THE IMPACT OF ACTIVATION OF THE RENIN-ANGIOTENSIN SYSTEM ON
INSULIN RESISTANCE AND NITRIC OXIDE PRODUCTION IN EXPERIMENTAL
MODELS OF OBESITY

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PROMOTER: PROF. BARBARA HUISAMEN
DECLARATION

I, the undersigned, hereby declare that the work contained 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: __________________________ Date: 28 November 2008.
Insulin stimulates the production of nitric oxide (NO) in endothelial cells and cardiac myocytes by a signalling pathway that involves the insulin receptor substrate (IRS)-1, phosphatidylinositol-3-kinase and protein kinase B (PKB/Akt). Physiological concentrations of NO play an important part in maintaining normal vascular function. It has been suggested that nitric oxide synthase (NOS) activity and NO production are chronically impaired in diabetes mellitus by an unknown mechanism. The renin-angiotensin system and subsequent production of angiotensin II (Ang II) are elevated in obesity and diabetes while antagonism of the AT1 receptor with Losartan has beneficial effects in patients with insulin resistance and type II diabetes. **Aims:** We therefore aimed to investigate (i) the effect of Ang II on myocardial insulin signalling with regards to key proteins (IRS-1, PKB/Akt, eNOS and p38 MAPK) in correlation with NO production, (ii) the effect of Losartan on these parameters. **Methods:** Hyperphagia-induced obese, insulin resistant rats (DIO=diet supplemented with sucrose and condensed milk) were compared to age-matched controls. Half the animals were treated with 10mg/kg Losartan per day for 1 week. Isolated hearts were perfused with or without 0.03 μIU/mL insulin for 15 min. Blood glucose, bodyweight, intraperitoneal fat and plasma insulin and Ang II were recorded. Proteins of interest and their phosphorylation were determined by Western blotting. NO production was flow cytometrically analyzed. ANOVA followed by the Bonferroni correction was used with a p< 0.05 considered significant. **Results:** DIO animals had significant elevated bodyweight, blood glucose, plasma insulin and Ang II levels. Our data showed that the hearts from the DIO animals are insulin resistant, ultimately reflected by the attenuated activation of the key proteins (IRS-1, PKB/Akt and eNOS) involved in insulin signalling as well as NO production. AT1 receptor antagonism improved NO
production in isolated adult ventricular myocytes from DIO animals while concurrently enhancing expression of eNOS, PKB/Akt and p38 MAPK. In contrast, NO production as well as expression of eNOS and PKB/Akt was attenuated in control animals after Losartan treatment. **Conclusion:** These results suggested that Ang II via AT1 or AT2 receptors, modulates protein expression of both PKB/Akt and eNOS. This encouraged us to investigate the involvement of AT2 receptors in the observed changes.

To investigate this we needed to establish a culture of neonatal rat cardiac myocytes treated with raised fatty acids and Ang II. If similar changes were induced as observed in the hearts of DIO animals, the involvement of the AT1 and AT2 receptors could be investigated using specific antagonists against these receptors. Primary cultured ventricular myocytes were isolated from 1-3 day old Wistar rat pups. They were cultured for 48 hours before the addition of palmitate and oleate at a concentration of 0.25 mM each and were treated with or without the fatty acids for a period of 4 days. After 18 hours of serum starvation, cells were stimulated with or without 10 nM insulin for 15 minutes. The effect of fatty acid treatment on cell viability and glucose uptake were assessed by trypan blue and propidium iodide staining and 2-deoxy-D-\(^{3}\)[H] glucose uptake respectively. Protein levels and phosphorylation of key proteins (PKB/Akt, PTEN and p38 MAPK) in insulin signalling was determined by Western blotting. 0.25 mM Fatty acids did not result in the loss of cell viability. Contrary to expectation, fatty acid treatment led to enhanced basal glucose uptake but lower Glut 1 protein expression. Basal protein expression of PPARα was, however, upregulated as was the expression of the phosphatase, PTEN. The latter could explain the lower PKB/Akt phosphorylation also documented.
From these results we conclude that neonatal cardiac myocytes, cultured in the presence of elevated fatty acids, did not respond in a similar manner as the intact hearts of our animals and further modifications of the system might be needed before it can be utilized as initially planned.
Insulien stimuleer die produksie van stikstofoksied (NO) in endoteel- en hartselle deur ’n seintransduksiepad wat die insulien reseptor substraatproteïen (IRS)-1, PI3-kinase en die proteïen kinase B (PKB/Akt) insluit. Fisiologiese konsentrasies NO speel ’n baie belangrike rol in die handhawing van normale vaskulêre funksie. Dit is voorgestel dat stikstofoksied sintase (NOS) aktiwiteit en die produksie van NO in diabetes mellitus versteur is as gevolg van ’n onbekende mekanisme. Die renien-angiotensien sisteem en die daaropvolgende produksie van angiotensien (Ang) II is verhoog in vetsug en diabetes terwyl antagonisme van die Ang II tipe I (AT1) met Losartan voordelige effekte het in pasiënte wat lei aan insulin weerstandigheid en tipe II diabetes. Die doel van hierdie studie was dus om (i) die effek van Ang II op insulin seintransduksie, veral met betrekking tot sleutel proteïene (IRS-1, PKB/Akt, eNOS and p38 MAPK) te bepaal in korrelasie met NO produksie en (i) die effek van Losartan op die betrokke parameters te ondersoek. Hiperfagie-geïndusseerde vetsugtige, insulin weerstandige rotte (DIO = dieet gesupplementeer met sukrose en kondensmelk) is vergelyk met ouderdomsgelyke kontroles. Die helfte van die diere is behandel met 10mg/kg Losartan vir ’n periode van 1 week. Geïsoleerde harte is geperfuseer met of sonder 0.03 μIU/mL insulien vir 15 min. Bloedglukose, liggaamsgewig, intraperitoneale vet, plasma insulien en Ang II vlakke is bepaal. Proteïen uitdrukking en fosforilering is bepaal deur middel van Western analises terwyl NO produksie bepaal is met behulp van vloeisitometrie. ANOVA gevolg deur ’n Bonferroni korreksie is gebruik om data te analiseer en ’n p < 0.05 is aanvaar as statisties beduidend. DIO diere het buidende verhoogde liggaamsgewigte, bloedglukose, plasma insulien- en Ang II vlakke gehad. Ons resultate het getoon dat die harte van DIO diere insulin weerstandig is, soos ook weerspieël deur die laer
aktivering van sleutel proteïene (IRS-1, PKB/Akt en eNOS) wat betrokke is by insulin seïntransduksie sowel as die produksie van NO. AT1 reseptor inhibisie (Losartan) het NO produksie in die geïsoleerde hartselle van DIO diere verbeter terwyl die proteïen uitdrukking van PKB/Akt, eNOS en p38 MAPK verhoog het. In teenstelling hiermee, het NO produksie sowel as die uitdrukking van eNOS en PKB/Akt in kontrole diere wat met Losartan behandel is, verlaag. Gevolglik dui on resultate daarop dat, Ang II via die AT1 of AT2 reseptore, proteïen uitdrukking van beide PKB/Akt en eNOS reguleer. Hierdie gevolgtrekking het ons genoodsaak om die rol van die AT2 reseptor verder te bestudeer.

Om die rol van die AT2 reseptor te kan bestudeer, moes daar van ‘n sel-gebaseerde sisteem gebruik gemaak word. ‘n Kultuur van neonatale kardiomiosiete, wat met verhoogde vetsure en Ang II behandel kon word, is dus daargestel. Indien soortgelyke veranderinge in proteïen uitdrukking waargeneem kon word, kon die rol van die AT1 en AT2 reseptore met spesifieke antagonistie, ondersoek word. ‘n Primêre kultuur van ventrikulêre miosiete is dus geïsoleer uit harte van 1-3 dag oud Wistar rotte. Die selle is vir 48 uur gekultuur voor die byvoeging van beide palmitaat en oleaat teen 0.25 mM elk by helfte van die selle vir ‘n periode van 4 dae. Na verwydering van alle serum vir 18 uur, is die selle met 10 nM insulien vir 15 min gestimuleer. Die effek van vetsuurbehandeling op seloorlewing en glukose opname is deur middel van trypan blou en propidiumjodaat kleuring en 2-deoxy-D-[^3[H] glukose opname onderskeidelik, bepaal. Proteïen uitdrukking en fosforilering van die sleutelproteïne in insulienseintransduksie (PKB/Akt, PTEN en p38 MAPK) is weereens met behulp van Western analises bepaal. 0.25 mM vetsure het geen effek op die oorlewing van die selle gehad nie. In teenstelling met ons verwagting, het vetsuur behandeling tot verhoogde basale glukose opname maar verlaagde Glut 1
uitdrukking geleid. Basale uitdrukking van PPAR$\alpha$ was opgereguleer asook die uitdrukking van die fosfatase, PTEN. Laasgenoemde kan moontlik die verlaagde PKB/Akt fosforilering wat gemeet is, verklaar.

Hierdie resultate lei tot die gevolgtrekking dat neonatale kardiale miosiete wat in kultuur aan verhoogde vetsure blootgestel is, nie soortgelyk reageer as die intakte hart van 'n dier op 'n hoë vet dieët nie. Verdere modifikasies aan die sisteem is nodig voordat die beplande studie verder uitgevoer kan word.
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### Units of Measurement

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<tbody>
<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<td>AU</td>
<td>arbitrary units</td>
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<tr>
<td>min</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
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<tr>
<td>mm²</td>
<td>cubic millimetres</td>
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<tr>
<td>N</td>
<td>normal</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>p</td>
<td>pico</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
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<tr>
<td>sec</td>
<td>seconds</td>
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<tr>
<td>μ</td>
<td>micro</td>
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</table>
μg/ml  micrograms per millilitre
μM  micromolar
μm  micrometer

**Molecular & Chemical Compounds**

- \( \cdot \text{O}_2^- \)  superoxide
- 2, 3-BDM  2, 3-butanedione monoxime
- 2DG  2-Deoxy-D-\[^{3}H\] glucose
- ACC  acetyl CoA carboxylase
- ACE  angiotensin converting enzyme
- ACS  acyl CoA synthase
- AMP  5'-adenosine monophosphate
- AMPK  5'-adenosine monophosphate kinase
- Ang II  angiotensin II
- Apaf-1  apoptotic protease activating factor-1
- APS  adaptor protein with plekstrin and Src
- ARB  angiotensin receptor blockers
- ARP-3  actin related protein-3
- AS160  160kDa substrate of PKB/Akt
- AT1  angiotensin II type 1 receptor
- AT2  angiotensin II type 2 receptor
- ATP  adenosine triphosphate
- Bcl-2  β-cell lymphoma-2 gene
- BSA  bovine serum albumin
- Ca\(^{2+}\)  calcium
- CaCl\(_2\)  calcium chloride
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>CAP</td>
<td>Cbl-associated protein</td>
</tr>
<tr>
<td>cGMP</td>
<td>3’, 5’- cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>copper sulphate</td>
</tr>
<tr>
<td>CPT-I</td>
<td>carnitine palmityltransferase</td>
</tr>
<tr>
<td>DAF/2A</td>
<td>diaminofluorescein-diacetate</td>
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<td>DAG</td>
<td>diacylglycerol</td>
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<td>DIO</td>
<td>diet-induced obesity</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>e.g.</td>
<td>for example</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>F-1, 6-P</td>
<td>fructose-1, 6 biphosphate</td>
</tr>
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<tr>
<td>FABP</td>
<td>fatty acid binding protein</td>
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<td>fatty acyl-CoA synthase</td>
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<td>fluorescence-activated cell sorter</td>
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<td>G-1-P</td>
<td>glucose-1-phosphate</td>
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<td>G-6-P</td>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
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Glut 1 glucose transporter 1
Glut 4 glucose transporter 4
GRK G-protein coupled receptor kinases
GS glycogen synthase
GSK 3 glycogen synthase kinase 3
GTP guanosine triphosphate
H₂O water
HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HUVEC human umbical vascular endothelial cells
i.e. that is
IAP inhibitor of apoptosis
Ins insulin
IP₃ inositol-1, 4, 5-triphosphate
IRAP insulin-responsive aminopeptidase
IRS insulin receptor substrate
JAK janus activated kinase
K⁺ potassium
KCI potassium chloride
KH₂PO₄ potassium dihydrogenphosphate
LCFAs long chain fatty acids
LDL low density lipoprotein
MAPK mitogen-activated protein kinase
MCD malonyl CoA decarboxylase
MgSO₄ magnesium sulphate
MLCK myosin light chain kinase
<table>
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<tr>
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<tr>
<td>MLCP</td>
<td>myosin light chain phosphatase</td>
</tr>
<tr>
<td>MPTP</td>
<td>mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NaHCO₃</td>
<td>sodium hydrogen carbonate</td>
</tr>
<tr>
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<td>sodium hydroxide</td>
</tr>
<tr>
<td>NBS</td>
<td>newborn calf serum</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
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<tr>
<td>P38 MAPK</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PARP</td>
<td>poly-(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDH</td>
<td>pyruvate dehydrogenase</td>
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<tr>
<td>PIP₃</td>
<td>phosphatidylinositol-3, 4, 5 triphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
</tr>
<tr>
<td>PLA₂</td>
<td>phospholipase A 2</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethyl sulphonyl fluoride</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase &amp; tensin homologue deleted from chromosome 10</td>
</tr>
<tr>
<td>PTP</td>
<td>protein tyrosine phosphatases</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
</tr>
<tr>
<td>RASMC</td>
<td>rat aortic smooth muscle cells</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>SHIP</td>
<td>Src homology 2 domain inositol phosphatase</td>
</tr>
<tr>
<td>Smac/DIABLO</td>
<td>second mitochondria-derived activator of caspase/direct inhibitor of IAP binding protein with low pl</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydromethyl) aminomethane hydrochloride</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine 5′-diphosphate</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>uridine-5′-diphosphate –glucose</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoproteins</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cells</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>βAR</td>
<td>β-Adrenergic receptors</td>
</tr>
</tbody>
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CHAPTER 1
LITERATURE REVIEW

1.1. PROBLEMS OF EPIDEMIC PROPORTION
In 1997, the World Health Organization (WHO) declared that obesity is becoming a major health problem in many developing countries [WHO 1998] and it is currently estimated that more than 1 billion individuals globally are overweight and 300 million individuals are obese. Over the past 10 years, health problems associated with overweight have received global recognition [Haslam & James 2005]. It is believed factors that contribute to this epidemic include global trends in diet, moving from traditional diets to those with increased refined foods, high free sugar and saturated fat in combination with reduced physical activity [WHO 2002].

Obesity is likely to be a major driving force for the increase in cardiovascular related deaths in developing countries. The increased risk of cardiovascular disease in obesity stems from a multitude of risk factors associated with obesity e.g. hypertension, diabetes & dyslipidemia that are the result of obesity [Lopaschuk et al., 2007]. It is well recognized that the incidence of obesity is also rising in South Africa, with 29.2 % of men and 56.6% of women being overweight or obese, in an African rural population group [Puoane et al., 2002]. Numerous epidemiological studies have shown that obesity is an important risk factor for the development of type II diabetes mellitus [Chan et al., 1994; Colditz et al., 1995]. Furthermore, obesity is associated with the development of coronary artery disease and is a major driver of the metabolic syndrome [Alberti & Zimmet 1998; NCEP 2002].

The metabolic syndrome is a cluster of metabolic disturbances that together define a progressive condition associated with development of type II diabetes mellitus and
cardiovascular disease [Reaven 1988; Grundy 2006]. It is estimated that the metabolic syndrome affects approximately one quarter of the population in developed countries [Alexander et al., 2003]. The National Cholesterol Education Program’s Adult Treatment Panel III (NCEP: ATP III) and the European Group of the Study of Insulin resistance, identified central–abdominal obesity, atherogenic dislipidaemia (hypertriglyceridaemia and reduced high-density lipoprotein-cholesterol), raised blood pressure, insulin resistance and glucose intolerance as essential components of the metabolic syndrome [Balkau & Charles 1999; NCEP 2002]. Furthermore, it has been reported that each component of the metabolic syndrome may be considered as an independent risk factor for cardiovascular disease [NCEP 2002]. Over the past two decades, the number of people with the metabolic syndrome has increased at an alarming rate. This increase is associated with the global epidemic of both obesity and diabetes [Zimmet et al., 2001].

Diabetes mellitus is affecting an increasing number of people worldwide and is a leading cause of blindness and non-traumatic amputations and it accounts for a significant proportion of end stage renal disease. Alarmingly, the incidence of diabetes mellitus is increasing in young adults as well as children [Bradshaw et al., 2007]. In 1998, the WHO estimated that there were 135 million people with diabetes [King et al., 1998] and this is projected to increase to 366 million in 2030 [Hogan et al., 2003; Wild et al., 2004]. It is postulated that this increase will occur in most developing countries with their increasing trend towards unhealthy diets, obesity and sedentary lifestyles resulting in late-onset diabetes (type II) [Green et al., 2003].

Diabetes has been classified into two forms namely type I and type II diabetes. Type I diabetes, caused by autoimmune destruction of pancreatic β-cells, accounts for 10% of all cases of diabetes. Ninety percent of diabetic individuals have type II (non-
insula dependent) diabetes which results from a combination of insulin resistance and \( \beta \)-cell dysfunction [Carley & Severson 2005]. Diabetes mellitus is a major risk factor predisposing to the development of ischaemic heart disease [Reaven 1988]. Ischaemic heart disease is projected to be one of the leading causes of death by the year 2020 [Murray & Lopez 1997].

Cardiovascular disease is a major cause of death in both obesity and diabetes mellitus and remains the number one killer in modern societies [Tang & Young 2001; Henriksen 2007]. In both obesity and diabetes, the cardiovascular complications develop without a specific symptomatic profile of disease. When type II diabetes, as a result of obesity, is therefore diagnosed, cardiovascular disease is mostly already present. Therefore prevention is no longer an option, only treatment. In order to develop treatment it is important to explore the possible mechanisms that ultimately result in cardiac disorders and subsequently heart failure. Numerous studies have reported that pathological cardiac disorders are associated with disturbance in cardiac energy metabolism [Barger & Kelly 2000; Davila-Roman et al., 2002].

1.2 CARDIAC ENERGY METABOLISM

The heart, which consists of specialized muscle cells, cardiac myocytes, is one of the most metabolically active organs in the body. Uninterrupted contraction is a unique feature of the heart [An & Rodriques 2006], therefore the cardiac muscle has a high demand for provision of energy in order to maintain cellular homeostasis and contractile function [Shah & Shannon 2003]. To accomplish this, under normal physiological conditions, the heart can utilize a multitude of substrates [An & Rodriques 2006], amongst which carbohydrates (glucose and lactate) and fatty acids are the major sources from which the heart derives its energy. Fatty acids account for about 70% of adenosine triphosphate (ATP) generation. In contrast, glucose and
lactate account for 30% [Saddik & Lopaschuk 1991]. The heart can rapidly switch substrate selection to accommodate different conditions, whether it is physiological or pathological [Rodrigues et al., 1995; Stanley et al., 1997; Buchanan et al., 2005]. Although substrate switching is necessary to ensure continuous ATP generation to maintain heart function, it has been associated with deleterious consequences (i.e. cardiac failure) [Young et al., 2002].

1.2.1 GLUCOSE METABOLISM

Glucose is a major substrate utilized by the heart under normal physiological conditions and enters mammalian cells through facilitative transporters (Gluts), which are 12 transmembrane domain-containing proteins [Mueckler 1990]. To date, about 13 Glut isoforms have been identified [Gould & Holman 1993]. With regards to cardiac glucose metabolism, Glut 1 and Glut 4 are both expressed in the heart and are the major contributors to glucose uptake. Basal cardiac glucose uptake is facilitated by Glut 1 that has a distinct sarcolemmal localization while Glut 4 is located in an intracellular storage pool. Translocation of these glucose transporters to the sarcolemmal membrane requires a stimulus e.g. insulin or contraction [Luiken et al., 2004]. Insulin is the predominant stimulant for glucose uptake after a meal. Insulin mediated glucose uptake will be discussed in more detail later (see section 1.4.2). However, Glut-mediated glucose uptake can also occur through insulin independent mechanisms.

Fairly recent studies have shown that translocation of Glut 4 to the sarcolemmal membrane is also stimulated by the activation of AMP-activated protein kinase (AMPK), a serine/threonine (Ser/Thr) kinase [Li et al., 2004a; Yang & Holman 2005]. AMPK has been proposed to be a metabolic energy sensor and is stimulated by the
increase of intracellular AMP/ATP ratio. AMPK is a heterotrimeric protein that consists of a catalytic subunit (α) and two regulatory subunits (β and γ). Both catalytic and regulatory subunits have two or more isoforms of which the AMPK α2 subunit is primarily expressed in the liver, skeletal muscle and the heart [Hardie & Carling 1997]. AMPK has been reported to play an imperative role in cellular metabolism for sustaining energy homeostasis. Its activation is coupled to switching off energy-consuming processes and promoting energy generating pathways. Enhanced activation of AMPK has been associated with stress conditions such as hypoxia and exercise in skeletal muscle and ischaemia in the heart [Marsin et al., 2000].

After entrance into a cell (cardiac myocytes or skeletal muscle cells), glucose is metabolized to be stored as glycogen, or undergoes glycolysis (Fig. 1.1 pg. 8). Glucose is phosphorylated to glucose-6-phosphate (G-6-P) by hexokinase. Secondly G-6-P is converted to glucose-1-phosphate (G-1-P) by phosphoglucomutase. G-1-P is converted to uridine-5’-diphosphate (UDP)-glucose in a reaction that involves UDP-glucose pyrophosphorylase. The final step in glycogen synthesis requires the polymerization of UDP-glucose to glycogen by glycogen synthase (GS), the rate-limiting enzyme of glycogen synthesis [Rothman et al., 1992]. The phosphatidylinositol 3-kinase/Protein kinase B (PI3K/PKB/Akt) signalling cascade has been reported to play a critical role in insulin mediated glucose uptake and glycogen synthesis in skeletal muscle [Saltiel & Kahn 2001] as well as the heart [Luiken et al., 2004]. GS is activated by dephosphorylation and inactivated by phosphorylation. Insulin is able to stimulate GS by dephosphorylation of serine residues that are phosphorylated by glycogen synthase kinase- 3 (GSK3), a downstream effector of PKB/Akt [Cohen 1999]. GSK3 is expressed as two isoforms, α and β, with a molecular weight of 51- and 47-kDa respectively [Woodgett 1990].
L6 myotubes, insulin induces phosphorylation at Ser$^{21}$ and Ser$^{9}$ for GSK3α and GSK3β respectively [Sutherland & Cohen 1994]. This suggests that loss of PKB/Akt activity, for example in a state of insulin resistance, may result in impaired GS activity and attenuation in glycogen synthesis.

Glucose is broken down through glycolysis in a 10 step reaction sequence to yield pyruvate. The first 5 steps of glycolysis require the utilization of ATP. The conversion of glucose to G-6-P by hexokinase and phosphofructokinase-1 (PFK-1) to produce fructose 1, 6 biphosphate (F-1, 6-P), consumes ATP. F-1, 6-P, a potent activator of PFK-1, is synthesized from fructose-6-phosphate when phosphorylated by phosphofructokinase-2 (PFK-2) [Hue & Rider 1987]. This is of interest, given that PFK-2 can be phosphorylated and activated by insulin [Deprez et al., 1997], AMPK and norepinephrine [Stanley et al., 2005]. Thus enhancing activation of PFK-2 by these mechanisms promotes glycolysis.

