Identification of the functional role of GSK3β in the *ex vivo* female rat heart at ischemic reperfusion

-with special reference to Akt-dependent signalling

by

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"I have the body of a weak and feeble woman, but the heart of a king"

Queen Elizabeth I (1533-1603)

Acknowledgement

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Abstract

Background: Coronary heart disease is the leading cause of death worldwide, arising mainly from ischemic heart disease. Currently, the most effective method for reducing mortality in patients suffering from a coronary occlusion is rapid reperfusion of the ischemic myocardium. Paradoxically, reperfusion *per se* can also increase cell death, a phenomenon termed lethal reperfusion injury. The increased understanding of this mechanism, has led to novel research aiming to develop protective interventions against lethal reperfusion injury.

Objective: The first aim of this study was to investigate whether treatment with insulin, the GSK3β-inhibitor SB-415286 or the combination of both, conferred cardioprotection in terms of reducing infarct size in the isolated *ex vivo* female heart, when administrated at the onset of ischemic-reperfusion. Secondly, the involvement of the prosurvival kinase Akt was explored to elucidate the intracellular signalling pathways involved in these cytoprotective treatments.

Methods: The effect of the various treatments was assessed using the *ex vivo* isolated Langendorff perfused rat heart model. Female rat hearts underwent 20 minutes of stabilisation, 30 minutes of regional ischemia followed by 120 minutes of reperfusion ± insulin (0.3 mU/mL), GSK3β-inhibitor (3μM) or insulin+GSK3β-inhibitor treatment for 15 minutes at the onset of reperfusion. Measurements of infarct size as percentage of risk area were performed in order to evaluate the cardioprotective effect. To explore phosphorylation status of the prosurvival kinase Akt in response to the various treatments administrated at ischemic reperfusion, cardiac tissue was isolated and homogenised, followed by protein detection using SDS-PAGE and Western Blotting.

Results: Administration of insulin, the GSK3β-inhibitor SB-415286 or the combination of the two, for 15 minutes from the onset of ischemic reperfusion, significantly reduced infarct size in the isolated *ex vivo* female rat heart as compared to control (Ins 29.9±5.5%, GSK3β-inh 29.4±2.7%, Ins+GSK3β-inh 27.1±5% vs. control 45.9±3.0%). No synergistic cardioprotective effect was observed with the combinational treatment, and also no difference in the response of female and male rat hearts was observed. Preliminary data suggest that phosphorylation of the prosurvival kinase Akt is involved in insulin mediated cardioprotection. However, its phosphorylation status is not affected by treatment with GSK3β-inhibitor.

Conclusion: Insulin, the GSK3β-inhibitor SB-415286 and insulin+GSK3β-inhibitor confers cardioprotection in terms of reducing infarct size in the isolated *ex vivo* female rat heart, when administrated for 15 minutes at the immediate onset of reperfusion. Our preliminary data suggests that the prosurvival kinase Akt is involved in insulin treatment administered at reperfusion.

1. Introduction

1.1 Background

Cardiovascular disease is the major cause of death worldwide, and is responsible for nearly 40% of the mortality in Europe before the age of 74 [1]. In Norway, 35% of all deaths in 2006 was a consequence of this diseased state, and of these approximately 40% were caused by ischemic heart diseases such as myocardial infarction, angina pectoris and arteriosclerosis [2]. Myocardial ischemia is caused by a coronary occlusion leading to reduced blood flow to the affected part of the heart, with increased risk of acute myocardial infarction (AMI) and irreversible tissue damage. In most cases, the occlusion is caused by a disruption in the vascular endothelium associated with unstable arteriosclerotic plaque. If the blood supply to the heart becomes too low to ensure adequate oxygen levels, the maintenance of normal oxidative metabolism will be impaired, leading to what is defined as an ischemic condition [3]. To reduce severe damage of ischemic myocardium, rapid restoration of coronary blood flow (reperfusion) is essential. In the clinic, this is normally achieved by the use of thrombolytic therapy, coronary artery bypass graft surgery, or primary percutaneous coronary intervention (PCI). Paradoxically, the reperfusion phase can simultaneously lead to increased cell death and myocardial damage, a phenomenon termed reperfusion induced injury.

Data from *Statistics Norway (Statistisk Sentralbyrå)* state that 48% of all deaths caused by ischemic heart disease in Norway are among women [2] ; however the majority of all cardiac research is performed on the male specie. Previous studies also show differences between genders in the response to myocardial ischemia, with premenopausal women appearing to be better protected [4-6]. To further investigate these findings, the main objective of this project was to explore whether pharmacological treatments with insulin, GKS3β- inhibitor and a combination of the two, could reduce the extent of reperfusion induced injury caused by AMI in the *ex vivo* female rat heart. Parallel experiments on male rats were performed by a colleague (Anita Wergeland), to investigate a potential sex-difference in the response of the various treatments.

1.2 Myocardial ischemia

Myocardial ischemia is defined as inadequate oxygen supply to myocardial tissue caused by a reduction in coronary blood flow. The two main consequences of ischemia are: A) lack of adequate amounts of oxygen and nutrients and B) reduced washout of metabolites such as lactate, protons, $NADH_2$ and CO_2 [7] (Fig.1-1).

The reduced amount of oxygen available to the mitochondria of the ischemic cells, inhibit their capacity to aerobically metabolize fatty acids (Fig. 1-1A). Consequently, a rapid decline in ATP will occur, which again will stimulate cells to initiate glycolysis, resulting in increased levels of lactate and protons (the latter from breakdown of ATP). This increase, together with accumulation of other metabolic waste products due to reduced washout, will inhibit glycolysis and further reduce the levels of ATP.

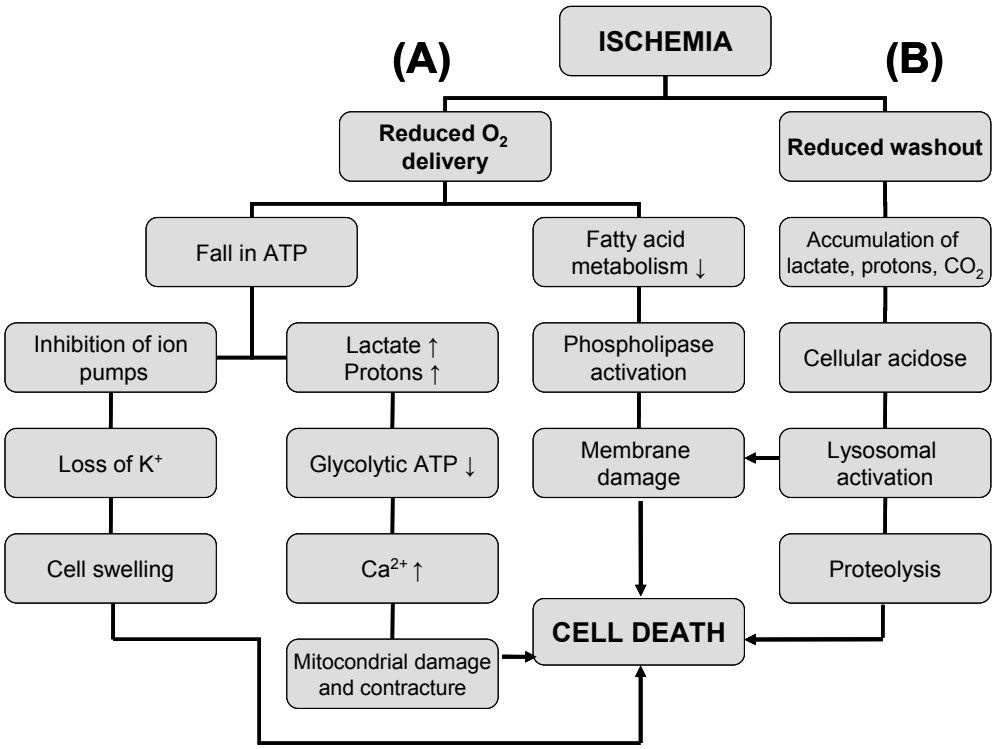

Figure 1-1: ***The main consequences of ischemia:*** (A)**:** Reduced O_2 delivery and (B) poor wash out of various metabolites are the major consequences of ischemia. A decrease in oxygen supply will down regulate oxidation of fatty acids and reduce the production of ATP. Accumulation of lactate, protons and CO_2 will occur due to reduced wash out, leading to cellular acidosis and proteolysis. Depletion of ATP will inhibit ATP dependent ion pumps resulting in potassium loss, water retention and cell swelling. As for the increase in lactate and proton levels, the glycolysis will be inhibited, causing a rise in Ca^{2+}-levels and damage of the mitochondria. The ischemic cascade culminates in cell death either by necrosis or apoptosis. (Figure modified from Opie 2004 [3])

Due to insufficient ATP levels, the contractile force of the myocardial cells will decrease markedly [8]. In addition, ATP-dependent ion pumps will be inhibited, leading to an ionic imbalance in the cell [7] (Fig. 1-1A). Potential consequences are damage of contractile proteins, activation of phospholipases, increased depolarization, ischemic contracture and mitochondrial damage [3]. Accumulation of lactate, protons and CO_2 will give rise to an acidic environment in the cell, which again will promote proteolysis and cell death [3] (Fig.1-1B).

It is important to address the metabolic differences between mild and severe ischemia. In a normal oxygenated heart, oxidation of fatty acids is the main source of energy and high levels of ATP and citrate will in this case inhibit glycolysis in myocardial cells. If the heart is subjected to mild ischemia, the depletion of ATP will cause stimulation of glycolysis. In contrast, glycolysis will be inhibited during severe ischemia due to reduced glucose delivery to the heart, as well as accumulation of lactate and protons. These metabolic changes will reduce the ability of the tissue to survive [3].

The severity of an ischemia is also determined by the position of the occlusion. A complete restriction of blood flow to the heart caused by an occlusion in aorta is defined as *global ischemia*. In contrast, if the occlusion is situated in the coronary arteries, only supplying blood flow to a specific area of the cardiac tissue, the condition is termed *regional ischemia.* As previously mentioned, the contractile force decrease markedly in ischemic cells, however, if ischemia persists less than 15 minutes, the myocardial cells are able to restore electric and mechanical activation [8]. On the other hand, if the myocardium is subjected to a longer period of ischemia, some of the cells will die [8]. In this project, *ex vivo* isolated female rat hearts were subjected to 30 minutes of regional ischemia by occluding the left anterior descending coronary artery (LAD).

1.3 Myocardial reperfusion injury

Reperfusion is defined as restoration of blood flow to a previous ischemic area, and is a necessary process to reduce tissue damage. Paradoxically, reperfusion can at the same time activate a cascade of reactions leading to increased myocardial damage [9], a phenomenon termed *myocardial reperfusion injury.* Jennings *et al.* [8] were the first to describe this, and later, four types of reperfusion injuries have been recognized: 1) myocardial stunning, 2) microvascular damage (reduced perfusion) 3) reperfusion arrhythmias and 4) lethal reperfusion injury [10]. Myocardial stunning refers to post-ischemic mechanical dysfunction despite restoration of normal blood flow and absence of irreversible damage [3, 9]. The condition is transient and the heart will normally recover fully within days or weeks. Microvascular damage is also a potential harm caused by reperfusion, and refers to impairment in blood delivery and hence reduced perfusion of some of the previous ischemic areas [11]. A third complication that can arise during reperfusion is arrhythmias. If left untreated, this is a lethal diseased state; however anti-arrhytmics are efficiently used to prevent this. Many researchers have investigated the three types of reperfusion damage described above, leaving lethal reperfusion injury as the more unexplored factor [10].

1.3.1 Lethal reperfusion injury

Lethal reperfusion injury is defined as death of myocardial cells caused by restoration of coronary blood flow after an ischemic event [10], e.g. after percutaneous coronary intervention (PCI) or in a surgical setting. An important point is that the affected myocardial cells are viable immediately before the reperfusion sets in, and consequently the possibility of rescuing these cells has received increased attention. Several studies have shown that administration of pharmacological agents at the immediate onset of reperfusion will reduce the extent of cell death after an ischemic episode [12-17], supporting the concept of lethal reperfusion injury as an important contributor to the total myocardial damage caused by an ischemic event. For patients suffering from AMI, this could therefore be a great step forward in developing new therapeutic strategies, aiming to salvage the reperfused myocardium.

