.6} group, and the Ins_{1.0} group respectively (See Fig.4.1.c, p82).

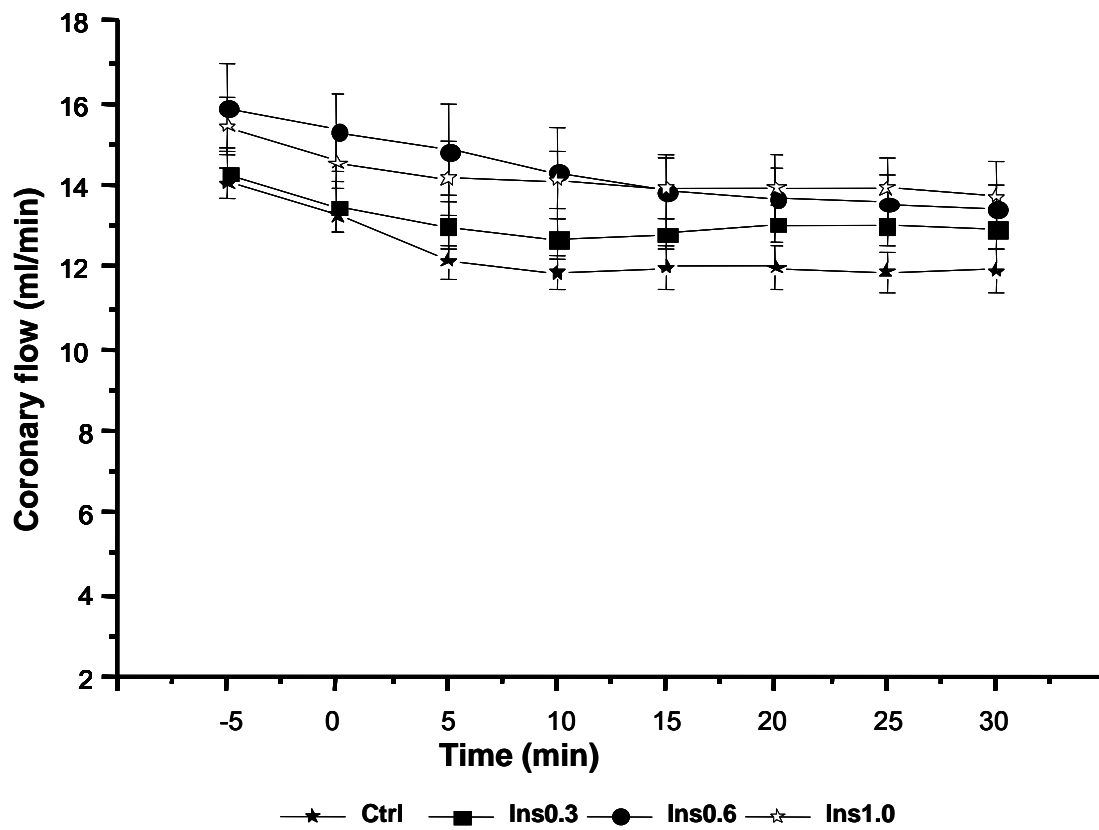


Figure 4.1.c: Effect of three doses of insulin on coronary flow

CFof hearts perfused with KH solution containing either 5 mM glucose (Control group, n = 14) or with 5 mM glucose and insulin at three different concentrations (0.3 mIU/mL (Ins_{0.3}) n = 12, 0.6 mIU/mL (Ins_{0.6}) n = 8, and 1.0 mIU/mL (Ins_{1.0}) n = 7).

EFFECTS OF SPECIFIC PATHWAY INHIBITORS ON CARDIAC FUNCTION

To investigate the role of the nitric oxide pathway in the inotropic response to insulin seen in the heart we used 10 μ M of L-NAME (Du Toit E.F., 1998), a non-specific inhibitor of nitric oxide synthase, for the implication of nitric oxide in the effect of insulin on the vasculature and the flow.

Further to this, in a preliminary study we investigated the role of the PI3-K pathway in the inotropic effect of insulin under normoxic conditions by using the PI3-K inhibitor wortmannin (100nm).

EFFECT OF L-NAME ON HEART RATE (FIGURE 4.2.A)

At the end of the stabilisation period, the HR's were 293.78 ± 7.48 bpm for the control group (Ctrl) $n = 25$, 296.09 ± 6.65 bpm for the insulin group (Ins) $n = 29$, and 299.20 ± 9.2 bpm for the L-NAME group (LNM) $n = 11$. Immediately after L-NAME administration, heart rate dropped to 229.8 ± 10.6 bpm for the group that received L-NAME. Following the 20 minutes perfusion with insulin after administration of L-NAME, the heart rate values were 253.2 ± 10.1 bpm. A drop in the heart rate was observed during administration of L-NAME and during the 10 minutes following L-NAME administration in the group that received L-NAME. There was a significant difference between the control and the insulin groups compared to the group that received L-NAME, $p < 0.05$ (See Fig. 4.2.a, p85).

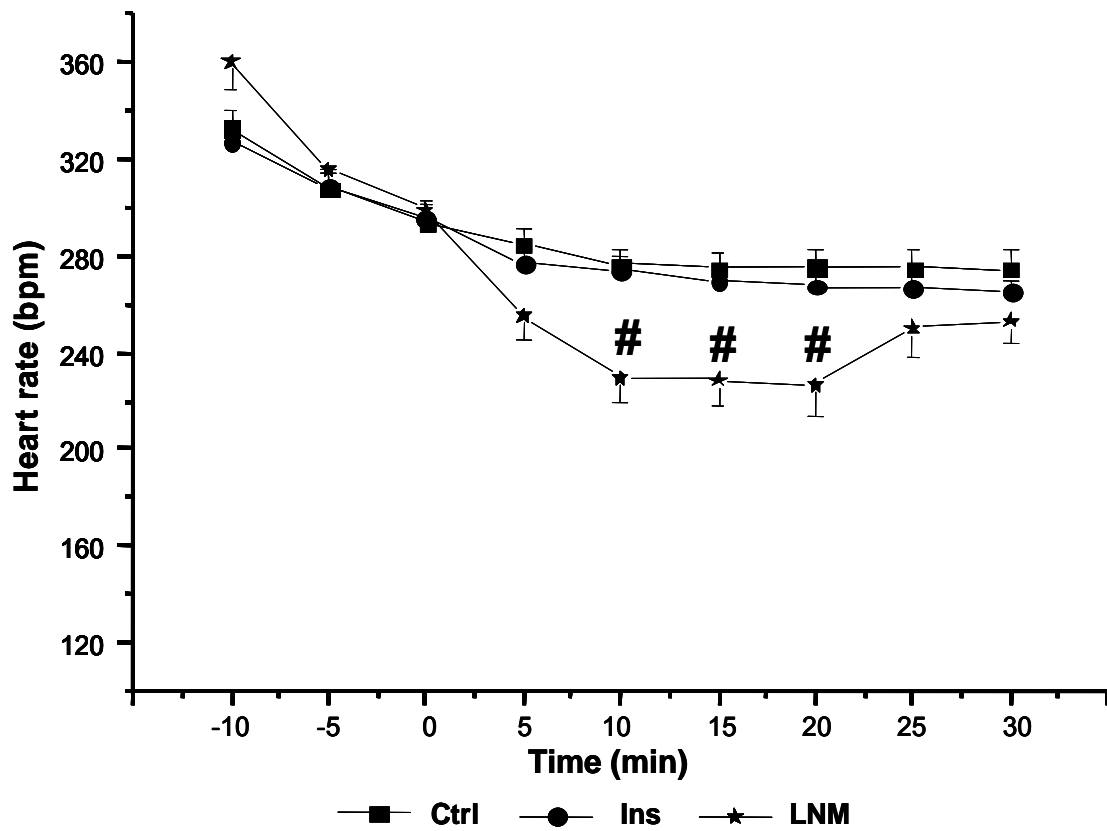


Figure 4.2.a: Effects of L-NAME on heart rate of isolated perfused rat hearts

Heart rate of hearts perfused with KH solution containing either 5 mM glucose (Control, n=25) or 5 mM glucose + 0.3 mIU/mL insulin (Ins group, n=29), or with 5 mM glucose + 0.3 mIU/mL insulin + 10 μ M L-NAME (LNM group, n=11), # p<0.05.

EFFECTS OF L-NAME ON LEFT VENTRICULAR DEVELOPED PRESSURE (FIGURE 4.2.B)

After the stabilisation time, the LVDevP was 84.69 ± 3.38 mmHg for the control group (n = 25), 78.05 ± 2.87 mmHg for Ins group (n = 29), and 87.90 ± 4.73 mmHg for LNM group (n = 11). Immediately after administration of L-NAME, LVDevP values were 84.16 ± 2.67 mmHg for the control group, 89.11 ± 2.74 mmHg for the Ins group, and 69.97 ± 11.07 mmHg for LNM group.

At the end of perfusion, the LVDevP values were 84.41 ± 2.96 mmHg for the control group, 99.23 ± 3.11 mmHg for the Ins group, and 84.16 ± 8.86 mmHg for the LNM group. Significant increase in LVDevP was observed in the group that received insulin compared to the control group. A decrease, but not significant, in the LVDevP was also observed during the L-NAME administration time in the group that received L-NAME (See Fig. 4.2.b, p87).

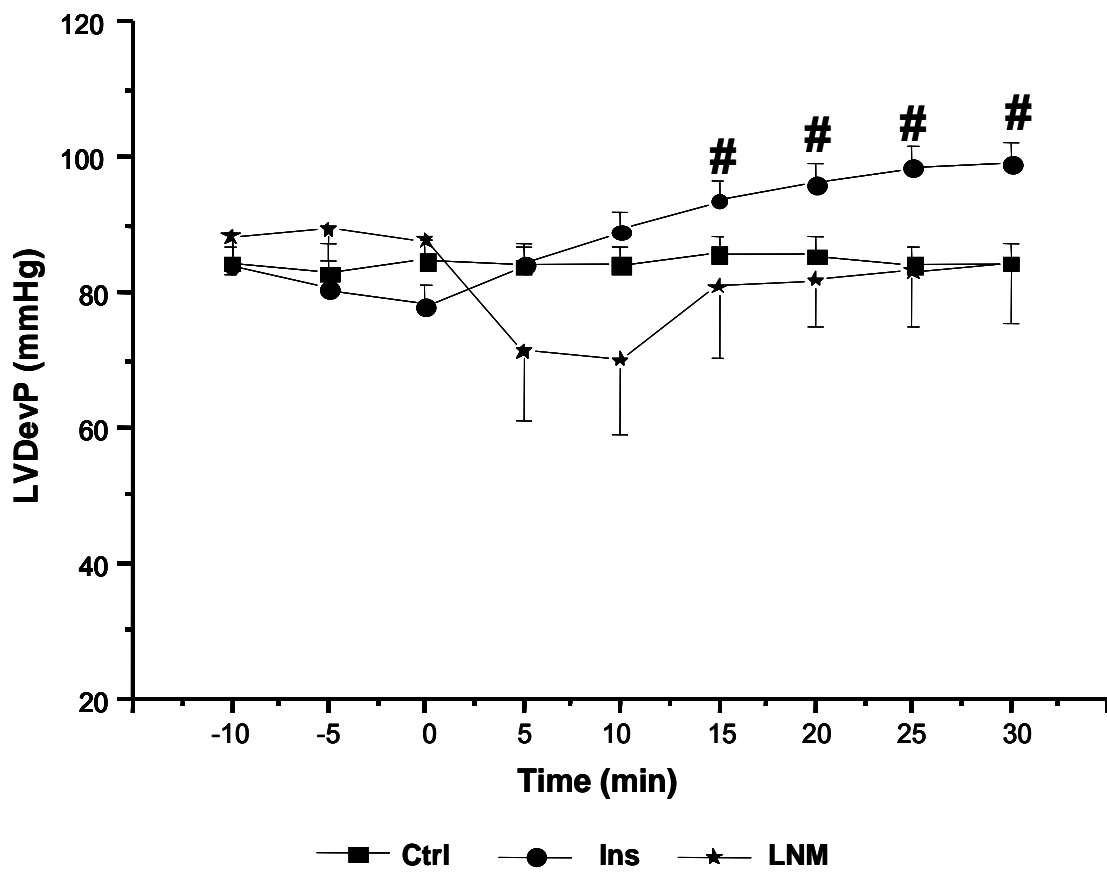


Figure 4.2.b: Effects of L-NAME on LVDevP of isolated perfused rat hearts

LVDevP of hearts perfused with KH solution containing either 5 mM glucose (Control, n=25) or 5 mM glucose + 0.3 mIU/mL insulin (Ins group, n=29), or with 5 mM glucose + 0.3 mIU/mL insulin + 10 μ M L-NAME (LNM group, n=11), # p<0.05.

EFFECT OF L-NAME ON CORONARY FLOW (FIGURE 4.2.C)

Coronary flow at the end of the stabilisation period was 13.55 ± 0.33 ml/min for the control group (n = 25), 13.77 ± 0.35 ml/min for the Ins group (n =29), and 14.33 ± 0.5 ml/min for the LNM group (n = 11). After 10 minutes of perfusion, there was a significant decrease of CF of the LNM group compared to Gluc₅ and Ins groups, $p < 0.05$. CF values 12.42 ± 0.34 ml/min for the control group, 12.81 ± 0.33 ml/min for the Ins group, and 6.8 ± 0.9 ml/min for the LNM group.

At the end of the experiment, CF was 12.50 ± 0.41 ml/min for the control group, 12.88 ± 0.30 ml/min for the Ins group, and 8.95 ± 0.8 ml/min for the LNM group (See Fig. 4.2.c, p89).

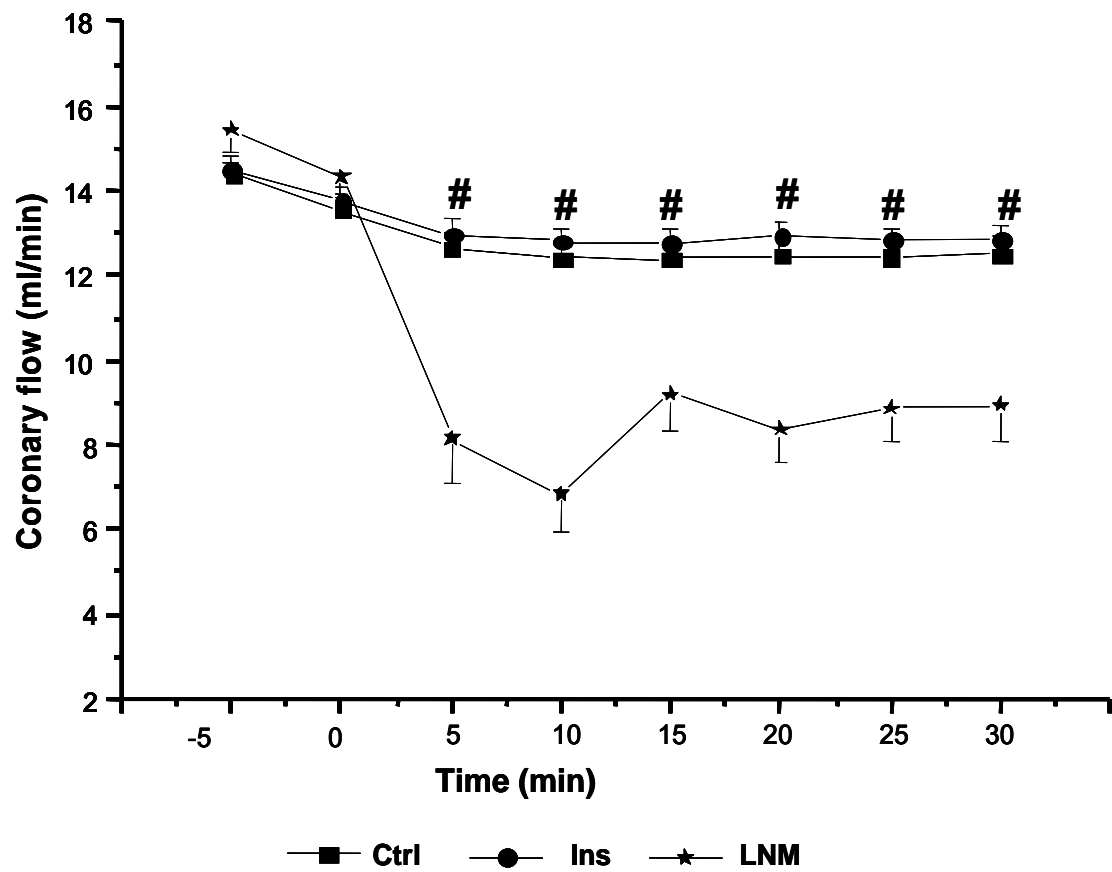


Figure 4.2.c: Effects of L-NAME on CF of isolated perfused rat hearts

CF of hearts perfused with KH solution containing either 5 mM glucose (Control, n=25) or 5 mM glucose + 0.3 mIU/mL insulin (Ins group, n=29), or with 5 mM glucose + 0.3 mIU/mL insulin + 10 μ M L-NAME (LNM group, n=11), # p<0.05.

PRELIMINARY WORTMANNIN STUDY

It has been widely demonstrated that the cardioprotection of insulin on the ischaemic and reperfused isolated heart is mediated by PI3-K. To investigate the implication of P3-K in the inotropic effect of insulin under normoxic conditions, we conducted a preliminary study where we used 100 nM of wortmannin, a specific inhibitor of PI3-K.