Pyruvate, the product of glycolysis, has three major end points: (i) converted to lactate, (ii) carboxylation to malate or oxaloacetate, or (iii) decarboxylation to acetyl CoA. Pyruvate is transported to the mitochondria and decarboxylated to acetyl-CoA by the enzymatic action of pyruvate dehydrogenase (PDH). PDH is a multi-enzyme complex that is located in the mitochondrial matrix [Randle 1986; Stanley et al., 2005]. The PDH complex is fairly active when tissues are well fed. However, suppression of the PDH complex is an important mechanism whereby substrates such as pyruvate is conserved for glucose synthesis by the liver under condition of fasting, starvation and exercise [Wu et al., 1998]. The PDH complex is the major target for substrate competition between glucose and fatty acids [Sugden & Hollness 1994]. The PDH complex activity is tightly regulated by dephosphorylation and
phosphorylation. PDH is inactivated when phosphorylated on the E1α subunit of the enzyme complex by pyruvate dehydrogenase kinase (PDK) and activated when dephosphorylated by PDH phosphatase [Randle 1986]. PDK is a serine kinase and to date four isoforms have been identified in humans and rodents (PDK1-PDK 4) [Popov et al., 1997]. The heart contains three PDK isoforms (PDK 1, PDK 2 and PDK 4) [Bowker-Kinley et al., 1998; Wu et al., 1998] but it was found that PDK 4 is the predominant form in the heart [Bowker-Kinley et al., 1998]. The PDH phosphatase dephosphorylates and activates PDH. The activity of PDH phosphatase is rapidly increased by Ca\(^{2+}\) and Mg\(^{2+}\) [McCormack & Denton 1989]. The products of fatty acid oxidation (acetyl CoA and NADH) promote PDK activity [Roche et al., 2001], while, pyruvate, produced from glycolysis, suppresses the activity of PDK [Popov et al., 1997; Bowker-Kinley et al., 1998]. Acetyl-CoA undergoes additional mitochondrial metabolism that results in the production of ATP by oxidative phosphorylation. Glycolysis and PDH activity in the heart are augmented by suppression of fatty acid oxidation [Stanley et al., 2005].
Figure 1.1 Simplified representation of glucose metabolism in cardiac myocytes. Adapted from Dyck & Lopashuck 2005

1.2.2 FATTY ACID METABOLISM

The importance of free fatty acids for myocardial metabolism was established more than 50 years ago by Bing’s pioneering studies showing that different substrates across the human heart differs in contribution to cardiac energy metabolism [Bing et al., 1953]. Physiologically, free fatty acids have been delineated as the predominant substrate for myocardial utilization and its use depends on the supply to the heart and regulation of cellular uptake [Lopaschuk et al., 1994]. Free fatty acids are highly hydrophobic and are either bound to plasma albumin or covalently bound in triglyceride contained within very low density lipoproteins (VLDL). In vivo these are the main sources of fatty acids for myocardial metabolism that are derived from adipose tissue lipolysis or hydrolyzed by lipoprotein lipase respectively [Braun & Severson 1992]. Albumin-bound long chain fatty acids (LCFAs) dissociate easily from albumin. Albumin serves as the transport vehicle of LCFAs through the aqueous
compartments while the LCFAs contained in VLDL have to be hydrolyzed by the enzymatic action of lipoprotein lipase [Van der Vusse et al., 2000]. The energy need of the heart is primarily provided by long chain saturated (palmitate) and monounsaturated (oleate) fatty acids [Van der Vusse et al., 1992]. The first step of fatty acid oxidation is cellular uptake. The mechanism by which fatty acids enter cardiac myocytes is either by passive or simple diffusion across the plasma membrane [Van der Vusse et al., 2000] or protein-mediated uptake. Protein-mediated uptake is believed to account for 80% of total fatty acid uptake. Protein-mediated uptake involves the fatty acid translocase (FAT) or the 43-kDa plasma membrane fatty acid binding protein (FABP). CD36, a 88- kDa FAT, which is abundantly expressed in both cardiac and skeletal muscle, has been found to be the major form of FAT in the heart. (Fig. 1.2 pg.12)

Briefly, once taken up, intracellular fatty acids bound to fatty acid binding protein (FABP) are activated to fatty acyl-CoA by fatty acyl-CoA synthase (FACS). It has been reported that insulin can stimulate translocation of FAT/CD36, much like translocation of the glucose transporter, Glut 4 [Luiken et al., 2002b]. Fatty acyl-CoA can be either transported to the mitochondria for β-oxidation or esterified to triglyceride by glycerolphosphate acyl transferase [Van der Vusse et al., 2002]. Triglyceride synthesis occurs by the Kennedy pathway in the heart by which fatty acyl-CoA acylates glycerol-3-phosphate followed by a cascade of acylation steps to finally yield triglyceride. Intracellular triglyceride stores can provide an endogenous source of fatty acids primarily by hydrolysis [Lewin & Coleman 2003]. For the occurrence of β-oxidation, fatty acyl-CoA should be transported to the mitochondria, dependent on the activity of carnitine palmitoyl transferase (CPT-I). CPT-I, the key enzyme in mitochondrial fatty acid uptake, is located on the outer mitochondrial
membrane and catalyzes fatty acyl-CoA to long chain acyl carnitine. CPT-II, located on the inner mitochondrial membrane exchange the carnitine to regenerate fatty acyl-CoA within the mitochondrial matrix [Lopaschuk et al., 1994]. Here fatty acids undergo β-oxidation and yields NADH, FADH$_2$ and acetyl-CoA for the citric acid cycle. The reducing equivalents NADH and FADH$_2$ primarily deliver electrons to the electron transport chain, which in the end, result in the formation of ATP by oxidative phosphorylation [Bing 1954].

Intracellular fatty acid oxidation is regulated at the level of mitochondrial uptake. The fate of CPT-I activity is largely dependent on malonyl CoA, key regulator of fatty acid oxidation and potent inhibitor of CPT-I [Hall et al., 1996; Dyck & Lopaschuk 2002]. To date, two isoforms of CPT-I have been identified, CPT-I α, that predominates in the liver and CPT-I β, the major isoform in the heart. CPT-I β has been found to be more susceptible to malonyl CoA inhibition than CPT-I α [Cook & Lappi 1992; McGarry & Brown 1997]. Elevation of cardiac malonyl CoA results in a decrease in mitochondrial fatty acid uptake and oxidation, while a fall in malonyl CoA increases fatty acid uptake and oxidation. The myocardial content of malonyl CoA is tightly regulated by two enzymes, acetyl CoA carboxylase (ACC) and malonyl CoA decarboxylase (MCD). Malonyl CoA in the heart is synthesized by the carboxylation of acetyl CoA by ACC, which is inhibited by phosphorylation by AMPK; therefore activation of AMPK reduces cytosolic levels of malonyl CoA and promotes fatty acid uptake and oxidation. Furthermore, malonyl CoA is degraded by MCD, resulting in acetyl CoA formation and accelerated fatty acid uptake and oxidation [Kudo et al., 1995; Hall et al., 1996; Dyck & Lopaschuk 2002]. (Fig. 1.2 pg.12)
The capacity of myocardial fatty acid oxidation can also be regulated at the level of gene expression through nuclear receptor signalling mechanisms to promote fatty acid utilization. The ligand-activated transcription factors, peroxisome proliferator-activated receptors (PPARs), form part of a family of nuclear receptors that modulate the expression of genes that encode the proteins involved in controlling fatty acid metabolism. PPARs have been found to regulate an array of target genes implicated in cellular lipid catabolism and storage [Berger & Moller 2002; Huss & Kelly 2004]. Thus far 3 isoforms of PPARs have been identified PPARα, PPARβ and PPARγ. PPARβ has been reported to be ubiquitously expressed in skeletal muscle, the heart and the brain. PPARγ is adipose-enriched and believed not to play a direct role in regulation of fatty acid oxidation [Briassant et al., 1996].

Interestingly, PPARα is highly expressed in tissues that favour fatty acid metabolism such as brown adipose tissue, slow-twitch skeletal muscle and the heart [Briassant et al., 1996; Berger & Moller 2002]. Free fatty acids are the endogenous, natural ligands for PPAR and LCFAs are believed to be among the activators of PPARα [Barger & Kelly 2000]. Once activated, PPARα form complexes with retinoid X receptors and bind specific response elements within the promoter region of a multitude of genes encoding enzymes involved in fatty acid uptake (FAT/CD36; FABP) as well as metabolism (acyl CoA synthase, CPT-I) [Huss & Kelly 2004]. Several studies with cultured cardiac myocytes depicted that overexpression of PPARα results in the acceleration of fatty acid uptake and oxidation [Brandt et al., 1998; Gilde et al., 2003]. Along with PPARα activation, CPT-I expression may also be augmented through an upregulation of MCD, which results in reduced malonyl-CoA levels. On the other hand, mice lacking PPARα display suppression of FA oxidation, accompanied by increased levels of malonyl-CoA [Campbell et al., 2002]. However, a cardiac-specific
PPARα knockout model is yet to be characterized [Madrazo & Kelly 2008]. Activation of PPARα is beneficial considering that fatty acid oxidation augments clearing fatty acids from the circulation, however this also may have detrimental effects on myocardial function. Recently it was demonstrated that agonism of PPARα compromises contractile function in mice exposed to repeated episodes of ischaemia [Dewald et al., 2005].

Figure 1.2 Schematic representation of the regulation of fatty acid oxidation in the heart under physiological conditions. Adapted from Dyck & Lopaschuk 2005.
1.2.3 CARDIAC SUBSTRATE PREFERENCE IN DISEASE STATES

Cardiac fatty acid utilization is tightly controlled in order to allow a metabolic switch when substrate supply to the heart is compromised. In conditions of metabolic stress such as ischaemia, diabetes, obesity and starvation, plasma free fatty acid concentration is markedly increased and cardiac substrate metabolism altered [Taegtmeyer et al., 2002; Young et al., 2002]. Due to the importance to the heart to maintain uninterrupted contractile function, it comes as no surprise that the heart is able to utilize different substrates to generate ATP under physiological and pathophysiological conditions [Taegtmeyer et al., 2002].

In most of the abovementioned pathophysiological states, the high circulating free fatty acids contribute to several cardiomyopathies. It is well documented that during obesity and diabetes, myocardial glucose utilization and oxidation is compromised at several points [An & Rodriques 2006]. Reduced glucose utilization may be a consequence of impaired insulin signalling together with reduced translocation of Glut 4 to the sarcolemmal membrane [Young et al., 2002]. In rodent models of diabetes (db/db mice), it has been reported that basal cardiac glucose uptake is unchanged. However, cardiac glycolysis and glucose oxidation were reduced. The overexpression of Glut 4 in the heart of db/db mice restored glucose utilization, thereby demonstrating that altered glucose utilization is likely due to reduced sarcolemmal Glut 4 content [Belke et al., 2000]. However it has also been postulated that glucose utilization is reduced by the high circulating free fatty acids rather than impaired insulin signalling. In several models of obesity, diabetes and insulin resistance, alterations at the level of fatty acid uptake and oxidation have been demonstrated. Streptozotocin (STZ)-induced diabetes, a model for type 1 diabetes [Luiken et al., 2002a], and Zucker rats, a model of type II diabetes [Luiken et al.,
2001], both displayed an increase in plasmalemmal FAT/CD36 and FABP accompanied by enhanced fatty acid uptake. In ob/ob and db/db mice, models of obesity and insulin resistance, a decrease in glucose utilization occurred at 4 weeks of age, even before the onset of impaired insulin signalling [Buchanan et al., 2005].

Elevated intracellular fatty acids increase cardiac PDK that results in phosphorylation and inhibition of PDH, rate limiting enzyme for glucose oxidation [Randle et al., 1963]. As previously mentioned, the heart contains three isoforms of PDK of which PDK 4 is predominant [Bowker-Kinley et al., 1998]. Enhanced PDK 4 activity is rapidly induced by diabetes and prolonged starvation. Potent stimulants for the activation of PDK 4 activity is increased levels of acetyl CoA and NADH, the products of fatty acid oxidation [Wu et al., 1998]. Fatty acids have been demonstrated to enhance protein expression of PDK 4 in slow-twitch skeletal muscle in response to high-fat diet [Holness et al., 2000]. Interestingly both diabetes and starvation are conditions associated with insulin deficiency and sustained cardiac fatty acid utilization [Sugden et al., 2000]. However, it seems that insulin deficiency is not a prerequisite for increased PDK 4 activity. PDK protein expression and activity is augmented in diet-induced insulin resistance despite the occurrence of high circulating insulin levels [Holness et al., 2000]. Furthermore, it has been reported that up regulation of PDK 4 protein expression during starvation is mediated by PPARα. In support of this, prolonged starvation of mice that lack PPARα resulted in impaired protein expression of PDK 4 in liver and kidney. This demonstrated the importance of PPARα in PDK 4 protein up regulation. In addition, it suggested a direct role for fatty acids in the induction of PDK 4 protein expression, considering that fatty acids are ligands for PPARα [Wu et al., 2001]. Reduced activity of the PDH complex results in an increase in lactate production and is accompanied by accelerated generation of
protons and accumulation of \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) that contribute to impaired cardiac efficiency \[\text{Liu et al., 1996}\]. On the other hand at the onset of cardiac hypertrophy and cardiac failure, myocardial substrate utilization switches from fatty acid to glucose metabolism \[\text{Davila-Roman et al., 2002}\].

Increased exposure to fatty acids may potentially result in the accumulation of fatty acid intermediates (ceramides and triacylglycerols) in the cell. This is referred to as cardiac lipotoxicity \[\text{Zhou et al., 2000}\]. Lipotoxicity has been associated with severe consequences on cellular functions such as cell death (apoptosis) and impaired contractile function \[\text{Taegtmeyer et al., 2002}\]. In a study done with obese Zucker diabetic rats, a marked accumulation of triacylglycerols occurred within the myocardium which was associated with elevated levels of ceramide and development of contractile dysfunction. In addition, factors that may contribute to lipotoxicity in these animals are the downregulation of PPARα and fatty acid oxidative enzymes \[\text{Zhou et al., 2000}\]. It has been proposed that in diabetes, the heart is exposed to excessive free fatty acid levels and that initially, the heart adapts by increasing fatty acid oxidative enzymes in order to maintain cardiac output. As diabetes progresses, free fatty acids availability exceeds the rate of oxidation and this causes lipid accumulation that is accompanied by decreased expression of PPARα \[\text{Young et al., 2002}\]. In contrast, the activation of PPARα in the heart of obese and diabetic animal models has been demonstrated with increased expression of genes involved in fatty acid oxidation accompanied by the suppression of genes involved in glucose utilization \[\text{Buchanan et al., 2005}\].

During myocardial energy stress AMPK is rapidly phosphorylated and activated. Activation of AMPK in STZ-induced diabetes, has been reported as the key
mechanism responsible for augmented fatty acid oxidation [Gamble & Lopaschuk 1997]. AMPK has been reported to act in response to metabolic stresses that deplete cellular ATP. Augmented activation of AMPK subsequently results in the phosphorylation and inhibition of ACC which is accompanied by maintained MCD activity. This ultimately results in reduced malonyl-CoA levels which alleviate the inhibition of CPT-I thus favouring mitochondrial fatty acid oxidation [Kudo et al., 1995; Dyck & Lopaschuk 2002]. In mice fed on a high fat diet, cardiac malonyl-CoA levels are depleted, accompanied by increased fatty acid oxidation and reduced insulin-sensitive glucose oxidation. It is not clear whether the decrease of malonyl-CoA is responsible for these increased fatty acid oxidation rates [Folmes & Lopaschuk 2007].

1.3. FATTY ACID INDUCED DAMAGE

More than a decade ago, it has been reported that excess fatty acids exert adverse effects on the mechanical function of the heart [Lopaschuk et al., 1994]. Cardiac failure in the human heart has been characterized as the progressive death of myocytes [Olivetti et al., 1997]. When a cell undergoes apoptosis, a variety of molecular and biochemical events occur which appear to be unique to apoptosis [Majno & Joris 1995]. In order to understand fatty acid mediated cell death, we should first explore the process of apoptosis.

1.3.1 APOPTOSIS

Apoptosis can be described as an active, physiological mode of cell death, in which the cells take part in their own demise. Apoptosis is associated with unique changes which can be used as biomarkers to identify this mode of cell death. Loss of intracellular water results in condensation of the cytoplasm and a change in cell size.
and shape. Furthermore, the condensation of nuclear chromatin and cleaving of DNA by endonucleases are also characteristics of apoptosis. The products of DNA cleavage and degradation are nucleosomal DNA fragments, which are multiples of 180 bp [Majno & Joris 1995]. These DNA fragments generate a characteristic “DNA ladder” pattern during agarose gel electrophoresis. The development of numerous cleavages in DNA has been reported in infarced myocardial cells [Itoh et al., 1995] and cultured cardiac myocytes subjected to palmitate [De Vries et al., 1997].

Apoptosis is viewed as an energy-dependent process, which is characterized by the preservation of mitochondria [Newmeyer & Ferguson-Miller 2003]. Mitochondria are key players in cell survival because of their role as source of energy and participation in apoptosis [Weiss et al., 2003].

Apoptosis is a tightly regulated system that involves two separate checkpoints, the regulatory (Bcl-2/Bax) proteins [Reed 1994] and the group of cysteine-aspartate-directed proteases, the caspases [Wolf & Green 1999]. The Bcl-2 regulatory proteins include both anti-apoptotic proteins (Bcl-2, Bcl-X) and pro-apoptotic proteins (Bad, Bax, Bak) [Adams & Cory 1998; Crow et al., 2004]. The Bcl-2 protein family is a group of key regulators of apoptosis that control the release of apoptotic factors from the mitochondria and the activation of caspases [Adams & Cory 1998]. To date 14 members of caspases have been identified and can be subdivided as upstream caspases (2, 8, 9, 10 & 12) and the downstream executioner caspases (3, 6 & 7) [Thornberry & Labeznik 1998; Stennicke et al., 1999]. In healthy cells, caspases are located in the cytosol as inactive procaspases that are cleaved and activated in response to apoptotic stimuli [Thornberry & Lazebnik 1998]. It has been reported that targets of caspases in the heart include proteins involved in contractile function such as actin, myosin heavy- and light chains and cardiac troponins [Communal et al.,
Most of these protein targets of caspases appear to be cleaved by the executioner caspases -3 and -7 [Crow et al., 2004]. Once these caspases are activated and cleave vital cellular components, apoptosis appears to be inevitable. Furthermore, another target for caspases includes the poly-(ADP-ribose) polymerase (PARP). However PARP cleavage ensure normal speed of apoptosis to occur and does not directly participate in the caspases activation cascade [Earnshaw 1995]. Therefore it is important to understand the initiating signals for the activation of caspases. In this regard, the mitochondria have received great attention for playing a vital role in the execution of apoptosis [Kluck et al., 1997; Weiss et al., 2003; Crow et al., 2004].

1.3.2 MITOCHONDRIAL DEATH PATHWAY

The immediate aims of apoptotic cell death are the activation of the caspases and inference with mitochondrial function [Thornberry & Lazebnik 1998]. Apoptosis is controlled by the interaction of various pro-survival and pro-death signals. The balance of Bcl-2 family of proteins, which may be anti-apoptotic (Bcl-2) or pro-apoptotic (Bad), determine the fate of a cell [Adams & Cory 1998] (Fig. 1.3 pg.20). Bad and Bax, the pro-apoptotic proteins, disrupt the integrity of the mitochondrial membrane and promote the release of apoptotic factors such as cytochrome c [Kluck et al., 1997]. Overexpression of Bcl-2, the anti-apoptotic protein in cardiac myocytes, inhibits the change in mitochondrial membrane electro-potential and attenuates the subsequent release of intermembrane proteins [Ryan et al., 2001]. However, certain regulators of apoptosis such as the inhibitor of apoptosis (IAP) physically interact with caspases and inhibit their function [Deveraux et al., 1998]. There are even proteins that counteract the actions of the caspase inhibitors e.g. Smac/DIABLO, the
mitochondrial protein that binds to IAP and enhances caspase activity [Du et al., 2000].

The mitochondrial death pathway, also known as the intrinsic pathway, is initiated by a variety of signals, however ultimately the result is the same in most cells. Whether a stimulus is extracellular e.g. radiation, nutrients and chemicals or intracellular e.g. oxidative stress and DNA damage, all these signals exert effects on the mitochondria. In due course these signals result in mitochondrial dysfunction accompanied by the release of pro-apoptotic proteins and subsequent caspase activity [Crow et al., 2004]. The earliest mitochondrial event is the release of cytochrome c into the cytosol [Kluck et al., 1997]. Cytochrome c participates in the activation of caspases together with apoptotic protease activating factor (Apaf) -1 and ATP. Apaf-1 interacts with cytochrome c and ATP to activate caspase 9 [Zou et al., 1997]. The activated and processed caspase-9 cleaves and activates caspase-3 which executes apoptosis [Bratton et al., 2001]. It has been postulated that cytochrome c release is triggered by changes in the mitochondrial permeability transition pore (MPTP) [Crow et al., 2004] or the rupture of the outer mitochondrial membrane. MPTP are multiprotein complexes that, upon cell death signals, are capable of forming large pores in the inner membrane of the mitochondria. The opening of MPTP is accompanied by the release of numerous pro-apoptotic proteins into the cytosol such as Smac/DIABLO, endonuclease and cytochrome c [Weiss et al., 2003]. Under normal conditions the mitochondrial membrane is relatively impermeable and maintains the proton- and osmotic gradient [Crow et al., 2004].
1.3.3 FATTY ACID INDUCED APOPTOSIS

De Vries and co-workers were the first to demonstrate that chronic exposure to the SFA, palmitate, induces apoptosis in neonatal rat ventricular myocytes. Palmitate, even at physiological concentrations, induces apoptosis of cardiac myocytes [De Vries et al., 1997]. Increased fatty acid accumulation in neonatal cardiac myocytes, leads to reduced utilization of fatty acids by these cells. However, this was accompanied by biomarkers of apoptosis, increased caspase-3-like activity and DNA laddering [De Vries et al., 1997]. The attenuation in fatty acid utilization in cardiac myocytes may initiate the signals for apoptotic cell death [Hickson-Bick et al., 2000]. In addition, palmitate has been reported to induce cytochrome c release, mitochondrial membrane loss and PARP cleavage [Sparagna et al., 2000].
1.4. METABOLIC ACTIONS OF INSULIN

Insulin is a potent anabolic hormone whose action is essential for appropriate tissue development: growth, proliferation and cell differentiation. In addition, it exerts a multitude of metabolic effects [Morisco et al., 2006] e.g. it is the primary hormone involved in regulating glucose homeostasis and glucose transport [Saltiel & Kahn 2001; White 2002]. Insulin, secreted by pancreatic β cells in response to increased circulating levels of glucose [DeFronzo 1988], reaches its target tissues, mainly the liver, adipose tissue and skeletal muscle, where it interacts with its receptor [Saltiel & Kahn 2001, White 2002]. It enhances fuel storage by reducing hepatic glucose production and increasing the rate of glucose uptake [DeFronzo 1988]. Additionally, insulin also affects lipid metabolism by increasing triglyceride synthesis and attenuating fatty acid release from the adipose tissue, primarily by inhibiting hormone-sensitive lipase [Lopaschuk et al., 1994]. Besides insulin’s multitude of metabolic effects, it has been demonstrated that insulin stimulation induces vasodilatation through nitric oxide production [Scherrer et al., 1994a]. In the heart, insulin regulates metabolism by modulating both glucose and lipid transport and metabolism as well as growth and apoptosis [Brownsey et al., 1997; Adel 2004].

1.4.1 FEATURES OF INSULIN SIGNAL TRANSDUCTION PATHWAYS

Circulating insulin binds to its cell surface receptor to elicit its biological actions [Elbina et al., 1985; Montagnani et al., 2001] (Fig. 1.4 pg.24). The binding of insulin to its heterotetrameric receptor, initiates the activation of complex signal transduction cascades that regulate diverse cellular functions [Nystrom & Quon 1999; Saltiel & Kahn 2001]. The insulin receptor is a widely expressed transmembrane tyrosine kinase consisting of two α- and β-subunits [Ebina et al., 1985]. After activation of it’s kinase activity by binding of insulin, the receptor phosphorylates several intracellular
substrates, including insulin receptor substrates (IRSs) [White 2002], Shc [Sasaoka et al., 1994], adaptor protein with plekstrin homology and Src homology 2 domain (APS) [Moodie et al., 1999] and GAB-1 [Holgado-Madruga et al., 1996] that serve as docking proteins for downstream signalling molecules. Tyrosine (Tyr) phosphorylation of IRS at multiple sites induces their binding to Src homology 2 (SH2) –domain-containing molecules, including phosphatidylinositol-3-kinase (PI3K) [Sowers 2004], Grb-2 [Nystrom & Quon 1999], Janus-activated kinase (JAK) and STAT [Marrero et al., 2004].