The mechanism leading to lethal reperfusion injury still remains incomplete, however several cellular events are described in the literature as possible theories: A) oxidative stress,

B) Calcium (Ca^{2+}) overload, C) restoration of physiologic pH and D) opening of the mitochondrial permeability transition pore (mPTP) [10] (Fig. 1-2). There is good evidence of increased oxidative stress in the reperfused ischemic myocardium, which itself can cause myocardial injury [18]. As presented in Figure 1-1, cell swelling followed by membrane and mitochondrial damage is a consequence of ischemia, and this can amplify during the reperfusion phase. The increased formation of oxygen-derived free radicals has shown to be an important factor leading to increased cellular damage and cell death. These highly reactive molecules have shown to combine with polyunsaturated fatty acids to form lipid peroxides and hydroperoxides, which in turn can inhibit enzymes, damage the sarcolemma and mitochondrial membrane, impair cellular calcium transport, and thereby cause further cellular damage [9].

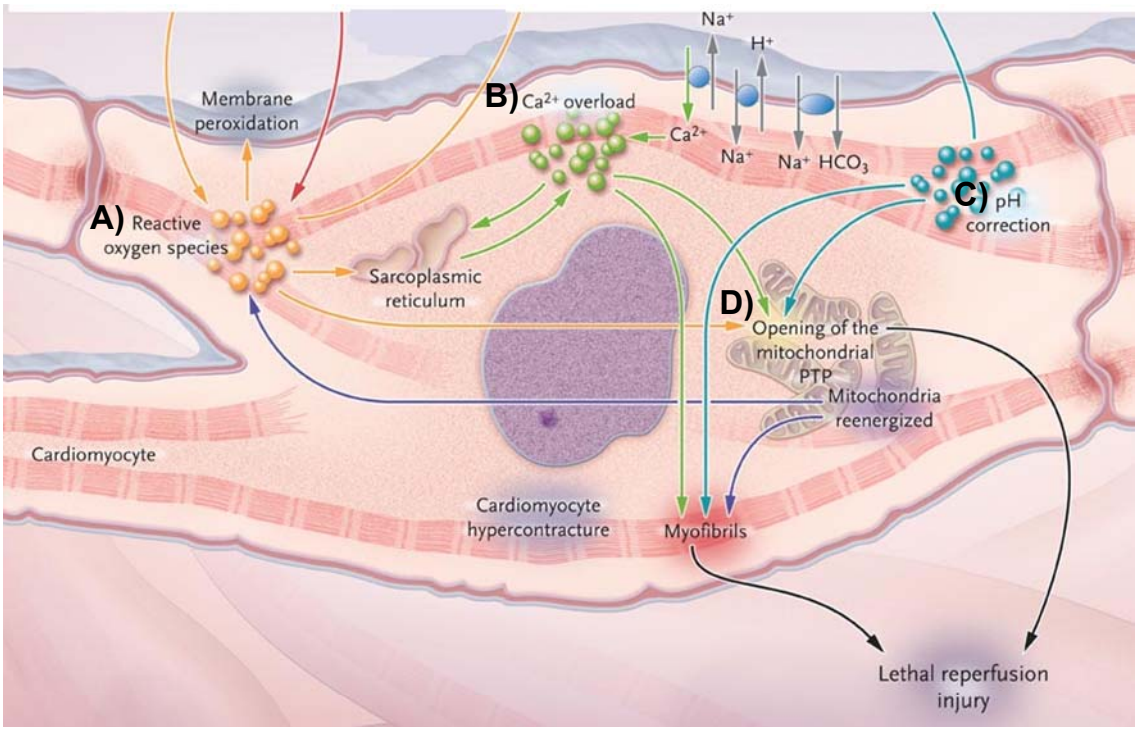

Figure 1-2: ***The main events of lethal reperfusion injury:*** During myocardial reperfusion an abrupt increase of Ca^{2+} (B, green) together with increased production of reactive oxygen species (A, yellow) and pH restoration (C, blue) interact to mediate increased cardiomyocyte death. The opening of the mPTP- channel (D) is mainly responsible for this by uncoupling oxidative phosphorylation and inducing mitochondrial swelling with the release of pro- apoptotic factors, (Figure modified from Yellon, 2008 [10]).

In addition to oxidative stress, an abrupt increase in Ca^{2+} concentration in the reperfusion phase has shown to be one of the main contributors to lethal reperfusion damage [10, 19]. Production of free radicals, activation of phospholipases with damage of the sarcolemma and mitochondrial membrane are consequences of this change, which again is associated with increased contracture, cell swelling and necrosis [9].

At the onset of reperfusion, the washout of metabolites in the heart, including lactate, gradually increases. Hence, the acidic environment arising during ischemia will restore to physiologic pH (~7.4). Activation of the sodium-hydrogen exchanger and the sodium-bicarbonate symporter will also contribute to this. Paradoxically, studies have shown that the return from acidic to normal pH after reperfusion accelerates cell death, thereby becoming another cause of lethal reperfusion injury [20].

As illustrated in Figure 1-2, the described oxidative stress, Ca^{2+} overload and pH correction arising in the reperfusion phase, all mediate opening of the mitochondrial permeability transition pore (mPTP), which are non-selective channels situated in the inner mitochondrial membrane. Opening of this pore leads to influx of solutes and water followed by swelling of the mitochondrial matrix. Subsequently, the outer mitochondrial membrane will burst, leaking various pro-apoptotic substances into the cytosol that will initiate apoptosis [21]. As expected, several studies have shown that inhibition of this mPTP- channel have a cardioprotective effect in reducing the extent of lethal reperfusion injury [21-23], from now on termed *reperfusion injury*.

1.3.2 Apoptosis versus necrosis

It is widely accepted that prolonged severe myocardial ischemia leads to cell death; however, the type of cell death contributing to this has been under intense investigation for many years. Today results are indicating that both *necrosis* and *apoptosis* are involved in myocardial ischemic- and reperfusion induced cell death. Necrosis is defined as cell death, most commonly caused by swelling of the cell and its organelles [24]. The cell will eventually burst (lysis) leading to the release of intracellular components, that will attract macrophages, activate fibroblast and hence give an inflammatory response (Fig. 1-3A).

Apoptosis is defined as a genetically programmed event, leading to cell shrinkage and degeneration of intracellular components without any rupture of the cell membrane [25]. The mitochondria remain intact in this process, whereas the nucleus is broken down, followed by cleavage of the DNA. When the cell shrinks, fragments are released in form of apoptotic bodies, which again are phagocytised by neighbouring cells (Fig. 1-3B). As distinct from necrosis, apoptosis do not result in any fibrosis, inflammation or scar formation [25].

Until recently, it was suggested that necrosis was the main event leading to ischemia-reperfusion induced injury. However, more recent studies also support apoptosis as an important contributor to this damaging process [26, 27]. As apoptosis is genomically regulated and can be manipulated, there has been an increased focus on the reperfusion phase as a target for cardioprotection both through pharmacological interventions and intentional variation of perfusion conditions (pre- and post-conditioning). Substantial research have been performed [12-17, 28, 29], but none of the experimental strategies have yet been routinely established in clinical practice [30].

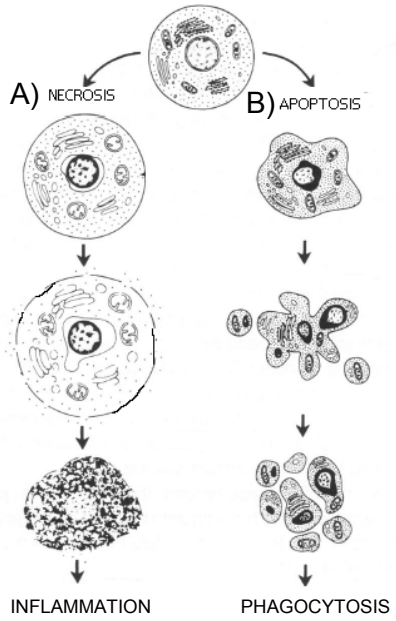

Figure 1-3: ***Necrosis versus apoptosis:*** Two types of cell death are involved in myocardial ischemic-reperfusion injury. A) Necrosis leads to rupture of the cell membrane, release of intracellular components and finally inflammatory reactions. B) Apoptosis is a more organized process with cell shrinkage and formation of apoptotic bodies which are phagocytised by surrounding cells (Figure modified from Opie, 2004 [25]).

1.3.3 Reperfusion therapy

As already mentioned, early reperfusion is important to limit the injury after an ischemic episode. Simultaneously, suppression of the cell death caused by reperfusion itself would be favourable to improve patient outcome. This is one of the main limitations of the reperfusion therapy used in the clinic today, as they do not influence the signalling pathways leading to increased cell death in the reperfusion phase. More recent approaches include pre- and post-conditioning where the heart is subjected to one or more episodes of sub-lethal ischemia followed by reperfusion, either prior (pre) or after (post) the main ischemic event. Both these techniques have shown to be powerful protectors against ischemic reperfusion injury by activating the reperfusion injury salvage kinase (RISK) cellular pathway [28, 29]. This pathway mainly activates two key proteins, namely Akt and Erk1/2 which both are known as prosurvival kinases [28]. Subsequently, the recruitment of the RISK pathway, for instance through pharmacological interventions, allow one to benefit from the cardioprotective effects seen in both pre- and post- conditioning.

1.3.3.1 Insulin therapy

One example of a pharmacological intervention used as reperfusion therapy is insulin administration. Insulin is a polypeptide hormone with extensive biological effects including regulations of glucose metabolism, protein and lipid synthesis, as well as gene transcription [31]. The biological effects are exerted by its binding to the α subunit of the heterodimeric insulin receptor situated in the cell membrane. Further on, the intrinsic tyrosine kinase activity of the α subunit induces phosphorylation of the β subunit of the receptor, leading to activation of various signalling pathways. Amongst these are the prosurvival phosphatidylinositol 3- kinase (PI3K) – Akt/ protein kinase B (PKB) signalling pathway, which is responsible for most of the metabolic actions of insulin [32]. Interestingly, this has also shown to be one of the main routes in the RISK signalling pathway [28]. As illustrated in Figure 1-4, phosphorylated Akt results in subsequent phosphorylation of various proteins, including p70 S6 kinase, mTOR and BAD. The overall effect of these cellular events is inhibition of apoptosis with increased cell survival [14].

The role of insulin as a promoter for cell survival, in the context of reperfusion injury, is investigated in several studies [14, 27]. Jonassen *et al.* showed that insulin administrated for

the first 15 minutes of reperfusion, significantly reduced infarct size in the isolated perfused male rat heart by activating the PI3K/Akt cell surviving signalling cascade [14]. In addition, the cytoprotective effect of insulin at reoxygenation were demonstrated to attenuate apoptosis in rat neonatal cardiomyocytes [27]. More recent studies by Tsang *et al.* describes a correlation between activation of the PI3K/Akt pathway and inhibition of mPTP opening [29], giving rise to a possible explanation of insulin's cardioprotective effect. As previously described, several factors (Ca^{2+}, ROS, pH) mediate opening of the mPTP during ischemia/reperfusion, however, insulin's direct effect on these factors are not yet fully understood. Still, opening of the mPTP have shown to induce the release of pro- apoptotic factors, and hence contribute to increased cardiomyocyte death. Together, this demonstrates the possibility to modify pro- apoptotic cellular pathways with pharmacological agents, such as insulin, in order to reduce to extent of reperfusion injury.