EFFECT OF WORTMANNIN ON HEART RATE (FIGURE 4.3.A)

At the end of the stabilisation period HR of the control group (Gluc₅, n = 25) was 293.78 ± 7.48 bpm, 296.09 ± 6.65 bpm for the insulin group (Ins, n = 29), and 297.70 ± 5.5 bpm for the wortmannin group (Wort, n = 26). At the end of the experiment, the heart rate values were 274.58 ± 7.69 bpm for the control, 265.18 ± 4.94 bpm for Ins, 250.58 ± 6.9 bpm for the Wort group. A significant decrease in heart rate of the hearts that received insulin + wortmannin was observed from 10 minutes after the initiation of perfusion with wortmannin until the end of the perfusion protocol, $p < 0.05$ (See Fig. 4.3.a, p 91).

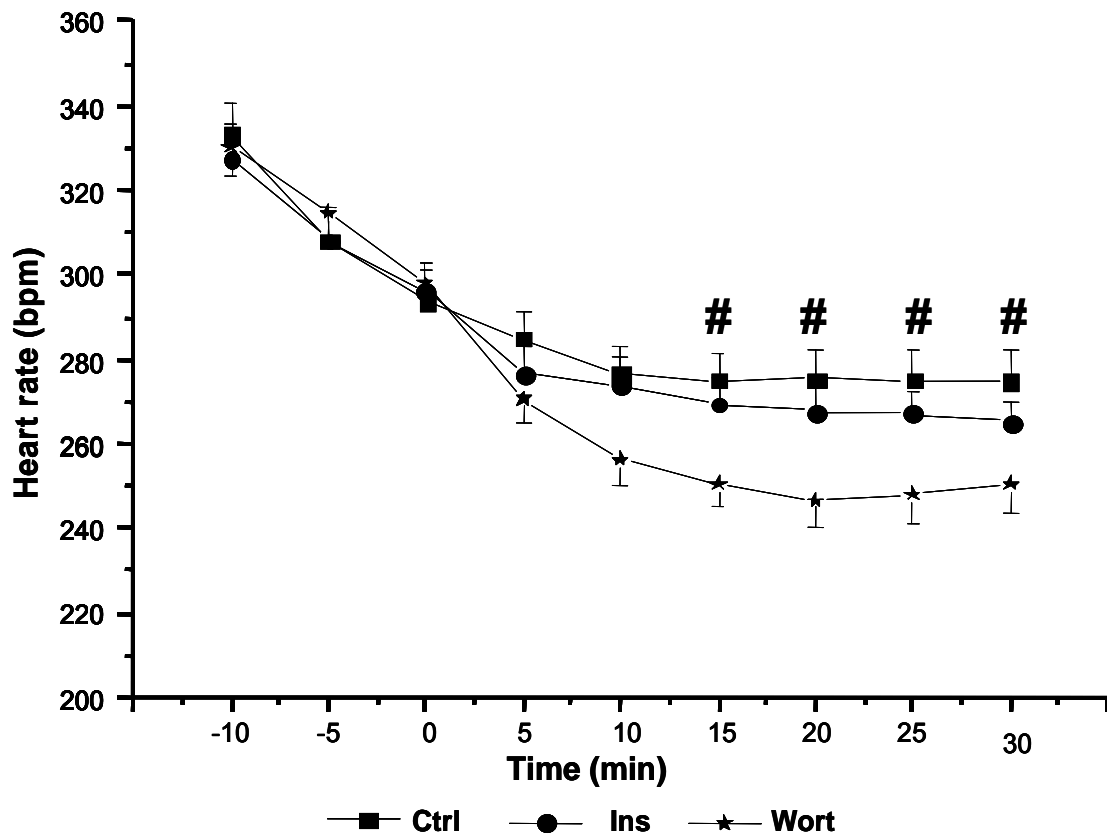


Figure 4.3.a: Effects of wortmannin on HR of isolated perfused rat hearts

HR of hearts perfused with KH solution containing either 5 mM glucose (Control, n=25) or 5 mM glucose + 0.3 mIU/mL insulin (Ins group, n=29), or with 5 mM glucose + 0.3 mIU/mL insulin + 100 nM wortmannin (Wort group, n=26), # p<0.05.

EFFECT OF WORTMANNIN ON LEFT VENTRICULAR DEVELOPED PRESSURE (FIGURE 4.3.B)

Left ventricular developed pressure (LVDevP) values at the end of the stabilisation period were 84.69 ± 3.38 mmHg for the control group (n = 25), 78.05 ± 2.87 mmHg for the Ins group (n = 29), and 87.62 ± 3.34 mmHg for the Wort group (n = 26). After 30 minutes of experiment, the LVDevP was 84.41 ± 2.96 mmHg for the control group, 99.23 ± 3.11 mmHg for the Ins group, and 61.90 ± 4.91 mmHg for the Wort group. Significant decrease in LVDevP was observed in the group that received wortmannin compared to the control and to the insulin groups, $p < 0.01$ (See Fig. 4.3.b, p93).

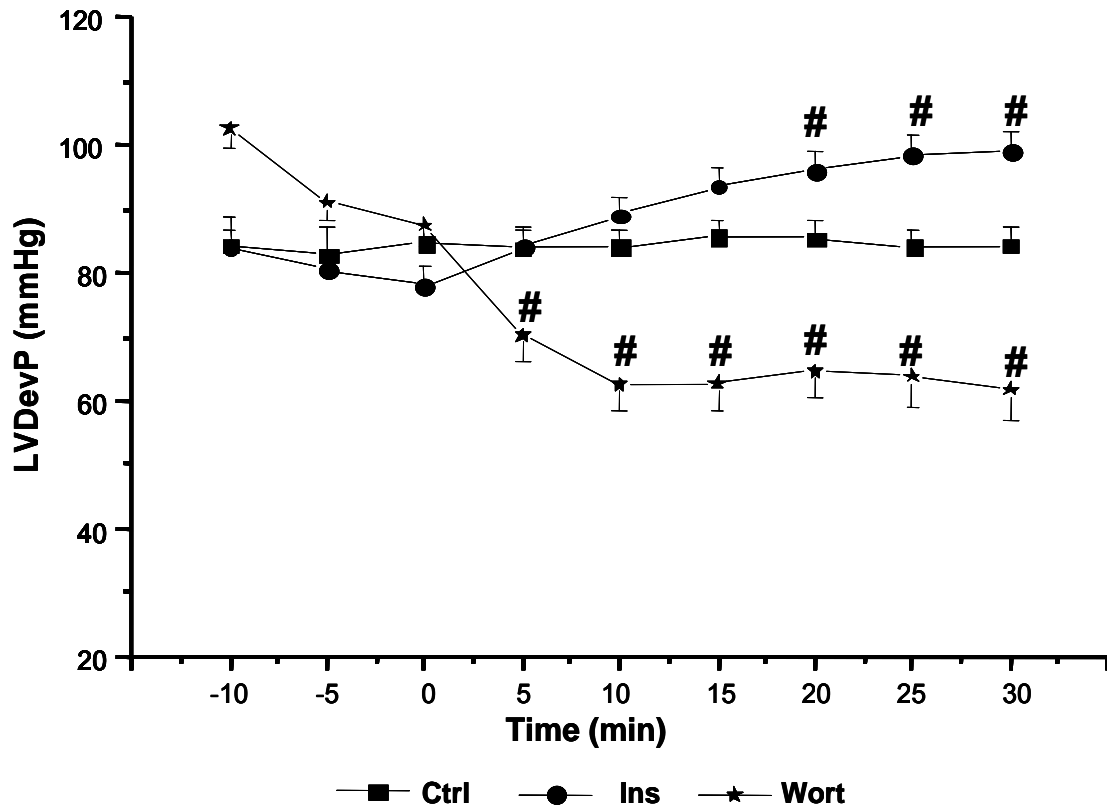


Figure 4.3.b: Effects of wortmannin on LVDevP of isolated perfused rat hearts
 LVDevP of hearts perfused with KH solution containing either 5 mM glucose (Control, n=25) or 5 mM glucose + 0.3 mIU/mL insulin (Ins group, n=29), or with 5 mM glucose + 0.3 mIU/mL insulin + 100 nM wortmannin (Wort group, n=26), # p<0.01.

EFFECT OF WORTMANNIN ON CORONARY FLOW (FIGURE 4.3C)

Coronary flow at the end of the stabilisation period was 13.55 ± 0.33 ml/min for the control group (n = 25), 13.77 ± 0.35 ml/min for the Ins group (n = 29), and 13.67 ± 0.4 ml/min for the Wort group (n = 26).

At the end of the experiment, CF was 12.50 ± 0.41 ml/min for the control group, 12.88 ± 0.30 ml/min for the Ins₃ group, and 8.92 ± 0.4 ml/min for the Wort₁₀₀ group. A rapid significant decrease in the CF of hearts that received wortmannin was observed immediately from the administration of wortmannin at the beginning of the experiment, $p < 0.01$. The low coronary flow was sustained until the end of the experiment time (See Fig. 4.3.c, p95).

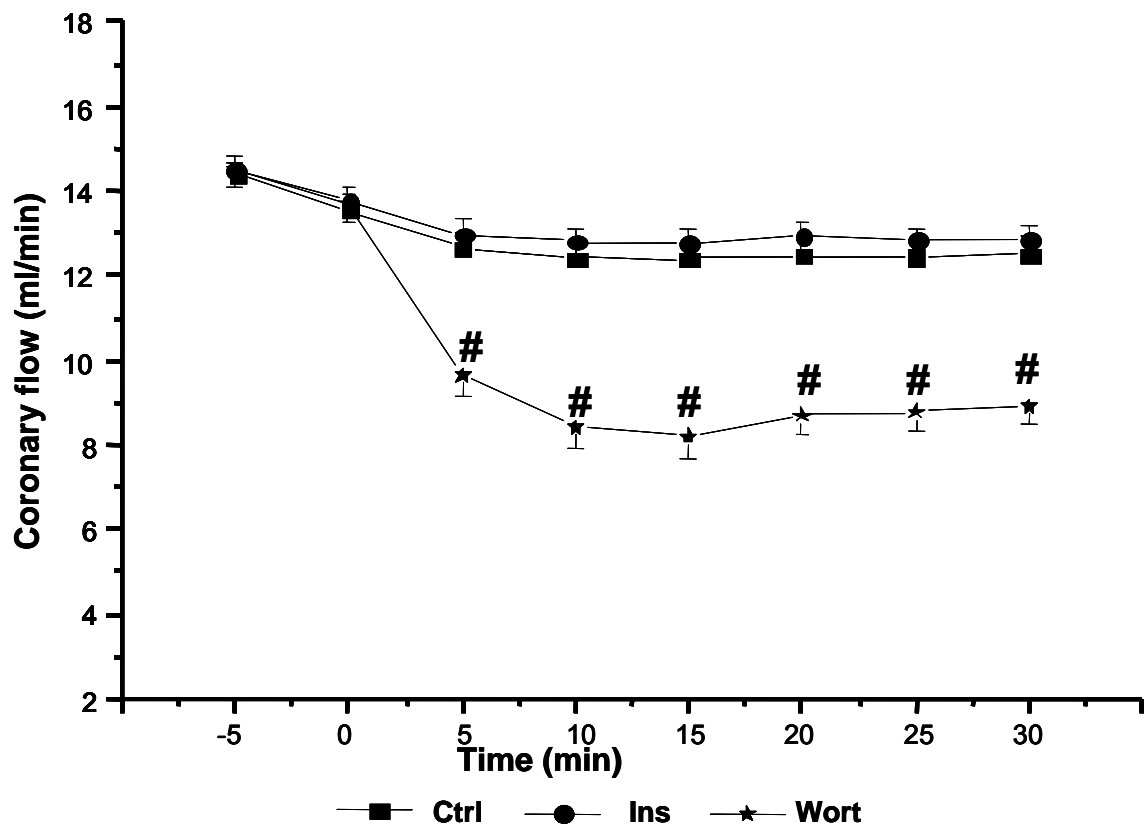


Figure 4.3.c: Effects of wortmannin on CF of isolated perfused rat hearts

CF of hearts perfused with KH solution containing either 5 mM glucose (Control, n=25) or 5 mM glucose + 0.3 mIU/mL insulin (Ins group, n=29), or with 5 mM glucose + 0.3 mIU/mL insulin + 100 nM wortmannin (Wort group, n=26), # p<0.01.

BIOCHEMICAL ANALYSIS DATA

EFFECT OF L-NAME ON PKB/Akt

TOTAL PKB/Akt

As expected, the content of total PKB/Akt of hearts in all groups was the same from the starting of the experiment up to the end of the experimental protocol.

AT 10 MIN

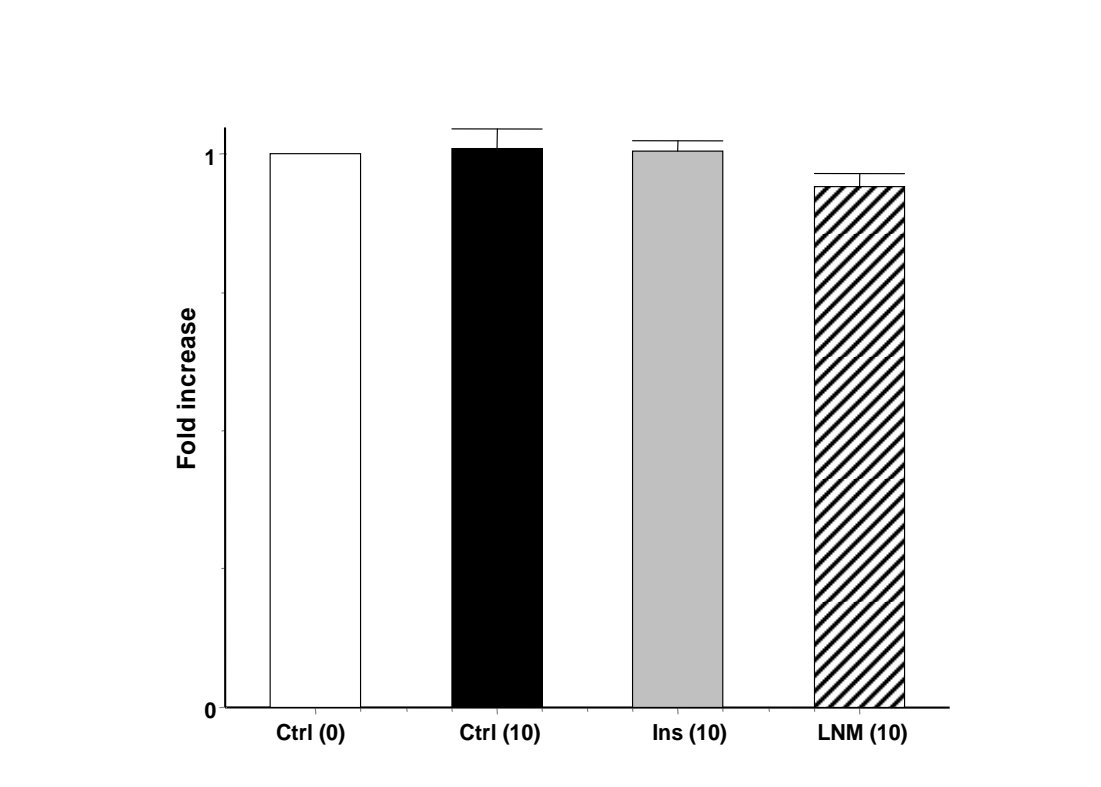


Figure 4.4.a: Total PKB/Akt of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (10): 5mM glucose measured 10 min after experiment starts.

Ins (10): 5mM glucose and 0.3mIU/mL insulin, measured 10 min after experiment starts.

LNM(10): 5mM glucose + 0.3mIU/mL insulin and 10 μ M L-NAME measured 10 min after experiment starts.

Total PKB/Akt was visualized with an appropriate primary antibody. n = 3 hearts per group.

AT 30 MIN

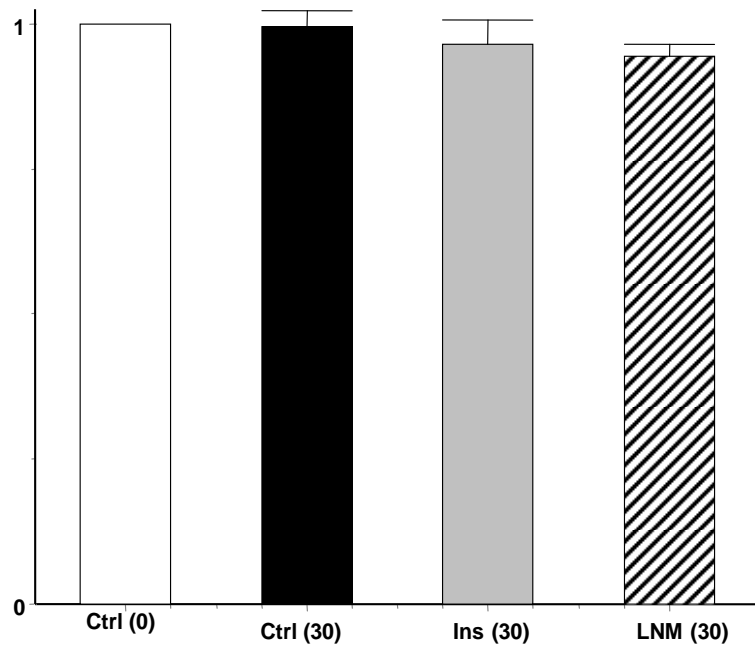


Figure 4.4.b: Total PKB/Akt of hearts perfused standard KH solution containing:
Ctrl (0): 5mM glucose measured immediately after stabilisation
Ctrl (30): 5mM glucose measured 30 min after experiment starts.
Ins (30): 5mM glucose and 0.3mIU/mL insulin, measured 30 min after experiment starts.
LNM (30): 5mM glucose + 0.3mIU/mL insulin and 10 μ M L-NAME measured 30 min after experiment starts.