PI3K is a heterodimer composed of a regulatory p85 subunit and a catalytic p110 subunit [Engelman et al., 2006]. When the SH2 domains of the p85 subunit of PI3K bind Tyr-phosphorylated motifs on IRS-1 [Cantley 2002], this activates the catalytic activity of the p110 subunit of PI3K [Sowers 2004]. Catalytic activity of the p110 subunit generates the lipid product of phosphatidylinositol 3, 4, 5-triphosphate (PIP₃) from the substrate phosphatidylinositol 4, 5 bisphosphate (PIP₂) [Cantley 2002]. PIP₃ levels are tightly regulated by the actions of phosphatases such as PTEN (Phosphatase and tensin homologue deleted from chromosome 10) and SHIP2 (inositol 5’ phosphatase) [Maehama & Dixon 1998]. PIP₃ serves as an allosteric regulator of the 3 phosphoinositide-dependent protein kinase-1 (PDK-1) that, once recruited; phosphorylates and activates the downstream Ser/Thr kinase Protein Kinase B (PKB) or Akt. PKB/Akt has originally emerged as a regulator of cell growth but its central role in signal transduction only became apparent when found that this enzyme lies downstream of PI3K [Franke et al., 1995]. It was first identified as a cellular homologue of the viral oncogene v-Akt, a protein to cause a form of leukemia in mice [Staal et al., 1977]. To date three isoforms of PKB/Akt have been identified in mammalian cells and are referred to as PKBα (Akt 1) [Coffer & Woodgett 1991], PKBβ (Akt 2) [Jones et al., 1991] and PKBγ (Akt 3) [Konishi et al., 1995].
PIP₃ facilitates the translocation of PKB/Akt to the plasma membrane and alters its conformation for subsequent phosphorylation by PDK-1. PDK-1 phosphorylation of PKBα/Akt 1 in the activation loop regulates access to the catalytic site of PKBα/Akt 1. Activation of this site at Thr^{308} therefore allows subsequent phosphorylation of the acceptor site at Ser^{473} by PDK-2 [Alessi et al., 1997; Vanhaesebroeck & Alessi 2000]. It has been reported that phosphorylation at site Thr^{308} partially activates PKB/Akt. However, full activation requires phosphorylation at the second site (Ser^{473}) but in most cases it seems that phosphorylation at these sites occur in tandem [Alessi et al., 1997]. This PI3K- dependent branch of the insulin-signalling pathway mediates most of the metabolic actions of insulin.

The tyrosine (Tyr) phosphorylated insulin receptor induces binding of the adaptor protein Shc to the receptor and subsequently allows binding of another adaptor protein, Grb-2, in response to proliferation signals. Grb-2 phosphorylation results in the recruitment of SOS, a GTP exchange factor that stimulates the exchange of GDP bound to Ras for GTP [Nystrom & Quon 1999]. Ras binds Raf kinase and facilitates translocation to the cell membrane where Raf is activated by several kinases including Protein kinase C (PKC) [Kolch et al., 1993]. Raf activates the downstream effector MEK that results in the activation of mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase (ERK) [Reusch et al., 1995]. The MAPK-dependent branch of insulin signalling pathways is involved in cell growth, differentiation and mitogenesis. Activation of these kinases takes place in cardiac myocytes, smooth muscle and endothelial cells [Bogoyevitch 2000].
1.4.2 INSULIN-INDUCED GLUCOSE UPTAKE

The predominant role of insulin is whole body glucose homeostasis but it was not until 1949 that the ability of insulin to stimulate glucose uptake was experimentally demonstrated [Levine et al., 1949]. Cardiac glucose uptake has been reported to be dependent on the transmembrane glucose gradient as well the content of sarcolemmal glucose transporters, Glut 1 and Glut 4 [Kraegen et al., 1993; An & Rodrigues 2006]. In skeletal and cardiac muscle, Glut 4 is considered to principally contribute to the regulation of glucose uptake by insulin [Adel 2004] while Glut 1 is responsible for basal glucose uptake.
1.4.2.1 GLUT 4 TRANSLOCATION

In the basal state, Glut 4 is found in specialized vesicle compartments within the cell. It is very well documented that insulin via PKB/Akt activation induces the translocation of Glut 4 from the intracellular storage vesicles to the plasma membrane to facilitate glucose entry [Slot 1991; Adel 2004] (Fig. 1.5 pg.29). Investigations into the mechanism of insulin stimulated glucose transport have revealed an intricate network of signalling from the insulin receptor to the intracellular pool of Glut 4 vesicles [Czech & Buxton 1993; Saltiel & Kahn 2001]. It has been reported that the Glut 4 compartment is enriched with a v-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) namely VAMP2 [Pessin et al., 1999] and the insulin-responsive aminopeptidase (IRAP) [Kandror & Pilch 1994; Keller et al., 1995] which contribute to the localization of Glut 4 to the plasma membrane. The translocation of Glut 4 involves a network of cellular interactions that couples to the actin cytoskeleton. There is an increasing body of evidence that support the role of actin in Glut 4 translocation [Tsakiridis et al., 1999; Omata et al., 2000]. The involvement of actin in Glut 4 translocation is best described in the event of membrane ruffling [Lu et al., 1996] in which the products (PIP$_2$ and PIP$_3$) of PI3K bind the cytoskeleton protein, profilin, and allow the remodelling of actin below the plasma membrane. The rearrangement of actin filaments involves the activation of proteins that modulate actin polymerization. These include the effectors of the Rho family of GTPases [Tsakiridis et al., 1999]. Apart from actin, it has been reported that Glut 4 vesicles move along a microtubule track to the cell surface [Olson et al., 2003]. Only recently, a novel 160kDa substrate of PKB/Akt, AS160, has been identified in 3T3-L1 adipocytes [Kane et al., 2002]. AS160 is a negative regulator of basal Glut 4 exocytosis. In the basal state it is associated with IRAP and in response to insulin disassociates and promotes Glut 4 translocation [Eguez et al., 2005; Larance et al.,]
2005]. AS160 contains a Rab GTPase-activating protein (GAP) domain that is necessary for vesicle trafficking and its phosphorylation favours insulin- induced Glut 4 translocation in adipocytes [Sano et al., 2003] and skeletal muscle [Kramer et al., 2006]. Phosphorylation of AS160 has also been reported to be augmented by insulin in cardiac myocytes to facilitate glucose uptake [Bertrand et al., 2008]. Sufficient glucose entry depends on the successful docking and fusion of the Glut 4 vesicles to the plasma membrane. It has been found that v-SNARE VAMP2 is required for insulin-dependent Glut 4 docking and fusion. The plasma membrane target for the Glut 4 vesicle has been reported to be the SNARE protein syntaxin4 in adipocytes [Cheatham et al., 1996; Volchuk et al., 1996]. Termination of glucose entry, in conditions of reduced insulin and blood glucose levels, results in the endocytosis of Glut 4 containing vesicles into the cytoplasm.

1.4.2.2 INSULIN-INDUCED PI3K/PKB/AKT PATHWAY

In heart muscle, insulin stimulates glucose uptake that involves the PI3K/PKB/Akt-dependent pathway [Franke et al., 1995; Alessi et al., 1997; Vanhaesebroeck & Alessi 2000; Sowers 2004]. The activation of these molecules has been reported to directly or indirectly participate in Glut 4 translocation and ultimately glucose uptake (Fig 1.5 pg.29). There is a substantial amount of evidence that demonstrate the crucial involvement of the products of PI3K (PIP₃ production) in insulin-mediated glucose transport. PIP₂, the substrate for PI3K, has been reported to be a regulator of the actin cytoskeleton and membrane trafficking [DiNubile & Huang 1997]. As mentioned before (see section 1.4.2.1), PIP₂ and PIP₃ are able to bind profilin, a cytoskeleton protein, that serves as a site for actin remodelling [Lu et al., 1996]. Furthermore, PIP₃ has been reported to be directly involved in Glut 4 vesicle trafficking, whereby it co-ordinates the membrane arrival and fusion of the vesicles in
L6 myoblasts [Ishiki et al., 2005]. However, Jiang and colleagues [1998] demonstrated that exogenous PIP₃ derivatives cannot mimic the physiological effects of insulin stimulated glucose uptake, thereby suggesting that insulin generates more than one signal to promote glucose uptake.

1.4.2.3 INSULIN INDUCED CBL-CAP-TC10 PATHWAY

In two independent studies [Wiese et al., 1995; Isakoff et al., 1995] it was reported that PI3K activation is necessary but not sufficient for insulin-induced glucose uptake, which indicated that additional signalling is required for this process (Fig. 1.5 pg.29). This second pathway revealed that signal initiation was isolated into discrete compartments in the plasma membrane, termed caveolae. Caveolae are small invaginations of the plasma membrane that consists of cholesterol and caveolin, the major structural protein. Caveolae are a subset of lipid-raft domains. These localized regions bind and organise a variety of signalling molecules [Smart et al., 1999]. It has been reported in adipocytes that insulin stimulates caveolin by tyrosine phosphorylation [Mastick et al., 1995] but further investigation confirmed the involvement of the insulin receptor substrate, Cbl [Ribon & Saltiel 1997]. The adaptor protein, APS serves as a docking site for Cbl phosphorylation that recruits it to the insulin receptor [Ahmed et al., 2000]. Cbl phosphorylation also requires the presence of the Cbl-associated protein (CAP). CAP is expressed in insulin sensitive tissues and is markedly increased in differentiation of 3T3-L1 adipocytes but not pre-adipocytes. The Cbl-CAP complex is recruited to the insulin receptor and subsequently translocated to lipid rafts in the plasma membrane and accumulates in a domain enriched in caveolae [Baumann et al., 2000]. Once phosphorylated by insulin, Cbl recruits the adapter protein CrkII along with the nucleotide-exchange protein C3G. C3G activates the GTPase TC10, a GTP-binding protein, by catalysing
the exchange of GTP for GDP resulting in the activation of TC10 within lipid rafts [Saltiel & Kahn 2001]. 3T3-L1 adipocytes incubated with wortmannin, a PI3K inhibitor, and stimulated with insulin showed complete attenuation of insulin stimulated PKB/Akt phosphorylation. However TC10 activity was unaffected by wortmannin, therefore it seems that TC10 activation occurs in tandem with the PI3K/PKB/Akt pathway [Chiang et al., 2001]. The Cbl/CAP/TC10 pathway serves as a crucial mediator of Glut 4 translocation whereby TC10 activation might directly regulate actin polymerization and rearrangement in adipocytes. However, it is unknown whether the actin rearrangement via TC10 activation occurs in muscle cells [Jebailey et al., 2004]. It has been reported that the actin related protein (ARP) -3 relays signalling from TC10 for actin polymerization or rearrangement to occur. Whether the Cbl/CAP/TC10 pathway exists in cardiac myocytes remained unknown until 2006 when Gupte and colleagues investigated the contribution of this pathway to insulin signalling in the heart. The presence of the Cbl/CAP/TC10 pathway in cardiac tissue isolated from Balb/C mice was demonstrated. Administration of insulin resulted in tyrosine phosphorylation of Cbl and activation of TC10. In addition, Cbl and TC10 activation is downregulated in ob/ob mice, an animal model of insulin resistance and obesity [Gupte & Mora 2006]. In addition, it is well established that the PI3K-dependent pathway is blunted during metabolic stress conditions such as insulin resistance, type 2- diabetes and obesity [Kim et al., 1999]. This may therefore be one of the mechanisms whereby glucose uptake is curtailed in insulin resistance.
**Figure 1.5** Simplified representation of insulin stimulated Glut 4 translocation and subsequent glucose uptake via PI3K/PKB/Akt pathway and Cbl/Cap/TC10 pathway. Adapted from Watson & Pessin 2006; He et al., 2007

### 1.4.4 TERMINATION OF INSULIN ACTION

The status of insulin signalling in cells depends on the balance between the ‘on’ signal induced by association with the receptor and the influence of ‘off’ signals that result in disassociation from the receptor. Along with disassociation of insulin from its receptor, the receptor as well as IRS-1, undergoes rapid dephosphorylation. This suggests the involvement of protein tyrosine phosphatases (PTP) [Drake & Posner 1998]. PTP1B has received a great deal of attention since the lack of the gene encoding for this enzyme in mice resulted in improved insulin sensitivity and enhanced tyrosine phosphorylation of insulin receptor and IRS in response to insulin. PTP1B knockout mice also develop resistance to diet-induced obesity, therefore suggesting a beneficial change in energy uptake and expenditure [Elchebly et al.,...
1999]. It has been demonstrated that insulin signalling can be regulated by lipid phosphatases that dephosphorylates PIP\(_3\). Administration of Src homology 2 domain inositol phosphatase (SHIP), a lipid phosphatase that hydrolyzes PIP\(_3\) to PIP\(_2\) by removal of the 5-OH-group, attenuates PKB/Akt activity and Glut 4 translocation to the plasma membrane in 3T3-L1 adipocytes. Attenuation of PI3K activity results in dephosphorylation of Ser\(^{473}\) and Thr\(^{308}\), along with loss of PKB/Akt activity [Vollenweider et al., 1999].

Another lipid phosphatase, PTEN, that dephosphorylates PIP\(_3\) specifically at the position 3-OH of the inositol ring, can also negatively regulate insulin signalling. Overexpression of PTEN has been shown to downregulate insulin-stimulated Glut 4 translocation in 3T3-L1 adipocytes [Ono et al., 2001]. Both lipid phosphatases seem to attenuate insulin signalling by reducing PIP\(_3\) levels. Ser/Thr phosphorylation of the insulin receptor and specifically IRS protein has been associated with termination or attenuation of insulin signalling. This represents a candidate mechanism by which insulin signalling is attenuated in a state of insulin resistance and will be fully discussed in 1.7.

1.5. NITRIC OXIDE: PHYSIOLOGICAL EFFECTS AND SIGNALLING

In the endothelium, nitric oxide (NO) is produced by eNOS, a Ca\(^{2+}\)-dependent enzyme that belongs to a family of arginine hydroxylases. eNOS catalyses the conversion of L-arginine to NO and L-citrulline, which also requires the involvement of molecular O\(_2\), NADPH, tetrahydrobiopterin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), calcium/calmodulin and heme [Nathan & Xie 1994]. The importance of NO, an endogenously produced cell signalling molecule and uncharged free radical with a very short half life, has been demonstrated in a wide
variety of physiological processes. Normal NO production plays an imperative role in preventing vascular disease because of its potent vasodilatory effects. In addition, eNOS and NO have been implicated in regulation of blood pressure and cardiac contractility. eNOS knockout mice have previously been reported to have increased blood pressure and lack endothelium-dependent vasodilatation [Huang et al., 1995]. Apart from this, NO has been found to regulate platelet aggregation and adhesion to the vascular wall. Furthermore, NO reduces endothelium permeability, controls expression of proteins involved in atherogenesis, reduces the rate of oxidation of low density lipoprotein (LDL) to its proatherogenic form and inhibits the proliferation vascular smooth muscle cells (VSMC) [Li & Forstermann 2000].

Cellular effects of NO can be 3’, 5’- cyclic guanosine monophosphate (cGMP) - dependent or cGMP- independent [Denninger & Marletta 1999]. The cGMP dependent mechanism, involves the activation of soluble guanylyl cyclase (sGC) by NO. sGC catalyzes the production of the second messenger cGMP. The effects of cGMP are frequently mediated by cGMP- dependent protein kinase (PKG). An increase in intracellular cGMP facilitates the removal of intracellular Ca$^{2+}$ that results in vascular relaxation [Vaandrager & de Jonge 1996]. The cGMP-independent mechanism is mediated by reactive nitrogen species, that are produced as a result of NO interaction with O$_2$ and superoxide (‘O$_2$’) but the importance of this mechanism still remains obscure [Denninger & Marletta 1999].

1.5.1 INSULIN- STIMULATED NITRIC OXIDE PRODUCTION

It has been demonstrated that insulin has direct effects on the endothelium to increase NO bioavailability [Ritchie et al., 2004]. A complete insulin signalling pathway regulating NO production has been elucidated in the endothelium [Zeng &
Insulin-stimulated NO production has been attributed to different mechanisms, including interaction with the sympathetic nervous system at the vascular level, the release of adenosine [McKay & Hester 1996], the activation of ion channels [Hasdai et al., 1998], and an increase in the generation of NO by the vascular endothelium [Scherrer et al., 1994a; Zeng & Quon 1996]. Insulin stimulation results in NO-dependent vasodilatation [Steinberg et al., 1994], enhanced blood flow accompanied by increased glucose uptake in skeletal muscle [Baron & Clark 1997]. This required the activation of the insulin signalling pathway that involves the PI3K/PKB/Akt-dependent branch.

It is very well documented that insulin stimulates the production of NO in endothelial cells as well as cardiac myocytes [Zeng et al., 2000]. Insulin binding to its membrane bound receptor [Ritchie et al., 2004] stimulates autophosphorylation of the receptor [Sowers JR. 2004]. In human umbilical vein endothelial cells (HUVEC), overexpression of the wild type of the insulin receptor results in a 3-fold increase in NO production. However this is not observed when HUVEC are transfected with a tyrosine kinase-deficient mutant insulin receptor [Zeng et al., 2000]. As described in 1.4.1, IRS-1, the major substrate of the insulin receptor, subsequently recruits and allows activation of PI3K [Ritchie et al., 2004]. Several studies have pointed out the importance of PI3K in mediating insulin-stimulated production of NO. Pre-incubation with a PI3K inhibitor, wortmannin, completely blocks NO production in response to insulin [Zeng & Quon 1996]. PKB/Akt phosphorylates human endothelial nitric oxide synthase (eNOS) at Ser^{1177}, equivalent to Ser^{1179} in bovine eNOS. This phosphorylation results in enhanced eNOS activity. In addition, overexpression of a dominant negative mutant of PKB/Akt in HUVEC inhibits production of NO in response to insulin [Zeng et al., 2000].
1.6. THE RENIN-ANGIOTENSIN SYSTEM

It is well known that the renin-angiotensin system (RAS) plays an imperative role in regulating physiological processes of the cardiovascular system [Geisterfer et al., 1988; Xi et al., 1999]. The RAS has long been recognized as an important regulator of systemic blood pressure, renal fluid and electrolyte homeostasis [Unger & Gohlke, 1990; Phillips et al., 1993]. Ang II, the primary biological active peptide, mediates the biological actions of the RAS [Henrion et al., 2001]. The RAS is viewed as a circulating endocrine system, whereby the protease renin, released from the juxtaglomerular cells of the kidney, cleaves the liver-derived macroglobulin precursor angiotensinogen, to produce the inactive decapeptide angiotensin I (Ang I). AngI is then converted to the active octapeptide Ang II by angiotensin-converting enzyme (ACE) within the pulmonary system [Campbell 1987; Johnston 1992; Dinh et al., 2001]. The RAS not only functions as an endocrine system, but also serves local paracrine and autocrine functions in tissues and organs [Metha & Griendling 2007]. Ang II has been found to affect most organs including the heart, kidneys, adrenals, vasculature, liver [Peach 1977] and brain with both beneficial and pathological effects [Xi et al., 1999]. Ang II e.g. has been found to be involved in cardiac hypertrophy, remodelling and hypertension [Baker et al., 1992].

The actions of Ang II are known to be mediated by at least two distinct Ang II receptor subtypes, designated AT1 and AT2 [de Gasparo et al., 1995]. It is very well documented that most of the physiological effects of Ang II are mediated via the AT1 receptor [Timmermans et al., 1993; Griendling et al., 1996]. The AT1 receptor is a member of the seven transmembrane-spanning G protein-coupled receptor family [Timmermans et al., 1993], consists of 359 amino acids and has a molecular mass of 41-kDa [Griendling et al., 1996]. AT1 receptors are widely distributed in target
tissues, including liver, kidney, heart, blood vessels, adrenal gland, brain and lung [Timmermans et al., 1993; Griendling et al., 1996; Dinh et al., 2001].

Ang II binds to the AT2 receptor with similar affinity as to the AT1 receptor [de Gasparo et al., 1995]. The effects elicited by signalling through the AT2 receptors have been reported to functionally antagonize AT1 receptors [Griendling et al., 1996]. The AT2 receptor, encoded by a 363 amino-acid protein, is also a seven transmembrane domain receptor with a molecular weight of 41-kDa but shares only 34% similarity with the AT1 receptor [Mukoyama et al., 1993]. The AT2 receptor is highly expressed in developing foetal tissue but rapidly declines after birth [Lazard et al., 1994], suggesting that it may play an important role in foetal development [Shanmungan et al., 1996].

1.6.1 CARDIAC RENIN-ANGIOTENSIN SYSTEM

Ang II has been reported to be involved in the pathophysiology of cardiac hypertrophy [Baker et al., 1992]. Considering this, it has been proposed that the RAS is activated in hypertrophy. It has been demonstrated that all the components required for Ang II production are present in the heart whereby Ang II is produced locally within the heart and modulates growth promoting effects. The production of Ang II in cardiac myocytes seems to be independent from the systemic RAS [Wollert & Drexler 1999]. Although the heart is geared for the production of Ang II, some of the components have been reported to be taken up from the systemic RAS. In several studies, renin mRNA and protein levels [Van Lutterotti et al., 1994; Danser et al., 1997; van Kesteren et al., 1999] have been measured, however all failed to confirm the existence of renin mRNA, protein levels and activity in cardiac myocytes. It has been proposed that presence of renin in cardiac cells is likely due to uptake
from the circulation, rather than de novo synthesis [Danser et al., 1997]. Similar to the findings regarding renin, it has been demonstrated that mRNA and protein levels of angiotensinogen are undetectable in cardiac tissue [van Kesteren et al., 1999]. In the systemic RAS, the release of renin and angiotensinogen are from the kidney and liver respectively [Campbell 1987; Johnston 1992; Dinh et al., 2001]. However, neonatal cardiac myocytes have been reported to synthesize ACE. When renin and angiotensinogen become available from the circulation through active uptake, ACE activity ensures production of Ang II. Both Ang I and II have been demonstrated in cardiac myocytes. When Ang I is additionally added to cardiac myocytes in culture, it is rapidly metabolized and converted to Ang II. This suggests that cardiac Ang II originate from local production [van Kesteren et al., 1999]. It has been pointed out that the systemic RAS may be concerned with the short-term regulation of cardiovascular homeostasis in response to acute changes in vascular tone or water reabsorption, while cardiac RAS plays a role in long-term regulation of chronic effects brought on by Ang II [Dzau 1988].

1.6.2 PHYSIOLOGICAL EFFECTS OF ANGIOTENSIN II

Ang II stimulation of the AT1 receptor results in the elevation of blood pressure and peripheral vascular tone [Hughes 1998]. Furthermore, Ang II has growth promoting effects, mediating growth and proliferation in cardiac myocytes and vascular smooth muscle cells [Huckle & Earp 1994]. Acute elevated levels of Ang II results in increased levels of AT1 receptor activation. However chronic exposure to Ang II decreases levels of its own receptor. Substantial evidence indicates that Ang II induces hypertrophy in cardiac myocytes [Baker et al., 1992; Sadoshima & Izumo 1993]. Transgenic mice over-expressing AT1 receptors in cardiac myocytes have been found to develop cardiac hypertrophy and remodelling [Paradis et al., 2000].
Similar to agonist-receptor systems, the effects of Ang II on its target tissues seem to be temporary; after stimulation of Ang II, tissue is desensitized and AT1 receptors are down regulated minutes after its activation [Griendling et al., 1986]. Desensitization of tissue to further Ang II stimulation requires receptor phosphorylation of several serine/threonine sites by G-protein coupled receptor kinases (GRK) which initiate and mediate the process of desensitization [Opperman et al., 1996]. Defects in the desensitization process have been reported in vascular disease which links Ang II to several pathological states, including insulin resistance, diabetes [Olsen et al., 2005], endothelial dysfunction [de Gasparo 2002], obesity and development of cardiovascular disease [Mehta & Griendling 2007].