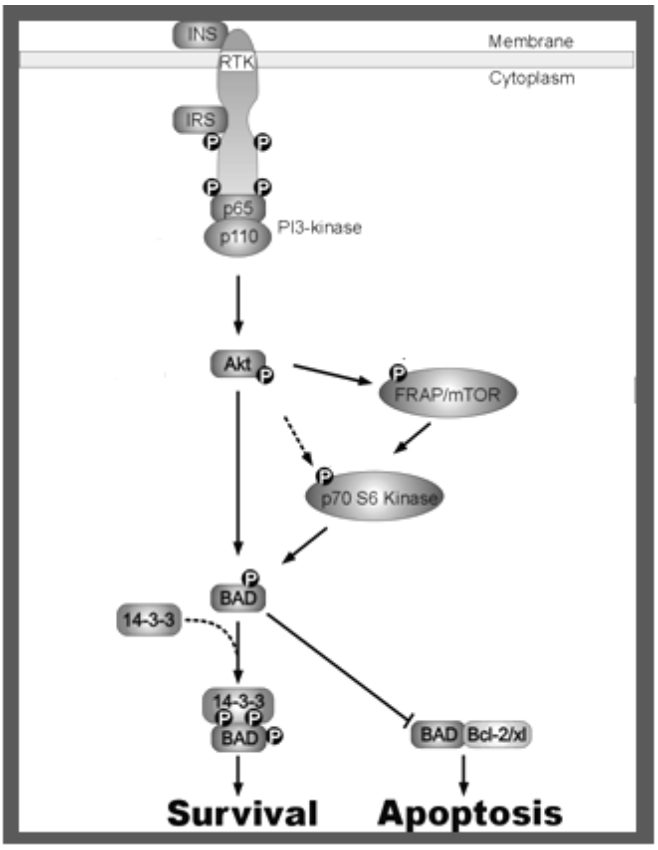

Figure 1-4: ***Insulin signalling:*** Insulin (INS) binds its tyrosine kinase receptor (RTK) in the cell membrane which then gets phosphorylated. This initiates the phosphorylation, and hence activation, of PI3-kinase and Akt with the following phosphorylation of various intracellular proteins; mTOR, p70S6 Kinase and BAD. The net effect of this signalling cascade is inhibition of pro- apoptotic pathways and increased cell survival (Figure modified from Jonassen, 2002 [33])

1.4 Glycogen Synthase Kinase 3β (GSK3β)

The present study focuses on a pathway for insulin mediated cell survival, compromising the multifunctional enzyme Glycogen synthase kinase 3 (GSK3). The name of the enzyme came from its ability to phosphorylate and thereby inactivate glycogen synthase, which is an important enzyme in the process of glycogen synthesis. There are two homologous isoforms of this enzyme, namely GSK3α and GSK3β, both exerting catalytic activity towards a number of intracellular substrates [22]. The two isoforms have 98% identity in their central catalytic domain [34], however the β isoform is reported to have a generally higher catalytic activity than the α isoform [35]. Moreover, studies with mice demonstrate that deletion of the GSK3β gene cause developmental arrest and embryonic lethality, thus indicating that GSK3α is unable to compensate for this gene loss [36]. Hence, GSK3β has been recognized as the most important isoform of the GSK3 enzyme.

GSK3β is a ubiquitously expressed and constitutively active enzyme, with the ability to phosphorylate and hence, regulate various cellular functions, including development, metabolism, gene transcription, protein translation, cell cycle and apoptosis [37]. As GSK3β is known to negatively regulate downstream signalling pathways, inhibition of this enzyme will stimulate these pathways, by removing the negative constrain imposed by GSK3β [38]. The active enzyme is described to facilitate apoptosis [39], a correlation that has been widely studied over the last years. An identified key mechanism is the ability for GSK3β to downregulate various transcriptional factors important for cell survival processes [37]. Additionally, GSK3β facilitates opening of the mPTP, resulting in the release of various pro-apoptotic factors from the mitochondria [22, 40].

1.4.1 GSK3β regulation

Various factors regulate GSK3β activity, including stimulatory tyrosine phosphorylation, formation of protein complexes and intracellular location. However, *inhibition* of the constitutively active enzyme through *phosphorylation* of the Ser-9 site has shown to be the most essential regulation mechanism in terms of anti-apoptotic actions (Fig. 1-5) [37]. Sutherland *et al.* were the first to identify GSK3β phosphorylation and showed this through *in vitro* phosphorylation of the enzyme both by MAPKAP kinase-1 (also called p90Rsk) and

p70S6 kinase [41]. A more recent study in cardiomyocytes describes even more pathways leading to inhibition of GSK3β, including activation of PI3K/Akt- , PKC- and PKA pathways [22]. To summarize, the literature describes numerous pathways all converging in the phosphorylation and hence inhibition of GSK3β (Fig. 1-5).

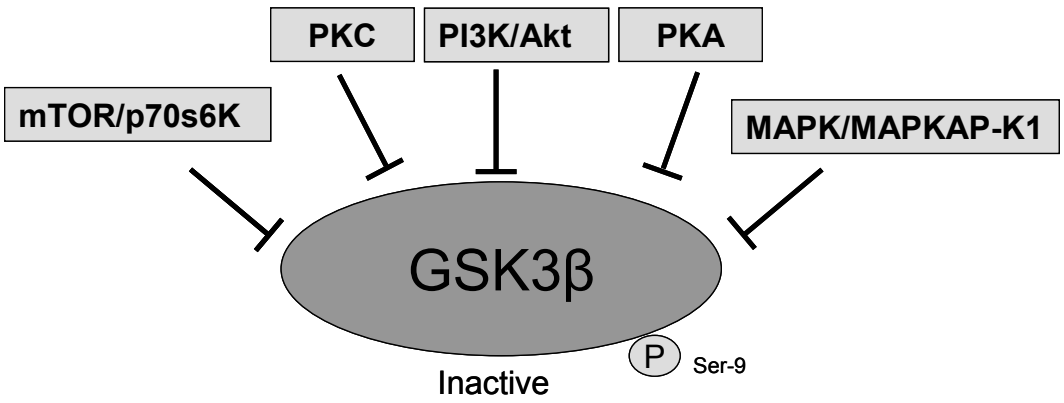

Figure 1-5: ***Regulation of GSK3β:*** Many upstream signalling kinases such as mTOR/p70s6K, protein kinase C (PKC), phosphatidylinositol 3- kinase (PI3K) / Akt, protein kinase A (PKA) and MAPK/MAPKAP-K1 are reported as inhibitors of GSK3β through phosphorylation at Ser-9. Based on Juhaszova *et al.*, 2004 [22].

1.4.2 GSK3β and cardioprotection

Several studies describe that inhibition of the constitutively active GSK3β through phosphorylation, leads to cardioprotection. Pre- and post-conditioning as described earlier [28], administration of opioids [42], erythropoietin [43] and propofol [44] are all treatments that have shown to be cardioprotective by increasing GSK3β phosphorylation. However, the so far most comprehensive study of GKS3β and cardioprotection, is the one performed by Juhaszova and colleagues [22]. Their data show a wide variety of drugs, including insulin, diazoxide and an N^+-H^+ exchanger inhibitor, exerting a protective effect in cardiomyocytes through increased phosphorylation of Ser-9 and thus inactivation of GSK3β with the following reduction in mPTP- opening and apoptosis. To verify these findings, additional

studies were performed on transgenic mice with a serine-9-to-alanine mutation, and hence a signal- resistant GSK3β enzyme. When treated with the same drugs as described above, no cardioprotection was observed. These results further prove the important role of GSK3β inhibition in the cell survival pathways facilitating cardioprotection [22].

Consistent with the findings of Juhaszova *et al.,* more recent studies by Gross *et al.* demonstrate that administration of the selective GSK3β – inhibitors SB-216763 or SB-415286 either 5 minutes before the onset of reperfusion or 10 minutes before the ischemic event, significantly reduced infarct size in the *in vivo* rat heart [23, 42]. Additional experiments also show that co-treatment with both SB-216763 and inhibitors of the PI3K/Akt and mTOR signalling cascade reduce infarct size in the *in vivo* rat heart, indicating that Akt and mTOR are upstream negative regulators of GSK3β (Fig. 1-5) and that SB-216763 is a direct inhibitor of GSK3β working independently of these pathways [42].

Based on the literature described, and the research interest of our group, this project was initiated to further investigate the involvement of GSK3β inhibition and insulin in cardioprotective reperfusion therapy in the female heart. As myocardial infarction is a sudden, acute condition, pharmacological treatment before the onset of ischemia is difficult. In contrast, drug administration in the reperfusion phase, which is when the occluded artery is mechanically or pharmacologically reopened, would be a more applicable approach. With the clinical aspect in mind and in contrast to the work done by Gross *et al.* [23, 42], we have administered drugs at the onset of reperfusion instead of prior or during the ischemic phase. More specifically, we wanted to investigate whether the GSK3β- inhibitor SB-415286 administrated for 15 minutes from the onset of reperfusion, offers cardioprotection after an ischemic event. Additionally, as insulin has been shown to phosphorylate GSK3β via the PI3K/Akt pathway, we wanted to explore whether combinational treatment of insulin and the GSK3β-inhibitor had any synergistic cardioprotective effect. An overview of the cellular pathways involved in this treatment is illustrated in Figure 1-6.

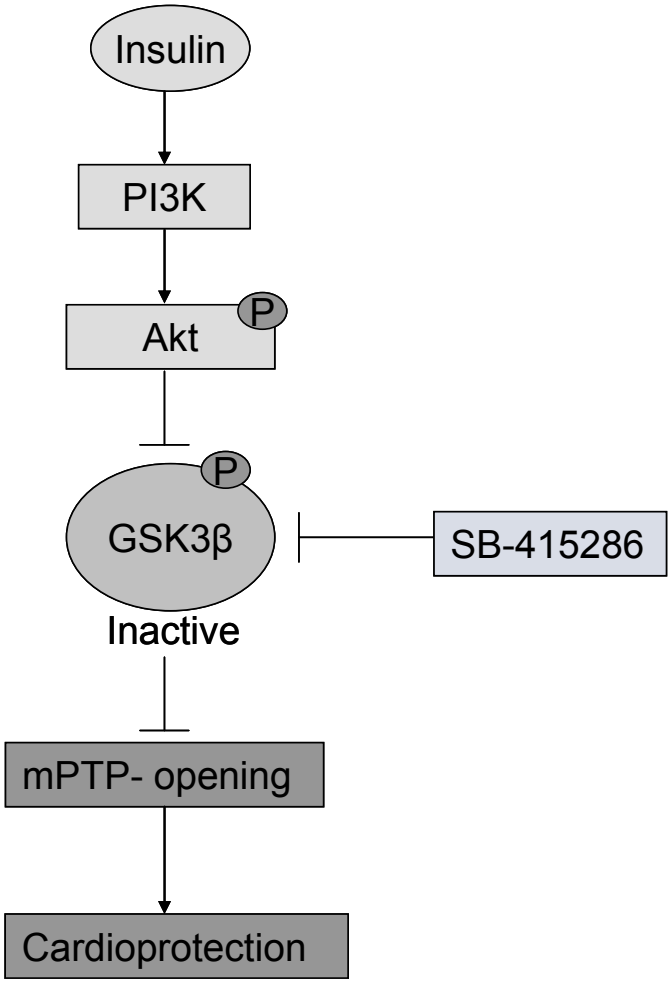

Figure 1-6: ***Insulin and GSK3β- inhibitor therapy:*** Insulin is a known activator of the PI3K/Akt pathway which leads to phosphorylation (P on Ser-9) and inhibition of GSK3β. SB- 415286 acts as a potent inhibitor of GSK3β by binding an important ATP site on the enzyme. The cardioprotective effect observed with GSK3β inhibition can be explained by reduced mPTP opening, less apoptosis and hence increased cell survival. By inhibiting the enzyme using both insulin and SB-415286, we hypothesise a synergistic cardioprotective effect.

1.5 Aims of study

The specific aims of this study are:

1) Explore if insulin therapy administrated at ischemic reperfusion also confer cardioprotection in the isolated *ex vivo* female rat heart.

2) Identify whether the GSK3β-inhibitor SB-415286 administrated for 15 minutes from the onset of reperfusion can reduce infarct size in the isolated *ex vivo* female rat heart.

3) Delineate whether there is a synergistic effect by combining insulin and GSK3β-inhibition (SB-415286) treatment at reperfusion in terms of reducing infarct size.

4) Investigate the phosphorylation status of Akt at early ischemic reperfusion in the isolated *ex vivo* female rat heart, after being exposed to the various treatments described above.

2. Materials and methods

Three distinct methods have been used in this project; perfusion of *ex vivo* isolated rat hearts in the Langendorff setup, isolation of heart tissue and finally Western Blotting. To evaluate the cardioprotective effect of our treatments, *ex vivo* isolated rat hearts were perfused through the Langendorff system, subjected to regional ischemia, treated with various agents at reperfusion and finally examined for determination of infarct size. Isolation and homogenisation of cardiac tissue, followed by protein detection with SDS- PAGE and Western Blotting was performed to investigate the role of the prosurvival kinase Akt in the various treatments.

2.1 The isolated e*x vivo* Langendorff perfused rat heart

2.1.1 Animal preparation

Female Wistar rats ordered from The Norwegian Institute of Public Health were used in the experiments (200 – 300 g, n=37). They were kept in the Animal Department at the Institute for Biomedicine, University of Bergen and were fed a standard diet with free access to water. All experiments were approved by the Norwegian State Commission for Laboratory Animals (id#1265 in "Forsøksdyrutvalgets tilsyns- og søknadssystem", FOTS), and carried out in accordance with the European Communities Council Directive of 1986 (86/609/EEC).

The rats were anesthetized with Pentobarbital 50mg/kg (i.p.) and simultaneously given 200IE (0.01mL) of the anticoagulant Heparin (Leo Pharma A/S). Before heart excision, both pedal and palpebral reflexes were checked to confirm adequate sedation.