Total PKB/Akt was visualized with an appropriate primary antibody. n = 3 hearts per group.

EFFECT OF L-NAME ON PHOSPHORYLATED PKB/Akt THREONINE

10 MIN

Phosphorylation of the threonine isoform of PKB/Akt was pronounced in the group that received L-NAME compared to the control and the insulin group. We did not find any significant, difference between all the groups.

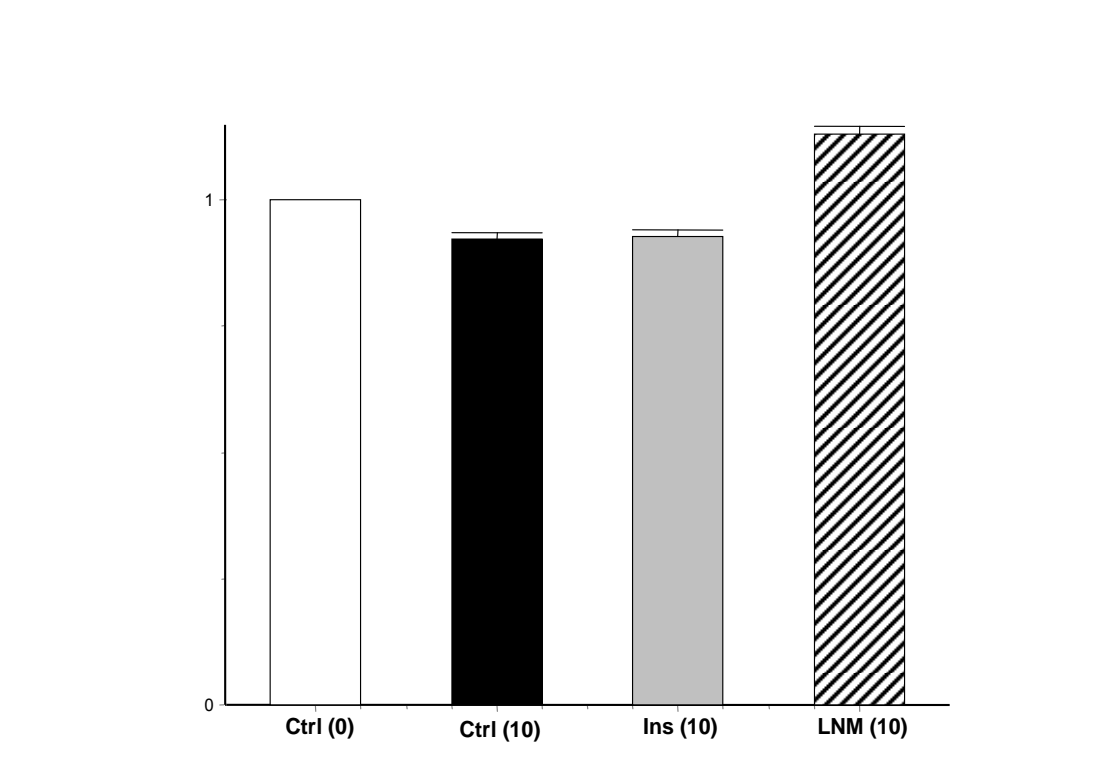


Figure 4.5.a: Phosphorylated PKB/Akt [Thr³⁰⁸] of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (10): 5mM glucose measured 10 min after experiment starts.

Ins (10): 5mM glucose and 0.3mIU/mL insulin, measured 10 min after experiment starts.

LNM (10): 5mM glucose + 0.3mIU/mL insulin and 10 μ M L-NAME measured 10 min after experiment starts.

Phosphorylated PKB/Akt was visualized with an appropriate primary phospho-Akt [Thr³⁰⁸] antibody. n = 3 hearts per group.

30 MIN

After 30 min of the experimental protocol, a decrease in phosphorylated PKB/Thr³⁰⁸ was observed in the group that received L-NAME. A slight decrease was also observed in the control group at the end of the experiment. There was no significant difference between the groups.

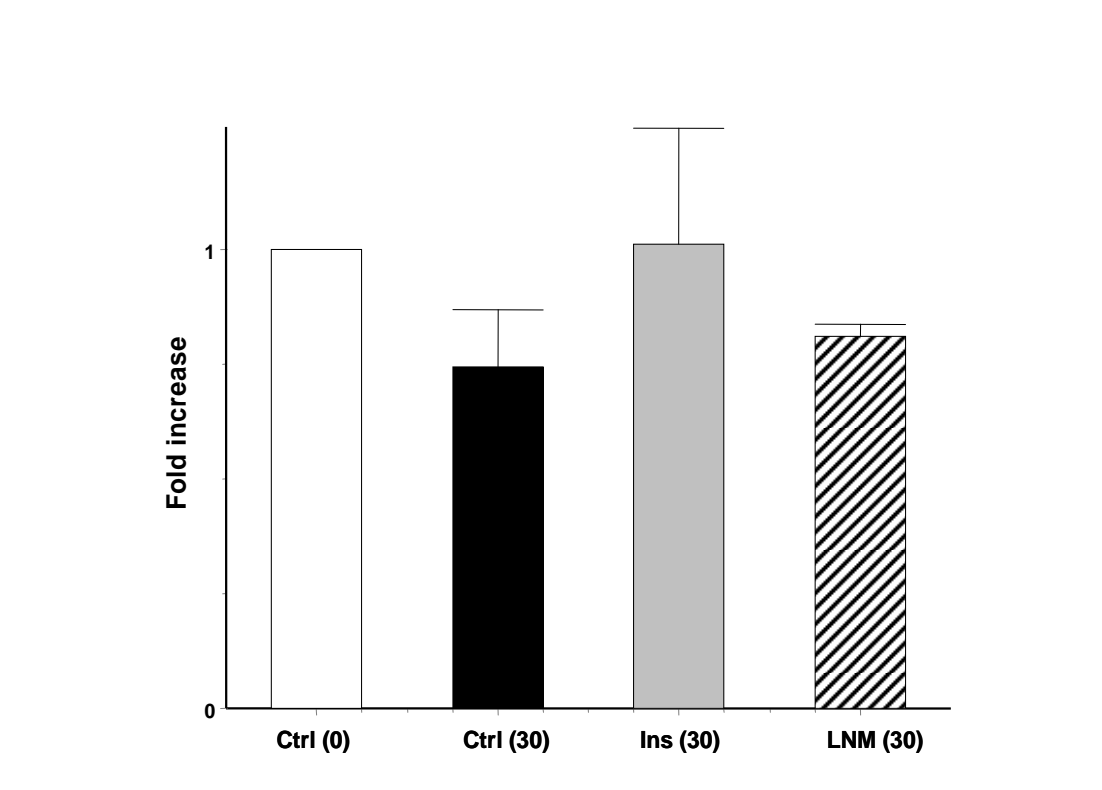


Figure 4.5.b: Phosphorylation of PKB/Akt [Thr³⁰⁸] of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (30): 5mM glucose measured 30 min after experiment starts.

Ins (30): 5mM glucose and 0.3mIU/mL insulin, measured 30 min after experiment starts.

LNM (30): 5mM glucose + 0.3mIU/mL insulin and 10 μ M L-NAME measured 30 min after experiment starts.

Phosphorylated PKB/Akt was visualized with an appropriate primary phospho-Akt [Thr³⁰⁸] antibody. n = 3 hearts per group.

EFFECT OF L-NAME ON PHOSPHORYLATED PKB/Akt SERINE

10 MIN

The content of phosphorylated PKB/Akt [Ser⁴⁷³] was slightly high in the control group and in the group that received insulin 10 minutes after the start of the experimental protocol compared to the control group at the beginning of the experiment. There was no significant difference in the content of phosphorylated PKB/Akt [Ser⁴⁷³] between the control group at the beginning of the experiment and the group that received L-NAME 10 min after the beginning of the experiment.

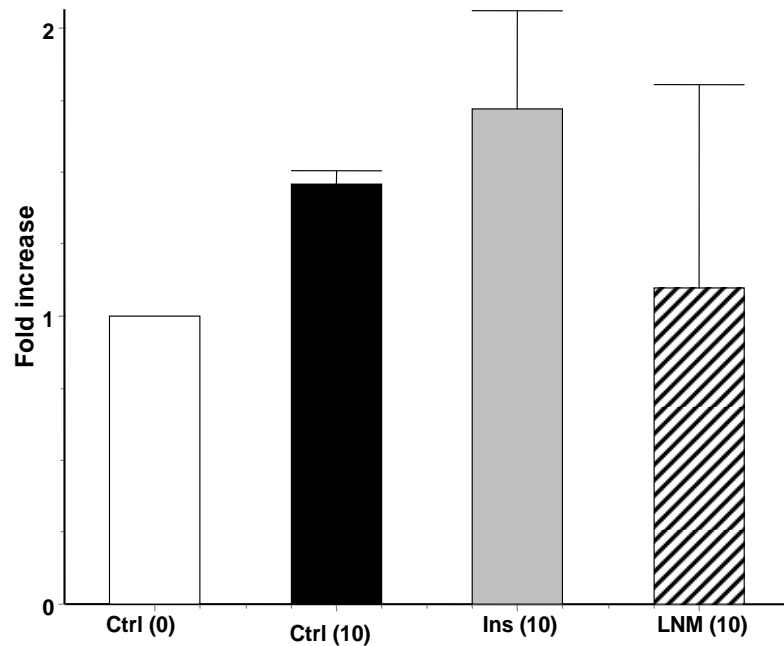


Figure 4.6.a: Phosphorylation of PKB/Akt [Ser⁴⁷³] of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (10): 5mM glucose measured 10 min after experiment starts.

Ins (10): 5mM glucose and 0.3mIU/mL insulin, measured 10 min after experiment starts.

LNM (10): 5mM glucose + 0.3mIU/mL insulin and 10 μ M L-NAME measured 10 min after experiment starts.

Phosphorylated PKB/Akt was visualized with an appropriate primary phospho-Akt [Ser⁴⁷³] antibody. n = 3 hearts per group.

30 MIN

Thirty minutes after the beginning of the experiment protocol, there was a decrease, but not significant, of phosphorylated PKB/Akt [Ser⁴⁷³] of the control group. An increase, but not significant, was observed in the group that received insulin compared to the other groups.

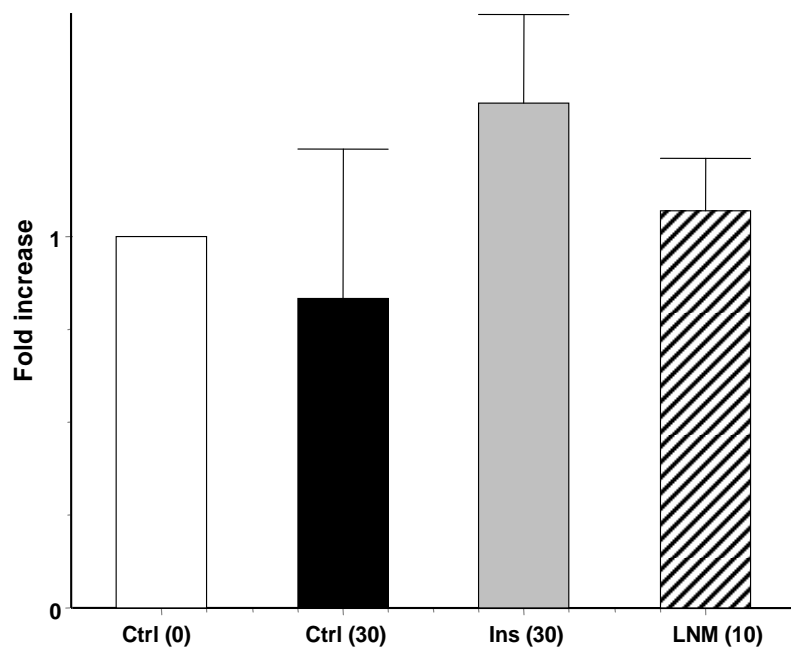


Figure 4.6.b: Phosphorylation of PKB/Akt [Ser⁴⁷³] of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (30): 5mM glucose measured 30 min after experiment starts.

Ins (30): 5mM glucose and 0.3mIU/mL insulin, measured 30 min after experiment starts.

LNM (30): 5mM glucose + 0.3mIU/mL insulin and 10 μ M L-NAME measured 30 min after experiment starts.

Phosphorylated PKB/Akt was visualized with an appropriate primary phospho-Akt [Ser⁴⁷³] antibody. n = 3 hearts per group.

EFFECT OF L-NAME ON PHOSPHORYLATED PI3-K

10MIN

As expected, an increase, but not significant, in phosphorylated PI3-K was observed in the group that received insulin compared to the other groups. There was no significant difference on the content of phosphorylated PI3-K between all groups.

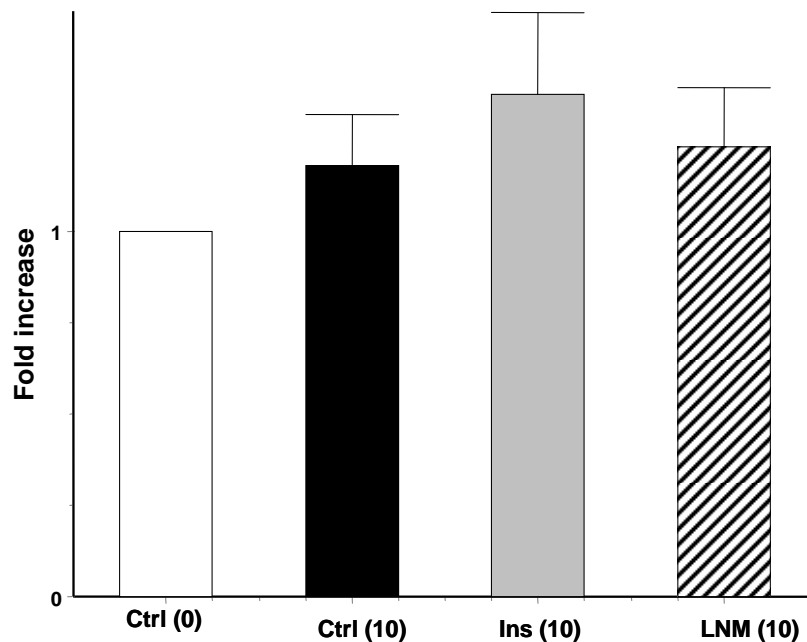


Figure 4.7.a: Activation of PI3-K-p-110 α of hearts perfused standard KH solution containing:
Ctrl (0): 5mM glucose measured immediately after stabilisation
Ctrl (10): 5mM glucose measured 10 min after experiment starts.
Ins (10): 5mM glucose and 0.3mIU/mL insulin, measured 10 min after experiment starts.
LNM (10): 5mM glucose + 0.3mIU/mL insulin and 10 μ M L-NAME measured 10 min after experiment starts.

Phosphorylated PI3-K was visualized with an appropriate primary phospho-PI3-K-p-110 α antibody. n = 3 hearts per group.

30MIN

At 30 minutes of experimental the protocol, an increase, but not significant, of phosphorylated PI3-K was observed in the group that received insulin. There was no change in the phosphorylated PI3-K content of the control group and the group that received L-NAME when compared to phosphorylated PI3-K content at 10 min of the experimental protocol.

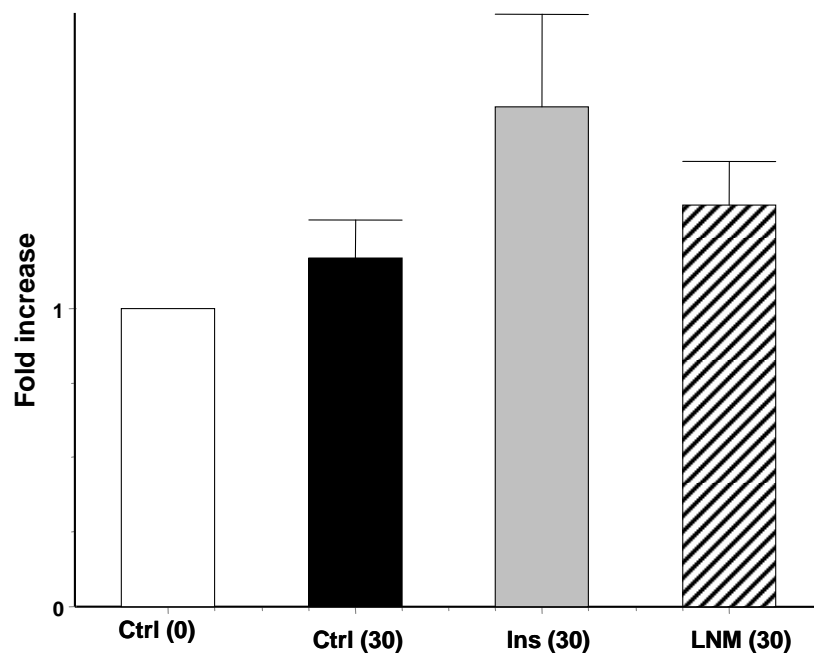


Figure 4.7.b: Activation of PI3-K-p-110 α of hearts perfused standard KH solution containing:
Ctrl (0): 5mM glucose measured immediately after stabilisation
Ctrl (30): 5mM glucose measured 30 min after experiment starts.
Ins (30): 5mM glucose and 0.3mIU/mL insulin, measured 30 min after experiment starts.
LNM (30): 5mM glucose + 0.3mIU/mL insulin and 10 μ M L-NAME measured 30 min after experiment starts.

Phosphorylated PI3-K was visualized with an appropriate primary phospho-PI3-K-p-110 α antibody. n = 3 hearts per group.

EFFECT OF WORTMANNIN ON PKB/Akt

EFFECT OF WORTMANNIN ON TOTAL PKB/Akt

10 MIN

Total PKB/Akt was the same in all groups at the beginning as well as at the end of the experimental protocol.