Cardiac myocytes express both AT1 and AT2 receptors [Booz & Baker 1996]. There is contradictory data concerning the role of AT2 receptors in the cardiovascular system. It is believed that AT2 receptors counteract the actions of AT1 receptors [Unger et al., 1996]. In support of this, AT2 receptor gene knockout mice displayed elevated blood pressure compared to wild type mice when intravenously infused with Ang II [Hein et al., 1995]. Furthermore, elevation in blood pressure and heart rate are significantly lower in transgenic mice with over-expressed cardiac AT2 receptors compared to wild type mice when infused with Ang II. According to Masaki et al [1998] Ang II mediates vasodilatory effects via AT2 receptors. In addition, activation of AT2 receptors has been reported to be anti-hypertrophic, suggesting that AT2 receptors have cardioprotective effects [Liu et al., 1997; Brede et al., 2003].
1.6.3 ANGIOTENSIN II SIGNALLING PATHWAYS

The functions of AT1 receptors have been well established in vascular smooth muscle cells (VSMC) while AT2 receptor mediated signalling pathways still remain under intense investigation [Yan et al., 2003]. Shear stress of VSMC is the endogenous stimulus for Ang II release [Sadohima et al., 1993]. It has been reported that Ang II via AT1 receptor subtype initiate a multitude of intracellular signalling cascades in neonatal cardiac myocytes, however the involvement of these signalling cascades in cardiac hypertrophy is not completely understood [Dinh et al., 2001; Mehta & Griendling 2007]. The cardiac AT1 receptor relays intracellular signalling cascades dependent on the activation of G proteins (Fig.1.6 pg.40). Traditionally, the G protein dependent pathway propagates the vasoactive actions of Ang II [Dinh et al., 2001]. In VSMC, activation of AT1 receptors, coupled to Gαq -protein results in activation of downstream effectors phospholipase A2 (PLA2), phospholipase C (PLC) and phospholipase D (PLD). Activation of PLC is the best characterized signalling pathway that produces the second messengers, inositol-1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG) within seconds after Ang II binding to AT1 receptors. IP3 binds to its receptor on the sarcoplasmic reticulum and stimulates the release of Ca2+ from intracellular stores into the cytoplasm. Ca2+ associates with calmodulin and subsequently activates myosin light chain kinase, which results in enhanced interaction between actin and myosin, leading to smooth muscle contraction. DAG allows activation of Protein Kinase C (PKC), which increases the pH during cell contraction [Griendling et al., 1996; Yan et al., 2003].

IP3 and DAG production by AT1 receptor stimulation is likely to contribute to activation of downstream Ser/Thr kinases, which include PKC and the MAPK. Ang II has been reported to activate, extracellular signal-regulated kinase (ERK1/2), c-Jun
N-terminal kinase (JNK) and the stress-activated protein kinase p38 (p38 MAPK) [Touyz & Schiffrin 2000]. MAPK have been implicated in VSMC differentiation and proliferation. It has been demonstrated that Ang II via AT1 receptor activates ERK1/2 only minutes after receptor activation in VSMC. Furthermore, ERK1/2 activation is likely to be Ca$^{2+}$ dependent because inhibition of PLC attenuates ERK activity in VSMC [Eguchi et al., 1996]. The activation of ERK has been reported to be involved in Ang II mediated cell growth and vasoconstriction. Circumstantial evidence implicates ERK activity in cardiac hypertrophy but limited information is available. In addition to ERK activation, JNK and p38 MAPK influence cell survival and proliferation and are mediators of Ang II-induced inflammation [Force et al., 1996].

In neonatal cardiac myocytes, Ang II via AT1 receptor induces the activation of the tyrosine kinases ERK [Sadoshima et al., 1995] and JNK [Kudoh et al., 1997]. However, Fischer and colleagues [1998] demonstrated that Ang II via the AT1 receptor in isolated adult cardiac myocytes does not result in increased activation of ERK. Despite the substantial evidence for the activation of the MAPK signalling cascades in cardiac myocytes, their role in Ang II-induced cardiac hypertrophy remains to be elucidated [Kim & Iwao 2001]. It is evident that Ang II is a mediator of oxidative stress, by activation of membrane-bound NADPH oxidase in VSMC [Griendling et al., 1996] and endothelial cells [Zhang et al., 1999]. Reactive oxygen species (ROS) such as superoxide (·O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are known to act as both intra- and intercellular second messengers that mediate signal transduction that cause hypertension and inflammation [Griendling et al., 2000].

AT2 receptor signalling involves activation of PLA$_2$, the NO-cGMP system and protein phosphatases [Dinh et al., 2001]. In various cell lines, AT2 receptors have been found to activate serine and tyrosine phosphatases that result in inhibition of
cell growth [Matsubara 1998]. MAPK phosphatase 1 (MKP-1) [Horiuchi et al., 1997], SH2-domain-containing phosphatase (SHP-1) [Bedecs et al., 1997] and Ser/Thr protein phosphatase 2A (PP2A) [Huang et al., 1996] are activated when AT2 receptors are stimulated and this results in the inactivation of ERK-mediated activity in VSMC. This activation of AT2 receptor resulting in the blockade of ERK activity has been best described in VSMC [Griendling et al., 1997; Berk 1999]. This is poorly investigated in cardiac myocytes. To date it is unknown whether AT2 receptors are coupled to a G-protein [Metha & Griendling 2007] but an inhibitory G-protein (Gı) has been reported to associate with AT2 receptors [Zhang & Pratt 1996]. According to Fischer et al [1998], Ang II stimulation increases MKP-1 mRNA levels that are subsequently attenuated by PD-123319, an AT2 receptor antagonist. Therefore, the increase in MKP-1 mRNA levels was mediated via the activation of AT2 receptors. This suggests that AT2 receptor, stimulation by increasing MKP-1 expression inhibits proliferative responses in cardiac myocytes by inhibiting ERK activation [Fischer et al., 1998].
Figure 1.6 Schematic representation of the role of Ang II in vascular smooth muscle cells (VSMC). Ang II mediates VSMC contraction via AT1 receptor. Adapted from Mehta & Griendling 2007.

1.6.4 FUNCTIONAL INTERACTION OF ANG II AND NO SIGNALLING PATHWAYS

There is a substantial amount of evidence reporting a functional interaction between Ang II and NO signalling pathways in the vascular system. It is has been documented that the principal mechanism of NO action in VSMC is to lower intracellular Ca\(^{2+}\) [Yan et al., 2003]. The activation of PLC and formation of IP\(_3\) are the early events in which Ang II induce Ca\(^{2+}\) release and subsequent VSMC contraction. NO via cGMP/PKG negatively regulates IP\(_3\) formation by directly inhibiting PLC activity [Wu et al., 2000]. Furthermore, the IP\(_3\) receptor on the sarcoplasmic reticulum (SR) is a well known substrate of PKG. It has been demonstrated in VSMC, that PKG phosphorylates the IP\(_3\) receptor resulting in a decreased Ca\(^{2+}\) release and subsequent smooth muscle
relaxation [Komalavilas & Lincoln 1996]. Moreover, cGMP can promote the uptake of Ca\(^{2+}\) into the SR through activation of the SR Ca\(^{2+}\)-pumping ATPase, regulated by phospholamban [Horowitz et al., 1996]. Phosphorylation of phospholamban by PKG, stimulates its activity and promote Ca\(^{2+}\) uptake into SR [Cornwell et al., 1991]. Furthermore, NO decreases \(\text{Ca}^{2+}\) influx into the cell by inhibition of the voltage-dependent activation of L-type Ca\(^{2+}\) channels via a cGMP/PKG mechanisms [Quignard et al., 1997].

Some studies have demonstrated that NO may directly modulate ACE activity. Intravenous administration of the precursor of NO, L-arginine, reduces the plasma concentration of Ang II by attenuating ACE activity [Higashi et al., 1995]. In addition, NO attenuates the actions of Ang II by downregulating the AT1 receptor mRNA expression in VSMC, suggesting that this may be one of the mechanisms by which NO exert its antihypertensive properties [Ichiki et al., 1998]. Ang II mediates NO generation by both AT1 and AT2 receptors, but activation of the AT2 receptor has been proposed to stimulate the production of NO in endothelial cells. However, in rat aorta, the AT2 receptor stimulated NO production in a kinin-dependent mechanism. Therefore it is important to determine whether the generation of NO by Ang II exerts beneficial or detrimental effects [Gohlke et al., 1998].

In endothelial cells, Ang II can stimulate the production of NO by activation of eNOS via its AT1 receptor [Saito et al., 1996; Suzuki et al., 2006] and this is believed to be a vascular protective mechanism [Olson et al., 1997]. Induction of NO production by Ang II via the AT1 receptor is achieved by Ser\(^{1179}\) phosphorylation of eNOS, accompanied by increased production of cGMP [Suzuki et al., 2006]. However, in VSMC, NO levels was decreased due to increases in \(\cdot\text{O}_2^-\) production induced by Ang
II activation of NADPH oxidase, suggesting that endothelial and VSMC respond differently to Ang II stimulation. Stimulation of NADPH oxidase is unique for Ang II, as it is not observed after nor-epinephrine stimulation [Rajagopalan et al., 1996]. Chronic exposure to Ang II results in impaired endothelial function that is likely mediated by the production of ROS. It has been shown that Ang II, in endothelial cells, regulates NO production and expression of eNOS mRNA [Schena et al., 1999]. In addition, in vivo infusion of Ang II, evokes increases in eNOS mRNA and protein levels; however, activation of eNOS by Ang II results in the uncoupling thereof causing ·O₂⁻ production rather than NO production [Mollnau et al., 2002]. Furthermore, introducing an adenovirus encoding for eNOS to VSMC, resulted in increased basal NO production that inhibited Ang II-induced hypertrophy of VSMC, providing evidence that production of NO mediated by Ang II, has beneficial effects in both endothelial and VSMC. In addition to its vasoconstrictive properties, Ang II has vasodilatory actions by the generation of NO [Suzuki et al., 2006].

It is well known that the endothelium is a source of both vasoconstrictors such as endothelin-1 and Ang II and vasodilators which include NO and prostacyclin. Alterations in which the actions of vasoconstrictors outweigh those of vasodilators can result in endothelial dysfunction. Endothelial dysfunction is characterized by decreased bioavailability of NO or reduced eNOS expression [Laight et al., 2000]. Endothelial dysfunction has been directly linked to increased oxidative stress that promotes alterations in vasorelaxation, smooth muscle proliferation and thrombosis. Although the principal target of Ang II is the VSMC, it also has various effects on the endothelium by induction of ROS and activation of apoptotic signalling pathways [Schena et al., 1999]. AT1 receptor-evoked oxidative stress has been implicated in all states of atherosclerosis. One of the consequences of increased superoxide
production in response to Ang II is inactivation of NO. Loss of NO via this mechanism propagates vascular lesion progression and ultimately impaired endothelium-dependent vasodilatation [Rajagopalan et al., 1996; Werner & Nickenig 2003].

1.6.5 BLOCKADE OF THE RENIN-ANGIOTENSIN SYSTEM
The role of Ang II in the pathophysiology of cardiovascular disease is well known. Inhibition of ACE activity and selective blockade of AT1 receptors are two pharmacological interruptions through which Ang II actions can be targeted [Givertz 2001]. ACE inhibitors have been extensively used clinically for the treatment of hypertension and heart failure [Urata et al., 1996]. AT1 receptor antagonists e.g. Losartan were developed clinically for the treatment of hypertension [Dinh et al., 2001]. However, ACE inhibitors and especially AT1 receptor antagonists exert different pharmacological actions when interrupting the RAS. It has been demonstrated that chronic treatment with an AT1 receptor antagonist results in increased plasma renin activity accompanied by high levels Ang II, due to interruption of a negative feedback mechanism of renin release [van den Meiracker et al., 1995; Shinozaki et al., 2004]. Increased plasma Ang II may cause the stimulation of exposed AT2 receptors. Since AT2 receptors antagonize the actions of AT1 receptors, this may suggest a possible mechanism by which AT2 receptors contribute to the anti-hypertensive and beneficial effects of AT1 receptor antagonists [Kim & Iwao 2000; Dinh et al., 2001].

Rats fed a high salt diet exhibit significantly higher blood pressure in response to Ang II infusion when co-administered with a selective AT2 receptor antagonist [Munzenmaier & Greene 1996]. The expression of AT2 receptors have been reported to be upregulated under certain pathological conditions such as heart failure,
myocardial infarction, vascular injury and postinfarct repair [Unger et al., 1996]. It has been reported that the ratio of AT2 to AT1 receptors are increased in the failing human hearts [Asano et al., 1997]. However, results from an autopsy of a non failing human heart showed that the heart contains approximately 50% AT2 receptors [Tsutsumi et al., 1998]. AT1 receptor antagonists have beneficial effects on cardiac function and hypertrophy after induction of myocardial infarct in rats [Weber 1997]. Cardiac hypertrophy, remodelling and cardiac dysfunction are not alleviated when treated with an AT2 receptor antagonist. This suggests that AT2 receptors have cardioprotective effects [Liu et al., 1997].

It is well documented that the insulin signalling pathway intersects with that of Ang II (will be discussed in section 1.7.3). Interruption in Ang II signalling may augment the insulin signalling cascade, resulting in improved insulin sensitivity and subsequent increased glucose uptake. This can be achieved primarily by antagonism of Ang II receptor blockers (ARB) or reducing Ang II levels by ACE inhibition [Folli et al., 1997]. The RAS and the subsequent production of Ang II are elevated in diabetes [Du et al., 2001]. Already in 1994, Sechi and co-workers found, in the hearts of STZ-induced diabetic rats, upregulation of AT1 receptors independent of alterations in renin concentration, suggesting activation of cardiac RAS in diabetes. Several clinical trials demonstrated that inhibition of the RAS was associated with reduced risk of developing new onset type II diabetes mellitus [Scheen 2004]. The mechanism of this protective effect is believed to involve improvement of both insulin sensitivity and secretion. AT1 receptor antagonists prevent the binding of Ang II and subsequently attenuate Ang II actions [Givertz 2001; Scheen 2004]. Losartan has been found to alleviate the inhibitory effects of Ang II on the insulin signalling cascade in endothelial cells [Andreozzi et al., 2004]. Rats fed a high fructose diet, displayed the
characteristics associated with the state of insulin resistance. Chronic treatment with Losartan not only resulted in elevated Ang II plasma levels but decreased Ang II-induced vasoconstriction, superoxide production and normalized blood pressure [Shinozaki et al., 2004]. ACE inhibitors and ARB are also used as treatment for endothelial dysfunction. These pharmacological treatments ultimately result in the improvement of endothelial function and blood pressure with reduced inflammation markers in the circulation [Shinozaki et al., 2004; Kim et al., 2006].

1.6.6 THE RENIN-ANGIOTENSIN SYSTEM & OBESITY

There is a considerable amount of evidence that links obesity to hypertension. It is well established that the RAS plays an important role in blood pressure control. Various studies from experimental animals and humans have suggested enhanced activation of the RAS associated with obesity and hypertension [Hall 2000]. In addition, substantial data supports the existence of the RAS in various tissues, playing an important role in the local production of Ang II [Engeli et al., 2000; Boustany et al., 2004]. Extensive studies have demonstrated, in rodents and humans, the presence of most components of the RAS necessary for Ang II production in adipose tissue [Engeli et al., 2000]. More than two decades ago the presence of angiotensinogen-mRNA was found in brown adipose tissue [Campbell & Habener 1987]. In humans angiotensinogen expression has been confirmed in adipose tissue [Karlsson et al., 1998; Engeli et al., 1999], primary cultured adipocytes [Jones et al., 1997; Engeli et al., 1999] and differentiating preadipocytes [Schling et al., 1999]. Angiotensinogen gene expression has been demonstrated to be augmented in obesity and reduced during starvation [Frederich et al., 1992]. Furthermore, enhanced expression and secretion of angiotensinogen is a characteristic feature of preadipocyte differentiation and can be considered as a late
marker of adipocyte differentiation [Schling et al., 1999]. Studies have demonstrated that overexpression of the angiotensinogen gene in adipose tissue of mice results in elevated plasma angiotensinogen and modest hypertension [Massiera et al., 2001]. Boustany and colleagues [Boustany et al., 2004] demonstrated that in a diet-induced obesity rat model, adipose-derived angiotensinogen contribute to the systemic RAS and influenced blood pressure.

In addition, renin-mRNA has been detected in human adipocytes [Karlsson et al., 1998]; however the origin of renin activity in adipose tissue, remains unsolved [Engeli et al., 2000]. The presence of renin in adipose tissue is likely due to the uptake of this enzyme from circulation, as reported for cardiac tissue [De Lannoy et al., 1997]. The production of Ang II, together with the presence ACE-mRNA [Karlsson et al., 1998; Engeli et al., 1999] has been reported to increase during differentiation of human preadipocytes [Schling et al., 1999]. It is evident that circulating Ang II is increased in diet-induced obese rats and correlates with mean arterial pressure, further demonstrating a link between obesity and hypertension [Boustany et al., 2004]. The presence of the AT1 receptor in adipose tissue was first identified in 1993 [Crandall et al., 1993] and since then mRNA, protein and functional levels of AT1 [Engeli et al., 1999; Schling et al., 1999] and AT2 receptors [Darimont et al., 2004] have been identified in rodent and human adipocytes. Presence of these components in adipose tissue confirms existence of adipose tissue RAS, which is likely to contribute to the development of insulin resistance [Engeli et al., 2003].

1.7. INSULIN RESISTANCE: AN INTRODUCTION

For many years, the term insulin resistance has been exclusively associated with the inability of insulin to regulate glucose metabolism without addressing other aspects of
insulin action [Scherrer et al., 1994b]. Insulin resistance has been described as a pathological state in which target tissues such skeletal muscle, adipose tissue and myocardium fail to respond to normal levels of circulating insulin levels [Saltiel 2001; White 2002]. It is described by attenuated insulin-stimulated glucose uptake in skeletal muscle and adipose tissue together with reduced ability of insulin to suppress glucose production by the liver [Saltiel 2001; White 2002; Gaul et al., 2005]. The pancreatic β cells, at the early stage of the pathology, first compensate for peripheral insulin resistance by augmenting insulin secretion to maintain euglycemia [Reaven 1988]. This hyperinsulinemia exacerbates insulin resistance and contributes to β cell failure [Saltiel 2001; White 2002]. In adipocytes, insulin resistance results in accelerated lipolysis with subsequent elevation in circulating free fatty acids [Reaven 1988]. Insulin resistance has been reported to be a hallmark in several diseases such as diabetes mellitus [Olefsky et al., 1973], obesity [Kolterman et al., 1980], hypertension [Reaven 1991], metabolic syndrome [NCEP 2002], and heart failure [Shah & Shannon 2003].

1.7.1 MOLECULAR MECHANISMS OF IMPAIRED INSULIN SIGNALLING

It has been suggested that abnormalities in insulin signalling may primarily account for insulin resistance [Lee & Pilch 1994]. Over the last decade, focus has been shifted towards IRS as an essential contributor to insulin resistance. Several mechanisms have been described as responsible for blunted tyrosine phosphorylation of IRS, such as phosphatase-mediated dephosphorylation [Goldstein et al., 1998], kinase-mediated Ser/Thr phosphorylation [Hotamisligil 1999] and proteasome-mediated degradation [Egawa et al., 2000]. The involvement of serine phosphorylation of IRS as desensitizer to insulin’s action has been pointed out [Abe et al., 1998; Sesti et al., 2001]. Enhanced serine phosphorylation of IRS-1, in
particular on Ser\textsuperscript{612}, causes dissociation of the p85 regulatory subunit of PI3K, inhibiting further signalling [Morisco \textit{et al.}, 2006]. A decrease in glucose uptake is linked to reduced tyrosine phosphorylation of IRS-1 and PI3K activation in animal models of insulin resistance and also type II diabetic patients [Sesti \textit{et al.}, 2001].

It has been suggested that several agents are able to induce insulin resistance by activation of Ser/Thr kinases that phosphorylate IRS-1 and inhibit its function [Gual \textit{et al.}, 2005]. These agents include tumor necrosis factor α (TNFα) [Kanety \textit{et al.}, 1995], fatty acids [Shulman 2000], Ang II [Folli \textit{et al.}, 1997] and hyperinsulinemia [Schmitz-Pheiffer & Whitehead 2003]. It is known that elevated levels of fatty acids [Shulman 2000] and Ang II [Sowers 2004] are evident in insulin resistant, obese and type II diabetic patients. Moreover, chronic elevation in insulin levels as a result of insulin resistance further promote the vicious cycle of insulin resistance. Insulin has been found to stimulate serine kinases that promote phosphorylation of IRS-1 [Griffin \textit{et al.}, 1999]. The inhibition of IRS-1 function may represent the unifying mechanistic link between all factors involved in insulin resistance [Gual \textit{et al.}, 2005].

1.7.2 THE FFA PARADIGM LINKING OBESITY TO INSULIN RESISTANCE

Several investigators have shown that obesity and elevated free fatty acids play a major role in the development of insulin resistance. In 1963, Randle and colleagues described the glucose-fatty acid cycle also known as the Randle-cycle that demonstrated that free fatty acids compete with glucose for substrate oxidation in rat muscle cells (Fig. 1.7 A pg.51). They proposed that elevated free fatty acids levels result in increased production of mitochondrial acetyl-CoA/CoA and NADH/NAD\textsuperscript{+} ratios, which inhibit PDH. This is accompanied by an increase in citrate levels that inhibit PFK-1 activity; the key enzyme that regulates glycolysis, resulting in G-6-P
build-up. Accumulation of G-6-P inhibits hexokinase activity and reduces glucose transport [Randle et al., 1963]. Free fatty acids as a mediator of insulin resistance has been supported by various studies, demonstrating that elevated levels of circulating free fatty acids can cause peripheral insulin resistance in both animals and humans. Lowering of free fatty acid levels in diabetic patients, results in the improvement of insulin sensitivity. This further provides evidence that fatty acids may play a causative role in the development of insulin resistance [Santomauro et al., 1997].

In 2000, Shulman and colleagues challenged the conventional Randle’s theory. By a series of studies using NMR spectroscopy, they investigated the cellular mechanisms of insulin resistance in humans. They demonstrated that elevated free fatty acids attenuated G-6-P concentration, suggesting that the initial impairment of FFA-induced insulin resistance occurs at the level of glucose transport, thus opposing the mechanism proposed by Randle’s theory [Shulman 2000] (Fig 1.7 B pg.51). Elevated plasma free fatty acids induce alterations in insulin signalling events by abolishing insulin stimulated IRS1- tyrosine phosphorylation and PI3K activation in humans [Dresner et al., 1999]. Elevated free fatty acids are accompanied by increased fatty acyl-CoA, ceramides and DAG, which upregulate PKC θ activity and, in turn, activate Ser/Thr kinases that induce serine phosphorylation of IRS-1 resulting in attenuated IRS-1 tyrosine activation [Griffin et al., 1999]. Aguirre and colleagues [2000] demonstrated that TNFα induce insulin resistance by enhanced Ser307 phosphorylation of IRS-1 in ovary cells. In support of this, it has been shown that fatty acids increase Ser307 phosphorylation of IRS-1 by a mechanism that requires activation of PKC θ. Fatty acids and TNFα follow the same pathway in the end to downregulate insulin signalling and promote insulin resistance [Yu et al., 2002]. In
3T3-L1 adipocytes and C2C12 myocytes isolated from C57BL/6J mice, PKC θ phosphorylates IRS-1 at Ser\(^{1101}\) that attenuated downstream activation of the PKB/Akt pathway. In addition, a point mutation of Ser\(^{1101}\) to alanine restores insulin signalling in these cultured cells. Ser\(^{1101}\) phosphorylation of IRS seems to be the mediator of the inhibitory effects of PKC θ [Li et al., 2004b]. Furthermore, PKC θ knockout mice are protected from fat-induced insulin resistance [Kim et al., 2004].
Figure 1.6 A schematic representation of the mechanisms of fatty acid-induced insulin resistance in skeletal muscle as proposed by Randle 1963 (A) and Shulman and colleagues (B). Adapted from Shulman 2000
1.7.3 ANGIOTENSIN II INDUCTION OF INSULIN RESISTANCE

Ang II, the biological active peptide of the RAS has been reported to interfere with insulin signalling. Crosstalk between insulin and Ang II signalling has received much attention since hypertension and insulin resistance often coexist and are leading risk factors for cardiovascular disease [Howard et al., 1996]. According to Nickenig et al [1998] insulin upregulates the gene expression of the AT1 receptor, further supporting the association between hypertension and insulin resistance. It has also been demonstrated that there is impaired insulin signalling in essential hypertension [McFarlane et al., 2001]. For instance, the presence of insulin resistance has been found in rodents with genetic hypertension such as Zucker obese and Goto-Kakizaki rats [Ouchi et al., 1996; Cheng et al., 2001]. In addition, overactivity of the renin-angiotensin system is a typical feature of hypertension and this may likely impair insulin signalling and contribute to development of insulin resistance [Yusuf et al., 2001]. Pharmacological blockade of the RAS with angiotensin receptor blockers (ARB) [Henriksen et al., 2001] and ACE inhibitors [Kudoh & Matsuki 2000] which are clinically used for the treatment of hypertension, have been found also to improve insulin sensitivity.