2.1.2 Langendorff perfusion setup

Isolated female rat hearts were cannulated by the aorta and perfused through the Langendorff perfusion setup, allowing us to study heart function *ex vivo* (Fig. 2-1). Two separated reservoirs placed above the perfusion cannula were heated (37°C), oxygenated (95% O_2, 5% CO_2) and filled with Krebs- Henseleit buffer (KHB) (see Appendix) with or

without supplementation of the various treatment agents. Before the perfusion fluid entered the reservoirs, it was filtered through a 5.0 μM Millipore filter to ensure a fluid free from particles. By placing the reservoirs 1 meter above the heart, a constant hydrostatic pressure of 80 mmHg was maintained as long as the reservoirs were filled up to a marked level.

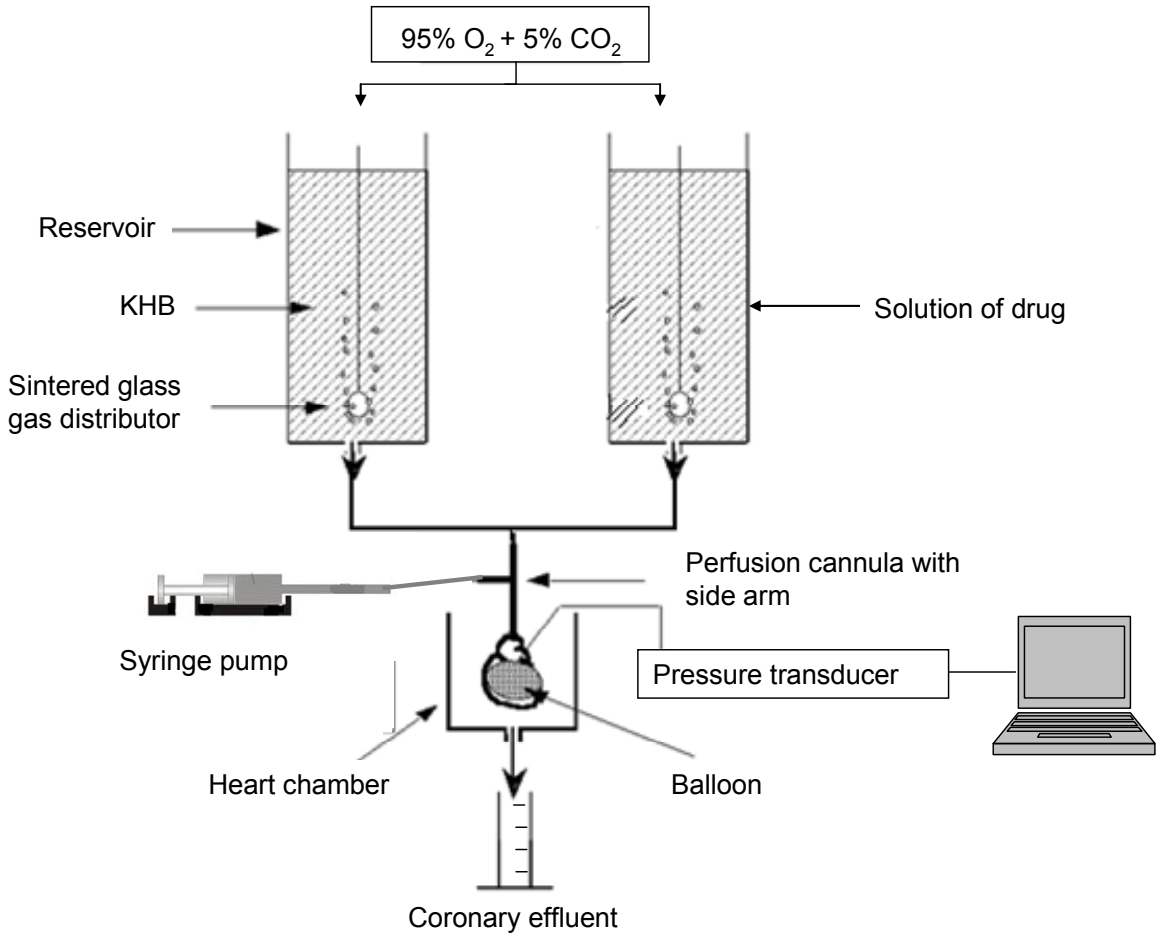

Figure 2-1: ***The Langendorff constant pressure isolated heart perfusion system.*** Two reservoirs connected to a perfusion cannula, ensured retrograde perfusion of the heart through aorta at a constant hydrostatic pressure (80 mmHg). One reservoir was filled with oxygenated Krebs-Henseleit buffer (KHB; left) while the other reservoir was filled with an oxygenated solution of drug (right). The side arm on the perfusion cannula allowed us to inject a solution of a second drug simultaneously, using a syringe pump. A temperature of 37 °C was maintained as the reservoir was surrounded by warm circulating water. To measure hemodynamic parameters such as heart rate and left ventricular developed pressure (LVDP), a water filled balloon was inserted into the left ventricle and connected to a pressure transducer coupled to a Power Lab recorder and a computer. The coronary flow could also be determined by timed measurements of the coronary effluent. (Figure modified from Sutherland *et al.*, 2000 [45]).

Perfusion fluid was led from the reservoirs, via rubber tubes and into a "bubble trap" with the function of removing/trapping air bubbles generated in the system. Further, the fluid was led through another tube and finally transferred via the cannulated aorta and into the heart. The perfusion cannula also contained a side arm, where administration of a second solution of drug could be performed using a syringe pump. A volume equivalent to 1/10 of the hearts coronary flow was introduced to the heart per minute, and by preparing a 10x concentration of the drug solution, the right dosage was ensured through the syringe pump. To monitor heart function, a small water filled latex-balloon connected to a pressure transducer (Edwards Lifescience LLC, CA, USA) was inserted into the left ventricle, hence giving measurements of both heart rate (HR) and left ventricular developed pressure (LVDP; LVDP= SP (Systolic pressure) – DP (Diastolic pressure)). The pressure transducer was calibrated with two known pressures (0 and 200 mmHg) before every experiment to ensure comparable measurements (see Appendix B for detailed description).

2.1.3 Langendorff perfusion procedure

Anesthetized rats were subjected to transabdominal incision and thorax was opened by cutting the ribs adjacent to sternum. The heart and a part of aorta were detached and transferred to ice-cold Krebs-Henseleit buffer (KHB), before being mounted onto the Langendorff perfusion system within 1 minute after excision (Fig. 2-1). This was performed by gently easing the aorta over the cannula, using a "bulldog" clamp to temporarily attach the heart, before tying a permanent silk suture around the aorta. Perfusion through the aorta was initiated and the heart was retrogradely perfused with oxygenated Krebs-Henseleit buffer (KHB). A constant hydrostatic pressure of 80mmHg ensured closure of the aortic valves and the heart was thereby perfused via the coronary ostia and drained into the right atrium through the coronary sinus [45].

Once the heart was adequately cannulated and subjected to perfusion buffer, normal heart rhythm and contractile function would re-establish within short time (seconds). A 3.0 silk suture attached to a small curved needle was then introduced to the heart and passed around the main branch of the left anterior descending artery (LAD; Fig. 2-3). The two ends of the suture were passed through a small plastic pipette tip to form a snare. By carefully tightening the suture, regional ischemia was introduced (Fig. 2-4), while reperfusion was achieved by releasing the snare.

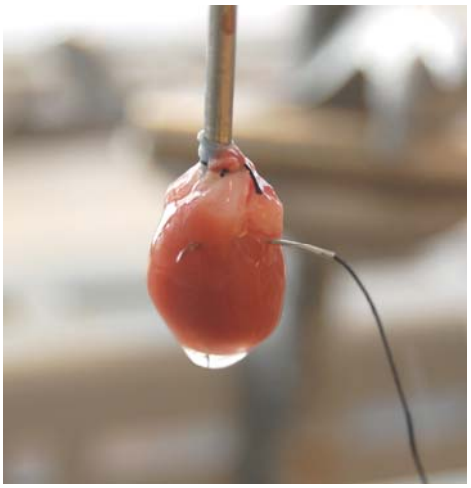

Figure 2-3: ***The isolated rat heart:*** The heart was cannulated via aorta and retrogradely perfused with KHB-buffer. To be able to induce regional ischemia, a silk suture attached to a curved needle was passed around the main branch of the left anterior descending artery (LAD).

To monitor cardiac function, a small pointed scissor was used to make a small opening in the left atrium in which a small compliant balloon of elastic rubber was inserted, passed through the mitral valve and placed into the left ventricle (Fig. 2-4). The balloon was connected via a water filled plastic catheter to a hydrostatic pressure transducer which was coupled to a Power Lab recorder (Power Lab System, AD Instruments). A microsyringe was used to fill the balloon with water until a left ventricular end diastolic pressure of 5-10 mmHg was obtained. In addition to left ventricular pressure measurements (diastolic, systolic and developed pressure) the balloon provided information about heart rate. All the mentioned data were recorded by the Power Lab and graphically expressed on the computerized program Chart-5 (AD Instruments) (Fig. 2-5).

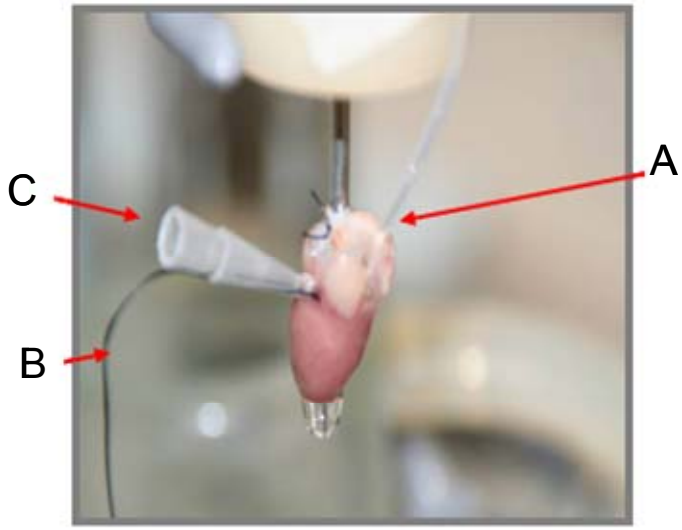

Figure 2-4: ***The isolated rat heart during regional ischemia***. A) To measure left ventricular pressure, a water filled latex balloon connected via a water filled catheter to a pressure transducer, was inserted into the left ventricle. B) A silk suture was passed around the left anterior descending artery (LAD) and C) two plastic pipettes were used to form a snare. By tightening the suture, regional ischemia was introduced, and reperfusion was achieved by releasing it.

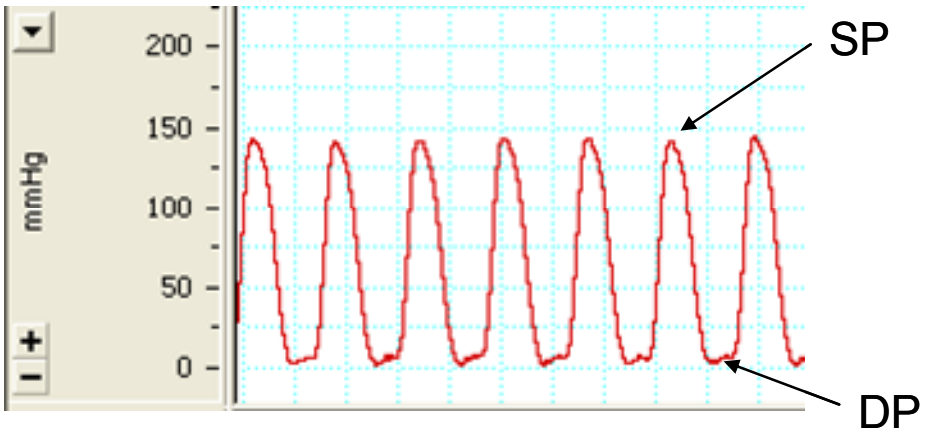

Figure 2-5: ***Data collection using the Power Lab.*** The graph demonstrates recordings of the left ventricular systolic and diastolic pressure (mmHg) during the stabilisation period. Peaks indicate the systolic pressure (SP) while the lowest points indicate diastolic pressure (DP). Left ventricular developed pressure (LVDP) is determined as: LVDP= SP-DP. The heart rate (HR) expressed in beats per minute is calculated by counting a minimum of 20 peaks (heartbeats) of the pressure curve and adjusting it in relation to time.