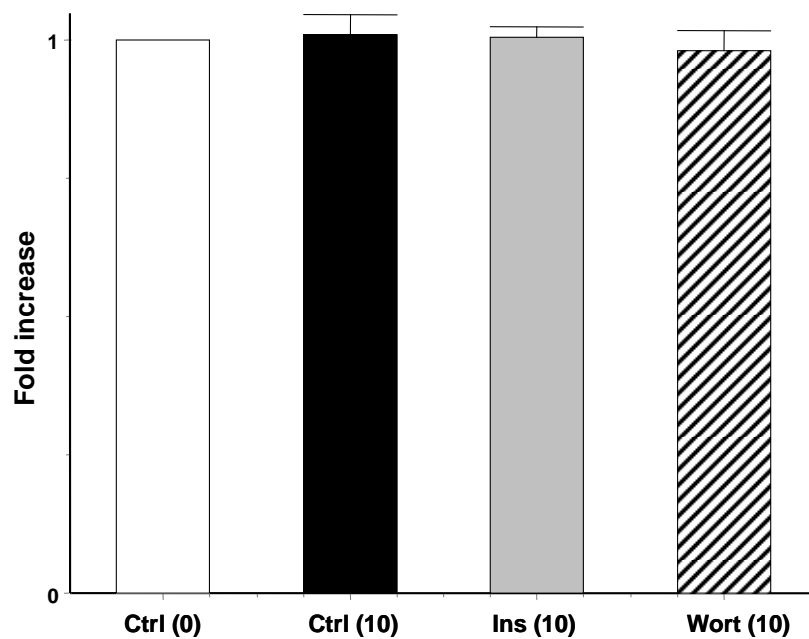


Figure 4.8.a: Total PKB/Akt of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (10): 5mM glucose measured 10 min after experiment starts.

Ins (10): 5mM glucose and 0.3mIU/mL insulin, measured 10 min after experiment starts.

Wort (10): 5mM glucose + 0.3mIU/mL insulin and 100mM wortmannin measured 10 min after experiment starts.

Total PKB/Akt was visualized with an appropriate primary antibody. n = 3 hearts per group.

30 MIN

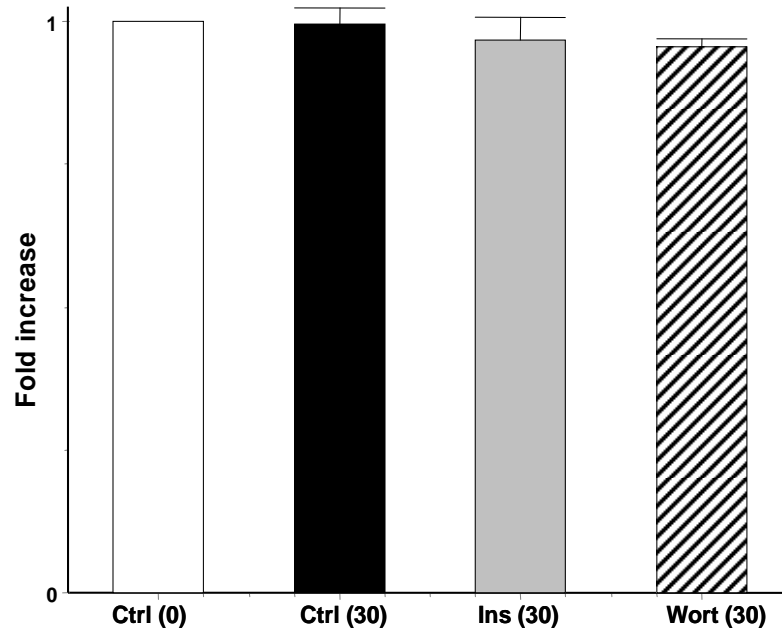


Figure 4.8.b: Total PKB/Akt of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (30): 5mM glucose measured 30 min after experiment starts.

Ins (30): 5mM glucose and 0.3mIU/mL insulin, measured 30 min after experiment starts.

Wort (30): 5mM glucose + 0.3mIU/mL insulin and 10 μ M Wortmannin measured 10 min after experiment starts.

Total PKB/Akt was visualized with an appropriate primary antibody. n = 3 hearts per group.

EFFECT OF WORTMANNIN ON PHOSPHORYLATED PKB/Akt THREONINE

10 MIN

After 10 minutes of the experimental protocol, the content of PKB/Akt [Thr³⁰⁸] was high in the group that received wortmannin compared to all other groups. No significant difference was observed between the groups.

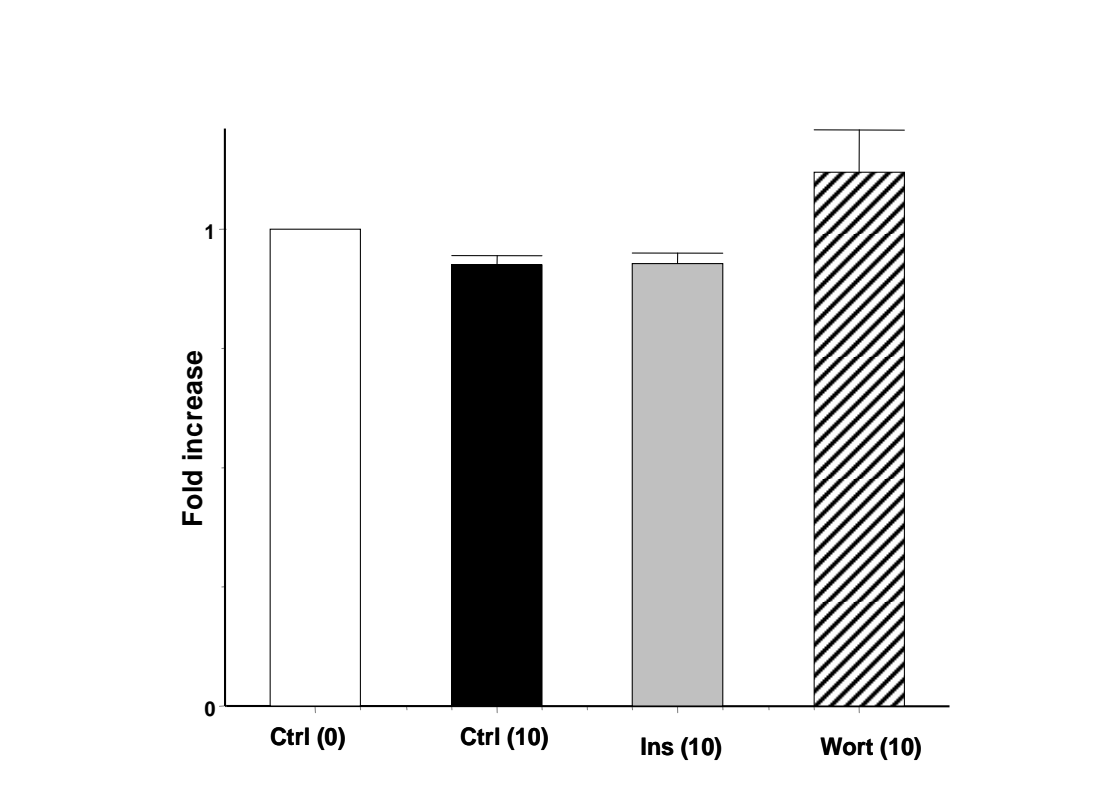


Figure 4.9.a: Phosphorylated PKB/Akt [Thr³⁰⁸] of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (10): 5mM glucose measured 10 min after experiment starts.

Ins (10): 5mM glucose and 0.3mIU/mL insulin, measured 10 min after experiment starts.

Wort (10): 5mM glucose + 0.3mIU/mL insulin and 100nM wortmannin measured 10 min after experiment starts.

Phosphorylated PKB/Akt was visualized with an appropriate primary phospho-Akt [Thr³⁰⁸] antibody. n = 3 hearts per group.

30 MIN

After 30 minutes of the experiment, there was a decrease in the content of PKB/Akt [Thr³⁰⁸] in the control group and in the group that received wortmannin, but it was not significant. There was no change in the content of PKB/Akt [Thr³⁰⁸] of the group that received insulin.

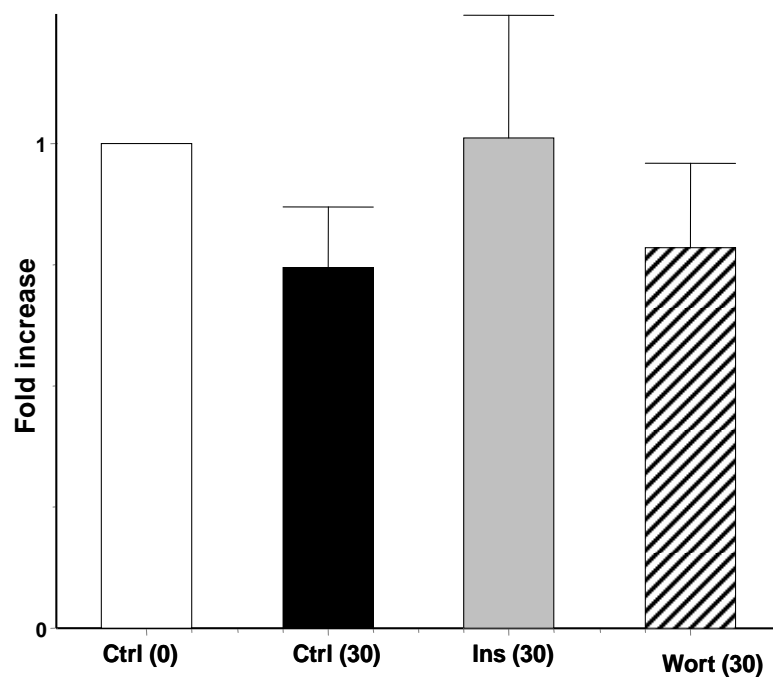


Figure 4.9.b: Phosphorylated PKB/Akt [Thr³⁰⁸] of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (30): 5mM glucose measured 30 min after experiment starts.

Ins (30): 5mM glucose and 0.3mIU/mL insulin, measured 30 min after experiment starts.

Wort (30): 5mM glucose + 0.3mIU/mL insulin and 100nM wortmannin measured 30 min after experiment starts.

Phosphorylated PKB/Akt was visualized with an appropriate primary phospho-Akt [Thr³⁰⁸] antibody. n = 3 hearts per group.

SERINE

10 MIN

After 10 minutes of the experimental protocol, the content of phosphorylated PKB/Akt [Ser⁴⁷³] was high in the control group and in the group that received insulin compared to the control group just after stabilisation. Phosphorylated PKB/Akt [Ser⁴⁷³] was lower in the group that received wortmannin.

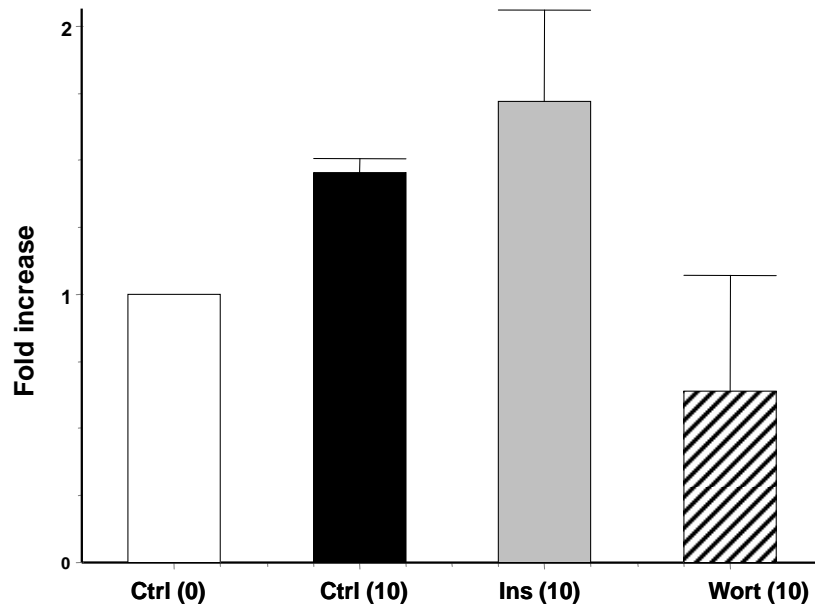


Figure 4.10.a: Phosphorylated PKB/Akt [Ser⁴⁷³] of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (10): 5mM glucose measured 10 min after experiment starts.

Ins (10): 5mM glucose and 0.3mIU/mL insulin, measured 10 min after experiment starts.

Wort (10): 5mM glucose + 0.3mIU/mL insulin and 100nM wortmannin measured 10 min after experiment starts.

Phosphorylated PKB/Akt was visualized with an appropriate primary phospho-Akt [Ser⁴⁷³] antibody. n = 3 hearts per group.

30 MIN

After 30 minutes of experiment, there was a slight decrease in phosphorylated PKB/Akt [Ser⁴⁷³] content of the control group. The content of phosphorylated PKB/Akt [Ser⁴⁷³] in the group that received wortmannin did not vary. No significant difference was observed between the groups.

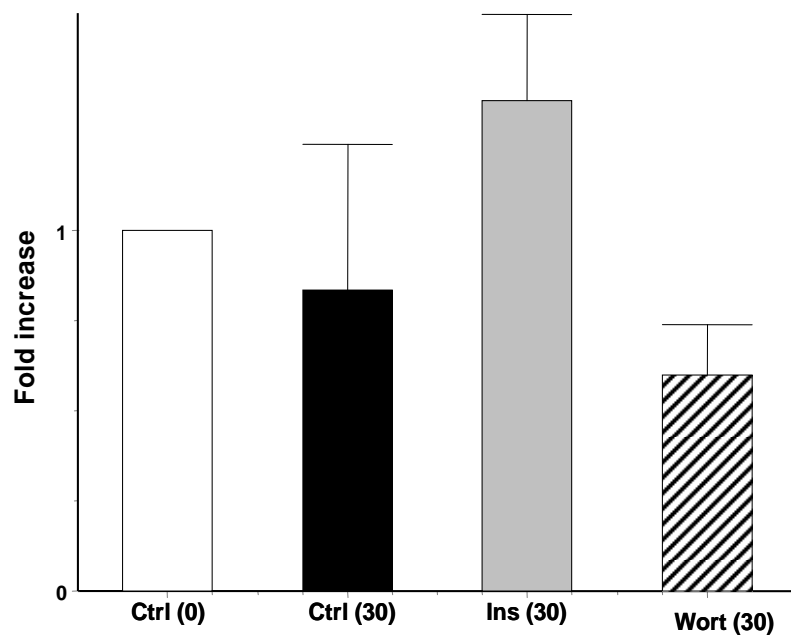


Figure 4.10.b: Phosphorylated PKB/Akt [Ser⁴⁷³] of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (30): 5mM glucose measured 30 min after experiment starts.

Ins (30): 5mM glucose and 0.3mIU/mL insulin, measured 30 min after experiment starts.

Wort (30): 5mM glucose + 0.3mIU/mL insulin and 100nM wortmannin measured 30 min after experiment starts.

Phosphorylated PKB/Akt was visualized with an appropriate primary phospho-Akt [Ser⁴⁷³] antibody. n = 3 hearts per group.

EFFECT OF WORTMANNIN ON PHOSPHORYLATED PI3K

10 MIN

At 10 minutes of the experimental protocol, the content of phosphorylated PI3-K was slightly high in the control group and in the group that received insulin, compared to the control group at the beginning of the experiment. The group that received wortmannin had a low content of PI3-K compared to the insulin and the control group. There was no significant difference between the groups.

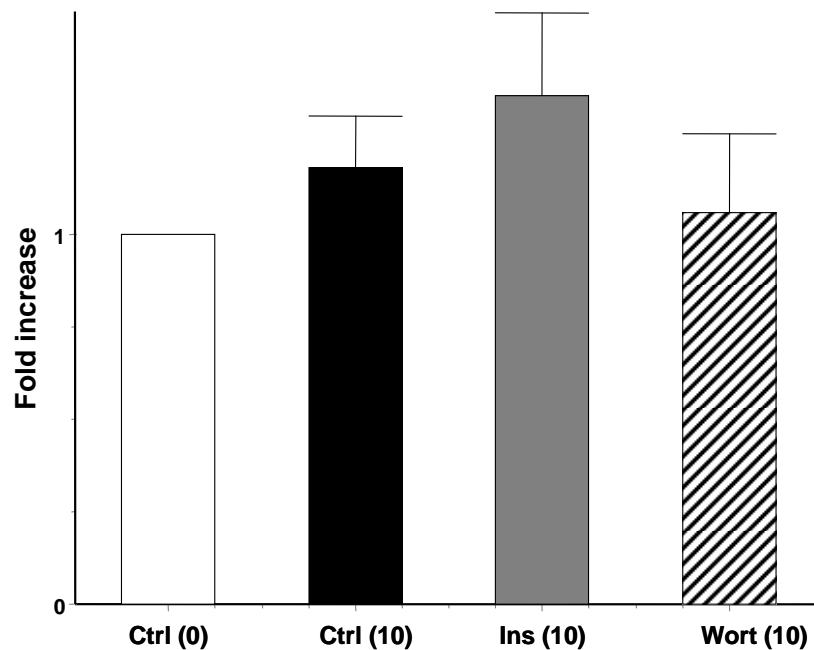


Figure 4.11.a: Phosphorylated PI3-K-p-110 α of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (10): 5mM glucose measured 10 min after experiment starts.

Ins (10): 5mM glucose and 0.3mIU/mL insulin, measured 10 min after experiment starts.

Wort (10): 5mM glucose + 0.3mIU/mL insulin and 100nM wortmannin measured 10 min after experiment starts.