Recent studies have demonstrated that Ang II negatively modulates insulin-mediated action by regulating multiple levels of the insulin signalling cascade. In cultured VSMC, it was demonstrated that Ang II increases serine phosphorylation of both the insulin receptor and IRS-1, thereby inhibiting insulin-stimulated tyrosine phosphorylation of IRS-1. Furthermore increased IRS-1 serine phosphorylation inhibits the interaction of IRS-1 with PI3K. In addition, Ang II increased serine phosphorylation of the p85 subunit of PI3K [Folli et al., 1997].
Ang II acting through its AT1 receptor is therefore likely to inhibit the actions of insulin in vascular tissue, in part, by interfering with insulin signalling. This downregulation of insulin signalling is likely to be mediated by the generation of reactive oxygen species (ROS), primarily through activation of membrane-bound NADPH oxidase [Griendling et al., 1994; Sowers 2004]. ROS are key intracellular second messengers that activate downstream signalling molecules, including PTP and protein tyrosine kinases [Touyz et al., 2003]. Treatment of rat aortic VSMC with an NADPH oxidase inhibitor, resulted in the inhibition of especially superoxide (\(\cdot O_2^-\)) production [Griendling et al., 1994]. Furthermore, PTP-1B has been demonstrated to play a role in Ang II receptor mediated signalling. Besides PTP-1B, several other phosphatases such as PTP\(\alpha\), CD45, LAR and SHP-2 have been found to dephosphorylate the insulin receptor. Marrero and colleagues [2004] demonstrated that Ang II induced the activation of PTP-1B in VSMC. Pre-incubation of VSMC with a PTP-1B antisense alleviated impaired insulin signalling induced by Ang II. This proposes that PTP-1B plays a key role in downregulation of the insulin signalling cascade induced by Ang II.

With this in mind, it has been demonstrated that, in VSMC isolated from rat thoracic aorta, Ang II extensively decreased IRS-1 protein levels by Ser\(^{307}\) phosphorylation of IRS-1 that subsequently result in proteasome-dependent degradation [Taniyama et al., 2005]. On the other hand, in endothelial cells, Ang II induced insulin resistance through the phosphorylation of IRS-1 at Ser\(^{312}\) and Ser\(^{616}\) via JNK- and ERK1/2-dependent mechanisms respectively. This compromises the interaction of IRS-1 with the p85 regulatory subunit of PI3K and results in downregulation of the insulin signalling pathway involving PI3K/PKB/Akt/eNOS and NO production [Andreozzi et al., 2004]. Furthermore in VSMC, Ang II inhibits insulin-induced PKB/Akt activation in a time and dose-dependent manner through increased phosphorylation of IRS-1.
which is dependent on PKCα. VSMC incubation with a PKC inhibitor relieved the attenuation of insulin-induced activation of PKB/Akt by Ang II, suggesting that upregulation of PKC is an alternative mechanism for the association between insulin resistance and hypertension [Motley et al., 2003]. In addition, Ang II inhibits insulin-stimulated glucose uptake in rat aortic smooth muscle by increased phosphorylation of IRS-1 at Ser$^{307}$ and Ser$^{616}$ via activation of ERK1/2 and JNK. Inhibition of ERK1/2 alleviated suppressed insulin-induced glucose uptake, suggesting that Ang II via ERK1/2 inhibits insulin stimulated glucose uptake [Izawa et al., 2005].

1.7.4 INSULIN RESISTANCE AND ENDOTHELIAL DYSFUNCTION

A decade ago, it had been demonstrated that insulin-induced glucose uptake in skeletal muscle and adipose tissue requires NO [Roy et al., 1998]. Mice that lack the gene for eNOS have been found to exert overt insulin resistance, suggesting that endogenous eNOS-NO may play an important role in insulin responsiveness and that dysregulation thereof contribute to the pathogenesis of insulin resistance and development of type II diabetes mellitus [Shankar et al., 2000]. Substantiating this conclusion, eNOS activity and subsequently NO-dependent vasodilatation, have been found to be chronically impaired in diabetic patients [Mäkimattila et al., 1996]. Insulin resistance is a prominent feature of metabolic disorders such as obesity and type II diabetes mellitus and a component of cardiovascular diseases including hypertension, atherosclerosis and coronary artery disease. Insulin resistance presents itself also as endothelial dysfunction in these mentioned cardiovascular disorders [Reaven 1993]. However, endothelial dysfunction is thought to precede the development of cardiovascular disease. A multitude of factors have been elucidated as contributors of insulin resistance and endothelial dysfunction, including glucotoxicity, lipotoxicity and inflammation [Kim et al., 2006]. Considering that the
PI3K/PKB/Akt pathway results in insulin stimulated glucose uptake and NO production; it is postulated that alterations in insulin signalling may represent the best mechanism underlying the association between endothelial dysfunction and insulin resistance [Ritchie et al., 2004].

1.8 MOTIVATION AND AIMS

In view of our lack knowledge regarding the influence of activation of the RAS in obesity on the development of myocardial insulin resistance and NO production, this study utilized the ARB, Losartan to dissect this problem. Intermediates and downstream signalling events of the insulin signal transduction pathway were studied in an animal model of diet-induced obesity presenting with elevated Ang II levels.

We hypothesized that the raised Ang II levels will negatively impact on PKB/Akt phosphorylation, eNOS activation and NO production that can be elicited by insulin in hearts from obese, insulin resistant rats and that treatment with Losartan will alleviate this.

Our initial aims were:

1. To characterize the effects of obesity on the myocardial insulin signaling pathway with specific reference to the proteins IRS-1, PKB/Akt, eNOS and p38 MAPK in correlation with NO production.

2. To investigate the effect of Losartan treatment on these parameters.

The outcome of this part of the study, pointed to the possible involvement of the AT2 receptors in the observed changes. However, due to financial implications, utilization of the available AT2 receptor blocker PD 123319 in the intact animal or in isolated,
perfused hearts was impossible. We therefore set out to develop and characterize a suitable cell-based model to address this problem. This was done by culturing neonatal cardiac myocytes in a high fat environment to simulate the changes documented in hearts from animals treated with the high caloric diet. If changes reflected the changes seen in the hearts from the animal model, it would enable us to use the AT2 blocker to answer the questions posed by the results of the first part of our study.

The aims of the second part of the study were therefore:

1. To establish a culture of neonatal rat cardiac myocytes
2. To treat these cardiac myocytes with raised fatty acids and determine the changes elicited by the treatment
3. If the cells became resistant to insulin stimulation, to subject them to a combination of raised fatty acids and Ang II to determine effects on insulin sensitivity, and
4. If similar changes were induced as seen in the intact animals, to dissect the involvement of the AT1 and the AT2 receptors using specific blockers of these receptors.
CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

HEPES, pyruvate, 2, 3-butanedionemonoxime (2, 3-BDM), propidium iodide, pancreatin, newborn calf serum (NBS), palmitic and oleic acid were obtained from Sigma-Aldrich (St Louis, MO). Dulbecco’s Modified Eagles Medium (DMEM), fetal calf serum (FCS), and penicillin/streptomycin/fungizone were purchased from Highveld Biological (Johannesburg). Collagenase Type II was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Bovine serum albumin (BSA) fraction V, fatty acid free was obtained from Roche (Cape Town). Commercially available primary antibodies against PKB/Akt Ser\(^{473}\), Akt, PTEN, PTEN Ser\(^{380}/\text{Thr}^{382}/\text{Tyr}^{383}\), AMPK, AMPK Thr\(^{172}\) and Glut 4 were obtained from Cell Signaling. In addition eNOS, eNOS Ser\(^{1177}\), IRS-1, IRS-1 Ser\(^{612}\) p38 MAPK and p38 Thr\(^{180}/\text{Tyr}^{182}\) were also purchased from Cell Signaling Technology (Beverly, MA). Antibodies against Glut 1 and PPAR α were obtained from Santa Cruz Biotechnology Inc. Horseradish-peroxidase-labelled secondary antibody and the ECL detection system were purchased from Amersham life Science. Losartan was a kind gift from Merck Biochemicals. Ang II (Euria-angiotensin II) and insulin (Coat-a-count\textsuperscript{®}) radioimmunoassay (RIA) kits were purchased from Euro Diagnostica, Malmoe, Sweden and Diagnostic Products respectively.
2.2 METHODS

2.2.1 ANIMAL MODEL

All animals received humane care in accordance with the National Institute of Health “Guide for the care and use of laboratory animals” (NIH publication no. 80-23, revised in 1985). The study was assessed and approved by the Committee for Experimental Animal Research of the Faculty of Health Sciences of the Stellenbosch University. Animals had free access to food and water and were kept in an AAALAC accredited facility, subjected to constant humidity at a regulated temperature of 25°C and a 12 h light/dark cycle.

In our laboratory we have an established model of diet-induced obesity (DIO) which displays several characteristics of the metabolic syndrome but these animals are pre-diabetic. Rats are placed on a high caloric diet (rat chow supplemented with sucrose and condensed milk) for a period of 16 weeks (see table 1). The control animals receive a standard rat chow diet for the 16 week period. Obesity is induced by means of hyperphagia [Pickavance et al., 1999]. DIO animals have a higher intake of calories per day compared to their age-matched controls.

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<td>Carbohydrates</td>
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</tr>
<tr>
<td>Protein</td>
<td>30 %</td>
<td>19 %</td>
</tr>
<tr>
<td></td>
<td>371 ± 18 kJ/day</td>
<td>570 ± 23 kJ/day</td>
</tr>
</tbody>
</table>
Male Wistar rats with an initial weight of 180 to 200g were randomly divided into two
groups and were placed on separate diets for a period of 16 weeks. One group
received the high caloric diet (DIO) and the control animals received the standard rat
chow. During week 16, half of each group received 10mg/kg/day Losartan, an AT1
receptor antagonist, for a period of 1 week in their drinking water. Following the 16
week period on the feeding program the rats were anaesthetized by intra-peritoneal
injection of sodium pentobarbital at a dose of 160 mg/kg. The body weights of fully
sedated animals were recorded. At the time of sacrifice, blood was collected for
blood glucose, plasma insulin and Ang II measurements. In addition, intra-peritoneal
fat was removed and the weight recorded. Blood glucose and plasma insulin and Ang
II were determined with a glucometer (GlucoPlus™) and RIA respectively.

2.2.2 ISOLATED HEART PERFUSIONS

The hearts were rapidly excised from fully sedated rats and arrested in ice-cold
Krebs- Henseleit buffer. The hearts were cannulated onto a perfusion rig and
perfused by retrograde Langendorff perfusion. Krebs-Henseleit bicarbonate buffer
containing in mM: NaCl 119; NaHCO$_3$ 24.9; KCl 4.74; KH$_2$PO$_4$ 1.19; MgSO$_4$ 0.6;
Na$_2$SO$_4$ 0.59; CaCl$_2$ 1.25; glucose 10 was used as perfusate. The buffer was
continuously gassed with 95% O$_2$/5% CO$_2$ during perfusion and kept at 37°C.

Hearts were either perfused for 30 min basal with the perfusate or stimulated for 15
min with 0.03μIU/mL insulin (15 min basal; 15 min insulin). Thereafter, hearts were
freeze-clamped with a pre-cooled Wollenberger tong, plunged into and stored in
liquid nitrogen until biochemical analyses (see section 2.7).
2.2.3 PREPARATION OF VENTRICULAR CARDIAC MYOCYTES

In a separate, independent group of animals, adult ventricular cardiomyocytes were isolated from animals on the high caloric diet and those who received the standard rat chow. The rats were anaesthetized as described earlier (section 2.2). Adult cardiac myocytes were isolated using a previously described method [Fischer et al., 1991] and subsequently modified in our laboratory [Huisamen et al., 2001]. Hearts were excised from fully sedated animals. After excision, hearts were cannulated via the aorta and retrogradely perfused (37°C, gassed with 100% O₂) in a calcium free, HEPES buffer (“Solution A”, containing in mM: KCl 6, Na₂HPO₄ 1, NaH₂PO₄ 0.2, MgSO₄ 1.4, NaCl 128, HEPES 10, D-glucose 5.5, and pyruvate 2, pH 7.4) for five minutes to rinse out the blood. This was followed by perfusion, in a re-circulating fashion, with a HEPES digestion buffer (“Solution B” solution A + 0.7 % BSA (fatty acid free) + 0.1 % collagenase + 15 mM 2,3 BDM) for 30 – 35 min. CaCl₂ was readministered at 20 and 25 min of total perfusion time to reach a total concentration of 200 μM. After digestion, hearts were removed from the perfusion apparatus and the ventricles carefully removed from atrial tissue and vascular remnants.

The ventricular tissue was then gently torn apart and incubated in a post-digestion buffer (“Solution C”: 1 part Solution A (containing 1% BSA, fatty acid free BSA) + 1 part Solution B + 200 μM CaCl₂) for 15 min at 37°C in a shaking waterbath (180 strokes/min). A step-wise readministration of calcium followed until the final concentration reached 1.25 mM. Thereafter, the tissue was filtered through a nylon mesh (200 x 200 μm) and gently centrifuged (100 rpm for 3 min). The cell pellet obtained was resuspended in an incubation buffer (“Solution D”: Solution A + 1.25 mM CaCl₂ + 2 % fatty acid free BSA) and left to stabilise on a slow rotator at room temperature for at least an hour. The average digested heart typically yielded approximately 3-5 million cardiac myocytes. Investigations were repeated on
myocytes preparations from different heart, with a sample size varying from 6-8. Light microscopic assessment reveal that the majority of cells (> 70%) isolated in this manner demonstrated typical rod-shaped morphology with clear striations and exclusion of trypan blue.

2.2.4 MEASUREMENT OF NITRIC OXIDE PRODUCTION IN ISOLATED CARDIAC MYOCYTES

To determine intracellular NO production in the isolated cardiac myocytes, the NO-specific fluorescent probe, diaminofluorescein-diacetate (DAF-2/DA), was used and mean fluorescence intensity determined by (Fluorescence-activated cell sorter) FACS analysis. This NO-detection technique has previously been developed and validated in our laboratory [Strijdom et al., 2004; Strijdom et al., 2006]. Control conditions were simulated by incubating isolated cardiac myocytes in solution D. At the beginning of the experiment (t = 0 min) the samples were incubated with non-limiting concentrations of the cell permeable DAF-2/DA (10μM in 1ml Solution D). DAF-2/DA was present for the full duration of the experiment (180 min) in both control and DIO samples. At the end of the experiments, cells were resuspended in probe-free solution D followed by FACS analysis. Light exposure was avoided as far as possible due to the light sensitivity of the fluorescent probe.

2.3 DETERMINATION OF PLASMA INSULIN LEVELS

The fasting blood samples were collected at the time of sacrifice (see section 2.2) and stored at -20°C. The insulin RIA relies on the principle that a fixed amount of ^125I-labeled insulin competes with insulin, present in the blood sample for sites on an insulin specific antibody. This antibody is immobilized to the wall of the polypropylene tubes. Decanting the supernatant from the tubes isolates the antibody bound fraction.
of the radiolabeled insulin. The radioactivity in the tubes is then counted in a gamma counter. Before the assay was performed, the components were brought to room temperature. All samples were done in duplicate. Uncoated polypropylene tubes were labeled for total counts (T) and non-specific binding (NSB) respectively. Insulin-antibody coated tubes were labelled for standards, controls and plasma samples. 200 µL of the zero calibrator A was pipetted into the NSB and A tubes. 200 µL of the remaining calibrator, control and sample were pipetted in the tubes prepared. 1mL of \(^{125}\text{I}\)-insulin was added to each tube and vortexed. The tubes were incubated for 18-24 h at room temperature. After the incubation period, the tubes were decanted (except for the total counts) using a foam decanting rack and the samples allowed to drain for 2-3 min. The removal of excess moisture enhances the precision of the assay. The radioactivity of each tube was determined in a gamma counter (Cobra II Auto Gamma, A.D.P, South Africa) for 1 min per tube. The standard curve generated was used to determine the concentration of the unknown samples.

2.4 DETERMINATION OF SERUM ANGIOTENSIN II LEVELS

Ang II was assessed by a competitive RIA. Ang II in the blood samples competes with \(^{125}\text{I}\)-labeled Ang II for binding to the antibodies. The bound \(^{125}\text{I}\)-Ang II is inversely proportional to the concentration of Ang II in the blood samples and standards. The bound and free phases are separated by a second antibody bound to the solid phase particles, followed by a centrifugation step. The radioactivity of the bound fractions was measured and a standard curve generated with known standards.

Before the assay, Ang II was enriched from the plasma using phenyl cartridges. These cartridges were prepared by washing with 1 ml of methanol, followed by 1 ml of H\(_2\)O. 2 ml of plasma were passed through the cartridges followed by a wash with 1
ml H$_2$O. The absorbed Ang II peptides were eluted with 0.5 ml of methanol. These extracts were freeze dried and stored. Afterwards all the extracts were reconstituted with 1mL assay buffer and thoroughly vortexed. The samples were placed on ice for the duration of the RIA. Polypropylene tubes were labelled in duplicate for determination of the total counts, standards, controls and the samples.

400 µl of the standard, control and the extracted samples were pipetted in duplicate in corresponding tubes. 400 µl and 600 µl of the assay buffer were pipetted to the 0 standards and NSB respectively. In the end, 200 µl of the Ang II antiserum was added to all the tubes (except the NSB and total counts), vortexed and incubated at 4°C for 6 hours.

Thereafter, the radiolabeled $^{125}$I-Ang II was added to all the tubes, vortexed and incubated at 4°C for 18-22 h. This was followed by the addition of the double antibody to all the tubes, except total counts, vortexed and incubated for a further 60 min. Finally the tubes were centrifuged at 1700xg for 15 min at 4°C. The supernatants were decanted and the residues in the tubes were counted for 2 min each in the gamma counter. The standard curve generated was used to determine Ang II levels in the plasma samples.

2.5 ISOLATION OF PRIMARY NEONATAL CARDIAC MYOCYTES

Wistar rat pups postnatal day 1 to 3 were used for all preparations. Animals were anaesthetized with sodium pentobarbital (40mg/kg), decontaminated with 70 % ethanol, and transferred to the Luminar flow hood. At least 25 neonatal rat hearts were used to prepare cardiac myocytes for one specific independent experiment.

Neonatal cardiac myocytes were originally isolated and cultured by Harary and Farley [1963] and modified ever since. We isolated the hearts from 1 to 3 day old Wistar neonatal rats as previously described by Pinson [1990] with minor
modifications. The beating hearts from sedated pups were surgically removed and kept in ADS buffer that contained in mM: NaCl 116; KCl 5; D-glucose 6; Na₂HPO₄·2H₂O 0.96; MgSO₄·7H₂O 0.81; HEPES 19. Submerging the hearts in ADS buffer ensured the removal of excess blood. The hearts were cut into small pieces of about 1-3 mm³ before digestion (Fig. 2.1).

One of the key steps for successful culture is the dissociation of cells from the heart tissue and this was achieved by an enzyme (collagenase) digestion step. The heart tissue was transferred to a 50 ml conical tissue flask that contained 10 ml steriley filtered collagenase solution (0.4 mg/ml collagenase and 0.6 mg/ml pancreatin in ADS buffer). The heart tissue was digested at 37°C in a water bath for 30 min, while subjected to constant shaking (150 strokes/min). The supernatant was carefully transferred to a 15 ml centrifuge tube and gently spun down at 30xg for 3 min. The cell pellet was suspended in 2 ml NBS, in order to deactivate any traces of collagenase activity and kept at 37°C. The remaining heart tissue was continuously digested by adding 5-10 ml fresh collagenase solution into the tissue flask. Depending on the amount of undigested tissue, digestion and centrifugation steps are repeated for two to three times for 30 min and the cells pooled.

The cell suspension (in NBS) from all digestion steps were centrifuged for 3 min at 30xg. The cells isolated from neonatal rat hearts contain a mixture of muscle as well as non-muscle cells. The second essential step in successful culture is the purification of muscle cells (myocytes) from non-muscle cells. The separation of the mixed cell population is achieved by a Percoll gradient method [Bick et al., 1998]. An alternating Percoll gradient solution with three different densities (in g/ml) (1.082, 1.062 and 1.050) was loaded onto the cell pellet obtained to separate non-myocytes from the myocytes. The cells suspended in Percoll were centrifuged for 20 min at
100xg. This resulted in formation of three different cell layers in which the cardiac myocytes were present in the 1.062 and 1.082 density layers (Fig 2.1 pg.65). Myocytes were carefully transferred to a 15 ml centrifuge tube and washed with ADS buffer, then centrifuged for 3 min at 30xg. The cell pellet was resuspended in 10 ml DMEM containing 20% heat inactivated FCS and 1 % penicillin/streptomycin in order to do a cell count with a Neubauer chamber. Finally the cells were plated onto tissue culture dishes pre-coated with fibronectin at a density of 0.80 x 10^6 cells/mm^2 and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Before experimental use, the cultured cardiac myocytes were allowed to reach confluence.

\[\text{Neonatal rats 1-4 days}\]

\[\text{Cardiac tissue Collagenase digestion}\]

\[\text{centrifugation}\]

Non-muscle cells

\[\text{Discontinuous Percoll gradient}\]

Myocytes

Figure 2.1 Schematic representation of the isolation of neonatal cardiac myocytes.
2.5.1 FATTY ACID TREATMENT OF NEONATAL CARDIAC MYOCYTES

The plasma from control and DIO animals as described in 2.2.1 were collected and analyzed by gas chromatography to determine which fatty acids were elevated by the high caloric diet. It was found that the saturated fatty acid, palmitic acid (palmitate) and the mono-unsaturated fatty acid, oleic acid (oleate) were significantly increased in the plasma of DIO rats.

Cardiac myocytes were cultured for 48 hours with the complete growth media before addition of palmitate and oleate at a ratio of 1:1. Palmitate and oleate were freshly made up from a 50mM stock solution (in 100% ethanol) kept at -80°C. 50 mM of palmitate and oleate were dissolved in DMEM that contains 20% FCS to yield a final concentration of 0.25 mM each. Fatty acids were supplemented to confluent, spontaneously contracting myocytes 48 hours after isolation. Similarly, a 100% ethanol was diluted and added to the control cells. Cells were treated with fatty acids for a period of 4 days at 37°C in a humidified atmosphere. About 18 hours prior to insulin stimulation or glucose uptake assay, serum-containing media was removed from cells treated with or without fatty acids and the cells incubated with DMEM.

2.5.2 INSULIN STIMULATION

The insulin was first prepared as a stock solution of 10 μM. On day 8, half of the cells were stimulated with 10nM of insulin for 15 min at 37°C. Immediately following insulin stimulation, media was removed and cells were placed on ice. Afterwards cells were washed with cold phosphate-buffered saline (PBS) (pH 7.4) and prepared for biochemical analysis via Western blotting as described in section 2.7. PBS contained in mM: NaCl 137, KCl 3, Na₂HPO₄.2H₂O 7.98 and KH₂PO₄ 1.47.
2.5.3 ASSESSMENT OF CARDIAC MYOCYTE VIABILITY

Cell viability of neonatal cardiac myocytes was assessed by two independent indices of viability, (i) propidium iodide (PI) and (ii) trypan blue exclusion staining. Serum-deprived cells were washed three times with PBS (pH 7.4). Afterwards, cells were detached from the plates with 2.5 ml of Trypsin-EDTA (Sigma-Aldrich) for 20-60 min. Detached cells were centrifuged at 30xg for 3 min, the supernatant discarded and the pellet suspended in 1ml ADS buffer. This was followed by assessment of cell viability by either trypan blue or PI staining.