Temperature of 37- 37.5 °C was maintained by placing a water circulated heat chamber made of glass around the isolated heart, and was monitored by inserting a small thermistor-probe into the pulmonary artery. A measuring cylinder was placed below this chamber where coronary effluent was collected, thus, the heart's coronary flow (CF) measured in millilitres per minute could be determined (Fig. 2-1).

2.1.4 Measurement of infarct size

At the end of the experimental protocol, measurements of infarct size and risk zone were carried out as described by Ytrehus *et al.* [46]. The silk suture, used to induce regional ischemia, was again securely tightened around the left coronary artery (LAD), followed by injection of Evans blue dye (0.2 % w/v) through the sidearm of the cannula. This last step ensured demarcation of the area subjected to ischemia (area at risk of infarction = non-stained by Evans Blue) and the non-ischemic part of the heart (stained by Evans Blue) (Fig. 2-6).

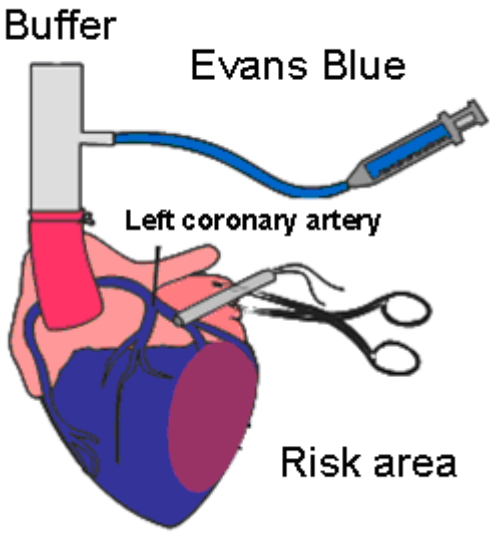

Figure 2-6: ***Determination of area at risk by injection of Evans Blue.*** The silk suture used to induce regional ischemia was re-tightened followed by injection of 2 ml of Evans Blue (0.2% w/v) via the sidearm of the cannula. Hence, only the area that had not been subjected to ischemia was perfused with the blue dye, whereas the ischemic area (area at risk) remained unstained.

Hearts were thereafter weighed, frozen and partly thawed, before being cut into 2 mm thick slices from the apex to the atrioventricular groove. Thawed heart slices were incubated in 1% triphenyl- tetrazolium- chloride (TTC; Sigma- Aldrich) solved in phosphate buffer (pH= 7.4, Appendix A) at 37°C for 20 minutes to ensured staining of the viable part of the risk zone (Fig. 2-7). A deep red colour develops as TTC reacts with NADH and dehydrogenase enzymes present in viable cells. Finally, the slices were immersed in 10% Formalin (Appendix A) in 1-3 days to enhance the contrast of stain between viable and non-viable areas (Fig. 2-7).

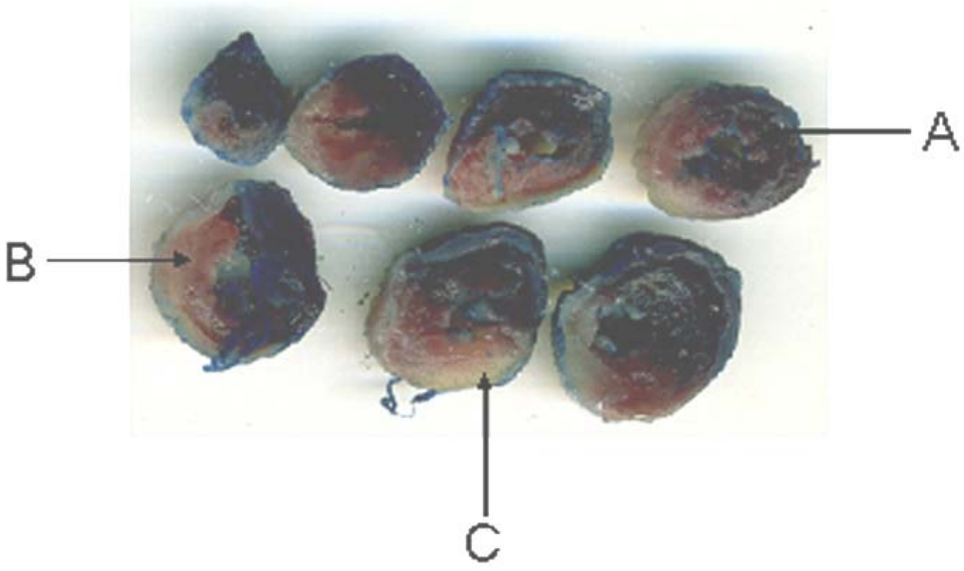

Figure 2-7: ***Triphenyl- tetrazolium- chloride (TTC) staining*****:** The hearts were cut into 2mm thick slices and stained with 1% TTC to separate viable from dead tissue. TTC staining was performed at 37°C for 20 minutes, followed by 1-3 days in formalin. A) The dark blue tissue represents the Evans blue staining, hence the area not subjected to ischemia. B) The red tissue marks the viable part of the ischemic area, while C) the white/grey tissue represents non-viable tissue.

Two glass plates separated by a 2 mm spacer ensured compression of the heart slices to a uniform thickness, and the slices were scanned (Epson Perfection 4490 Photo) to further analyze our result. Determination of the infarcted area (tetrazolium negative, grey), the viable risk zone (tetrazolium positive, red) and the area not subjected to ischemia (blue) was performed using the computerized programme *Planimetry*$^{+}$ (Traasdahl, University of

Tromsø, Norway). Tracing of the various areas with a digitalized tracing pad (Acedad III, Graphics Tablet) allowed us to analyse pixel density (Fig. 2-8). Hence, heart-volume, infarcted-volume and risk zone-volume, all expressed in mm^3, could be calculated by multiplying each area (mm^2) with the slice thickness (2mm) and then summing the products of each slice. The size of the infarction could then be expressed as a percentage of the area at risk (infarct/risk ratio (%)).

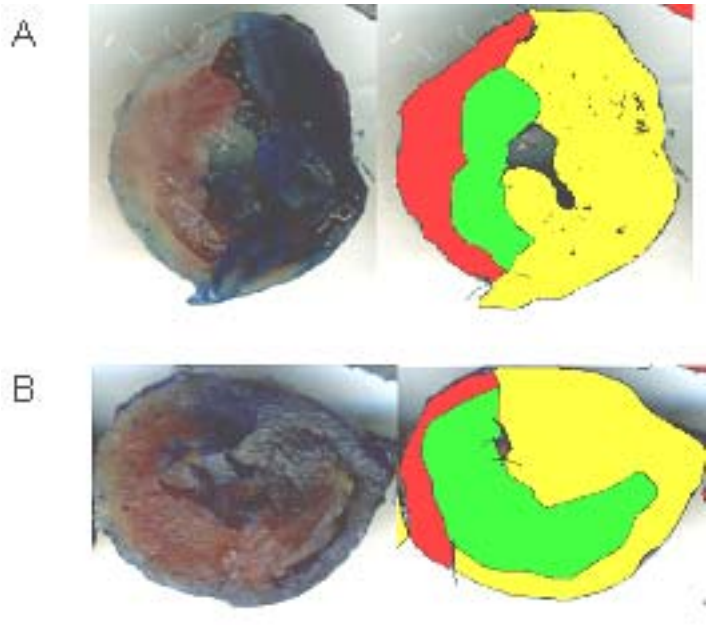

Figure 2-8: ***Example of digitalized tracing analysis using Planimetry*****+:** The different areas of the heart were traced using a digitalized tracing pad. Separation of the areas was done by using different colour codes: Red = non-viable area, green = viable part of the area at risk, yellow = area not at risk. A pixel density analysis calibrated to represent mm^3, was then performed to calculate the % infarct area/ area at risk ratio. A) Control heart perfused with KHB B) Heart co-treated with insulin and GSK3β-inhibitor.

2.1.5 Experimental protocol

The experimental protocol is shown in Figure 2-9. All hearts were stabilized for 20 minutes before being subjected to 30 minutes of regional ischemia (RI) and 120 minutes of reperfusion. Four groups of isolated hearts were studied: 1) Control (n=13), 2) Insulin (0.3 mU/mL, n=7), 3) GSK3β- inhibitor (3μM, n=8) and 4) Insulin + GSK3β- inhibitor (n=9). The control group was perfused with Krebs Henseleit Buffer (KHB) throughout the experiment, while the three following groups were respectively treated with Insulin (Novo Nordisk A/S, Denmark), the GSK3β- inhibitor SB-415286 (Tocris Bioscience, UK) or the combination of insulin and the GSK3β- inhibitor for 15 minutes given at the onset of reperfusion (Fig. 2-9).

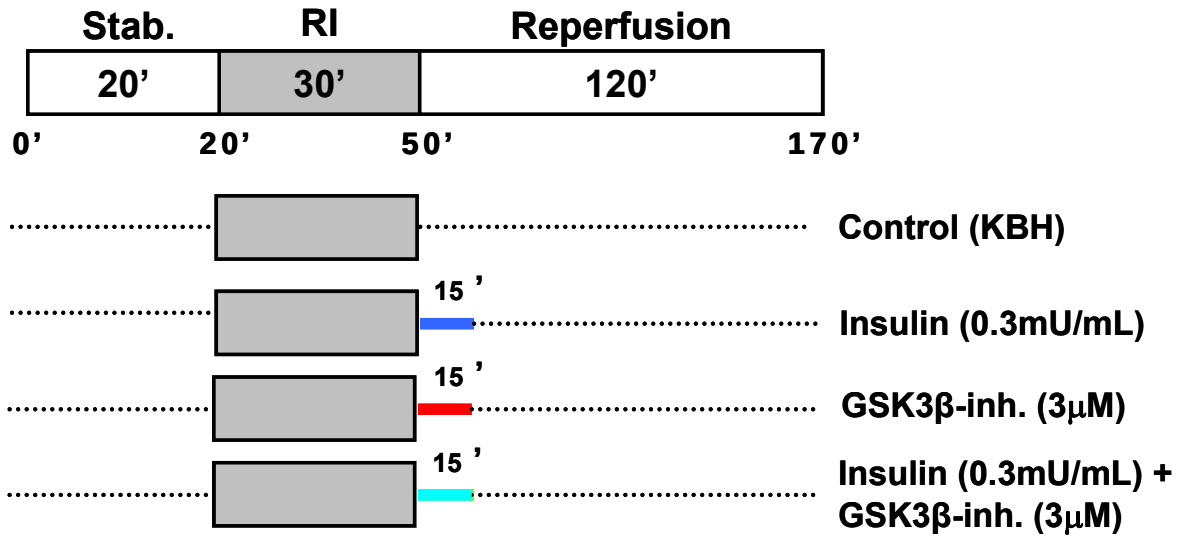

Figure 2-9: ***Experimental protocol for the isolated rat hearts subjected to regional ischemia and reperfusion.*** All hearts were stabilized for 20 minutes prior to 30 minutes of regional ischemia (RI) and 120 minutes of reperfusion. The control group was administrated Krebs-Henseleit buffer (KHB; dotted lines) throughout the entire protocol (for 170 minutes), while the three other groups received a 15 minutes treatment of either insulin (blue line), GSK3β- inhibitor (red line) or a combination of the two (turquoise line), from the onset of reperfusion.

2.2 Immunoblotting

To investigate which intracellular pathways insulin and the GSK3β inhibitor activate when administrated at ischemic-reperfusion, cardiac tissue was isolated and homogenised, followed by protein detection with SDS- PAGE and Western Blotting.

2.2.1 Tissue isolation

Hearts (n=25) were mounted onto the Langendorff perfusion setup and underwent protocols as presented in Figure 2-10. Baseline groups were subjected to a stabilisation period of 20 minutes, perfused with either KHB ($Control_{BL}$), 0.3mU/mL insulin ($Insulin_{BL}$) or 3μM GSK3β- inhibitor ($GSK3\beta\text{-}inh_{BL}$). The other hearts went through the ischemia-reperfusion period; however, the collection of tissue was performed after 15 minutes of reperfusion and injection of Evans Blue dye (Fig. 2-10). Hearts were immediately put on ice followed by removal of the right ventricle. The left ventricle of the baseline hearts were cut into small pieces, while hearts being exposed to regional ischemia, were divided based on stained (area non at risk = Evans Blue positive) and non-stained (area at risk) areas. The tissue samples were snap frozen in liquid N_2, and then stored at -80°C.