Phosphorylated PI3-K was visualized with an appropriate primary phospho- PI3-K-p-110 α antibody. n = 3 hearts per group.

30 MIN

PI3-K content was still high in the group that received insulin compared to the group that received wortmannin and to the control group, 30 minutes after the experiment. No significant difference was observed.

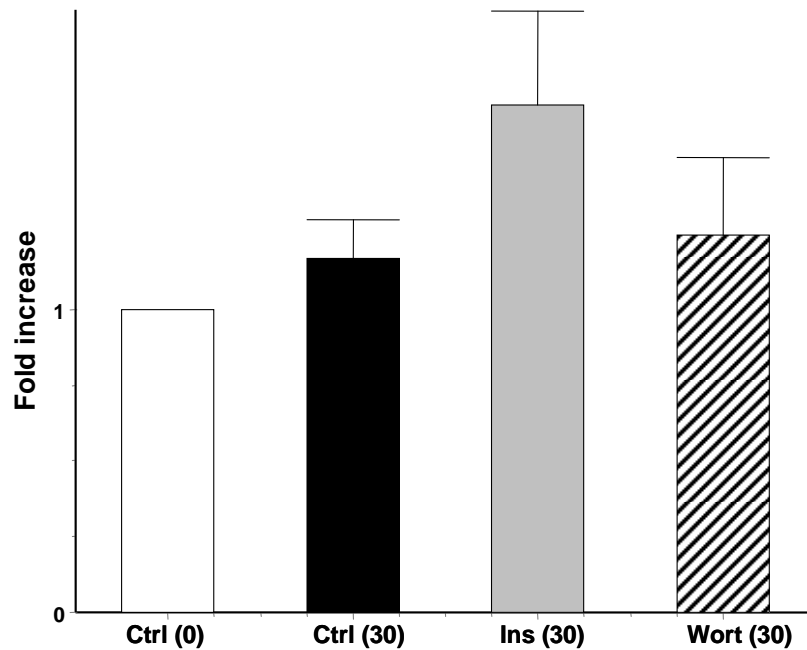


Figure 4.11.b: Phosphorylated PI3-K-p-110 α of hearts perfused standard KH solution containing:

Ctrl (0): 5mM glucose measured immediately after stabilisation

Ctrl (30): 5mM glucose measured 30 min after experiment starts.

Ins (30): 5mM glucose and 0.3mIU/mL insulin, measured 30 min after experiment starts.

Wort (30): 5mM glucose + 0.3mIU/mL insulin and 100nM wortmannin measured 30 min after experiment starts.

Phosphorylated PI3-K was visualized with an appropriate primary phospho PI3-K-p-110 α antibody. n = 3 hearts per group.

WESTERN BLOTS

10 MIN

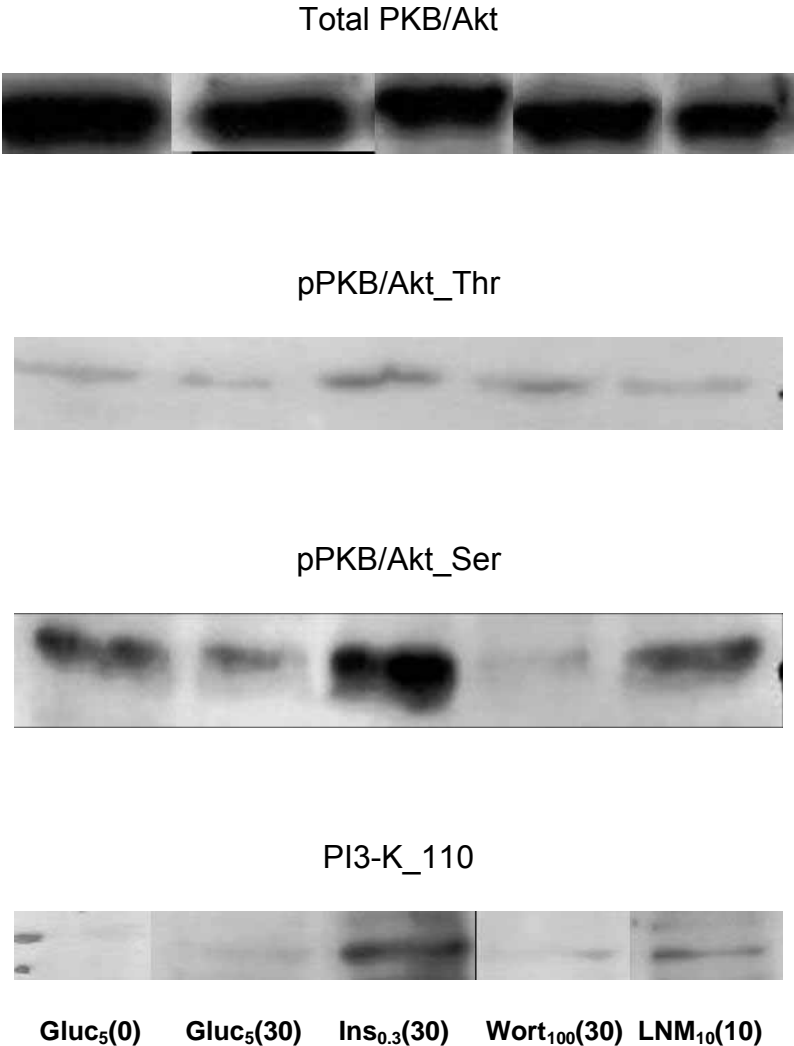


Figure 4.12: Representative Western blots of biochemical data

Chapter 5

DISCUSSION

It is well documented that insulin protects the heart against ischaemia. Sodi-Pallares *et al.* were the first in 1962 to perform the pioneering work on the cardioprotective effect of insulin against ischaemia. The positive effect of insulin on the functional recovery and survival of the ischaemic heart has been the focus of several studies. Amongst the major findings are: a reduction in infarct size with insulin given at the onset of reperfusion (Jonassen *et al.*, 2000), the improved cardiac output recovery during reperfusion (Van Rooyen *et al.*, 1998), and the attenuation of apoptosis in rat neonatal cardiomyocytes when insulin was administered during reoxygenation (Jonassen *et al.*, 2000).

It was long believed that the effects of insulin involved in its cardioprotection such as (i) the enhancement of glycogen synthesis (Lawrence, 1992), (ii) the improved protein synthesis (Reif *et al.*, 1997), (iii) the stimulation of glycolysis (Depre *et al.*, 1998), (iv) the recruitment of glucose transporters to enhance glucose entry into the cells (Coffer *et al.*, 1998), and (v) the positive inotropic and positive chronotropic properties (Kearney *et al.*, 1998) were of metabolic origin, but lately the emphasis has shifted towards the signal transduction pathways downstream of the insulin receptor. The beneficial effects of insulin on ischaemia/reperfusion induced injury as well as the pathways involved have been studied extensively, however little is known about the effect(s) of insulin

and the involvement of the various signalling pathways in the responses seen to insulin in the healthy heart under normoxic conditions.

This study was therefore designed to investigate the pathways involved in the positive effects of insulin on cardiac function in an isolated healthy heart under normoxic conditions with a special regard of the possible involvement of the nitric oxide pathway in the effect of insulin on the vasculature and a possible involvement of the PI3-K – PKB/Akt pathway on cardiac function, compared to the effects of insulin in the isolated ischaemic/reperfused heart model.

PRELIMINARY STUDY

INSULIN EFFECT ON CARDIAC FUNCTION (HR AND LVDEV_P)

In order to determine a suitable protocol with insulin that best shows the beneficial effects of insulin on the heart, a preliminary study was performed to determine the suitable dose of insulin to be administered to the heart, *via* the perfusion solution. In previous work done in our laboratory, Louw (Louw 2001, Unpublished results, MSc thesis) found that insulin increased cardiac contractility before ischaemia. We wanted thus to examine whether her results could be repeated, and to see if insulin, at different concentrations, was able to exert a protective effect under normoxic conditions without any stressful events such as ischaemia. Therefore, we added insulin to the perfusion solution at the concentration of 0.3 mIU/mL in one group, 0.6 mIU/mL to a second group and 1.0 mIU/mL to a third group. We observed that the hearts where insulin was

added to the perfusion solution at the concentration of 0.3 mIU/mL gave us a constant significant increase in the LVDevP compared to the control group. The LVDevP of all insulin groups was higher than the control group (Fig. 4.1.b, p77). From the results obtained in our study and on the basis of previous studies that used insulin in their protocols (Rosenzweig *et al.* 1980; Aulbach *et al.* 1999; Jonassen *et al.* 2001; Louw 2001, Unpublished results, MSc thesis), we assumed that insulin at the concentration of 0.3 mIU/mL was the suitable dose to be used and we conducted all further experimentation with insulin at the dose of 0.3 mIU/mL added to the perfusion solution after a 10 minutes stabilisation period of the perfusion protocol. We also hypothesized that the increase in the LVDevP that was observed in the groups that received insulin could be attributed to the positive inotropic ability of insulin that allows improved contraction of the myocardium (Kearney *et al.*, 1998), and thus a better developed pressure.

Previous studies showed that the vasodilatory effects of insulin decrease the rate of contraction of the myocardium by increasing the vascular tone and the left ventricular end diastolic pressure (Oltman *et al.* 2000; Sundell *et al.* 2002; Westerbacka *et al.* 2001). Other studies also showed that PI3-K played a role in increased contractility in cardiomyocytes (Pitcher *et al.* 1998; Balligand 1999; Koch *et al.* 2000; Perry & Lefkowitz 2002; Claing *et al.* 2002; Rockman *et al.* 2002) and it is accepted that the cardiomyocytes contraction mediates the function of the left ventricle. In those studies, the argument was that the β -

adrenergic pathway and PKA linked to Ca^{2+} release from the sarcoplasmic reticulum may be involved in the increase of the contraction. Although our model and study design were different, it is tempting to assume that in our experiment, insulin had similar effects in stimulating the cardiomyocytes leading therefore to the increased contraction in hearts receiving insulin alone.

INSULIN EFFECT ON CORONARY FLOW

It is generally recognised and accepted that insulin is a vasodilator. In our preliminary protocol, the coronary flow (CF) of the control group was lower than that of the groups that received insulin throughout the experiment. Although not significant within the groups where insulin was administered, a dose-dependent variation of the coronary flow was observed, with the lower value of CF being attributed to the lower concentration of insulin and the higher dose of insulin resulting in a higher coronary flow. This means that the vasodilatory effect of insulin on the vasculature is dose dependent. These results are consistent with that of Sundell and Knuuti (2003) where insulin has been found to be a slow vasodilator and that it induces vasodilatation in a time-dependent manner (Sundell & Knuuti 2003).

Results of the present study are in accordance with Sundell *et al.* (2002) who demonstrated that insulin induces a dose-dependent increase in hyperaemic myocardial blood flow in healthy men. They found that physiological hyperinsulinaemia (serum insulin ~65 mU/L) significantly increased myocardial

blood flow and that supra-physiological hyperinsulinaemia (serum insulin ~460 mU/L) further enhanced hyperaemic myocardial blood flow (Sundell *et al.* 2002). This confirms that the vasodilatation properties of insulin are dose-dependent.

EFFECT OF L-NAME ON FUNCTION OF HEARTS PERFUSED WITH INSULIN

In the current study, treatment of the hearts with the nitric oxide synthase inhibitor L-NAME resulted in a rapid decrease in coronary flow as well as a rapid and reversible decrease in heart rate and left ventricular developed pressure. This indicates that nitric oxide mediates the effects of insulin on cardiac function. Insulin is known to improve cardiac function (Jonassen *et al.* 2001; Louw 2001; Van Rooyen *et al.* 2002) The present study also suggests that L-NAME, by inhibiting nitric oxide synthase (NOS), overrides the vasodilatory properties of insulin. These results are consistent with previous work where coronary infusion of NOS inhibitors in isolated rat hearts or in humans with normal cardiac function resulted in a small but significant reduction in left ventricle contractility (Kojda *et al.* 1997; Cotton *et al.* 2001)..

L-NAME is a non selective inhibitor of all three NOS isoforms. In the heart, NO is constitutively produced by vascular and endocardial endothelium, the cardiomyocytes, and the autonomic nerves (Casadei & Sears 2003). Endothelial NOS (eNOS) is responsible for vasodilatation and possesses a positive inotropic effect under normal conditions (Wunderlich *et al.* 2003). Studies have

demonstrated that basal endogenous NO is an important vasodilator and NOS inhibition could increase vascular tone and thus reduce the coronary flow (Lieberthal *et al.* 1991; Naseem *et al.* 1995). Consistent with these studies, results in the present study confirmed the impairment of cardiac function after inhibition of NOS by L-NAME. This provisory evidence provides a platform to suggest that the inotropic effect of insulin may be linked to the involvement of NO in the regulation of the vasculature in our model.

Zeng and Quon have shown that insulin induces vasodilatation via endothelium dependent mechanisms including the L-arginine nitric oxide pathway in human umbilical vein endothelial cells (Zeng & Quon 1996). They argued that in peripheral vasculature insulin rapidly and dose-dependently stimulates NO production in human endothelial cells and that insulin-induced vasodilatation can be abolished by inhibitors of eNOS. In accordance with this, administration of L-NAME together with insulin to hearts resulted in a decrease in coronary flow in the present study. This indicates that insulin may stimulate NOS activity in the heart under normoxic conditions.

Other findings of the present study were that addition of L-NAME in the insulin perfusate had a time dependence effect and that decreases in HR and in LVDevP were reversible after cessation of L-NAME administration. The CF did not return to normal values at 30 min. This led us to believe that reduction in CF was not responsible for the decreases in HR and in LVDevP of hearts that

received L-NAME. L-NAME is a non-specific inhibitor of NOS therefore, it will inhibit nNOS in the heart. The decrease in function that was observed in the present study may also implies that NO is implicated in the inotropic effects of insulin on isolated perfused rat heart since inhibition of nNOS will decrease LVDevP; and that impairments of function with NOS inhibitors may be reversible in the healthy heart.

EFFECT OF WORTMANNIN ON HEARTS PERFUSED WITH INSULIN

The addition of wortmannin to the perfusion solutions containing insulin resulted in a significant impairment of HR, LVDevP and CF. It might be possible that wortmannin could abolish the positive inotropic effect of insulin on the myocardium, and subsequently induce the loss of the protection offered by insulin due to the inhibition of the down-stream signalling pathways leading from the insulin receptor (in particular the PI-3-kinase pathway).

PKB

The metabolic action of insulin is involved in all aspects of the cell. These aspects include the increase of glucose uptake from the blood, enhanced conversion to glycogen and triacylglycerol, the inhibition of glycogen and fat breakdown, and the promotion of protein synthesis (Moule & Denton, 1997). The cardioprotective properties of insulin are well known, and the main effects consist of a reduction in infarct size when given at the onset of reperfusion (Jonassen, 2000), an improved

cardiac output recovery during reperfusion (Van Rooyen, 1998), and the attenuation of apoptosis (Jonassen, 2000).

PKB is an important mediator of the physiological effects of insulin. Administration of insulin to cells can lead to a 20- to 50-fold activation of kinase activity and phosphorylation of PKB (Hemmings, 1997).

In the present study, administration of L-NAME in the insulin perfusion solution resulted in an increase in phosphorylation of PKB/Akt [Thr³⁰⁸], immediately after L-NAME administration, more than that observed in the group that received insulin alone. Phosphorylation of PKB/Akt [Thr³⁰⁸] decreased 20 min later after the cessation of administration of L-NAME and perfusion with insulin. This may suggest an upstream crosstalk between the NO and PKB/Akt pathways. Our results are in accordance with those of Brown *et al.* (2001) where they found that high concentrations of NO led to significant increases in cGMP in aortic smooth muscle rat cells. The increase in cGMP led to an increase of Src homology 2 phosphatase 2 (SHP2) activity. However, SHP2 is a phosphatase that will dephosphorylate PKB/Akt. Therefore, inhibition of NOS with L-NAME induces the decrease in NO and this may lead to an increase in PKB/Akt phosphorylation.

Administration of wortmannin to the insulin perfusate impaired cardiac function. However no difference was observed in the content of phosphorylated PKB/Akt

[Thr³⁰⁸] in the group that received wortmannin compared to the insulin and control groups. Contrary to phosphorylated PKB/Akt [Thr³⁰⁸], a decrease in the content phosphorylated PKB/Akt [Ser⁴⁷³] in the group that received wortmannin was more pronounced; although no significant difference could be found.

Several studies indicate that PI3-K is involved in the regulation of PKB. Findings from those studies indicate that wortmannin, a specific inhibitor of PI3-K, inhibits insulin-induced activation of PKB (Franke et al., 1995; Burgering & Coffey, 1995; Kohn et al., 1995).

In agreement with the results that indicated a role for PI3-K in the activation of PKB (Franke et al., 1995; Burgering & Coffey, 1995; Kohn et al., 1995), the current study showed that wortmannin reduced the phosphorylation of PKB. Although not significant, we concluded that in healthy hearts, phosphorylation of PKB is also mediated via a PI3-K pathway like in the reperfused hearts.

Although addition of wortmannin to insulin resulted in a complete decrease in the LVDevP and in the coronary flow, it did not abolish phosphorylation of PI3-K in the present study. This indicates that the increased inotropic effects could be mediated via a pathway different from the insulin induced PI3-K activation. Previous studies have shown that PKB/Akt could be activated by increases in intracellular cAMP in a manner that is independent of PI3-K (Sable *et al.* 1997; Konishi *et al.* 1997; Filippa *et al.* 1999). Agents that could increase intracellular

levels of cAMP could activate PKB/Akt in transfected 293 cells (Sable *et al.* 1997). Filippa *et al.* (1999) demonstrated that the kinase responsible for this activation was the protein kinase A (PKA). This is not surprising, since virtually all the effects of cAMP are mediated by PKA (Filippa *et al.* 1999).