**PI staining:** Cell membrane permeability and nuclear staining by PI was assessed by FACS analysis. Cardiac myocytes were incubated with 10μM PI for 15 min before FACS analysis. Data are expressed as mean fluorescence intensity as % of control.

**Trypan blue:** The yield and viability of cells were determined by using a 0.4 % trypan blue solution (in PBS). A 1:1 ratio of cell suspension (e.g. 30μl) to trypan blue (30 μl) was used. Cells that excluded the trypan blue dye were considered as viable. Cell viability was expressed as a percentage of the viable cells over the total number of cells (including trypan blue stained cells). Assessment of trypan blue staining was performed under light microscopy using a haemocytometer.
2.6 DETERMINATION OF 2-DEOXY-D-\(^{3}\)[H] GLUCOSE (2DG) UPTAKE BY NEONATAL CARDIAC MYOCYTES

2DG uptake was measured as described previously [Donthi et al., 2000; Huisamen et al., 2001]. Following the 4 day treatment with or without fatty acids, cardiac myocytes were serum deprived overnight and cultured in DMEM (Fig 2.2). At the end of this period, cells were deprived of all substrates that might be present in the medium. The cells were incubated with 2 ml of Solution E, containing in mM: KCl 6, Na\(_2\)HPO\(_4\) 1, NaH\(_2\)PO\(_4\) 0.2, MgSO\(_4\) 1.4, NaCl 128, HEPES 10; CaCl\(_2\) 1.25 and 2% fatty acid free BSA for 3 hours at 37°C in a humidified atmosphere. Determination of myocyte glucose uptake was measured in solution E. Cells were stimulated with or without 1, 10 or 100nM insulin for 15 min in duplicate where after they were incubated with 1.5 μCi/ml 2DG (PerkinElmer, Boston) in a final concentration of 1.8 μM deoxyglucose for 30 min. The reaction was stopped by the addition of 400 μM phloretin in order to stop carrier-mediated (Glut 1 and Glut 4) glucose uptake. Following this, medium containing 2DG was aspirated and cells were washed twice with a basic buffer that contained in mM: KCl 6, Na\(_2\)HPO\(_4\) 1, NaH\(_2\)PO\(_4\) 0.2, MgSO\(_4\) 1.4, NaCl 128 and HEPES 10. Cells were lysed in 250 μl of 1N NaOH at 70°C in a water bath for 30 to 40 min. 250 μl dH\(_2\)O were added to lysed cells to yield a concentration of 0.5 N NaOH. In order to determine cell-associated radioactivity, 100 μl of the cell lysate was mixed with 3ml of scintillation fluid and kept overnight in the dark before counting in a scintillation counter (Beckman). 2DG uptake was presented as pmol 2DG/mg protein/30min. The remaining 400 μl of cell lysate was used for determination of protein content by the method of Lowry [Lowry et al., 1951].
For protein content determination by the method of Lowry [Lowry et al., 1951]; three BSA protein standards of known concentration [0.238 mg/ml; 0.476 mg/ml and 0.952 mg/ml] were used and 0.5 N NaOH used as the blank. Directly before the assay, the reaction buffer, which contained 2 % Na$_2$CO$_3$, 1% CuSO$_4$•5H$_2$O and 2% NaK$^+$ tartrate was prepared. The assay was done in duplicate and 50 μl of blank, standards and samples were used to perform the protein assay. 1 ml of reaction buffer was added to blank, standards and samples, rapidly vortexed and allowed to stand at room temperature for 10 min. Afterwards 0.1 ml Folin-Ciocalteu’s phenol reagent (1:2 dilution) was added, vortexed and permitted to stand for 30 min. This resulted in a colour development of which the absorbance was read at 750 nm against the blank. The standard curve was used to determine the unknown protein concentrations.

![Figure 2.2 Illustration of the assessment of 2DG uptake in neonatal cardiac myocytes.](image-url)
2.7 WESTERN BLOT ANALYSIS

2.7.1 PROTEIN EXTRACTION

The proteins of interest were extracted with a lysis buffer. The lysis buffer, contained (in mM) Tris 20, EGTA 1 (pH 7.5), 1mM sodium orthovanadate (Na$_3$VO$_4$), NaCl 150, Triton-X-100 1%, phenylmethyl sulfonyl fluoride (PMSF) 1, aprotinin 10 μg/ml, leupeptin 10 μg/ml, a complete phosphatase and protease inhibitor cocktail (see addendum). Frozen tissue sample (section 2.2.2) were homogenized (Polytron PT-10 homogenizer, 2x4sec, setting 4) in 0.8 ml lysis buffer and centrifuged at 1000xg for 15 min at 4°C. The supernatant was separated from the cell pellet, kept on ice and used for protein determination according to the method of Bradford [1976].

Isolated adult cardiac myocytes (section 2.2.3) were lysed with 0.1 ml lysis buffer, sonicated twice for 5 sec and centrifuged at 1000xg for 15 min at 4°C. The supernatant was removed from the cell pellet for protein determination [Bradford 1976].

Neonatal cardiac myocytes (section 2.5.1) or cells after 15 min insulin stimulation (section 2.5.2) were placed on ice and washed with PBS. These cells were used for protein determination and quantification analysis. They were lysed with 0.1 ml lysis buffer, sonicated twice for 5 sec and centrifuged at 1000xg for 15 min at 4°C. The supernatant was removed from the cell pellet for protein assessment.

The supernatant from these independent protocols were removed from insoluble material and the protein concentration quantified using the Bradford method [Bradford 1976]. This method is suitable for measuring fractions with low protein concentration. The supernatants were diluted 1:10 in distilled H$_2$O in order to dilute
traces of the detergents (e.g. Triton-X 100) that may interfere with the protein assay. A standard curve is used to determine the unknown protein concentrations of the samples. BSA protein standards containing 1 to 20 μg were made up to a final volume of 0.1 ml before the addition of 0.9 ml Bradford reagent. The Bradford reagent was diluted 1:5 with distilled H₂O before use. An appropriate volume of sample was adjusted to 0.1 ml before addition of the Bradford reagent. All the samples were vortexed after the addition of the Bradford reagent. After 15 min, the absorbance was read at 595nm against a blank (0.1 ml H₂O and 0.9 ml Bradford reagent) in a spectrophotometer. The rest of the supernatants were diluted in Laemmlli sample buffer, boiled for 5 min and stored at -80°C.

2.7.2 PROTEIN SEPARATION

All stored samples were boiled for 5 minutes and centrifuged at 1500 rpm for 2min to remove particulate material. Normalized proteins were subjected to sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (see addendum). Proteins were separated according to their molecular weight using the standard Bio-RAD Mini-Protean III system. The molecular weights of the proteins separated on the SDS-PAGE gel were identified with a protein ladder marker obtained from Fermentas Life Sciences. The proteins resolved by electrophoresis were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon™ P, Millipore) with an applied electrical current (200mA/gel for 1 hour) in a tank filled with transfer buffer. The transfer buffer contained in mM: Tris 25, Glycine 192 and methanol 20%. Afterwards, membranes were immersed in fresh methanol (to dehydrate membrane) and air dried in order to be stained with Ponçéau Red (a reversible staining) for visualization of proteins and monitoring equal loading of samples. The Ponçéau Red was rinsed off where after the non-specific binding sites
on the membrane were blocked by immersing the membrane in 5% fat-free milk made up in a Tris-buffered saline (TBS) + 0.1 % Tween 20 for at least 1 hour at room temperature on a shaker. After the membranes were blocked with the 5% fat-free milk solution, they were washed with three changes of TBS-Tween for 5 min each. These membranes were immuno-blotted with primary antibodies overnight at 4°C.

**IRS-1 and IRS-1 phospho-Ser$^{612}$**

We evaluated IRS-1 and phospho- IRS-1 in the isolated adult cardiac myocytes (prepared in section 2.2.3). 80μg protein was loaded on a 4 % stacking gel and separated on a 7.5 % SDS-PAGE gel. The primary antibody was used at a 1: 1000 dilution in TBS-Tween overnight at 4°C.

**PKB/Akt & Phospho- Ser$^{473}$ PKB/Akt & PTEN, Phospho-Ser$^{380}$/Thr$^{382}$/Thr$^{383}$ PTEN, AMPKα and Phospho-Thr$^{172}$ AMPKα**

30 μg protein of each sample was loaded in a 4 % stacking polyacrylamide gel and separated on a 10 % SDS-PAGE gel. Primary antibodies were used at a 1: 1000 dilution in TBS-Tween. However the primary phospho-Thr$^{172}$ AMPK antibody was used at a 1: 500 dilution in TBS-Tween.

**eNOS and phospho-Ser$^{1177}$ eNOS**

80μg protein was loaded on a 4 % stacking gel and separated on a 7.5 % SDS-PAGE gel. The primary antibody was also used at a 1: 1000 dilution in TBS-Tween overnight at 4°C.
**P38 MAPK and phospho-Thr\textsuperscript{180}/Tyr\textsuperscript{182} p38 MAPK**

30 μg protein was loaded on a 4 % stacking gel and resolved on a 12 % SDS-PAGE gel.

**Glut 1, Glut 4, PPARα**

70μg protein was separated on a 10 % SDS-PAGE gel. Glut 1 and PPARα primary antibodies were used at a 1:250 dilution in 5 % milk solution. Glut 4 primary antibody was used at a 1:1000 dilution in TBS-Tween.

**2.7.3 IMMUNODETECTION OF PROTEINS**

After the overnight incubation with the primary antibodies, the membranes were thoroughly washed in TBS-Tween and incubated with an anti-rabbit immunoglobulin G, horseradish-peroxidase conjugated secondary antibody (from donkey) for 1 hour at room temperature. The secondary antibody was used at a 1:4000 dilution in 2.5 % (for PKB/Akt, Glut 1, Glut 4, eNOS and PPARα) or 5 % (for AMPKα and PTEN) milk-TBS-Tween solution. However, secondary antibody for both p38 MAPK and phospho-p38 MAPK was used at a 1:4000 dilution in TBS-Tween. After 1 hour incubation, membranes were rinsed 3 times for 5 min each with TBS-Tween. Proteins were detected by enhanced chemiluminescence by making use of the ECL detection reagents (Amersham life science) for 1 minute (Fig. 2.3). The excess ECL detection reagent was drained off the membrane and light emission from the membrane then captured on autoradiography film, Hyperfilm™ ECL (Amersham life science). The ECL reagents react with the horseradish-peroxidase. The protein band density was quantified by laser scanning densitometry making use of the program UN-SCAN-IT (Silkscience) to analyse the bands. The normalized data is expressed
as arbitrary densitometry units (AU). Care was always taken to include a control reference on the blots to normalize the data.

Figure 2.3 Illustration of enhanced chemiluminescence. Depicted from Amersham Life Science product booklet.

2.8 STATISTICAL ANALYSIS

Unless stated otherwise, all data are presented as mean ± S.E.M. Statistical significance between groups was assessed by one way analysis of variance (ANOVA) or 2 way- ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. Alternatively an unpaired Student T test was used to compare statistical difference between two groups (Control vs. fatty acid treated cells); P < 0.05 is considered as statistically significant. Statistical analysis of data was performed using GraphPad Prism 5.
2.9 ADDENDUM: Chapter 2

Preparation of palmitate and oleate stock solutions

Palmitate: 0.38 g was dissolved in 30 ml 100% ethanol (subjected to heat)

Oleate: 0.5 ml was dissolved in 30 ml 100% ethanol (subjected to heat)

Aliquot fatty acids separately in dark eppendorfs

Before storage at -80°C gas fatty acids with nitrogen in order to prevent oxidation.

Lysis buffer for protein extraction

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<th>Reagent</th>
<th>Concentration</th>
</tr>
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<tr>
<td>NaCl</td>
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**SDS polyacrylamide gel**

<table>
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<tr>
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<td>50 µl</td>
<td>50 µl</td>
</tr>
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<td>20 µl</td>
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</table>
3.1 EXPERIMENTAL ANIMALS: CHARACTERISTICS

Table 2. Characteristics of the experimental animals after the 16 week feeding program.

<table>
<thead>
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<th>Parameters</th>
<th>Control</th>
<th>DIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>350 ± 11.13 g</td>
<td>395 ± 7.57 g **</td>
</tr>
<tr>
<td>Intraperitoneal fat</td>
<td>10.60 ± 0.61 g</td>
<td>17.78 ± 1.13 g ***</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>4.59 ± 0.11 mmol/l</td>
<td>5.36 ± 0.22 mmol/l **</td>
</tr>
<tr>
<td>Plasma insulin level</td>
<td>31.38 ± 2.84 μIU/mL</td>
<td>49.47 ± 6.17 μIU/mL**</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>0.021 ± 0.003 nM</td>
<td>0.042 ± 0.002 nM**</td>
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</table>

We found that, after the 16 week feeding program, the rats on the high caloric diet displayed a significant increase (395 ± 7.57 g vs. 350 ± 11.13 g; p < 0.01) in body weight (became obese) compared to the control animals. Obesity was associated with significant elevation in plasma insulin levels (49.47 ± 6.17 μIU/mL vs. 31.38 ± 2.84; p < 0.01) that suggests that our animals became insulin resistant. We found that there was an apparent increase in intraperitoneal fat (10.60 ± 0.61 g vs. 17.78 ± 1.13 g; p < 0.0001) and blood glucose levels (5.36 ± 0.22 mmol/l vs. 4.59 ± 0.11; p < 0.01) in DIO compared to the control animals. Furthermore, the DIO animals had significantly elevated Ang II levels. This suggests that, in our DIO animals, elevated insulin levels were needed to maintain normal blood glucose levels, indicating a state of insulin resistance.
3.2 MYOCARDIAL IRS-1 CONTENT

We evaluated the total protein content of IRS-1 in isolated adult cardiac myocytes from control and DIO animals (Fig. 3.1). There was no apparent difference in IRS-1 content.

3.2.1 SERINE PHOSPHORYLATION OF IRS-1

It is very well documented that Ser phosphorylation of IRS-1 results in attenuated insulin signalling events [Shulman 2000; Andreozzi et al., 2004]. We measured the phosphorylated state of IRS-1 in adult cardiac myocytes (Fig 3.2). There was a significant increase in Ser$^{612}$ phosphorylation in myocytes from DIO, compared to the control animals, underscoring our deduction that the DIO animals were insulin resistant.
Figure 3.1 Myocardial IRS-1 protein content in isolated cardiac myocytes from control and DIO animals were determined via Western blotting as described in material and methods (Chapter 2). The insert is a representative blot. Data expressed as mean ± SEM. (n = 6 individual cell isolates, assayed in duplicate)

Figure 3.2 Serine phosphorylation of IRS-1 in isolated adult cardiac myocytes from control and DIO animals. Ser^{612} phosphorylation was determined via Western blotting as described in material and methods. The insert is representative blot. Data presented as mean ± SEM *p < 0.05 (n = 6 individual cell isolates, assayed in duplicate).
3.3 MYOCARDIAL TOTAL PKB/AKT

We evaluated the total protein content of PKB/Akt in hearts from the different groups of animals (Fig. 3.3). Under basal conditions, the expression of PKB/Akt was significantly higher in hearts from the DIO animals. When animals were treated with Losartan, we found that in control hearts, PKB/Akt protein content was significantly attenuated compared to untreated animals. In the DIO animals, the PKB/Akt protein content showed a diametrically opposite reaction with significantly more protein expressed in hearts from Losartan treated animals than in hearts from untreated animals. Stimulation of the hearts with insulin did not alter this profile. These results suggest that the AT1 receptor modulates PKB/Akt protein expression.

3.3.1 PHOSPHORYLATION OF PKB/AKT

In view of the elevated plasma insulin levels observed in our DIO animals (see table 2) and the indications of development of insulin resistance, we investigated whether the insulin signalling cascade was downregulated by measuring the phosphorylation state of PKB/Akt, the key mediator of the metabolic effects of insulin [Kim et al., 1999] (Fig. 3.4). There was no significant difference in the basal phosphorylation of PKB/Akt on Ser\(^{473}\) between control and DIO hearts. However perfusion with insulin for 15 min elicited a significant increase in PKB/Akt phosphorylation (5.83 ± 1.89 vs. 1.28 ± 0.09 AU; p < 0.05) in control animals but this is not seen in the DIO animals. In support of the elevated plasma insulin levels in DIO animals and the higher Ser\(^{612}\) phosphorylation of IRS-1, the inability of insulin to induce PKB/Akt phosphorylation in DIO animals demonstrated that these animals are insulin resistant. Treatment with Losartan had no affect on phosphorylation of PKB/Akt either in the presence of the absence of insulin.
Figure 3.3 Total protein levels of PKB/Akt in control and DIO animals were determined via Western blotting as described in material and methods. The inserts are representative bands from Western blots. Data presented as mean ± SEM. * p < 0.05; **p < 0.01 (n = 4 individual hearts, assayed in duplicate)

Figure 3.4 Phosphorylation of PKB at Ser\textsuperscript{473} in the control and DIO animals were determined via Western blotting as described in material and methods. The inserts are representative bands from Western blots. Data presented as mean ± S.E.M. * p < 0.05; ***p< 0.001 (n = 4 individual hearts, assayed in duplicate)
3.4 MYOCARDIAL TOTAL eNOS

We evaluated the total protein content of eNOS in all the different groups (Fig. 3.5). There was not difference in the protein content of eNOS between control and DIO animals. However when animals were treated with the AT1 receptor antagonist, Losartan, we found that in the control animals, there was a significant attenuation (0.68 ± 0.09 vs. 1.32 ± 0.11 AU; p < 0.01) of eNOS protein content compared to the untreated group. In contrast, eNOS protein content was significantly augmented in the DIO animals when treated with Losartan. Stimulation with insulin did not alter this profile. Similar to PKB/Akt (Fig 3.1), this result indicates that the AT1 receptor modulates the expression of eNOS.

3.4.1 PHOSPHORYLATION OF eNOS

PKB/Akt phosphorylates eNOS on Ser1177, and in turn, eNOS activation results in the production of NO in cardiac myocytes [Zeng et al., 2000]. We measured the phosphorylated state of eNOS in the presence and absence of insulin in groups treated with or without Losartan (Fig. 3.6). Stimulation with insulin resulted in increased phosphorylation of eNOS (1.99 ± 0.27 vs. 1.34 ± 0.21 AU; p < 0.05) in the control animals but this is not seen in the DIO animals. Treatment with Losartan could not improve this.
Figure 3.5 Total protein levels of eNOS in control and DIO animals were determined via Western blotting as described in material and methods. The insert is a representative blot. Data expressed as mean ± SEM. * p< 0.05 **; p < 0.01 ***; p < 0.001 (n = 4 individual hearts, assayed in duplicate)

Figure 3.6 Phosphorylation of eNOS in control and DIO animals were determined via Western blotting as described in material and methods. The insert is a representative blot. Data presented as mean ± SEM *p < 0.05; **P < 0.01 (n = 4 individual hearts, assayed in duplicate)
3.5 MYOCARDIAL P38 MAPK

Because of the alleged importance of the MAPK cascade in insulin signalling, we also evaluated the total myocardial protein content of p38 MAPK in all the groups (Fig. 3.7). Total protein content of p38 MAPK was noticeably increased in the DIO animals in all groups compared to the control animals (p < 0.05).

3.5.1 P38 MAPK PHOSPHORYLATION

In addition, we measured the phosphorylation of p38 MAPK in control and DIO animals (Fig. 3.8). Insulin stimulation did not affect the phosphorylation of p38 MAPK either in control or in DIO animals. Losartan treatment resulted in enhanced phosphorylation of p38 MAPK in both control and DIO animals compared to basal values.
Figure 3.7 Total p38 MAPK in control and DIO animals were determined via Western blotting as described in material and methods. The insert is a representative blot. Data expressed as the mean ± SEM. *p < 0.05 (n = 4 individual hearts, assayed in duplicate)

Figure 3.8 Phosphorylation of p38 MAPK in control and DIO animals were determined via Western blotting as described in material and methods. The insert is a representative blot. Data expressed as the mean ± SEM *p < 0.05; ** p < 0.01 (n = 4 individual hearts, assayed in duplicate)
3.6 NO PRODUCTION IN ISOLATED CARDIAC MYOCYTES

To determine whether the insulin resistant state that we documented in our DIO animals, translated also into changes in NO production, we determined the ability of cardiac myocytes from Losartan treated vs. untreated animals to produce NO. The DAF/2A probe used as a direct measure of NO production in isolated cells, showed that in cardiac myocytes from the DIO animals, basal NO production was significantly lower compared to the control cells (Fig 3.9). Losartan resulted in attenuated NO production in control animals. In contrast, Losartan treatment augmented NO production in the cells from DIO animals. These results clearly underscore the eNOS protein expression found in the hearts of control and DIO animal treated with Losartan (Fig 3.5).

Figure 3.9 NO production in control and DIO animals with the DAF/2A probe as described in materials and methods. The data is presented as the mean ± SEM * p< 0.05 (n = 6 individual cell preparations, assayed in duplicate)
3.7 DISCUSSION

It is well recognized that the increased incidence and prevalence of obesity is a major contributor to the increased prevalence of diabetes [Kopelman & Hitman 1998]. One of the hallmarks of diabetes is insulin resistance and this, as well as obesity, are risk factors for the development of cardiovascular disease. It is suggested that NOS activity and NO production are chronically impaired in diabetic patients. Furthermore, the RAS and the subsequent production of Ang II have been reported to be significantly elevated in diabetes [Du et al., 2001]. The activation of the RAS and elevated Ang II production have negative consequences on conditions such insulin resistance and type II diabetes. This is supported by the observations that AT1 receptor antagonist, Losartan, elicits significant improvements in insulin sensitivity in conditions such as insulin resistance and type II diabetes [Andreozzi et al., 2004; Shinozaki et al., 2004].

In this present study we aimed to address the importance of these changes in cardiovascular pathology using a rat model of diet-induced obesity. We (i) characterized the expression and activation of key proteins IRS-1, PKB/Akt and eNOS in the heart that ultimately lead to NO production, and correlated this with the measurement of NO in cardiac myocytes and (ii) evaluated the effect of Losartan on these parameters.

3.7.1 IMPAIRED INSULIN SIGNALLING

Our data showed that our rat model of diet-induced obesity resulted in myocardial insulin resistance. We documented elevated blood glucose, plasma insulin, plasma Ang II levels (Table 2) and an altered phosphorylation state of key proteins in insulin signalling events (Fig 3.2, 3.4 & Fig 3.6). A considerable amount of evidence indicates that augmented serine phosphorylation of IRS-1 results in the
downregulation of the insulin signalling cascade [Folli et al., 1997; Shulman 2000; Andreozzi et al., 2004]. We attempted to investigate the serine phosphorylation of IRS-1 in whole hearts but could not detect differences with the available antibodies (data not shown). However, in isolated adult cardiac myocytes from DIO animals (Fig. 3.2), Ser$^{612}$ phosphorylation was significantly augmented compared to the control animals, without changes in total myocardial IRS-1 protein (Fig 3.1). Increased serine IRS-1 phosphorylation is postulated to sterically hinder tyrosine phosphorylation, therefore activation of IRS-1. This therefore suggests attenuation of signalling events induced by insulin at the level of IRS-1. Increased serine phosphorylation of IRS-1 has previously been described in C2C12 myocytes and 3T3-L1 cells under conditions of elevated fatty acids [Griffin et al., 1999]. As discussed in 1.7.2, TNFα and PKCθ mediate negative effects not through serine phosphorylation on Ser$^{612}$-IRS-1. Therefore, the obesity-induced serine phosphorylation of IRS-1 in our model is because of a different mechanism. According to Andreozzi et al [2004], Ang II impairs insulin signalling events at Ser$^{616}$ in HUVEC, analogous to Ser$^{612}$ in rat IRS-1. Therefore, obesity-induced serine phosphorylation of IRS-1 in our model is likely due to the elevated Ang II levels. The work done by Folli et al [1997] demonstrated that Ang II inhibits the insulin signaling events at multiple levels. Besides increased serine phosphorylation of IRS-1; Ang II stimulation of rat aortic smooth muscle cells (RASMC) also resulted in augmented serine phosphorylation of the insulin receptor. Furthermore, Ang II prevented IRS-1 and PI3K association [Folli et al., 1997].
3.7.1.1 PKB/AKT AND eNOS

In view of increased serine phosphorylation of IRS-1 in isolated cardiac myocytes (Fig 3.2) and previous work done in our laboratory demonstrating that insulin stimulated PKB/Akt phosphorylation is impaired in isolated rat cardiac myocytes from Zucker obese rats [Huisamen et al., 2001], we expected insulin signalling events to be impaired in DIO hearts. Physiological concentrations (0.03 μIU/ml) of insulin could only stimulate an increase in phosphorylation of PKB/Akt (Fig 3.4) and eNOS (Fig 3.6) in control animals. PKB/Akt phosphorylation is an important checkpoint for eNOS activation in response to insulin [Zeng et al., 2000]. The inability of insulin to increase PKB/Akt and eNOS phosphorylation underscores that our DIO animals were insulin resistant.