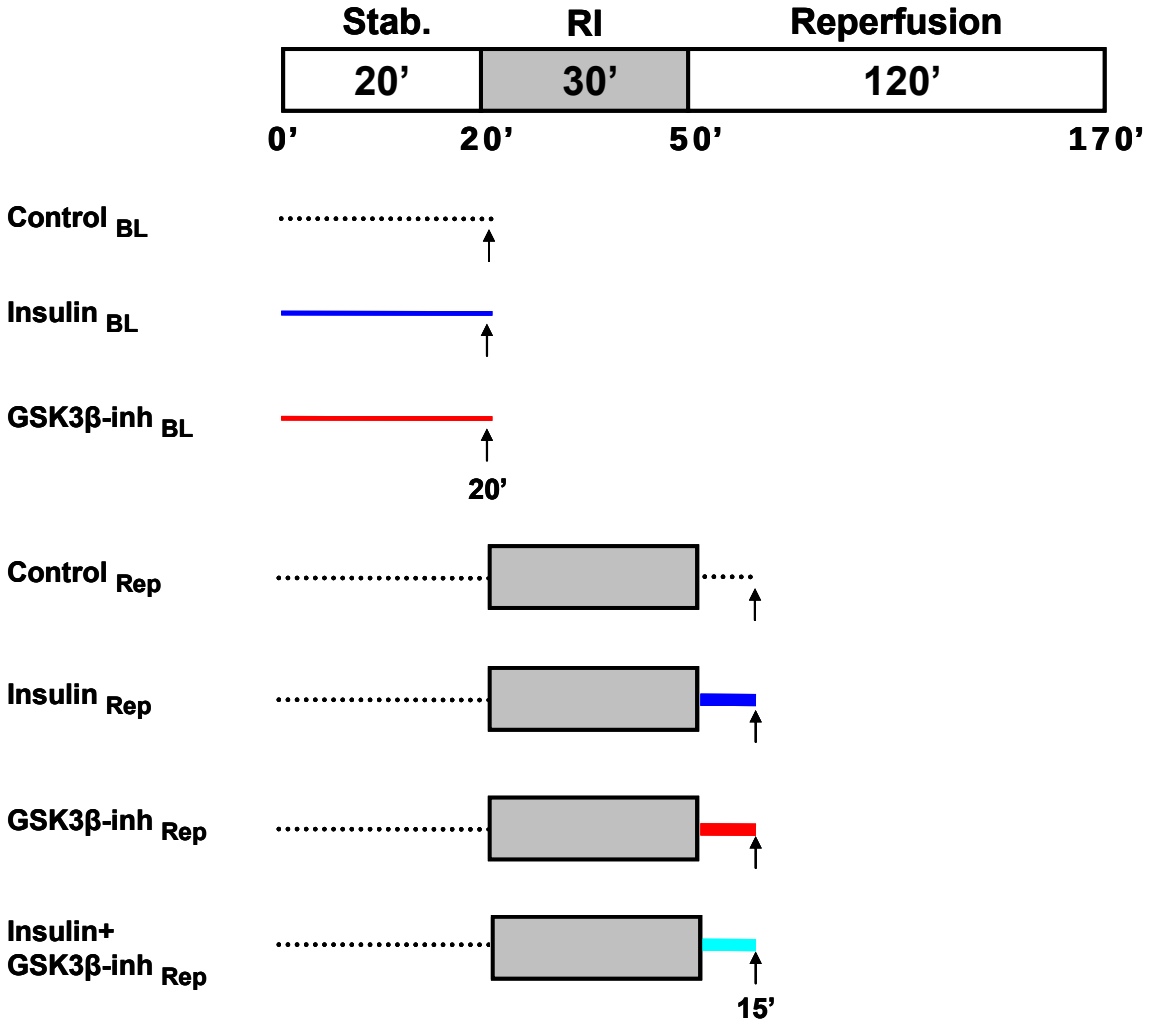

Figure 2-10: ***Protocol for tissue isolation using the Langendorff perfusion setup*****:** Baseline (BL) hearts were perfused with either Krebs Henseleit buffer ($Control_{BL}$; dotted line), 0.3 mU/mL insulin ($Insulin_{BL}$; blue line) or 3 µM GSK3β-inhibitor ($GSK3\beta\text{-}inh_{BL}$; red line) and collected after 20 minutes. 4 other groups underwent 20 minutes of stabilisation, followed by 30 minutes of regional ischemia (RI). At the onset of reperfusion the different groups were respectively perfused with KHB ($Control_{Rep}$), insulin ($Insulin_{Rep}$), GSK3β inhibitor ($GSK3\beta\text{-}inh_{Rep}$) or the combination of the two (Insulin + GSK3β- inh_{Rep}, turquoise line) for 15 minutes, before the tissue isolation was performed.

2.2.2 Tissue homogenisation and protein estimation

Cardiac ventricular heart tissue was homogenised in lysis buffer (Appendix A) using the automated tissue homogeniser Precellys 24 (Bertin Technologies), programmed to 3x15 seconds with 10 seconds break in between. Samples were then centrifuged (4°C, 14000 rpm, 10 min) to remove cell debris, leaving a supernatant containing the cytosolic fraction. Protein concentration of this fraction was determined as described in the applied BCA Protein Assay Kit (Thermo Scientific), and by measuring absorbance of the samples using a microplate spectrophotometer (VERSAmax^{TM}). Samples of known protein concentrations and their correspondent absorbance were expressed in a standardcurve (Fig. 2-11), and the unknown concentration of our samples could then be determined by extrapolation on this curve. Based on the estimated protein concentrations, the amount of sample (µL) loaded onto the gel could be calculated, to ensure equal amounts of proteins (µg) in each lane. Before final storage at -20°C, the homogenised samples were mixed with Sample buffer (Bio-Rad) in ratio 3:1.

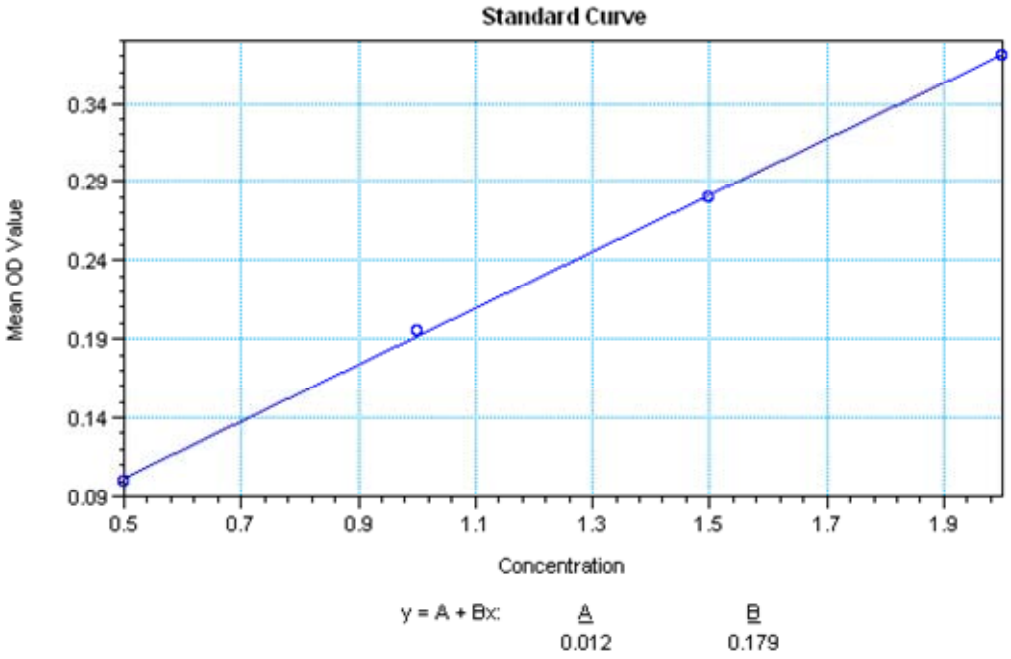

Figure 2-11: ***Standardcurve for determination of protein concentrations*****:** 4 standard samples of known protein concentration (0.5, 1, 1.5, 2 mg/mL) were mixed with the protein reacting BCA followed by measurement of light intensity using a spectrophotometer. These known concentrations were plotted against optical density (OD) in a linear standardcurve (y = A + Bx). The unknown protein concentrations of our samples could then be determined by mixing them with the same BCA, measuring optical density and calculating the x (concentration) in the linear standard function.

2.2.3 SDS- PAGE and Western Blotting

Myocardial phosphorylated Akt in the area at risk of infarction was determined by SDS-PAGE and Western Blotting using antibodies against p-Akt 473 (Cell Signalling) and total-Akt (Santa Cruz).

One dimensional SDS-PAGE (Sodium dodecyl sulphate - polyacrylamide gel electrophoresis; 12.5%) was used to separate the proteins by size. Samples containing Sample Buffer were slowly de-frozen on ice followed by 5 minutes boiling at 90°C to denaturise all proteins. 40µg of sample protein was loaded onto the gel in parallel lanes to a protein standard marker (Precision Plus Protein^{TM}, Bio-Rad) containing 10 peptides of various sizes, thus, serving as reference to easier detect the size of our protein of interest. The electrophoresis was carried out in Running Buffer (Appendix A) at 95V for 15 minutes to completely stack the proteins in the gel, and then at 120V for approximately 1 hour. Proteins were then transferred from the gel to a methanol pre-activated Hypobond^{TM}- ECL^{TM} PVDF Membrane (Amersham Bioscience), using a Bio-Rad Transblot "sandwich" immersed in Transfer Buffer (Appendix A). This process was performed at 100V for 1 hour with stirring and in a cold environment to prevent the gel from melting. Further, the membrane was activated in methanol a second time and air-dried, followed by incubation with 5% non-fat dried milk (Marvel Dried Skimmed Milk) in Tris-Buffered Saline 0.1% Tween-20 (TBS-T; Appendix A) for 2 hours. The milk proteins saturate the membrane and prevent unspecific binding of antibodies. TBS-T was used to wash the membrane (2x2 minutes by hand and 2x10 minutes on an orbital shaker) followed by incubation of primary antibody (p-Akt 1:500) over night at 4°C on an orbital shaker. The following day, the membrane was again washed with TBS-T, probed with the appropriate secondary antibody (1:10000; anti- rabbit against p-Akt) for 1 hour at room temperature and finally washed one last time. Secondary antibodies used in this experiment are conjugated to a horseradish peroxidase enzyme (HRP) with the ability to amplify signals and increase detectability of our protein. Addition of the chemiluminescent agent ECL Western Blotting Substrate (Pierce) to the membrane ensured an electrochemical reaction with the HRP bound 2° antibody, producing luminescence which is directly proportional to the amount of probed protein (Fig. 2-12). A highly advanced CCD- camera (The Bio-Rad Gel Doc XR) was used to capture the strength of this light emission, expressing it as a digital image of the western blot. The Bio-Rad Quantity One®

software could finally optimize and analyse the images by quantifying the relative amount of protein based on its optical density (densometric reading).

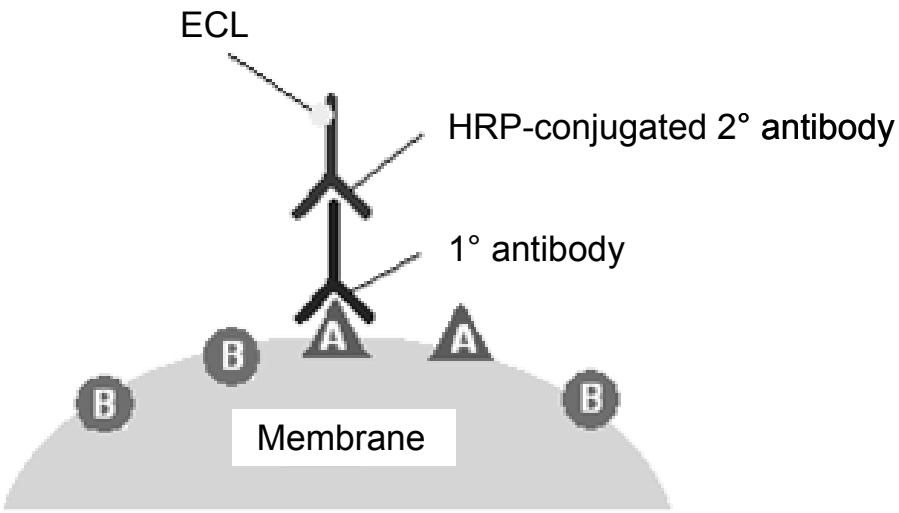

Figure 2-12: ***Immunohistochemical reactions on the protein containing membrane*****:** The membrane containing the protein of interest (A) is probed with a 1° antibody at 4°C over night. Incubation of a horseradish peroxidase (HRP) conjugated 2° antibody for 1 hour at room temperature, ensures binding to the 1° antibody. To detect the protein of interest, an electrochemiluminescent agent (ECL) is added, causing a light emitting reaction with the HRP enzyme, being directly proportional to the amount of protein A on the membrane. Based on the strength of this reaction, protein quantification can be performed.