In the present study, the decrease in PI3-K activation was associated with an increase in phosphorylated PKB/Akt [Ser⁴⁷³] in hearts that received insulin + L-NAME. One would expect PKB/Akt [Ser⁴⁷³] to be decreased if PI3-K activation is decreased (Jonassen *et al.* 2001; Louw 2001, Unpublished results, MSc thesis; Jonassen *et al.* 2004). However, cAMP may also phosphorylate PKB/Akt (Sable *et al.* 1997; Konishi *et al.* 1997; Filippa *et al.* 1999). Louw (2001) found in her study that insulin increased cAMP during low flow ischaemia. She demonstrated that there was a cross signalling from the insulin cascade to production of cAMP, a messenger normally associated with β -adrenergic stimulation (Louw 2001). Therefore, we suggest that PKB/Akt may be phosphorylated by cAMP and also via the NO/cGMP pathway in our model.

Although cAMP was not measured in the present study, it is tempting to speculate that cAMP stimulation of PKA led to phosphorylation of PKB/Akt.

It has been demonstrated in previous studies that insulin increased the cAMP and cGMP content in human vascular smooth muscle cells (Trovati *et al.* 1995) and that insulin and EGF activate the adenylyl cyclase system in a bivalve mollusc (Pertseva *et al.* 1995). Based on this information, we speculate that the increase

in contractility in the group where insulin was administered alone could result not only from the direct insulin effect on LVDevP but also from the NO mediated increase in contractility via cGMP or from the β -adrenergic stimulation of cAMP that stimulates PKA and induces PKB/Akt phosphorylation. From the observed results in the present study, we also suggest that the increase in contractility in hearts that received insulin alone may also result from additive effects of all above mentioned pathways, including cAMP, NO and cGMP, which mediate the increase in contraction in hearts receiving insulin alone.

The mechanisms of insulin-induced vasodilatation are well characterized. They have been studied mainly in peripheral vasculature (Laakso *et al.* 1990; Baron 1994; Utriainen *et al.* 1995). We were unable to find any documented data on the signalling pathways of insulin on normoxic perfused isolated rat heart function or data describing the mechanisms of the effect of insulin-induced vasodilatation on the heart. To the best of our knowledge, this is the first study that set a protocol to investigate insulin pathways in normoxic perfused isolated rat hearts. Therefore, further research should be carried out in this regard in the attempt of clarifying the signalling pathways involved in insulin cardioprotection in hearts under normoxic condition.

Limitations of our study

Although this study set out to investigate the insulin pathways involved in the cardioprotection in normoxic conditions, our protocols showed some limitations.

L-NAME is an inhibitor of NOS. Therefore, inhibition of NOS leads to a decrease in NO, compared to the baseline values. The decrease in NO induces a decrease in coronary flow, thus a decrease in oxygen and glucose provision to the heart. We therefore perfused with L-NAME for 10 minutes because we speculated that NOS was sufficiently inhibited. The best way would have been to provide L-NAME throughout the protocol. Also the fact that we did not measure PI3-K and PKB/Akt constantly at several different time points might not have allowed us to draw a definitive conclusion. Phosphorylation of PI3-K and PKB/Akt may occur within the first 5 minutes after the beginning of the experiment while we investigated only the baseline state and the values of phosphorylated PI3-K and PKB/Akt at the end of our protocol.

CONCLUSIONS

The results obtained in the present study showed that a dose of 0.3 mIU/mL provided a constant positive inotropic effect in the heart. The beneficial effects of insulin were observed here by a constant increase in the left ventricular developed pressure (LVDevP). In this study, L-NAME exerted a negative effect on cardiac function by decreasing the heart rate (HR) and LVDevP as well as the coronary flow (CF). In the insulin+L-NAME group, the negative inotropic effects were reversed during reperfusion with insulin. This may indicate that nitric oxide (NO) has a role in the effect of insulin on cardiac function. The present study confirmed the detrimental effect of wortmannin on hearts since the LVDevP and the CF were decreased in the group that received wortmannin.

We furthermore conclude that, in contrast to the role that the PI3-K – PKB/Akt – p70^{S6k} pathway plays in the protective effects of insulin during ischaemia/reperfusion, activation of NOS and production of NO is the dominant signalling pathway of the effects of insulin during normoxic perfusion.

REFERENCES

1. Abel,E.D., Kaulbach,H.C., Tian,R., Hopkins,J.C., Duffy,J., Doetschman,T., Minnemann,T., Boers,M.E., Hadro,E., Oberste-Berghaus,C., Quist,W., Lowell,B.B., Ingwall,J.S., & Kahn,B.B. (1999) Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT4 from the heart. *J.Clin.Invest* 104, 1703-1714.
2. Ahmed,S.S., Lee,C.H., Oldewurtel,H.A., & Regan,T.J. (1978) Sustained effect of glucose-insulin-potassium on myocardial performance during regional ischemia. Role of free fatty acid and osmolality. *J.Clin.Invest* 61, 1123-1135.
3. Aikawa,R., Nawano,M., Gu,Y., Katagiri,H., Asano,T., Zhu,W., Nagai,R., & Komuro,I. (2000) Insulin prevents cardiomyocytes from oxidative stress-induced apoptosis through activation of PI3 kinase/Akt. *Circulation* 102, 2873-2879.
4. Alessi,D.R., Andjelkovic,M., Caudwell,B., Cron,P., Morrice,N., Cohen,P., & Hemmings,B.A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* 15, 6541-6551.
5. Alloatti,G., Levi,R., Malan,D., Del Sorbo,L., Bosco,O., Barberis,L., Marcantoni,A., Bedendi,I., Penna,C., Azzolino,O., Altruda,F., Wymann,M., Hirsch,E., & Montrucchio,G. (2003) Phosphoinositide 3-kinase gamma-deficient hearts are protected from the PAF-dependent depression of cardiac contractility. *Cardiovasc.Res.* 60, 242-249.
6. Andjelkovic,M., Maira,S.M., Cron,P., Parker,P.J., & Hemmings,B.A. (1999) Domain swapping used to investigate the mechanism of protein kinase B

regulation by 3-phosphoinositide-dependent protein kinase 1 and Ser473 kinase. *Mol.Cell Biol.* 19, 5061-5072.

7. Apstein,C.S., Gravino,F.N., & Haudenschild,C.C. (1983) Determinants of a protective effect of glucose and insulin on the ischemic myocardium. Effects on contractile function, diastolic compliance, metabolism, and ultrastructure during ischemia and reperfusion. *Circ.Res.* 52, 515-526.
8. Apstein,C.S. (1998) Glucose-insulin-potassium for acute myocardial infarction: remarkable results from a new prospective, randomized trial. *Circulation* 98, 2223-2226.
9. Arcaro,A. & Wymann,M.P. (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem.J.* 296 (Pt 2), 297-301.
10. Ashley,E.A., Sears,C.E., Bryant,S.M., Watkins,H.C., & Casadei,B. (2002) Cardiac nitric oxide synthase 1 regulates basal and beta-adrenergic contractility in murine ventricular myocytes. *Circulation* 105, 3011-3016.
11. Aulbach,F., Simm,A., Maier,S., Langenfeld,H., Walter,U., Kersting,U., & Kirstein,M. (1999) Insulin stimulates the L-type Ca²⁺ current in rat cardiac myocytes. *Cardiovasc.Res.* 42, 113-120.
12. Awan,M.M. & Saggerson,E.D. (1993) Malonyl-CoA metabolism in cardiac myocytes and its relevance to the control of fatty acid oxidation. *Biochem.J.* 295 (Pt 1), 61-66.
13. Baines,C.P., Wang,L., Cohen,M.V., & Downey,J.M. (1999) Myocardial protection by insulin is dependent on phosphatidylinositol 3-kinase but not

- protein kinase C or KATP channels in the isolated rabbit heart. *Basic Res. Cardiol.* 94, 188-198.
14. Balligand, J.L., Ungureanu, D., Kelly, R.A., Kobzik, L., Pimental, D., Michel, T., & Smith, T.W. (1993) Abnormal contractile function due to induction of nitric oxide synthesis in rat cardiac myocytes follows exposure to activated macrophage-conditioned medium. *J. Clin. Invest* 91, 2314-2319.
 15. Balligand, J.L. & Cannon, P.J. (1997) Nitric oxide synthases and cardiac muscle. Autocrine and paracrine influences. *Arterioscler. Thromb. Vasc. Biol.* 17, 1846-1858.
 16. Balligand, J.L. (1999) Regulation of cardiac beta-adrenergic response by nitric oxide. *Cardiovasc. Res.* 43, 607-620.
 17. Bandyopadhyay, G., Standaert, M.L., Zhao, L., Yu, B., Avignon, A., Galloway, L., Karnam, P., Moscat, J., & Farese, R.V. (1997) Activation of protein kinase C (alpha, beta, and zeta) by insulin in 3T3/L1 cells. Transfection studies suggest a role for PKC-zeta in glucose transport. *J. Biol. Chem.* 272, 2551-2558.
 18. Bandyopadhyay, G., Kanoh, Y., Sajan, M.P., Standaert, M.L., & Farese, R.V. (2000) Effects of adenoviral gene transfer of wild-type, constitutively active, and kinase-defective protein kinase C-lambda on insulin-stimulated glucose transport in L6 myotubes. *Endocrinology* 141, 4120-4127.
 19. Baron, A.D. (1994) Hemodynamic actions of insulin. *Am. J. Physiol* 267, E187-E202.
 20. Belhassen, L., Kelly, R.A., Smith, T.W., & Balligand, J.L. (1996) Nitric oxide synthase (NOS3) and contractile responsiveness to adrenergic and

- cholinergic agonists in the heart. Regulation of NOS3 transcription in vitro and in vivo by cyclic adenosine monophosphate in rat cardiac myocytes. *J.Clin.Invest* 97, 1908-1915.
21. Belke, D.D., Larsen, T.S., Gibbs, E.M., & Severson, D.L. (2001) Glucose metabolism in perfused mouse hearts overexpressing human GLUT-4 glucose transporter. *Am.J.Physiol Endocrinol.Metab* 280, E420-E427.
22. Bolli, R. (1991) Oxygen-derived free radicals and myocardial reperfusion injury: an overview. *Cardiovasc.Drugs Ther.* 5 Suppl 2, 249-268.
23. Borthwick, A.C., Wells, A.M., Rochford, J.J., Hurel, S.J., Turnbull, D.M., & Yeaman, S.J. (1995) Inhibition of glycogen synthase kinase-3 by insulin in cultured human skeletal muscle myoblasts. *Biochem.Biophys.Res.Commun.* 210, 738-745.
24. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.* 72, 248-254.
25. Braimbridge, M.V., Clement, A.J., Brown, A.H., Sabar, E., & Mendel, D. (1969) Triple Starr valve replacement. *Br.Med.J.* 3, 683-688.
26. Brazil, D.P. & Hemmings, B.A. (2001) Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem.Sci.* 26, 657-664.
27. Bredt, D.S. & Snyder, S.H. (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc.Natl.Acad.Sci.U.S.A* 86, 9030-9033.

28. Bredt, D.S. & Snyder, S.H. (1992) Nitric oxide, a novel neuronal messenger. *Neuron* 8, 3-11.
29. Brilla, C.G., Janicki, J.S. and Weber, K.T. (1991) Impaired diastolic function and coronary reserve in genetic hypertension. Role of interstitial fibrosis and medial thickening of intramyocardial coronary arteries. *Circ.Res.* 69 (1):107-115.
30. Brown, C., Lin, Y. and Hassid, A. (2001) Requirement of protein tyrosine phosphatase SHP2 for NO-stimulated vascular smooth muscle cell motility. *Am.J.Physiol Heart Circ.Physiol* 281 (4):H1598-H1605.
- Brownsey, R.W., Boone, A.N., & Allard, M.F. (1997) Actions of insulin on the mammalian heart: metabolism, pathology and biochemical mechanisms. *Cardiovasc.Res.* 34, 3-24.
31. Brunner, F., Andrew, P., Wolkart, G., Zechner, R., & Mayer, B. (2001) Myocardial contractile function and heart rate in mice with myocyte-specific overexpression of endothelial nitric oxide synthase. *Circulation* 104, 3097-3102.
32. Burgering, B.M. & Coffey, P.J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 376, 599-602.
33. Cantley, L.C. (2002) The phosphoinositide 3-kinase pathway. *Science* 296, 1655-1657.
34. Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S., & Reed, J.C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282, 1318-1321.

35. Casadei, B. & Sears, C.E. (2003) Nitric-oxide-mediated regulation of cardiac contractility and stretch responses. *Prog. Biophys. Mol. Biol.* 82, 67-80.
36. Charron, M.J. & Katz, E.B. (1998) Metabolic and therapeutic lessons from genetic manipulation of GLUT4. *Mol. Cell Biochem.* 182, 143-152.
37. Chesley, A., Lundberg, M.S., Asai, T., Xiao, R.P., Ohtani, S., Lakatta, E.G., & Crow, M.T. (2000) The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3'-kinase. *Circ. Res.* 87, 1172-1179.
38. Claing, A., Laporte, S.A., Caron, M.G., & Lefkowitz, R.J. (2002) Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog. Neurobiol.* 66, 61-79.
39. Coffey, P.J., Jin, J., & Woodgett, J.R. (1998) Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation 1. *Biochem. J.* 335 (Pt 1), 1-13.
40. Condorelli, G., Drusco, A., Stassi, G., Bellacosa, A., Roncarati, R., Iaccarino, G., Russo, M.A., Gu, Y., Dalton, N., Chung, C., Latronico, M.V., Napoli, C., Sadoshima, J., Croce, C.M., & Ross, J., Jr. (2002) Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. *Proc. Natl. Acad. Sci. U.S.A* 99, 12333-12338.
41. Cotton, J.M., Kearney, M.T., MacCarthy, P.A., Grocott-Mason, R.M., McClean, D.R., Heymes, C., Richardson, P.J., & Shah, A.M. (2001) Effects of nitric oxide synthase inhibition on Basal function and the force-frequency relationship in the normal and failing human heart in vivo. *Circulation* 104, 2318-2323.

42. Crackower, M.A., Oudit, G.Y., Kozieradzki, I., Sarao, R., Sun, H., Sasaki, T., Hirsch, E., Suzuki, A., Shioi, T., Irie-Sasaki, J., Sah, R., Cheng, H.Y., Rybin, V.O., Lembo, G., Fratta, L., Oliveira-dos-Santos, A.J., Benovic, J.L., Kahn, C.R., Izumo, S., Steinberg, S.F., Wymann, M.P., Backx, P.H., & Penninger, J.M. (2002) Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. *Cell* 110, 737-749.
43. Cross, D.A., Alessi, D.R., Vandenheede, J.R., McDowell, H.E., Hundal, H.S., & Cohen, P. (1994) The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem.J.* 303 (Pt 1), 21-26.
44. Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., & Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91, 231-241.
45. DeFronzo, R.A. (1981) The effect of insulin on renal sodium metabolism. A review with clinical implications. *Diabetologia* 21, 165-171.
46. Del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., & Nunez, G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 278, 687-689.
47. Depre, C., Rider, M.H., & Hue, L. (1998) Mechanisms of control of heart glycolysis.
48. Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M.J., Gout, I., Totty, N.F., Truong, O., Vicendo, P., Yonezawa, K., & . (1994) PI 3-kinase is a dual

specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. *EMBO J.* 13, 522-533.

49. Diaz,R., Paolasso,E.A., Piegas,L.S., Tajer,C.D., Moreno,M.G., Corvalan,R., Isea,J.E., & Romero,G. (1998) Metabolic modulation of acute myocardial infarction. The ECLA (Estudios Cardiológicos Latinoamerica) Collaborative Group. *Circulation* 98 , 2227-2234.
50. Doenst,T., Richwine,R.T., Bray,M.S., Goodwin,G.W., Frazier,O.H., & Taegtmeyer,H. (1999) Insulin improves functional and metabolic recovery of reperfused working rat heart. *Ann.Thorac.Surg.* 67, 1682-1688.
51. Doenst,T., Bothe,W., & Beyersdorf,F. (2003) Therapy with insulin in cardiac surgery: controversies and possible solutions. *Ann.Thorac.Surg.* 75, S721-S728.
52. Downward,J. (1998) Mechanisms and consequences of activation of protein kinase B/Akt. *Curr.Opin.Cell Biol.* 10, 262-267.
53. Du Toit,E.F., McCarthy,J., Miyashiro,J., Opie,L.H., & Brunner,F. (1998) Effect of nitrovasodilators and inhibitors of nitric oxide synthase on ischaemic and reperfusion function of rat isolated hearts. *Br.J.Pharmacol* 123, 1159-1167.
54. Dudek,H., Datta,S.R., Franke,T.F., Birnbaum,M.J., Yao,R., Cooper,G.M., Segal,R.A., Kaplan,D.R., & Greenberg,M.E. (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* 275, 661-665.
55. Edwards,E., Geng,L., Tan,J., Onishko,H., Donnelly,E., & Hallahan,D.E. (2002) Phosphatidylinositol 3-kinase/Akt signaling in the response of vascular endothelium to ionizing radiation. *Cancer Res.* 62, 4671-4677.