Although there is considerable evidence linking Ang II to the induction of insulin resistance, the mechanism by which RAS inhibition results in improved insulin sensitivity remains poorly understood [Scheen 2004]. Few studies have reported on the effects of Losartan on myocardial insulin-resistance. In this present study, Losartan at a dose of 10 mg/kg/day for a period of 1 week was unable to improve myocardial insulin signalling events. In aortic smooth muscle cells, it was also found that Losartan could not alleviate Ang II inhibitory effects on IRS-1 and PI3K association. However, the use of Saralasin, a different AT1 antagonist, blocked the inhibitory effects of Ang II on insulin signalling [Folli et al., 1997]. Furthermore, Losartan has also been unable to decrease IRS-1 serine phosphorylation in skeletal muscle; whereas ACE inhibition improved insulin sensitivity by increasing insulin stimulated IRS-1 tyrosine phosphorylation [Jacobs et al., 1996]. Wu and co-workers [2004] demonstrated in skeletal muscle from fat-fed, STZ-induced diabetic rats that 4mg/kg/day Losartan for 6 weeks resulted in improved
Insulin sensitivity determined by oral glucose tolerance test. However, this treatment was unable to promote insulin signalling events (i.e. IRS-1 tyrosine phosphorylation and PKB/Akt activation) and did not effect changes in protein levels [Wu et al., 2004].

A substantial amount of evidence in human studies shows that Losartan improves insulin sensitivity [Moan et al., 1996; Justiniani & Messerli 1997]. However, it now seems controversial whether Losartan can improve insulin signalling in states of insulin resistance [Folli et al., 1996; Nakagawa et al., 1999; Wu et al., 2004].

In a previous study done in insulin resistant rats fed a diet high in fructose [Shinozaki et al., 2004], 30mg/kg/day Losartan improved parameters associated with vascular dysfunction. Losartan, at this dose, for a period of 8 weeks was sufficient to normalize blood pressure and vascular NADPH oxidase activity and attenuated superoxide production. Ang II has been reported to mediate vasoconstrictive effects and impaired eNOS action by the activation of vascular NADPH oxidase [Rajagopalan et al., 1996]. Shinozaki and colleagues [2004] demonstrated increased eNOS activity in an insulin resistant model after Losartan treatment. Improved insulin sensitivity induced by Losartan might therefore be dose and time dependent.

In this study, we used 10mg/kg/day Losartan to explore effects on insulin signalling because Losartan at a dose of 1mg/kg/day has been reported to already improve blood pressure [Navarro-Cid et al., 1995].

It has previously been demonstrated that AT2 receptors do not contribute to impaired insulin signalling events, considering that antagonism with PD123319 did not alleviate Ang II inhibitory effects on insulin signalling cascade [Folli et al., 1997]. However, according to Unger et al [1996], activation of the AT2 receptors counteracts the actions induced by AT1 receptor activation. Therefore it has been proposed that
the AT2 receptor signalling effects are unmasked in the presence of AT1 receptor antagonism.

3.7.1.2 p38 MAPK

In our model of DIO, the myocardial content of p38 MAPK was significantly increased, irrespective of the Losartan treatment (Fig 3.7). One documented study showed no differences in total p38 MAPK protein levels in the hearts of rats on a high fat diet [Li et al., 2005]. The cause and consequence of increased p38 MAPK in our DIO animal model remain to be elucidated. This is the first evidence of increased p38 MAPK in the heart in obesity and corroborates the finding of Főldes et al [2006] that rats fed a high-fat diet together with Ang II infusion for 24 h have significantly increased p38 MAPK gene expression.

It is noteworthy that p38 MAPK can be activated by insulin stimulation [Somwar et al., 2000]. A hallmark of insulin resistant conditions (i.e. obesity & type II diabetes) is impairment of the PI3K-dependent signalling pathway, whereas the MAPK pathway is unaffected in these conditions [Jiang et al., 1999; Cusi et al., 2000]. It has been reported that p38 MAPK activity was augmented in the hearts from rats fed a high-fat diet [Li et al., 2005]. This increase in the MAPK-dependent signalling may have pathophysiological implications because activation of this pathway promotes the secretion of the vasoconstrictor endothelin (ET)-1 in the endothelium. Insulin resistant patients have been reported to have elevated ET-1 plasma levels [Piatti et al., 1996]. The p38 MAPKα isoform is associated with apoptosis in the heart [Wang et al., 1998].
In the present study we found no differences in p38 MAPK phosphorylation in the absence and presence of physiological levels of insulin in both control and DIO animals (Fig 3.8). However, Losartan treatment resulted in increased p38 MAPK phosphorylation both in control and DIO animals. The precise role of Ang II receptors on the MAPK activation in cardiac tissue remains controversial. Previously it has been reported that Ang II via the AT1 receptor activates the stress-activated p38 MAPK in VSMC [Touyz & Schriffin 2000], human cardiac myocytes [Wei et al., 2000] and RASMC [Kyaw et al., 2001]. Despite the large amount of evidence that the AT1 receptor mediated signalling results in the activation of p38 MAPK, an inability of Ang II to elicit p38 MAPK activation has also been demonstrated previously [Izawa et al., 2005]. Similarly, our data suggest that p38 MAPK in the heart is not activated by Ang II acting through the AT1 receptor. As discussed in section 1.7.5, treatment with Losartan results in elevated Ang II that may stimulate exposed AT2 receptors [van den Meiracker et al., 1995; Shinozaki et al., 2004]. Whether increased p38 MAPK phosphorylation results from activation of AT2 receptor remains to be elucidated.

The blockade of the RAS has been associated with improved insulin sensitivity. This is manifested by improved insulin signalling [Folli et al., 1997] and glucose uptake [Henriksen et al., 1996], however by a mechanism that is poorly understood [Scheen 2004]. Interestingly, p38 MAPK has been proposed to play a role in insulin-stimulated glucose transport [Sweeney et al., 1999; Somwar et al., 2000]. These results were further substantiated by Somwar and colleagues [2001] who showed that a dominant negative p38 MAPK and selective inhibitors reduced insulin-stimulated glucose uptake, therefore, proposing that p38 MAPK signalling regulates stimulation of glucose transport. Furthermore, p38 MAPK has been suggested to be involved in the intrinsic activity of Glut 4 transporters [Kandror 2003]. p38 MAPK activity has been
previously reported to be increased by Losartan [Keisuke et al., 2001]. Similar to the work done by Keisuke and co-workers, we also found that Losartan treatment resulted in increased p38 MAPK activation. We can only speculate that increased p38 MAPK phosphorylation (Fig 3.8) might be one of the mechanisms of by which Losartan may improve glucose uptake without direct effects on insulin signalling events (i.e. PKB/Akt phosphorylation) (Fig 3.4). We did not perform glucose uptake in the isolated cardiac myocytes from these animals. This may be done in future to investigate this mechanism.

3.7.2 SIGNALLING INDUCED BY ANGIOTENSIN II

We documented higher basal protein levels of PKB/Akt in the hearts from DIO animals (Fig 3.3). However; DIO did not change basal expression of eNOS (Fig 3.5). Protein levels of both PKB/Akt and eNOS were altered in control and DIO animals when treated with Losartan. In control animals, Losartan resulted in attenuated protein expression of both PKB/Akt and eNOS. This suggests that both PKB/Akt and eNOS protein expression is regulated by Ang II via the AT1 receptor. A substantial amount of evidence demonstrates that Ang II via AT1 and AT2 receptors regulate the expression of eNOS in endothelial cells [Saito et al., 1996] accompanied by an increase in NO production [Suzuki et al., 2006].

It has recently been identified that AT2 receptors induce eNOS expression in rat cardiac myocytes [Ritter et al., 2003]. In addition, eNOS expression has been reported to be decreased in AT2 receptor deficient mice [Brede et al., 2003]. We currently have no explanation why AT1 antagonism with Losartan, which should elevate Ang II levels and stimulate the unopposed AT2 receptors, resulted in lower levels of eNOS protein in the hearts from control animals. In this study, the expression levels of the AT1 and AT2 receptors were not determined and the role of
AT2 receptors in this should be examined. To the best of our knowledge this is the first study to demonstrate that Ang II via either the AT1 or AT2 receptor regulates PKB/Akt expression.

The regulation of Ang II on PKB/Akt and eNOS protein expression seems to be impaired in the DIO animals. Losartan treatment resulted in increased eNOS protein expression in the DIO animals compared to the basal expression (Fig 3.5). Increased cardiac eNOS protein expression and activity have been previously demonstrated in patients with coronary artery disease and heart failure receiving ACE inhibitor therapy [Morawietz et al., 2006]. Cardiac upregulation of eNOS expression in the response to ACE inhibition has been reported in the failing hearts of Dahl salt sensitive hypertensive rats [Kobayashi et al., 1999]. It should be remembered that ACE inhibitors prevent the production of Ang II and do not exert effects at the level of the receptor.

Earlier reports on Losartan mediated increased eNOS protein expression are rare. Increased AT2 receptor activation and expression have been very well documented in pathological conditions such as vascular injury, myocardial infarction and cardiac failure [Unger et al., 1996]. Whether AT2 receptor expression is upregulated or downregulated in the state of insulin resistance, coupled to high Ang II levels, is unknown. However, AT2 receptor activation should be unopposed during Losartan treatment, leading to enhanced eNOS expression. The report that PKB/Akt protein expression is augmented in the hearts from DIO animals after Losartan treatment is novel.
3.7.3 NO PRODUCTION

It is very well documented that Ang II via AT1 and AT2 receptor signalling pathways regulate eNOS expression and consequently NO production [Saito et al., 1996; Gohlke et al., 1998]. The activation of the NO signalling cascade by AT2 receptors mediates its biological actions [Carey et al., 2000]. The ability of cardiac myocytes from control and DIO animals to produce NO was measured with DAF/2A. We found that basal NO production of cardiac myocytes from DIO animals was significantly reduced vs. cells from control animals (Fig 3.9). This lower NO production in the cells from the DIO animals can be (i) because both downregulation of the AT1 receptors due to elevated Ang II levels [Baker et al., 1992; Sadoshima & Izumo 1993] as well as insulin resistance [Nickenig et al., 1998] leads to lower expression of the AT1 receptors therefore attenuated signalling. (ii) Activation of the NADPH oxidase system may result in elevated ROS production therefore scavenging of NO resulting in lower bioavailability of NO [Rajagopalan et al., 1996]. Interestingly, Losartan treatment resulted in attenuated NO production in control animals, whereas the ability of the cells from DIO animals to produce NO was improved by Losartan treatment. In animals treated with Losartan, increased levels of Ang II are expected, accompanied by downregulation of the AT1 receptor. Furthermore, the inhibitory actions of signalling induced by activation of the AT2 receptor may be acerbated under these conditions. Together, this may be the cause of the lower NO production measured in cells from control animals treated with Losartan.

The NO production clearly underscores the protein expression of eNOS in the hearts from control and DIO animals (Fig 3.5). Increased eNOS protein expression and NO production with Losartan treatment in DIO animals occurred without changes in eNOS Ser\textsuperscript{1177} phosphorylation (Fig 3.4). It should be noted that various kinases
including PKB/Akt [Dimmeler et al., 1999], PKC and PKA [Michell et al., 2001] share the ability to phosphorylate the Ser^{1177} site.

As with eNOS expression, the observed increased NO production in DIO animals with Losartan treatment might be due to the activation of the unopposed AT2 receptors. These receptors have been previously reported to stimulate NO production in a bradykinin-dependent fashion in endothelial cells [Gohlke et al., 1998]. Ang II plays a central role in the production of ROS by stimulating the activity of NADPH oxidase in VSMC [Griedling et al., 1994] and endothelial cells [Zhang et al., 1999]. Losartan has been reported to decrease NAPDH oxidase mediated ROS production [Shinozaki et al., 2004]. As superoxide can readily scavenge NO, we can argue that increased NO production may also be attributed to reduced oxidative stress.

Interestingly, p38 MAPK has been reported to regulate eNOS expression [Xing et al., 2006], however p38 MAPK signalling pathway activation results in the downregulation of eNOS expression in human endothelial cells. The role of p38 MAPK on eNOS expression in cardiac myocytes is unclear but we can argue that the increased p38 MAPK phosphorylation (Fig 3.8) with Losartan treatment in control animals may contribute to the attenuation of eNOS protein levels but that this regulatory effect is impaired in DIO animals, where Losartan treatment also elevated phosphorylation p38 MAPK.
Our initial results strongly indicated the involvement of the AT2 receptors in the documented changes. To investigate this we needed to (i) establish a culture of neonatal rat cardiac myocytes with (ii) elevated fatty acids and Ang II. If similar changes were induced as observed in the hearts of DIO animals (Chapter 3), the involvement of the AT1 and AT2 receptors could be investigated using specific antagonists against these receptors.

To simulate the changes brought about in the DIO animals in our cell culture model, we determined the changes in fatty acid composition in the plasma of the animals after 16 weeks on the high caloric diet. Plasma was collected and analyzed by gas chromatography. These results indicated that the two major fatty acids elevated in the animals were palmitate and oleate. After establishing a culture of rat neonatal cardiac myocytes, we therefore supplemented the medium with a concentration of palmitate and oleate that did not affect the viability of the cells over the time period necessary to conduct the experiment.
4.1. EFFECT OF FATTY ACIDS ON CELL VIABILITY

A substantial amount of evidence indicates that prolonged exposure of cardiac myocytes to fatty acids may have detrimental effects [De Vries et al., 1997]. Conditions (obesity and type II diabetes) associated with high circulating levels of fatty acid portray alterations in cardiac substrate utilization [Taegtmeyer et al., 2002] and impaired insulin signal transduction [Shulman 2000]. Elevated levels of fatty acids are furthermore associated with induction of apoptosis.

To determine whether palmitate and oleate exert adverse effects on neonatal cardiac myocyte viability, two independent indices of cell viability were assessed. This was accomplished by measuring viability with propidium iodide (PI) and the use of FACS analysis (Fig 4.1A) and trypan blue staining (light microscopy) (Figure 4.1B). The viability data is expressed as a % of control (adjusted to 100%). We found that the co-administration of palmitate and oleate, both at a concentration of 0.25 mM and a period of 4 days in culture media had no significant effect on the cell viability when assessed by PI (Fig. 4.1A) and trypan blue staining (Fig 4.1B).
Figure 4.1 The effect of palmitate and oleate co-administration on cell viability assessed by 10 μM PI via FACS analysis (A) and trypan blue staining via microscopy (B) (n = 2 individual cell preparations)
4.2 EFFECT OF FATTY ACIDS ON CARDIAC GLUCOSE UPTAKE

We evaluated whether the prolonged exposure to palmitate and oleate at the concentration of 0.25 mM in culture media affected 2DG uptake in cardiac myocytes. Cardiac myocytes, cultured in the absence or the presence of 0.25 mM fatty acids, were stimulated for 15 min with or without 1nM, 10nM or 100nM insulin and 2DG uptake measure over a time period of 30 min. (Fig. 4.2). Due to differences in basal glucose uptake, data is expressed as a ratio of basal control (adjusted to 1). In the absence of insulin, basal glucose uptake was significantly (1.87 ± 0.123 vs. 1 pmol/mgprot/30min; p < 0.01) increased in the cells after exposure to fatty acids compared to control cells. In groups stimulated with 1 nM insulin, 2DG uptake also was significantly (3.19 ± 0.18 vs. 1.91 ± 0.30 pmol/mgprot/30min; p < 0.05) increased in the cells exposed to fatty acids compared to control cells. No differences were found at higher insulin concentrations.

![Figure 4.2](image)

*Figure 4.2 The effect of prolonged exposure of fatty acids on neonatal cardiac myocyte glucose uptake, when stimulated with or without 1, 10 an 100nM insulin for 15 min before the administration of 2DG for a further 30 min. Data expressed as mean ± S.E.M and as a ratio of basal control * p < 0.05, ** p < 0.01 (n = 3 individual cell preparations, assayed in duplicate)*
4.2.1 GLUT 4

Glut 4 is known to be the insulin-responsive glucose transporter [Watson & Pessin 2001]. We evaluated total Glut 4 protein levels in the control and fatty acid treated groups. This was accomplished by Western blotting using a 45-kDa Glut 4 antibody from Cell Signaling Technology. However, the separation of proteins on the SDS-PAGE gel resulted in two distinct bands of 55- and 45-kDa respectively that bound to the antibody. We quantified both these proteins but found no difference in the expression levels of either the Glut 55-kDa (Fig 4.3 A) or Glut 45-kDa (Fig 4.3 B) proteins, in the control and fatty acid treated cells.
Figure 4.3 Glut 4 protein levels were determined by Western blotting as described in materials and methods. (A) Glut 4 55-kDa, (B) Glut 45-kDa data is presented as mean ± SEM. The insert is of a representative blot.

(n = 5)
4.2.2 GLUT 1

Glut 1 transporters, with distinct sacrolemmal localization predominantly facilitates basal cardiac glucose uptake [Luiken et al., 2004]. Glut 1 protein levels were significantly suppressed (0.95 ± 0.11 AU vs. 1.35 ± 0.11; p < 0.05) in fatty acid treated cardiomyocytes (Fig 4.4). This is contrary to what we expected when we consider the measurement of basal 2DG uptake (Fig 4.2), where the cells exposed to fatty acids showed a significant increase in 2DG uptake. The levels of Glut 1 protein therefore does not reflect or explain the elevated basal 2DG uptake measured in the cells after exposure to fatty acids.

Figure 4.4 Glut 1 expression after exposure to fatty acids were determined by Western blotting as described in materials and methods. The insert is of a representative blot. Data represented as the mean ± SEM. *p < 0.05 (n = 5)
4.3 PPARα CONTENT

The rate of myocardial fatty acid oxidation vs. glucose oxidation can also be regulated at the level of gene expression. PPARα is a nuclear receptor that is involved in the transcriptional regulation of fatty acid metabolism [Berger & Moller 2002; Huss & Kelly 2004]. We evaluated the expression of PPARα in control and fatty acid treated cells as a measurement of fatty acid oxidation. PPARα expression was significantly (1.58 ± 0.12 vs. 1.2 ± 0.07 AU; p < 0.05) augmented in the fatty acid treated cells compared to control cells (Fig 4.5), demonstrating that fatty acids were taken up by the cells and exerting biological effects.

![Graph showing total PPARα expression after exposure of neonatal cardiac myocytes to fatty acids determined by Western blotting as described in materials and methods. Data expressed as the mean ± SEM. The insert is of a representative blot. (n = 6)](image)

*Figure 4.5 Total PPARα expression after exposure of neonatal cardiac myocytes to fatty acids were determined by Western blotting as described in materials and methods. Data expressed as the mean ± SEM. The insert is of a representative blot. (n = 6)*
4.4 AMPKα CONTENT

AMPKα, metabolic sensor of energy homeostasis, switches off energy-consuming processes and promotes energy generating pathways. Its activation will result in enhanced fatty oxidation [Hardie & Carling 1997]. We evaluated the total protein content of AMPKα in both control and fatty acid treated cells. We observed no differences in both groups in the absence of insulin. The stimulation with 10 nM insulin did not change this (Fig 4.6).

4.4.1 AMPKα ACTIVATION

AMPKα, a Ser/Thr kinase, has been reported to act in response to metabolic stress in conditions of depleted ATP [Gamble & Lopaschuk 1997]. AMPKα has also been implicated in glucose uptake [Li et al., 2004; Yang & Holman 2005]. We investigated activation of AMPKα by measuring its phosphorylation on Thr\textsuperscript{172} in control and fatty acid treated cells. It was apparent that no differences in activation of AMPKα were observed in cells exposed to fatty acids vs. control cells. The presence of 10 nM insulin did not change this (Fig 4.7).
Figure 4.6 Total AMPKα expression after the exposure of neonatal cardiac myocytes to fatty acids and stimulated with 10nM insulin for 15 min, was determined by Western blotting as described in materials and methods. The insert is of a representative blot. Data is expressed as mean ± SEM (n = 2 individual cell preparations, assayed in duplicate).

Figure 4.7 AMPKα phosphorylation after exposure of neonatal cardiac myocytes to fatty acids when stimulated with or without 10 nM insulin for 15 min. Data expressed as mean ± SEM. The insert is of a representative blot. (n = 5)
4.5 TOTAL PKB/AKT

PKB/Akt is the key mediator of the metabolic actions of insulin, including glucose uptake [Kim et al., 1999]. We evaluated total protein levels of PKB/Akt in both groups in the absence and presence of 10 nM insulin. No alterations were found in the expression level of the protein (Fig 4.8).

4.5.1 PKB/AKT PHOSPHORYLATION

Since there was a noticeable increase in insulin stimulated glucose uptake (Fig 4.2) in both groups, this prompted us to investigate the effect of prolonged exposure of fatty acid on insulin signalling by measuring phosphorylation of PKB/Akt on Ser^{473} (Fig 4.9). We observed that in the absence of insulin, basal PKB/Akt phosphorylation was significantly (0.27 ± 0.09 AU vs. 0.92 ± 0.08 AU; p < 0.01) attenuated in the fatty acid treated cells. This decreased phosphorylation of PKB/Akt occurred without any changes in total PKB/Akt content. The efficacy of 10 nM insulin to stimulate PKB/Akt is clearly demonstrated by the significant increase in phosphorylation in control cells (1.78 ± 0.21 vs. 0.92 ± 0.08 AU; p < 0.05) and fatty acid treated cells (1.70 ± 0.189 vs. 0.27 ± 0.09 AU; p < 0.001). The extent of this phosphorylation induced by insulin was much greater in the cells previously exposed to fatty acids, namely a 6 fold increase vs. a 1.9 fold increase.
Figure 4.8 Total proteins levels of PKB/Akt in control and fatty acid treated cells when stimulated with or without 10 nM insulin for 15 min, were determined by Western blotting. The insert is of a representative blot. Data expressed as mean ± SEM. (n = 6)

Figure 4.9 The phosphorylation PKB/Akt in control and fatty acid treated cells when stimulated with or without 10 nM insulin for 15 min, data expressed as mean ± SEM. The insert is of a representative blot. *p < 0.05, **p < 0.01, ***p < 0.001 (n = 6)
4.6 PTEN PROTEIN LEVELS

PTEN, a potent lipid phosphatase, negatively regulates the PI3K/PKB/Akt pathway and its activation results in impaired insulin signalling [Maehama & Dixon 1998]. In light of the increased glucose uptake (Fig 4.2) and activation of PKB/Akt (Fig 4.9), we evaluated the content of PTEN in both groups (Fig 4.11). PTEN protein levels were significantly suppressed (0.84 ± 0.06 vs. 1.03 ± 0.02 AU; p < 0.01) in fatty acid treated cells compared to control cells. Stimulation with 10 nM insulin did not change this, with PTEN protein levels still attenuated (0.73 ± 0.11 vs. 0.98 ± 0.02 AU; p < 0.05) in the fatty acid treated cells compared to the control cells.