2.3 Statistical analysis

Values are presented as mean ± standard error of mean (SEM). One way analysis of variance (ANOVA), followed by a Fisher's post hoc test, was used to test for significant differences between and within groups (Minitab 15, Minitab Inc, USA). $P \leq 0.05$ was considered statistically significant.

3. Results

3.1 The isolated e*x vivo* Langendorff perfused rat heart

3.1.1 Heart and body weight

To exclude variation, the body weight of the rats was measured, as well as heart weight after completed protocol and infusion of Evans Blue dye. No statistical significant difference was found in either rat weight (200-300g) or heart weight (1- 1.7g) between the experimental groups.

3.1.2 Infarct Size

Infarct size was determined as the ratio between the volume of the infarcted area and the volume of area at risk, expressed in percentage (Fig. 3-1). Treatments with insulin, GSK3β-inhibitor or the combination of both for 15 minutes at the onset of reperfusion, reduced infarct size with respectively 35%, 36% and 41% as compared to control (Ctr = 45.9±3.0% vs. Insulin= 29.9±5.5%, GSK3β-inh.= 29.4±2.7%, Insulin+GSK3β-inh.= 27.1±5%, $p<0.05$). However, no significant difference was observed in infarct size when comparing the insulin-, GSK3β- inhibitor- and insulin + GSK3β inhibitor groups (Fig.3-1).

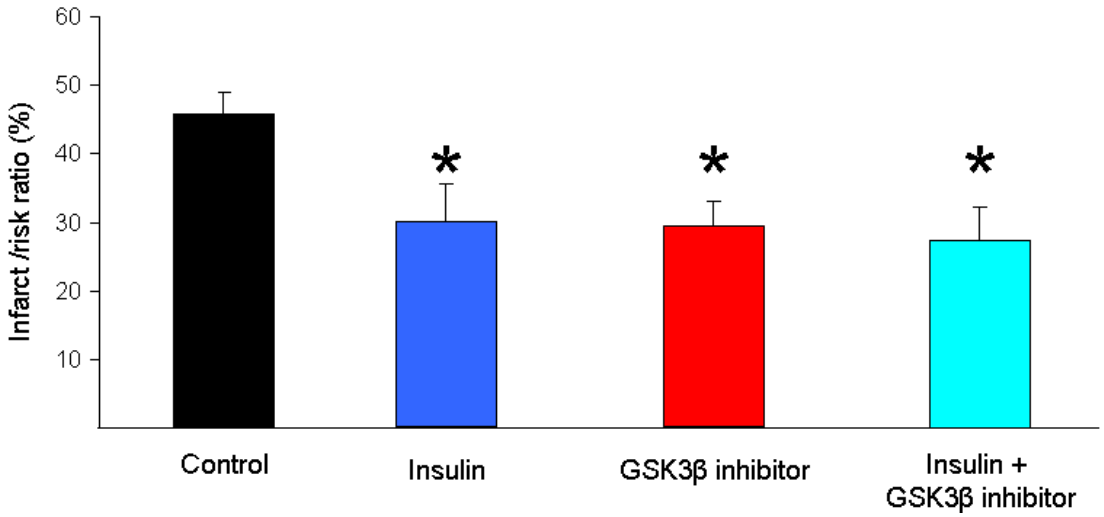

Figure 3-1: ***Infarct size expressed as percentage of area at risk*:** Infarct size is expressed as percentage of the area at risk of infarction. Treatment with either Insulin (blue, n=7), GSK3β- inhibitor (red, n=8) or the combination of the two (turquoise, n=9) for 15 minutes at the onset of reperfusion, significantly reduced infarct size as compared to control (black, n=13). All values are presented as mean ± SEM. * $p<0.05$.

In order to evaluate variations in size of the areas at risk, volumes in the various groups were compared. There was no statistical differences in the volumes of the risk areas between the various experimental groups (control 2475±279 mm^3, insulin 2311±356 mm^3, GSK3β-inh 1924±217 mm^3, insulin + GSK3β-inh 1550±126 mm^3, $p<0.05$). Volume of infarct size (mm^3) versus volume of area at risk (mm^3) is presented in Figure 3-2, and reflects that all treated hearts had a lower infarcted volume compared to control hearts, although being in the same range of volume of area at risk (~1000-3000mm^3). This implies that the infarct reducing effect of the various drug treatments was not a consequence of variation in area at risk, but rather a result of the treatment itself.

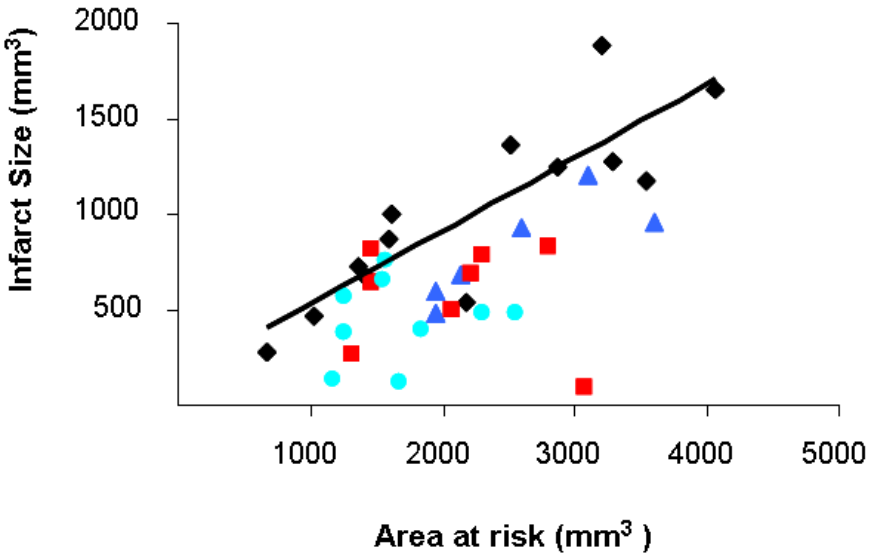

Figure 3-2: ***Infarct size (mm^3) versus size of area at risk (mm^3)*****:** The infarcted volume (mm^3) in the ◆ control hearts is clearly higher than the corresponding volume in the ▲ insulin-, ■ GSK3β-inhibitor- and the ● insulin + GSK3β inhibitor treated hearts, as they all lie under the regression line for the control hearts. Although there are variations in the area at risk, most hearts are concentrated between area of risk volumes of 1000 and 3000 mm^3. The infarct reducing effects of the treatments is not a consequence of variations in the size of the area at risk.

3.1.3 Hemodynamic variables

Measurements of coronary flow (CF), left ventricular developed pressure (LVDP) and heart rate (HR) were performed at regular intervals during the experimental protocol (Fig. 3-3). The four experimental groups showed no significant difference in either coronary flow or left ventricular developed pressure at any time point during the experiment. Still, regional ischemia clearly reduced cardiac function in all groups as confirmed by a substantial decrease in both CF (mean fall by 46%) and LVDP (mean fall by 49%), while appropriate reperfusion was confirmed by an increase of the same variables (Fig. 3-3). Heart rate did not differ significantly between the groups at any time during the experiments, with mean values of 278 beats/minute (BPM) at the end of stabilisation, 236 BPM at 25 minutes of ischemia and 259 BPM at the end of reperfusion.

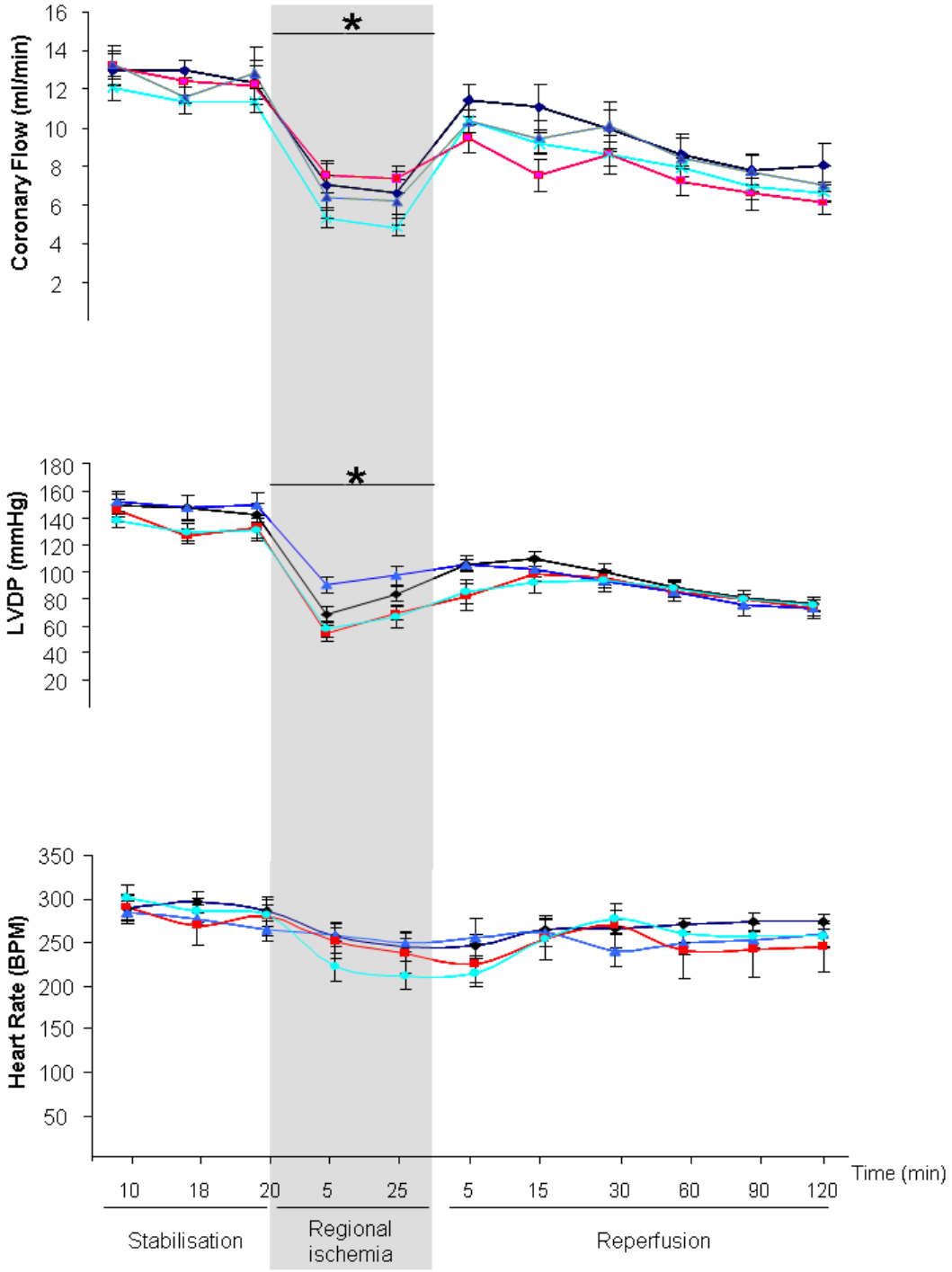

Figure 3-3: ***Coronary flow, left ventricular developed pressure and heart rate during the experiments*****:** The four represented groups are: ◆Control, ▲15 min insulin at reperfusion, ■ 15 min GSK3β inhibitor at reperfusion, ●15 min insulin + GSK3β inhibitor at reperfusion. No statistical difference was found between the four groups in either coronary flow (CF), left ventricular developed pressure (LVDP) or heart rate (HR) at any timepoint of the experiment. However, a significant decrease in both CF and LVDP in the regional ischemic phase was observed for all groups (*$p<0.05$), followed by an increase of the same variables upon reperfusion. Heart rate remained relatively stabile throughout the whole experiments in all four groups. Although a slight decrease in HR during the ischemic phase was observed, the differences were not statistical significant.