56. Egert, S., Nguyen, N., & Schwaiger, M. (1999) Contribution of alpha-adrenergic and beta-adrenergic stimulation to ischemia-induced glucose transporter (GLUT) 4 and GLUT1 translocation in the isolated perfused rat heart. *Circ.Res.* 84, 1407-1415.
57. Elfering, S.L., Sarkela, T.M., & Giulivi, C. (2002) Biochemistry of mitochondrial nitric-oxide synthase. *J.Biol.Chem.* 277, 38079-38086.
58. Fath-Ordoubadi, F. & Beatt, K.J. (1997) Glucose-insulin-potassium therapy for treatment of acute myocardial infarction: an overview of randomized placebo-controlled trials. *Circulation* 96, 1152-1156.
59. Filippa, N., Sable, C.L., Filloux, C., Hemmings, B., & Van Obberghen, E. (1999) Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase. *Mol.Cell Biol.* 19, 4989-5000.
60. Fischer-Rasokat, U. & Doenst, T. (2003) Insulin-induced improvement of postischemic recovery is abolished by inhibition of protein kinase C in rat heart. *J.Thorac.Cardiovasc.Surg.* 126, 1806-1812.
61. Flaim, K.E., Kochel, P.J., Kira, Y., Kobayashi, K., Fossel, E.T., Jefferson, L.S., & Morgan, H.E. (1983) Insulin effects on protein synthesis are independent of glucose and energy metabolism. *Am.J.Physiol* 245, C133-C143.
62. Franke, T.F., Yang, S.I., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R., & Tsichlis, P.N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81, 727-736.
63. Franke, T.F., Kaplan, D.R., & Cantley, L.C. (1997) PI3K: downstream AKTion blocks apoptosis. *Cell* 88, 435-437.

64. Fujio, Y., Guo, K., Mano, T., Mitsuuchi, Y., Testa, J.R., & Walsh, K. (1999) Cell cycle withdrawal promotes myogenic induction of Akt, a positive modulator of myocyte survival. *Mol. Cell Biol.* 19, 5073-5082.
65. Gao, F., Gao, E., Yue, T.L., Ohlstein, E.H., Lopez, B.L., Christopher, T.A., & Ma, X.L. (2002) Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. *Circulation* 105, 1497-1502.
66. Garthwaite, J., Charles, S.L., & Chess-Williams, R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 336, 385-388.
67. Garthwaite, J. (1991) Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci.* 14, 60-67.
68. Hafezi-Moghadam, A., Simoncini, T., Yang, Z., Limbourg, F.P., Plumier, J.C., Rebsamen, M.C., Hsieh, C.M., Chui, D.S., Thomas, K.L., Prorock, A.J., Laubach, V.E., Moskowitz, M.A., French, B.A., Ley, K., & Liao, J.K. (2002) Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nat. Med.* 8, 473-479.
69. Hemmings, B.A. (1997) Akt signaling: linking membrane events to life and death decisions. *Science* 275, 628-630.
70. Heusch, G., Post, H., Michel, M.C., Kelm, M., & Schulz, R. (2000) Endogenous nitric oxide and myocardial adaptation to ischemia. *Circ. Res.* 87, 146-152.

71. Hill, M.M., Clark, S.F., Tucker, D.F., Birnbaum, M.J., James, D.E., & Macaulay, S.L. (1999) A role for protein kinase Bbeta/Akt2 in insulin-stimulated GLUT4 translocation in adipocytes. *Mol. Cell Biol.* 19, 7771-7781.
72. Hue, L., Beauloye, C., Marsin, A.S., Bertrand, L., Horman, S., & Rider, M.H. (2002) Insulin and ischemia stimulate glycolysis by acting on the same targets through different and opposing signaling pathways. *J. Mol. Cell Cardiol.* 34, 1091-1097.
73. Huisamen, B., van Zyl, M., Keyser, A., & Lochner, A. (2001) The effects of insulin and beta-adrenergic stimulation on glucose transport, GLUT4 and PKB activation in the myocardium of lean and obese non-insulin dependent diabetes mellitus rats. *Mol. Cell Biochem.* 223, 15-25.
74. Hurel, S.J., Rochford, J.J., Borthwick, A.C., Wells, A.M., Vandenhede, J.R., Turnbull, D.M., & Yeaman, S.J. (1996) Insulin action in cultured human myoblasts: contribution of different signalling pathways to regulation of glycogen synthesis. *Biochem. J.* 320 (Pt 3), 871-877.
75. Ignarro, L.J. (1990) Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu. Rev. Pharmacol. Toxicol.* 30, 535-560.
76. Jagasia, D., Whiting, J.M., Concato, J., Pfau, S., & McNulty, P.H. (2001) Effect of non-insulin-dependent diabetes mellitus on myocardial insulin responsiveness in patients with ischemic heart disease. *Circulation* 103, 1734-1739.
77. Ji, G.J., Fleischmann, B.K., Bloch, W., Feelisch, M., Andressen, C., Addicks, K., & Hescheler, J. (1999) Regulation of the L-type Ca²⁺ channel during cardiomyogenesis: switch from NO to adenylyl cyclase-mediated inhibition. *FASEB J.* 13, 313-324.

78. Jo, S.H., Leblais, V., Wang, P.H., Crow, M.T., & Xiao, R.P. (2002) Phosphatidylinositol 3-kinase functionally compartmentalizes the concurrent G(s) signaling during beta2-adrenergic stimulation. *Circ.Res.* 91, 46-53.
79. Johnson, L.R. (1988) Regulation of gastrointestinal mucosal growth. *Physiol Rev.* 68, 456-502.
80. Jonassen, A.K., Aasum, E., Riemersma, R.A., Mjos, O.D., & Larsen, T.S. (2000) Glucose-insulin-potassium reduces infarct size when administered during reperfusion. *Cardiovasc. Drugs Ther.* 14, 615-623.
81. Jonassen, A.K., Brar, B.K., Mjos, O.D., Sack, M.N., Latchman, D.S., & Yellon, D.M. (2000) Insulin administered at reoxygenation exerts a cardioprotective effect in myocytes by a possible anti-apoptotic mechanism. *J.Mol.Cell Cardiol.* 32, 757-764.
82. Jonassen, A.K., Sack, M.N., Mjos, O.D., & Yellon, D.M. (2001) Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ.Res.* 89, 1191-1198.
83. Jonassen, A.K., Mjos, O.D., & Sack, M.N. (2004) p70s6 kinase is a functional target of insulin activated Akt cell-survival signaling. *Biochem.Biophys.Res.Commun.* 315, 160-165.
84. Jones, P.F., Jakubowicz, T., Pitossi, F.J., Maurer, F., & Hemmings, B.A. (1991) Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc.Natl.Acad.Sci.U.S.A* 88, 4171-4175.

85. Jung,F., Palmer,L.A., Zhou,N., & Johns,R.A. (2000) Hypoxic regulation of inducible nitric oxide synthase via hypoxia inducible factor-1 in cardiac myocytes. *Circ.Res.* 86, 319-325.
86. Kearney,M.T., Cowley,A.J., Stubbs,T.A., Evans,A., & Macdonald,I.A. (1998) Depressor action of insulin on skeletal muscle vasculature: a novel mechanism for postprandial hypotension in the elderly. *J.Am.Coll.Cardiol.* 31, 209-216.
87. Khan,S.A., Skaf,M.W., Harrison,R.W., Lee,K., Minhas,K.M., Kumar,A., Fradley,M., Shoukas,A.A., Berkowitz,D.E., & Hare,J.M. (2003) Nitric oxide regulation of myocardial contractility and calcium cycling: independent impact of neuronal and endothelial nitric oxide synthases. *Circ.Res.* 92, 1322-1329.
88. King,L.M. & Opie,L.H. (1998) Glucose and glycogen utilisation in myocardial ischemia--changes in metabolism and consequences for the myocyte. *Mol.Cell Biochem.* 180, 3-26.
89. Kitamura,T., Ogawa,W., Sakaue,H., Hino,Y., Kuroda,S., Takata,M., Matsumoto,M., Maeda,T., Konishi,H., Kikkawa,U., & Kasuga,M. (1998) Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Mol.Cell Biol.* 18, 3708-3717.
90. Koch,W.J., Lefkowitz,R.J., & Rockman,H.A. (2000) Functional consequences of altering myocardial adrenergic receptor signaling. *Annu.Rev.Physiol* 62, 237-260.
91. Kohn, A. D. K., K.S.; Roth,R.A. (1995). "Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing ser/thr kinase." *EMBO J.* 14(17): 4288-4295.

92. Kohn, A.D., Summers, S.A., Birnbaum, M.J., & Roth, R.A. (1996) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J.Biol.Chem.* 271, 31372-31378.
93. Kojda, G., Kottenberg, K., & Noack, E. (1997) Inhibition of nitric oxide synthase and soluble guanylate cyclase induces cardiodepressive effects in normal rat hearts. *Eur.J.Pharmacol.* 334, 181-190.
94. Kolter, T., Uphues, I., Wichelhaus, A., Reinauer, H., & Eckel, J. (1992) Contraction-induced translocation of the glucose transporter Glut4 in isolated ventricular cardiomyocytes. *Biochem.Biophys.Res.Commun.* 189, 1207-1214.
95. Konishi, H., Matsuzaki, H., Tanaka, M., Takemura, Y., Kuroda, S., Ono, Y., & Kikkawa, U. (1997) Activation of protein kinase B (Akt/RAC-protein kinase) by cellular stress and its association with heat shock protein Hsp27. *FEBS Lett.* 410, 493-498.
96. Koss, K.L. & Kranias, E.G. (1996) Phospholamban: a prominent regulator of myocardial contractility. *Circ.Res.* 79, 1059-1063.
97. Laakso, M., Edelman, S.V., Brechtel, G., & Baron, A.D. (1990) Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man. A novel mechanism for insulin resistance. *J.Clin.Invest* 85, 1844-1852.
98. Laughlin, M.R., Taylor, J.F., Chesnick, A.S., & Balaban, R.S. (1992) Regulation of glycogen metabolism in canine myocardium: effects of insulin and epinephrine in vivo. *Am.J.Physiol* 262, E875-E883.

99. Lavan, B.E., Fantin, V.R., Chang, E.T., Lane, W.S., Keller, S.R., & Lienhard, G.E. (1997a) A novel 160-kDa phosphotyrosine protein in insulin-treated embryonic kidney cells is a new member of the insulin receptor substrate family. *J. Biol. Chem.* 272, 21403-21407.
100. Lavan, B.E., Lane, W.S., & Lienhard, G.E. (1997b) The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J. Biol. Chem.* 272, 11439-11443.
101. Lawrence, J.C., Jr. (1992) Signal transduction and protein phosphorylation in the regulation of cellular metabolism by insulin. *Annu. Rev. Physiol.* 54, 177-193.
102. Legtenberg, R.J., Houston, R.J., Oeseburg, B., & Smits, P. (2002) Physiological insulin concentrations protect against ischemia-induced loss of cardiac function in rats. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 132, 161-167.
103. Lieberthal, W., McGarry, A.E., Sheils, J., & Valeri, C.R. (1991) Nitric oxide inhibition in rats improves blood pressure and renal function during hypovolemic shock. *Am. J. Physiol.* 261, F868-F872.
104. Louw, R. (2001) The signaling pathways involved in the cardioprotection offered by insulin to the global low flow ischaemic/reperfused myocardium. MSc thesis. Physiological Sciences. Stellenbosch, University of Stellenbosch.
105. Malmberg, K., Ryden, L., Efendic, S., Herlitz, J., Nicol, P., Waldenstrom, A., Wedel, H., & Welin, L. (1995) Randomized trial of insulin-glucose infusion followed by subcutaneous insulin treatment in diabetic patients with acute

myocardial infarction (DIGAMI study): effects on mortality at 1 year. *J.Am.Coll.Cardiol.* 26, 57-65.

106. Maroko,P.R., Libby,P., Sobel,B.E., Bloor,C.M., Sybers,H.D., Shell,W.E., Covell,J.W., & Braunwald,E. (1972) Effect of glucose-insulin-potassium infusion on myocardial infarction following experimental coronary artery occlusion. *Circulation* 45, 1160-1175.
107. Massion,P.B., Feron,O., Dessy,C., & Balligand,J.L. (2003) Nitric oxide and cardiac function: ten years after, and continuing. *Circ.Res.* 93, 388-398.
108. Mather,K., Anderson,T.J., & Verma,S. (2001) Insulin action in the vasculature: physiology and pathophysiology. *J.Vasc.Res.* 38, 415-422.
109. Matsui,T., Li,L., del,M., Fukui,Y., Franke,T.F., Hajjar,R.J., & Rosenzweig,A. (1999) Adenoviral gene transfer of activated phosphatidylinositol 3'-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. *Circulation* 100, 2373-2379.
110. Matsui,T., Tao,J., del Monte,F., Lee,K.H., Li,L., Picard,M., Force,T.L., Franke,T.F., Hajjar,R.J., & Rosenzweig,A. (2001) Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation* 104, 330-335.
111. McClellan,G., Weisberg,A., Lin,L.E., Rose,D., Ramaciotti,C., & Winegrad,S. (1993) Endothelial cells are required for the cAMP regulation of cardiac contractile proteins. *Proc.Natl.Acad.Sci.U.S.A* 90, 2885-2889.
112. Medical Research Council Working Party on the Treatment of Myocardial Infarction (1968) Potassium, glucose, and insulin treatment for acute myocardial infarction. *Lancet* 2, 1355-1360.

113. Meier,R., Alessi,D.R., Cron,P., Andjelkovic,M., & Hemmings,B.A. (1997) Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bbeta. *J.Biol.Chem.* 272, 30491-30497.
114. Mockridge,J.W., Punn,A., Latchman,D.S., Marber,M.S., & Heads,R.J. (2000) PKC-dependent delayed metabolic preconditioning is independent of transient MAPK activation. *Am.J.Physiol Heart Circ.Physiol* 279, H492-H501.
115. Moncada,S., Palmer,R.M., & Higgs,E.A. (1989) Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem.Pharmacol.* 38, 1709-1715.
116. Moniotte,S., Kobzik,L., Feron,O., Trochu,J.N., Gauthier,C., & Balligand,J.L. (2001) Upregulation of beta(3)-adrenoceptors and altered contractile response to inotropic amines in human failing myocardium. *Circulation* 103, 1649-1655.
117. Moule,S.K. & Denton,R.M. (1997) Multiple signaling pathways involved in the metabolic effects of insulin. *Am.J.Cardiol.* 80, 41A-49A.
118. Naga Prasad,S.V., Esposito,G., Mao,L., Koch,W.J., & Rockman,H.A. (2000) Gbetagamma-dependent phosphoinositide 3-kinase activation in hearts with in vivo pressure overload hypertrophy. *J.Biol.Chem.* 275, 4693-4698.
119. Naga Prasad,S.V., Laporte,S.A., Chamberlain,D., Caron,M.G., Barak,L., & Rockman,H.A. (2002) Phosphoinositide 3-kinase regulates beta2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/beta-arrestin complex. *J.Cell Biol.* 158, 563-575.

120. Naseem,S.A., Kontos,M.C., Rao,P.S., Jesse,R.L., Hess,M.L., & Kukreja,R.C. (1995) Sustained inhibition of nitric oxide by NG-nitro-L-arginine improves myocardial function following ischemia/reperfusion in isolated perfused rat heart. *J.Mol.Cell Cardiol.* 27, 419-426.
121. Neely,J.R. & Grotyohann,L.W. (1984) Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. *Circ.Res.* 55, 816-824.
122. Negoro,S., Oh,H., Tone,E., Kunisada,K., Fujio,Y., Walsh,K., Kishimoto,T., & Yamauchi-Takahara,K. (2001) Glycoprotein 130 regulates cardiac myocyte survival in doxorubicin-induced apoptosis through phosphatidylinositol 3-kinase/Akt phosphorylation and Bcl-xL/caspase-3 interaction. *Circulation* 103, 555-561.
123. Nicholson,K.M. & Anderson,N.G. (2002) The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal.* 14, 381-395.
124. Okada,D. (1992) Two pathways of cyclic GMP production through glutamate receptor-mediated nitric oxide synthesis. *J.Neurochem.* 59, 1203-1210.
125. Oliver,M.F. & Opie,L.H. (1994) Effects of glucose and fatty acids on myocardial ischaemia and arrhythmias. *Lancet* 343, 155-158.
126. Oltman,C.L., Kane,N.L., Gutterman,D.D., Bar,R.S., & Dellsperger,K.C. (2000) Mechanism of coronary vasodilation to insulin and insulin-like growth factor I is dependent on vessel size. *Am.J.Physiol Endocrinol.Metab* 279, E176-E181.

127. Onoda,T., Iinuma,H., Sasaki,Y., Hamada,M., Isshiki,K., Naganawa,H., Takeuchi,T., Tatsuta,K., & Umezawa,K. (1989) Isolation of a novel tyrosine kinase inhibitor, lavendustin A, from *Streptomyces griseolavendus*. *J.Nat.Prod.* 52, 1252-1257.
128. Opie L.H (1998) *The heart: Physiology, from cell to circulation*, 3rd Edition, Philadelphia. Lippincott-Raven.
129. Opie,L.H. & Owen,P. (1976) Effect of glucose-insulin-potassium infusions on arteriovenous differences of glucose of free fatty acids and on tissue metabolic changes in dogs with developing myocardial infarction. *Am.J.Cardiol.* 38, 310-321.
130. Patti,M.E. & Kahn,C.R. (1998) The insulin receptor--a critical link in glucose homeostasis and insulin action. *J.Basic Clin.Physiol Pharmacol.* 9, 89-109.
131. Perry,S.J. & Lefkowitz,R.J. (2002) Arresting developments in heptahelical receptor signaling and regulation. *Trends Cell Biol.* 12, 130-138.
132. Pertseva,M.N., Plesneva,S.A., Shpakov,A.O., Rusakov,Y., & Kuznetsova,L.A. (1995) Involvement of the adenylyl cyclase signaling system in the action of insulin and mollusk insulin-like peptide. *Comp Biochem.Physiol B Biochem.Mol.Biol.* 112, 689-695.
133. Pitcher,J.A., Hall,R.A., Daaka,Y., Zhang,J., Ferguson,S.S., Hester,S., Miller,S., Caron,M.G., Lefkowitz,R.J., & Barak,L.S. (1998) The G protein-coupled receptor kinase 2 is a microtubule-associated protein kinase that phosphorylates tubulin. *J.Biol.Chem.* 273, 12316-12324.