4.6.1 PTEN PHOSPHORYLATION

The activity of PTEN was evaluated by measuring phosphorylation on Ser<sup>380</sup>, Thr<sup>382</sup>, and Thr<sup>383</sup> (Fig 4.11). Basal phosphorylation of PTEN was significantly (0.80 ± 0.05 vs. 1.02 ± 0.01 AU; p < 0.05) downregulated in the fatty acid treated cells in comparison with the control cells. This suggested increased PTEN activity in the fatty acid treated cells that may account for the low basal levels of phosphorylated PKB/Akt phosphorylation. Stimulation with 10 nM insulin for 15 min resulted in an evident attenuation of PTEN phosphorylation in control cells (0.85 ± 0.05 vs. 1.02 ± 0.01 AU; p < 0.05) rendering PTEN more active.
Figure 4.10 Total PTEN protein levels in control and fatty acid treated cells when stimulated with or without 10 nM insulin for 15 min. Data expressed as mean ± SEM. The insert is of a representative blot. *p < 0.05, **p < 0.01 (n = 5)

Figure 4.11 Phosphorylation of PTEN in control and fatty acid treated cells when stimulated with or without 10 nM insulin for 15 min, data expressed as mean ± SEM. The insert is of a representative blot. *p < 0.05 (n = 5)
4.7 p38 MAPK

The MAPK signalling pathway has been reported to mediate the growth promoting effects of insulin [Reusch et al., 1995]. We determined the total protein p38 MAPK content in both groups. We observed that p38 MAPK protein content was significantly (0.80 ± 0.05 vs. 1.04 ± 0.03 AU; p < 0.05) decreased in fatty acid treated cells compared to the control cells (Fig. 4.12). Stimulation of 10 nM insulin, led to no apparent difference between the groups.

4.7.1 PHOSPHORYLATION OF p38 MAPK

We investigated activation of MAPK by measuring its phosphorylation on Thr\(^{180}/\)Tyr\(^{182}\) in control and fatty acid treated cells. It was apparent that no differences in phosphorylation of p38 MAPK were observed in the absence insulin (Fig. 4.13). However, addition of insulin to fatty acid treated cells resulted in a noticeable increase (3.85 ± 0.18 vs. 1.14 ± 0.42 AU; p < 0.001) in p38 MAPK phosphorylation compared to fatty acid basal values, having no effect in control cells.
Figure 4.12 Total proteins levels of p38 MAPK in control and fatty acid treated cells when stimulated with or without 10nM insulin for 15 min, data expressed as mean ± SEM. The insert is of a representative blot. **p < 0.05 (n = 5)

Figure 4.13 Phosphorylation of p38 MAPK in control and fatty acid treated cells when stimulated with or without 10nM insulin for 15 min, data expressed as mean ± SEM. **p < 0.01, ***p < 0.001 (n = 5)
4.8 DISCUSSION

Free fatty acids are considered the primary source of energy for the heart. Neonatal cardiac myocytes already have the capacity to utilize fatty acids at a significant rate. This suggests that neonatal cardiac myocytes can extract and metabolize exogenous fatty acids [Ross & McCarl 1984; De Vries et al., 1997]. In the present study, we investigated the impact of chronic exposure of neonatal cardiac myocytes to fatty acids and the subsequent molecular events that might have been altered.

4.8.1 FATTY ACIDS ON CELL VIABILITY

Our data indicates that co-administration of palmitate and oleate at a concentration of 0.25 mM for a period of 4 days had no effect on cell viability (Fig 4.1 A & B). To the best of our knowledge, this is the first study to demonstrate the effects of long term exposure of neonatal cardiac myocytes to palmitate and oleate. Previously, cultured neonatal rat cardiac myocytes have been incubated for 6 days with polyunsaturated fatty acids (eicosapentaenoic acid and docosahexaenoic acid). This has been found to have cardioprotective effects on neonatal cardiac myocytes [Bordoni et al., 2007]. Previous studies demonstrated that especially the saturated fatty acid, palmitate, can induce significant loss of cell viability in various cell types [Rosenthal 1981; Zhang et al., 1992; De Vries et al., 1997; Miller et al., 2005]. In most cases, fatty acid-induced apoptosis have been attributed to saturated fatty acids rather than unsaturated fatty acids [De Vries et al., 1997]. The mechanisms by which palmitate induce apoptosis became clear in the past decade and has been attributed to the ability of palmitate to induce ceramide formation [Hickson-Bick 2000 & 2002]. However, in previously mentioned studies [Hickson-Bick 2000 & 2002] palmitate was used at a concentration of 0.5 mM at a period of not longer than 20 hours. De Vries and colleagues [1997] demonstrated that 48 hours incubation with palmitate resulted in total loss of cells.
Decreasing the concentration from 0.5 mM to 0.25 and even 0.1 mM did not prevent cell death. In our hands, incubation of neonatal cardiac myocytes with 0.25 mM palmitate for 4 days did not result in the loss of cell viability. One explanation for this difference may be the use of serum in our study. The studies of De Vries et al [1997]; Hickson-Bick et al [2000 & 2002] did not make use of serum when cardiac myocytes were incubated with fatty acids.

4.8.1.1 THE ROLE OF SERUM-RICH MEDIA

FCS contains a rich variety of proteins enabling survival, growth and proliferation of cells in culture [Even et al., 2006]. It has been reported that the removal of serum factors from the media is sufficient to induce apoptosis in cell culture [Galli & Fratelli 1993]. In support of this, serum-deprivation in Balb/c 3T3 fibroblasts exhibited features that are characteristic of physiological cell death (apoptosis) [Kulkarni & McCulloch 1994]. Cultured cardiac myocytes can also be added to this list. Serum-deprivation and incubation with saturated fatty acids accelerate the process of apoptosis [De Vries et al., 1997]. We incubated palmitate and oleate in the presence of serum with the expectation not to have severe loss of cell viability. We aimed to investigate the effects of these fatty acids on insulin signalling rather than the process of apoptosis. We found that incubation with fatty acids in the presence of serum preserved cell viability (Fig. 4.1).

It has been previously reported that the presence of serum together with palmitate results in the inhibition of palmitate oxidation [Sleboda et al., 2001]. However, Sleboda and colleagues did not show whether, as a consequence of reduced palmitate oxidation there were any alterations in glucose oxidation.
A second reason for the survival of fatty acid treated cells may be the absence of BSA complexed to fatty acids, as described in previous studies [De Vries et al., 1997; Hickson-Bick et al., 2000; Wang et al., 2006]. Three decades ago, Paris and co-workers [1978] demonstrated that the rate of long chain fatty acid uptake in cultured cardiac cells was increased by albumin. They suggested that albumin cannot be replaced by other serum FABP. Increased survival in our cells might be due to the inhibition of fatty acid uptake and oxidation by the absence of albumin. However, several studies favour the view that fatty acid uptake occur solely on the unbound fraction and that cell-surface albumin receptors facilitate the dissociation of fatty acid-albumin complexes [Popov et al., 1992; Elmadhoun et al., 2001].

### 4.8.1.2 THE ROLE OF OLEATE

A considerable amount of evidence indicates that mono-unsaturated fatty acids such as oleate counteract the apoptotic inducing effects seen by palmitate. Co-administration of oleate with palmitate has been demonstrated to inhibit palmitate-induced apoptosis [De Vries et al., 1997; Miller et al., 2005]. De Vries et al [1997] hypothesized that the co-administration of oleate rather than lowering of the concentration of palmitate (from 0.5 mM to 0.25 mM) preserved cell viability. Palmitate at a concentration of 0.25 mM was also harmful to neonatal cardiac myocytes. Miller et al [2005] demonstrated that oleate at a concentration of 0.1 mM was able to prevent 0.5 mM palmitate induced apoptosis. Taking this into account, we can speculate that in the present study, oleate contributed to preserved cell viability.
4.8.1.3 THE ROLE OF KINASES

The intrinsic capacity of cells to undergo apoptosis can be suppressed by survival signals from factors within their environment. PKB/Akt is a critical regulator of a wide variety of cellular processes, including apoptosis, proliferation and differentiation [Staal et al., 1977]. Over-expression of the wild type or activated form of PKB/Akt has the ability to attenuate the process of apoptosis induced by stress signals [Kauffman-Zeh et al., 1997; Kennedy et al., 1997]. Our data showed no difference in the protein expression of PKB/Akt in both control and fatty acid treated cells (Fig. 4.8). Although basal PKB/Akt phosphorylation was suppressed in the fatty acid treated cells (Fig 4.9), this was not associated with loss of cell viability. PKB/Akt promotes cell survival through direct phosphorylation of transcriptions factors that regulate pro (Bad) - and anti-apoptotic (Bcl-2) genes [Skorski et al., 1997; Pugazhenthi et al., 2000].

The activation of p38 MAPK is associated with cardiac myocytes cell death during ischaemia because inhibition of p38 MAPK protects against cell death [Mackay & Mochly-Rosen 1997; Saurin et al., 2000]. These findings were further substantiated in our laboratory in neonatal cardiac myocytes. Engelbrecht et al [2004] demonstrated that the activation of p38 MAPK is associated with increased caspase-3 activation and PARP cleavage. Wang and colleagues [1998] reported that a distinct isoform of p38 MAPK was involved in cell death namely p38α. In contrast, p38β may mediate myocardial survival. Basal p38 MAPK protein levels were significantly decreased in fatty acid treated cells; however, we found no significant difference in the basal p38 MAPK phosphorylation (Fig. 4.13). Therefore we can suggest that PKB/Akt and p38 MAPK may have further contributed to cell survival.
4.8.2 PPARα EXPRESSION IN NEONATAL CARDIAC MYOCYTES

The capacity of neonatal cardiac myocytes to produce energy from the oxidation of fatty acids is controlled at the level of nuclear gene transcription by PPARα, encoding enzyme involved in fatty acid oxidation [Barger & Kelly 2000]. Endogenous long chain fatty acids, such as palmitate are ligands for the activation of PPARα [Huss & Kelly 2004]. We showed that PPARα expression was significantly increased in the fatty acid treated cells (Fig 4.5). PPARα regulates the expression of enzymes involved in glucose (PDK4) and fatty acid oxidation (FABP, FACS, CD36/FAT & CPT-1) [Stanley et al., 2005]. PPARα activation in cardiac myocytes by increasing fatty oxidation was shown to limit palmitate-induced apoptosis [Kong & Rabkin 2004]. We can further argue that increased PPARα expression resulted in decreased intracellular accumulation of fatty acid intermediates (i.e. ceramide) and the subsequent palmitate-induced cell death.

4.8.3 CARDIAC GLUCOSE UPTAKE

Our data showed that cardiac fatty acid supplementation resulted in an increase in glucose uptake compared to control conditions (Fig 4.2). In both the control and fatty acid treated cells there was a noticeable, dose-dependent increase in 2DG uptake in response to insulin. In the work done by Hickson-Bick et al [2005], alterations in fatty acid metabolism in neonatal cardiac myocytes were likely mediated by palmitate after 20 h exposure. They observed a small but significant increase in glucose oxidation in neonatal cardiac myocytes pre-incubated with palmitate for 20 h. However, in our study cardiac 2DG uptake measurement does not reflect glucose oxidation by cardiac myocytes but rather carrier-mediated glucose transport. Due to the significant basal increase in 2DG uptake it was of interest to evaluate the expression of Glut 1 (Fig 4.4). However, we observed a decrease in Glut 1
expression in fatty acid treated cells compared to the control conditions while we anticipated an increase to account for the high basal levels of glucose uptake.

4.8.3.1 THE EXPRESSION OF GLUT 4

We measured Glut 4 protein levels in the absence of insulin. Interestingly, we observed two distinct bands after Western blot analysis, of 55- and 45-kDa (Fig 4.3) respectively in both control and fatty acid treated cells, with no difference in expression. Little research has been done measuring Glut 4 protein content in neonatal cardiac myocytes. In a fairly recent publication, [Guan et al., 2008], Western blot analysis of subcellular and membrane fractions were positive for Glut 4 protein with a size of approximately 48-kDa in neonatal cardiac myocytes. Glut 4 protein with a size of 55-kDa has been previously identified in 3T3-L1 adipocytes [Sargeant & Pâquet 1993] and adult rat heart [Bahr et al., 1995]. In contrast to the results of Guan et al [2008], we demonstrated that neonatal cardiac myocytes express two isoforms of Glut 4, namely a 55-kDa and 45-kDa isoforms.

Glut 4 expression has been demonstrated to be compromised in type II diabetes likely due to the elevated fatty acids [Armoni et al., 2005]. Our data showed that the basal as well as in the presence of 1nM insulin, 2DG uptake was significantly augmented in the fatty acid treated cells (Fig. 4.2). The expression of the two isoforms of Glut 4 in neonatal cardiac myocytes might explain the increased 2DG uptake in fatty acid treated cells. However, these isoforms are also expressed in the control cells. Previously it has been demonstrated that Glut 4 protein content was not significantly different in the muscle from rats fed a high fat diet and STZ treated compared to control animals [Wu et al., 2004], while the amounts of Glut 4 in the membrane differed. As we did not measure Glut 4 translocation, it may still be
possible that cells treated with fatty acids contained more Glut 4 in the cell membrane.

4.8.3.2 AMPKα

Insulin is the main stimulus for glucose uptake after a meal but glucose uptake also occurs independent of insulin stimulation. This is well described in conditions such as exercise and ischaemia and mediated by the activation of AMPKα [Gamble & Lopaschuk 1997]. We have observed no differences in total AMPKα content (Fig 4.6) or activation (Fig. 4.7). Activation of AMPKα is associated with increased fatty acid oxidation [Hardie & Carling 1997; Hickson-Bick et al., 2004]. Although PPARα expression was significantly increased in the fatty acid treated cells, this was without changes in AMPKα activity. Reduced AMPKα activation has been associated with reduced fatty acid oxidation in neonatal cardiac myocytes chronically exposed to palmitate [Hickson-Bick et al., 2000]. This allows us to speculate that fatty acid treatment for the period of 4 days did not result in the need to increase ATP-generating pathways suggesting that these cells were not metabolically stressed. In the work done by Pimenta [2008], 8 h exposure of skeletal muscle to palmitate resulted in decreased palmitate oxidation but increased AMPKα activation. The elevated glucose uptake that we measured in the cells after exposure to fatty acids can therefore not be ascribed to activation of AMPKα.

4.8.4 INSULIN SIGNALLING EVENTS

A large amount of evidence implicates fatty acids as one of the major contributors to insulin resistance [Griffin et al., 1999; Dresner et al., 1999; Shulman 2000] as discussed in preceding literature review (see section 1.7.2).
4.8.4.1 PKB/Akt

PKB/Akt is an important downstream kinase of post insulin receptor signalling and has been reported to be the ultimate reflection of insulin activity. Basal phosphorylation of PKB/Akt in fatty acid treated cells is impaired (Fig. 4.9). From our results, we can argue that the increased basal PTEN activity (Fig. 4.11) may account for this. 10 nM insulin was sufficient to correct this and resulted in PKB/Akt phosphorylation to the same degree as in control cells. Insulin stimulation in fatty acid treated cells leads to a six fold increase in PKB/Akt phosphorylation. This suggests that palmitate and oleate at a concentration of 0.25 mM did not result in impaired insulin signalling. In two separate studies, palmitate [Coll et al., 2008] and oleate [Liu et al., 2007] resulted in reduced insulin stimulated Ser\textsuperscript{473} PKB phosphorylation in muscle cells and hepatocytes respectively. In cultured mouse cardiac muscle HL-1 cells, insulin stimulation significantly increased PKB/Akt phosphorylation which was blunted by pre- and co-treatment with 1.2 mM palmitate. Palmitate and ceramide did not accelerate PKB/Akt dephosphorylation beyond that with the removal of insulin alone [Soltys et al., 2002]. Again, in the study by Soltys, palmitate administration to cardiac muscle was performed in the absence of serum. However, similar to our findings on cell survival, the combination of palmitate and oleate did not reduce insulin signalling of the phosphorylation of PKB/Akt. Conflicting data exist with respect to the ability of palmitate to attenuate insulin signalling events [Usui et al., 1997; Schitz-Pheiffer et al., 1999]

4.8.4.2 p38 MAPK

Palmitate induces multiple forms of cell stress in isolated cardiac myocytes which result in the activation of stress associated signalling pathways and apoptosis [Miller et al., 2005]. We found that basal p38 MAPK content was reduced in fatty acid
treated cells (Fig. 4.12) although p38 MAPK phosphorylation was intact (Fig. 4.13). Fatty acid pre-treatment resulted in a significant increase in p38 MAPK phosphorylation after insulin stimulation. Insulin has been reported to increase p38 MAPK phosphorylation in a variety of cell types in the absence of fatty acids [Somwar et al., 2000 & 2001]. In hepatocytes [Liu et al., 2007], oleate resulted in impaired insulin signalling through a p38 MAPK-dependent manner. Hepatocytes incubated for 16 h with oleate, stimulated with 10 nM insulin resulted in an increase in p38 MAPK phosphorylation in response to increases in oleate concentration. Our data showed that irrespective of the co-administration of fatty acids, insulin stimulation resulted in significant augmented p38 MAPK phosphorylation in cardiac myocytes, corroborating the findings of Liu and colleagues [2007]. Increased p38 MAPK in our fatty acid treated cells did not result in insulin resistance.

4.8.4.3 THE INVOLVEMENT OF PTEN

PTEN activation depends on dephosphorylation at Ser\(^{380}\)/Thr\(^{382}\)/Thr\(^{383}\) of the COOH-terminal tail [Vazquez et al., 2001]. Interestingly, PTEN content (Fig 4.10) and phosphorylated PTEN (Fig. 4.11) was significantly reduced in fatty acid treated cells. It is very well documented that PTEN negatively regulates the PI3K/PKB/Akt pathway [Maehama & Dixon 1998]. Over-expression of PTEN in 3T3-L1 adipocytes inhibits PKB/Akt activation and glucose uptake [Nakashima et al., 2000]. Decreased PTEN activity in fatty acid treated cells (Fig. 4.11) may explain the downregulation of basal PKB/Akt phosphorylation (Fig 4.9). Palmitate [Wang et al., 2006] and oleate [Liu et al., 2007] have been demonstrated to induce insulin resistance through the upregulation of PTEN. Our data suggest that the co-administration of palmitate and oleate to cardiac myocytes resulted in significant activation of PTEN compared to the control conditions. In this model, contrary to what we expected, stimulation with
insulin resulted in significant activation of PTEN in control cells, however insulin did not change the status of PTEN phosphorylation in fatty acid treated cells (Fig. 4.11). We can argue that the reduced PTEN protein levels observed are due to its activation by the fatty acids. Augmented PTEN activity has been associated with increased p38 MAPK activity in endothelial cells [Wang et al., 2006] and hepatocytes [Liu et al., 2007]. In contrast, in our model, PTEN activity was not associated with increased p38 MAPK activity in the fatty acid treated cardiac myocytes.

4.9 ROLE OF KINASES AND PHOSPHATASES IN GLUCOSE UPTAKE
PKB/Akt phosphorylation is very well associated with glucose uptake; the ultimate reflection of insulin action. Diminished phosphatase activity (i.e. PTEN) has been reported to promote insulin-stimulated PKB/Akt phosphorylation and subsequent glucose uptake [Tang et al., 2005]. Somwar and colleagues [2001] demonstrated a role for p38 MAPK in insulin-stimulated glucose uptake. A p38 MAPK inhibitor (SB203580) resulted in reduced insulin-stimulated glucose uptake in adipocytes and muscle cells without the diminishing Glut 1 or Glut 4 at the plasma membrane [Sweeney et al., 1999]. Taking into account basal PKB/Akt phosphorylation (Fig 4.9), basal PTEN phosphorylation (Fig. 4.11) and basal p38 MAPK phosphorylation (Fig 4.13) in fatty acid treated cells the basal increase in 2DG uptake seems to be paradoxical and unexplained. Stimulation with 10 nM insulin resulted in the significant increase in PKB/Akt and p38 MAPK phosphorylation in fatty acid treated cells. This mirrors the effect of insulin mediated 2DG uptake in fatty acid treated cells.
4.10 FATTY ACID INCUBATION TIME

In most cases, neonatal rat cardiac myocytes are cultured between 48 to 60 h in the complete media (as discussed in section 2.5) Generally after this period, cells are serum-deprived [De Vries et al., 1997; Hickson-Bick et al., 2002] and changed in a medium that only contains the fatty acids for the incubation period. This is directly followed by the assessment of interested parameters (i.e. glucose uptake). However, we removed the fatty acid containing media overnight before biochemical analysis. This led us to speculate that the absence of fatty acids might contribute to enhanced 2DG uptake and the insulin signalling events.

We therefore repeated these experiments without removing the fatty acids from the medium. We found that the increase 2DG uptake previously observed (Fig. 4.2) in fatty acid treated cells was abolished by the presence of fatty acid in the glucose assay media (Fig. 4.14). 2DG uptake in fatty acid treated cells with 1 nM insulin was significantly attenuated (21.58 ± 4.15 vs. 69.86 ± 4.44 pmol/mg protein/30min; p < 0.05) compared to the control cells. Similar, insulin stimulation was unable to alleviate PKB/Akt phosphorylation in fatty acid treated cells (Fig. 4.15) as previously observed (Fig. 4.9) PTEN phosphorylation was noticeably decreased in basal fatty acid treated cells, suggesting increased PTEN activity (Fig. 4.16). PTEN basal activity was unchanged by the presence of fatty acid during biochemical analysis. Fatty acid treatment still resulted in increased PTEN activation that can be held accountable for the decreased basal PKB/Akt activation (Fig. 4.15). No significant difference was found in the groups treated with insulin.
Figure 4.14 The effect of fatty acids on the measurement of 2 DG uptake when present in Sol E; cells stimulated with or without 1, 10 or 100nM insulin before incubation with 2DG uptake for 30 min. Data presented as the mean ± SEM p < 0.05 (n = 2)

Figure 4.15 The effect of fatty acids on the phosphorylation of PKB/Akt when present in Sol E; cells stimulated with or without 1, 10 or 100nM insulin. The insert is of a representative blot. Data presented as the mean ± SEM **p < 0.01; ***p < 0.01 (n = 2)
Figure 4.16 The effect of fatty acids on the phosphorylation of PTEN when present in Sol E; cells stimulated with or without 1, 10 or 100nM insulin. The insert is of a representative blot. Data presented as the mean ± SEM **p < 0.01 (n = 2)
From the first part of the study, we conclude that animals placed on the high caloric diet became obese and ultimately insulin resistant (Chapter 3, table 2). Insulin resistance in the DIO animals was reflected by (i) increased serine IRS-1 phosphorylation (ii) reduced ability of insulin to stimulate PKB/Akt and eNOS phosphorylation and (iii) reduced NO production. We furthermore concluded that signalling via either the AT1 or the AT2 receptor, modulated the expression of both PKB/Akt and eNOS. To the best of our knowledge, this is the first demonstration that Ang II affects PKB/Akt protein expression in our model of DIO. We furthermore clearly showed that Losartan treatment can increase NO production by adult cardiomyocytes. However, due to financial constrains, the role of AT2 receptor could not be dissected in the intact animal or in isolated perfused hearts. To investigate this we needed to establish a cell based model representing the heart. We therefore set up a culture of neonatal cardiac myocytes to treat with elevated fatty acids and Ang II.

We supplemented this culture with a concentration of palmitate and oleate without a significant loss of cell viability. To the best of our knowledge this is the first study to show that prolonged exposure of neonatal cardiomyocytes to a combination of palmitate and oleate can be accomplished without a significant effect on the cell viability.

We furthermore concluded from our results, that fatty acids must be present to exert negative effects on glucose uptake and insulin signalling. If they are removed, even for a short while (18 hours in our study), the metabolic behaviour of the cells with
regards to glucose uptake and insulin sensitivity, is diametrically opposite to the expected effects, raising the question of the roles of signal transduction versus altered protein expression in the effects that are observed.

The shortcomings of the present study are certainly that we were not able to utilize the AT2 receptor antagonist in vivo to better understand the effects of Ang II on protein expression. Furthermore, we did not measure activation of the NADPH oxidase in this study and therefore cannot comment on the role of scavenging of NO to lower its bioavailability.

In the study using the neonatal cardiomyocytes, the results that we obtained were not similar to what we observed in the intact, isolated hearts. The model will therefore need to be modified to continue with a study of the role of Ang II and the AT1 and AT2 receptors.
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