134. Prasad,S.V., Perrino,C., & Rockman,H.A. (2003) Role of phosphoinositide 3-kinase in cardiac function and heart failure. *Trends Cardiovasc.Med.* 13, 206-212.
135. Prendergast,B.D., Sagach,V.F., & Shah,A.M. (1997) Basal release of nitric oxide augments the Frank-Starling response in the isolated heart. *Circulation* 96, 1320-1329.
136. Puybasset,L., Bea,M.L., Ghaleh,B., Giudicelli,J.F., & Berdeaux,A. (1996) Coronary and systemic hemodynamic effects of sustained inhibition of nitric oxide synthesis in conscious dogs. Evidence for cross talk between nitric oxide and cyclooxygenase in coronary vessels. *Circ.Res.* 79, 343-357.
137. Raitakari,M., Knuuti,M.J., Ruotsalainen,U., Laine,H., Makea,P., Teras,M., Sipila,H., Niskanen,T., Raitakari,O.T., Iida,H., & . (1995) Insulin increases blood volume in human skeletal muscle: studies using [15O]CO and positron emission tomography. *Am.J.Physiol* 269, E1000-E1005.
138. Recchia,F.A., Osorio,J.C., Chandler,M.P., Xu,X., Panchal,A.R., Lopaschuk,G.D., Hintze,T.H., & Stanley,W.C. (2002) Reduced synthesis of NO causes marked alterations in myocardial substrate metabolism in conscious dogs. *Am.J.Physiol Endocrinol.Metab* 282, E197-E206.
139. Reif,K., Burgering,B.M., & Cantrell,D.A. (1997) Phosphatidylinositol 3-kinase links the interleukin-2 receptor to protein kinase B and p70 S6 kinase. *J.Biol.Chem.* 272, 14426-14433.
140. Reimer,K.A., Vander Heide,R.S., & Richard,V.J. (1993) Reperfusion in acute myocardial infarction: effect of timing and modulating factors in experimental models. *Am.J.Cardiol.* 72, 13G-21G.

141. Rockman,H.A., Koch,W.J., & Lefkowitz,R.J. (2002) Seven-transmembrane-spanning receptors and heart function. *Nature* 415, 206-212.
142. Rosenzweig,J.L., Havrankova,J., Lesniak,M.A., Brownstein,M., & Roth,J. (1980) Insulin is ubiquitous in extrapancreatic tissues of rats and humans. *Proc.Natl.Acad.Sci.U.S.A* 77, 572-576.
143. Sable,C.L., Filippa,N., Hemmings,B., & Van Obberghen,E. (1997) cAMP stimulates protein kinase B in a Wortmannin-insensitive manner. *FEBS Lett.* 409, 253-257.
144. Scheid,M.P. & Woodgett,J.R. (2001) PKB/AKT: functional insights from genetic models. *Nat.Rev.Mol.Cell Biol.* 2, 760-768.
145. Scheid,M.P., Huber,M., Damen,J.E., Hughes,M., Kang,V., Neilsen,P., Prestwich,G.D., Krystal,G., & Duronio,V. (2002) Phosphatidylinositol (3,4,5)P3 is essential but not sufficient for protein kinase B (PKB) activation; phosphatidylinositol (3,4)P2 is required for PKB phosphorylation at Ser-473: studies using cells from SH2-containing inositol-5-phosphatase knockout mice. *J.Biol.Chem.* 277, 9027-9035.
146. Sears,C.E., Bryant,S.M., Ashley,E.A., Lygate,C.A., Rakovic,S., Wallis,H.L., Neubauer,S., Terrar,D.A., & Casadei,B. (2003) Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. *Circ.Res.* 92, e52-e59.
147. Seger,R. & Krebs,E.G. (1995) The MAPK signaling cascade. *FASEB J.* 9, 726-735.

148. Shepherd,P.R., Nave,B.T., & O'rahilly,S. (1996) The role of phosphoinositide 3-kinase in insulin signalling. *J.Mol.Endocrinol.* 17, 175-184.
149. Shioi,T., McMullen,J.R., Kang,P.M., Douglas,P.S., Obata,T., Franke,T.F., Cantley,L.C., & Izumo,S. (2002) Akt/protein kinase B promotes organ growth in transgenic mice. *Mol.Cell Biol.* 22, 2799-2809.
150. Sidossis,L.S., Stuart,C.A., Shulman,G.I., Lopaschuk,G.D., & Wolfe,R.R. (1996) Glucose plus insulin regulate fat oxidation by controlling the rate of fatty acid entry into the mitochondria. *J.Clin.Invest* 98, 2244-2250.
151. Slot,J.W., Geuze,H.J., Gigengack,S., James,D.E., & Lienhard,G.E. (1991) Translocation of the glucose transporter GLUT4 in cardiac myocytes of the rat. *Proc.Natl.Acad.Sci.U.S.A* 88, 7815-7819.
152. Sodi-Pallares,D., Testelli,M.R., Fishleder,B.L., Bisteni,A., Medrano,G.A., Friedland,C., & De Micheli,A. (1962) Effects of an intravenous infusion of a potassium-glucose-insulin solution on the electrocardiographic signs of myocardial infarction. A preliminary clinical report. *Am.J.Cardiol.* 9, 166-181.
153. Srivastava,A.K. (1998) Use of pharmacological agents in elucidating the mechanism of insulin action. *Trends Pharmacol.Sci.* 19, 205-209.
154. Stanley,W.C., Hall,J.L., Hacker,T.A., Hernandez,L.A., & Whitesell,L.F. (1997) Decreased myocardial glucose uptake during ischemia in diabetic swine. *Metabolism* 46, 168-172.
155. Stool,S.E., Eavey,R.D., Stein,N.L., & Sharrar,W.G. (1977) The "chubby puffer" syndrome. Upper airway obstruction and obesity, with intermittent

somnolence and cardiorespiratory embarrassment. *Clin.Pediatr.(Phila)* 16, 43-50

156. Suhara,T., Magrane,J., Rosen,K., Christensen,R., Kim,H.S., Zheng,B., McPhie,D.L., Walsh,K., & Querfurth,H. (2003) Abeta42 generation is toxic to endothelial cells and inhibits eNOS function through an Akt/GSK-3beta signaling-dependent mechanism. *Neurobiol.Aging* 24, 437-451.
157. Sun,X.J., Rothenberg,P., Kahn,C.R., Backer,J.M., Araki,E., Wilden,P.A., Cahill,D.A., Goldstein,B.J., & White,M.F. (1991) Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* 352, 73-77.
158. Sun,X.J., Wang,L.M., Zhang,Y., Yenush,L., Myers,M.G., Jr., Glasheen,E., Lane,W.S., Pierce,J.H., & White,M.F. (1995) Role of IRS-2 in insulin and cytokine signalling. *Nature* 377, 173-177.
159. Sundell,J., Nuutila,P., Laine,H., Luotolahti,M., Kalliokoski,K., Raitakari,O., & Knuuti,J. (2002) Dose-dependent vasodilating effects of insulin on adenosine-stimulated myocardial blood flow. *Diabetes* 51, 1125-1130
160. Sundell,J. & Knuuti,J. (2003) Insulin and myocardial blood flow. *Cardiovasc.Res.* 57, 312-319.
161. Sutherland,C. & Cohen,P. (1994) The alpha-isoform of glycogen synthase kinase-3 from rabbit skeletal muscle is inactivated by p70 S6 kinase or MAP kinase-activated protein kinase-1 in vitro. *FEBS Lett.* 338, 37-42.
162. Taegtmeyer,H. & Villalobos,D.H. (1995) Metabolic support for the postischaemic heart. *Lancet* 345, 1552-1555.

163. Takata,M., Ogawa,W., Kitamura,T., Hino,Y., Kuroda,S., Kotani,K., Klip,A., Gingras,A.C., Sonenberg,N., & Kasuga,M. (1999) Requirement for Akt (protein kinase B) in insulin-induced activation of glycogen synthase and phosphorylation of 4E-BP1 (PHAS-1). *J.Biol.Chem.* 274, 20611-20618.
164. Tennant R & Wiggers CJ (1935) Effect of coronary occlusion on myocardial contraction. *Am.J.Physiol* 112, 351-361.
165. Tomai,F., Crea,F., Chiariello,L., & Gioffre,P.A. (1999) Ischemic preconditioning in humans: models, mediators, and clinical relevance. *Circulation* 100, 559-563.
166. Trovati,M., Massucco,P., Mattiello,L., Cavalot,F., Mularoni,E., Hahn,A., & Anfossi,G. (1995) Insulin increases cyclic nucleotide content in human vascular smooth muscle cells: a mechanism potentially involved in insulin-induced modulation of vascular tone. *Diabetologia* 38, 936-941.
167. Uphues,I., Kolter,T., Goud,B., & Eckel,J. (1994) Insulin-induced translocation of the glucose transporter GLUT4 in cardiac muscle: studies on the role of small-molecular-mass GTP-binding proteins. *Biochem.J.* 301 (Pt 1), 177-182.
168. Utriainen,T., Malmstrom,R., Makimattila,S., & Yki-Jarvinen,H. (1995) Methodological aspects, dose-response characteristics and causes of interindividual variation in insulin stimulation of limb blood flow in normal subjects. *Diabetologia* 38, 555-564.
169. Van Rooyen,J., McCarthy,J., & Opie,L.H. (2002) Increased glycolysis during ischaemia mediates the protective effect of glucose and insulin in the isolated rat heart despite the presence of cardiodepressant exogenous substrates. *Cardiovasc.J.S.Afr.* 13, 103-109.

170. Vanhaesebroeck,B., Leever,S.J., Ahmadi,K., Timms,J., Katso,R., Driscoll,P.C., Woscholski,R., Parker,P.J., & Waterfield,M.D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu.Rev.Biochem.* 70, 535-602.
171. Vila-Petroff,M.G., Younes,A., Egan,J., Lakatta,E.G., & Sollott,S.J. (1999) Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ.Res.* 84, 1020-1031.
172. Wang,Q., Wang,X., Hernandez,A., Hellmich,M.R., Gatalica,Z., & Evers,B.M. (2002) Regulation of TRAIL expression by the phosphatidylinositol 3-kinase/Akt/GSK-3 pathway in human colon cancer cells. *J.Biol.Chem.* 277, 36602-36610.
173. Watanabe,T., Smith,M.M., Robinson,F.W., & Kono,T. (1984) Insulin action on glucose transport in cardiac muscle. *J.Biol.Chem.* 259, 13117-13122.
174. Welsh,G.I. & Proud,C.G. (1993) Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochem.J.* 294 (Pt 3), 625-629.
175. Westerbacka,J., Seppala-Lindroos,A., & Yki-Jarvinen,H. (2001) Resistance to acute insulin induced decreases in large artery stiffness accompanies the insulin resistance syndrome. *J.Clin.Endocrinol.Metab* 86, 5262-5268.
176. White,M.F. & Kahn,C.R. (1994) The insulin signaling system. *J.Biol.Chem.* 269, 1-4.
177. Wijkander,J., Landstrom,T.R., Manganiello,V., Belfrage,P., & Degerman,E. (1998) Insulin-induced phosphorylation and activation of

phosphodiesterase 3B in rat adipocytes: possible role for protein kinase B but not mitogen-activated protein kinase or p70 S6 kinase. *Endocrinology* 139, 219-227.

178. Wunderlich,C., Flogel,U., Godecke,A., Heger,J., & Schrader,J. (2003) Acute inhibition of myoglobin impairs contractility and energy state of iNOS-overexpressing hearts. *Circ.Res.* 92, 1352-1358.
179. Xiao,R.P., Cheng,H., Zhou,Y.Y., Kuschel,M., & Lakatta,E.G. (1999) Recent advances in cardiac beta(2)-adrenergic signal transduction. *Circ.Res.* 85, 1092-1100.
180. Yamada,K. & Nabeshima,T. (1997a) Simultaneous measurement of nitrite and nitrate levels as indices of nitric oxide release in the cerebellum of conscious rats. *J.Neurochem.* 68, 1234-1243.
181. Yamada,K. & Nabeshima,T. (1997b) Two pathways of nitric oxide production through glutamate receptors in the rat cerebellum in vivo. *Neurosci.Res.* 28, 93-102.
182. Yamashita,K., Kajstura,J., Discher,D.J., Wasserlauf,B.J., Bishopric,N.H., Anversa,P., & Webster,K.A. (2001) Reperfusion-activated Akt kinase prevents apoptosis in transgenic mouse hearts overexpressing insulin-like growth factor-1. *Circ.Res.* 88, 609-614.
183. Zeng,G. & Quon,M.J. (1996) Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. *J.Clin.Invest* 98, 894-898.
184. Ziolo,M.T., Kato,H., & Bers,D.M. (2001) Expression of inducible nitric oxide synthase depresses beta-adrenergic-stimulated calcium release from

the sarcoplasmic reticulum in intact ventricular myocytes. *Circulation* 104, 2961-2966

185. Ziolo, M.T. & Bers, D.M. (2003) The real estate of NOS signaling: location, location, location. *Circ.Res.* 92, 1279-1281.

186. Zou, L.B., Yamada, K., Tanaka, T., Kameyama, T., & Nabeshima, T. (1998) Nitric oxide synthase inhibitors impair reference memory formation in a radial arm maze task in rats. *Neuropharmacology* 37, 323-330.

APPENDIX 1

SALT BUFFERS

PERFUSION BUFFER: KREBS HENSELEIT

Reagents	Concentrations
NaCl	119 mM
NaHCO ₃	25 mM
KH ₂ PO ₄	1.2 mM
KCl	4.7 mM
MgSO ₄ -7H ₂ O	0.59 mM
CaCl ₂ -2H ₂ O	1.25 mM
Glucose	5 mM

PROTEINS SEPARATION BUFFERS

➤ LYSIS BUFFER

Reagents	Concentrations
Tris-Base	20 mM
pNPP	20 mM
NaF	50 mM
DTT	1 mM
EGTA	1 mM
PMSF	1 mM

NaVO ₄	0.1 mM
Aprotinin	10 μg/ml
Leupeptin	10μg/ml

➤ **LAEMMLI (SAMPLE BUFFER)**

Reagents	Concentrations
Tris	0.5M
SDS	0.4%
Glycerol	
Bromo phenol Blue	
β-mercaptoethanol	

➤ **RUNNING BUFFER**

Reagents	Concentrations
Tris	50mM
Glycine	384mM
SDS	10%

➤ **BLOTTING BUFFER**

Reagents	Concentrations
Tris	25mM
Glycine	192mM
Methanol	20%

GELS BUFFERS

Mini-gel system

Reagents	Concentrations
Tris-HCl (pH 8.8)	1.5M
Tris-HCl (pH 6.6)	0.5M
SDS	10%
Acrylamide	40%
APS	10%
Temed	

APPENDIX 2

ABBREVIATION

β-AR	Beta-adrenergic receptor
β-ARK1	Beta-adrenergic receptor kinase-1
β-FGF	Beta fibroblast growth factor
A	Agonist
AC	Adenyl cyclase
ADP	Adenosine di-phosphate
AMI	Acute myocardial infarction
ANOVA	Analysis of variance
AP-2	Adaptor protein-2
aPKC	Atypical protein kinase C
APS	Ammonium persulfate
AR	Adreno-receptor
ATP	Adenosine tri-phosphate
cAMP	Cyclic adenosine monophosphate
CF	Coronary flow
cGMP	Cyclic guanosine monophosphate
DP	Diastolic pressure
DTT	dithiotreitol
EC	Excitation–contraction
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-related kinases

FFA	Free fatty acids
GF	Growth factor
GIK	Glucose-insulin-potassium
Gluc	Glucose
GLUT-1	Glucose transporter 1
GLUT-4	Glucose transporter 4
GPCR	G protein coupled receptor
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase-3
HBGF	Heparin binding growth factor
HR	Heart rate
hsp90	Heat-shock protein (90kDa)
IGF-1	Insulin-like growth factor 1
IGF-IR	Insulin-like growth factor 1-insulin receptor
iNOS	Inducible nitric oxide synthase
Ins	Insulin
IR	Insulin receptor
IRS-1/2	Insulin receptor substrate-1/2
JNK	c-Jun N-terminal protein kinase
KH	Krebs-Henseleit
L-NAME	N ^o - nitro - L -arginine-methyl ester
LNM	L-NAME
LPS	Lipopolysaccharide

LVDevP	Left ventricular developed pressure
MAP	Mitogen activated kinase
mTOR	Mammalian target of Rapamycin
NADPH (reduced form)	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
NaVO₂	Sodium orthovanadate
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
p38 MAP	p38 mitogen activated protein
PCD	Programmed cell death
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PDK-1/2	Phosphoinositide-dependent protein kinase-1/2
PFK2	Phosphofructokinase-1/2
PH	Pleckstrin homology
PI3-K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase

PLB	Phospholamban
PMSF	phenylmethyl sulphonyl fluoride
pNPP	p-nitrophenylphosphate
PP-1	Protein phosphatase-1
PtdIns	Phosphatidylinositols
PVDF	Polyvinylidenedifluoride
RyR2	Ryanodine receptor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide electrophoresis
SEM	Standard error of the mean
SERCA	Sarco(endoplasmic reticulum Ca ²⁺ ATPase
SHP2	Src homology 2 phosphatase 2
SP	Systolic pressure
SR	Sarcoplasmic reticulum
Wort	Wortmannin

APPENDIX 3

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