3.2 Immunoblot analysis – phosphorylation of Akt

As already described, insulin is known to phosphorylate the prosurvival kinase Akt at Ser 473. Baseline hearts perfused with 0.3mU/mL insulin for 20 minutes ($Insulin_{BL}$) showed a high degree of Akt phosphorylation compared to baseline control hearts perfused with Krebs-Henseleit buffer ($Control_{BL}$), as demonstrated in the Western Blot in Figure 3-4A. GSK3β-inhibitor baseline hearts were perfused with 3μM GSK3β-inhibitor for 20 minutes, resulting in a very low Akt phosphorylation. Regional ischemia followed by 15 minutes of reperfusion, clearly increases the level of phospho- Akt (p- Akt) in control hearts ($Control_{Rep}$), with an even stronger activation in the various groups given treatment for 15 minutes at the onset of reperfusion ($Insulin_{Rep}$, GSK3β-inh_{Rep} and Insulin+GSK3β- inh_{Rep}). Figure 3-4B is an example of a densometric analysis of the Western Blot bands. Due to time limitations, the Western Blot data were not completed, and further tissue isolation and immunoblotting is therefore required to draw any precise conclusion.

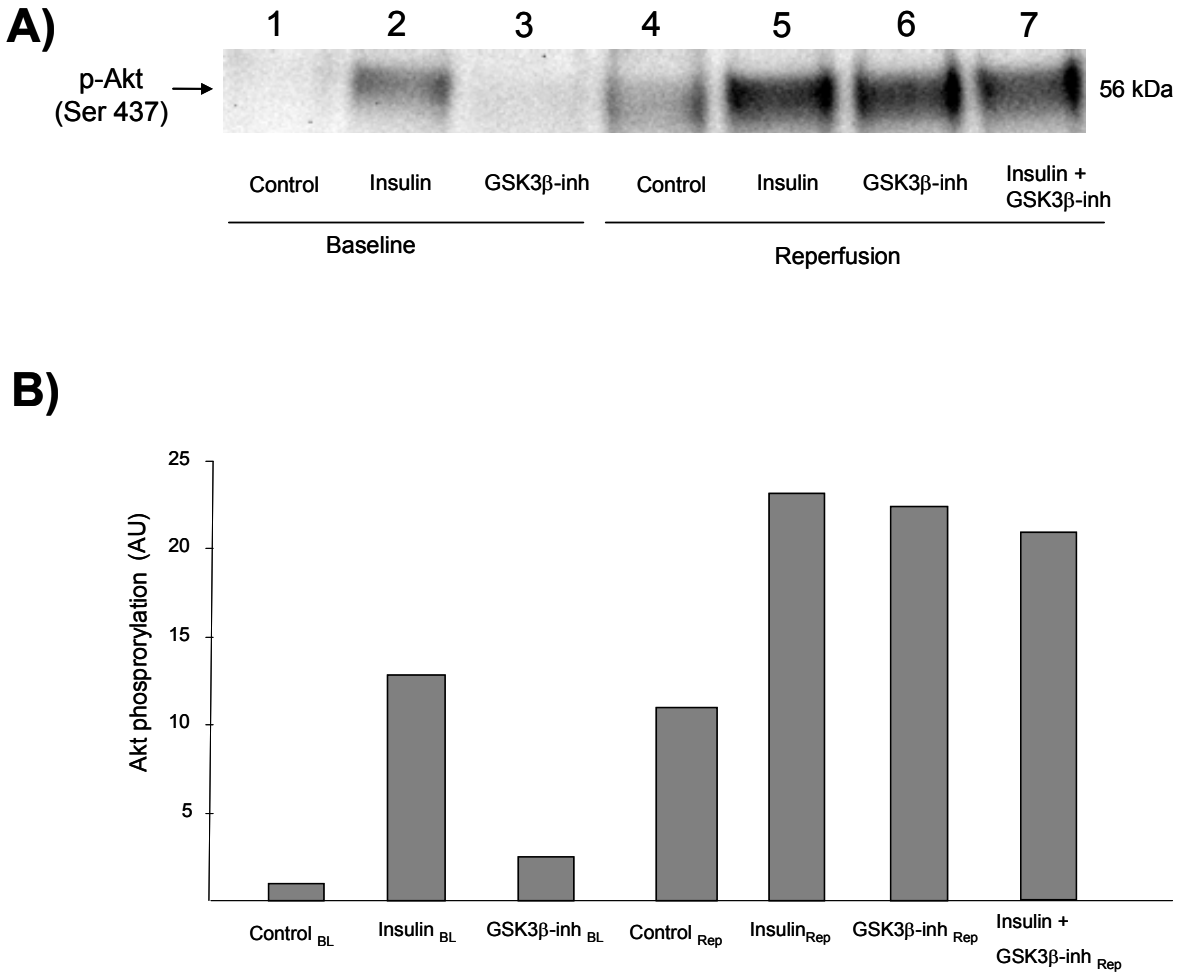

Figure 3-4: ***The effect of insulin and GSK3β inhibition on Akt phosphorylation*****:** A): A representative Western Blot showing the effect of insulin and GSK3β inhibition on Akt phosphorylation. Baseline (BL) hearts were perfused with either Krebs-Henseleit buffer ($Control_{BL}$), 0.3 mU/ml Insulin ($Insulin_{BL}$) or 3μM GSK3β inhibitor for 20 minutes. Reperfused (Rep) hearts underwent 30 min of regional ischemia, followed by 15 min of reperfusion with either Krebs-Henseleit buffer ($Control_{Rep}$), insulin ($Insulin_{Rep}$, 0.3mU/mL), GSK3β inhibitor ($GSK3β\text{-}inh_{Rep}$, 3μM) or insulin + GSK3β inhibitor (Insulin + GSK3β- inh_{Rep}). B): Densometric analysis of Western Blot showing Akt phosphorylation, expressed in arbitrary units (AU) with $Control_{BL}$ = 1.

4. Discussion

This discussion section has been divided into two parts, where of the first part will concern the central findings of the present study and the second part comprises methodological considerations.

4.1 Discussion of central findings

4.1.1 Summary of main results

Although reperfusion is required to salvage ischemic myocardial cells from cell death, reperfusion of the occluded artery *per se* can induce myocardial damage, a phenomenon termed *lethal reperfusion injury*. In this study we aimed to test whether three various pharmacological treatments could reduce the extent of reperfusion injury caused by myocardial ischemia in female rat hearts. We demonstrate that either administration of insulin, the GSK3β inhibitor (SB-415286) or the combination of both, offers cardioprotection in terms of reducing infarct size by 35, 36 and 41% in the *ex vivo* isolated female rat heart. In order to reveal the signalling pathways involved in this cardioprotection, phosphorylation status of the prosurvival kinase Akt was investigated, suggesting that Akt is involved in the intracellular cytoprotective cascade following insulin therapy at ischemic reperfusion.

4.1.2 The cardioprotective effect of insulin

Dosage (0.3 mU/mL) and timing of our insulin administration was taken from previous dose-response studies in our group performed on the male rat heart [14]. Administration of insulin for 15 minutes at the onset of reperfusion, following an ischemic event, induces a significant reduction in infarct size in the *ex vivo* isolated female rat heart by 35% as compared to control. The same degree of cardioprotection is observed in male rat hearts (unpublished data by A.Wergeland performed in parallel to the current study). In addition, previous work by Jonassen *et al.* demonstrates the cardioprotective effect of insulin both in cardiomyocytes, Girardi cells and in *ex vivo* isolated male rat hearts [14, 16, 17, 27].

Consequently, the main finding in this part of the study is that insulin appears to protect the female myocardium to the same extent as the male rat hearts.

The mechanism by which insulin exerts its cardioprotective effect is investigated by many researchers [14, 47, 48]. As previously described, myocardial ischemia causes an abrupt increase in [Ca^{2+}] followed by mitochondrial damage and myocardial contracture [3]. One of the underlying causes is known as increased release of endogenous catecholamines in the ischemic myocardium, stimulating β-adrenergic receptors [49]. A study by Yu *et al.* suggests that the cardioprotective effect of insulin is due to its ability to counteract this β- adrenergic action and thereby preventing the increase in intracellular [Ca^{2+}] [47]. Moreover, insulin has been shown to increase glucose uptake in the ischemic myocardium [48], and by this contributing to glycolysis which is important to achieve functional and metabolic recovery after an ischemic event [50]. However, studies by Jonassen *et al.* suggest that insulin-mediated cardioprotection is independent of glucose at reperfusion [14]. Furthermore, results from the same study demonstrate that insulin has to be present at the immediate onset of reperfusion to exert its cardioprotection, and that this effect is mediated via the anti-apoptotic PI3K/Akt signalling pathway [14]. Our study supports these findings by conferring cardioprotection also in female rat hearts and by demonstrating the involvement of the prosurvival kinase Akt when insulin was administered at the onset of reperfusion.

Results presented in Figure 3-4 illustrate that perfusion of the isolated female rat heart with insulin both at baseline ($Insulin_{BL}$) and reperfusion ($Insulin_{Rep}$), stimulates phosphorylation of Akt compared to their respective controls. However, previous studies in our group show a higher degree of Akt phosphorylation in insulin treated baseline male hearts [14], which serve as the main aberration from our results. Possible reasons for this difference remain uncertain, although one could speculate that female hearts are less responsive to insulin activated Akt phosphorylation at baseline. However, contradictory results from Camper-Kirby *et al.* demonstrate indeed a higher level of phosphorylated Akt in female *mice* compared to male [51]. Thus, it is likely to believe that other factors, such as methodological issues or too few parallels (n), contribute to the reduced insulin mediated Akt phosphorylation in our experiments, compared to previous studies. Clearly, further experiments needs to be done to elucidate this aberration.

Interestingly, our results demonstrate that ischemia/reperfusion in itself phosphorylates Akt ($Control_{Rep}$), which is also supported by previous studies [14, 28]. Comparison of the $Control_{Rep}$–hearts with the insulin treated hearts ($Insulin_{Rep}$) show a higher extent of Akt phosphorylation in the latter group, hence, we can deduce that insulin causes an additional activation of the PI3K/Akt signalling pathway compared to ischemia/reperfusion in itself. Although the downstream targets of this pathway are not fully understood, its prosurvival effect has been demonstrated, and thus represents an explanation of insulin's cardioprotective effect.

4.1.3 The cardioprotective effect of GSK3β- inhibition

Results from the present study demonstrate that administration of the GSK3β-inhibitor SB-415286 for 15 minutes at the onset of reperfusion, significantly reduce infarct size in the *ex vivo* isolated female rat heart by 36% as compared to control. The same degree of cardioprotection is observed in male rat hearts (unpublished data by A.Wergeland). The GSK3β-inhibitor SB-415286 dose was set to 3μM, which compared to other studies [22, 52], is a lower dose (i.e. Das *et al.* used 10μM at Langendorff perfused rat hearts, although not investigating effects on infarct size). However, as our dose of 3μM conferred cardioprotection we continued to use it throughout the experiments. Recent and independent studies have also shown that inhibition of GSK3β offers cardioprotection in cardiomyocytes [22], *in situ* rat hearts [43] and in isolated *ex-vivo* guinea pig hearts [44]. Consistent with these findings, and in concordance to the studies done by Gross *et al.* on *in vivo* male rat hearts [23, 42], we demonstrated the ability for the GSK3β-inhibitor SB-415286, to reduce infarct size in the *ex vivo* isolated female rat heart.

Results illustrated in Figure 3-4 demonstrate that administration of the GSK3β inhibitor SB-415286 to female baseline rat hearts ($GSK3\beta\text{-}inh._{BL}$) causes low Akt phosphorylation. This result was expected, as GSK3β is known to be downstream of Akt [42], and therefore the phosphorylation status of Akt will not be influenced by direct inhibition of GSK3β. On the other hand, female rat hearts subjected to regional ischemia followed by 15 minutes of GSK3β inhibitor treatment ($GSK3\beta\text{-}inh._{Rep}$) showed an increased Akt phosphorylation compared to GSK3β-inhibitor baseline hearts. It is reasonable to believe that this effect is mediated by ischemia/reperfusion itself, as we have shown that GSK3β inhibition do not influence the phosphorylation status of Akt. Hence, we would expect that hearts treated with

GSK3β-inhibitor for 15 minutes at reperfusion (GSK3β-inh.$_{Rep}$) would have the same level of phosphorylated Akt as control hearts only being subjected to regional ischemia (Control$_{Rep}$). The explanation why our GSK3β-inh.$_{Rep}$ hearts show a higher degree of Akt phosphorylation (almost as high as Insulin$_{Rep}$-hearts) compared to Control$_{Rep}$-hearts is uncertain, yet it is likely to believe that methodological factors might have influenced the results (discussed later).

The knowledge about the precise mechanism by which GSK3β inhibition is cardioprotective remains incomplete, however, the mitochondrial permeability transition pore (mPTP) is hypothesized as an important target [22, 40]. The mPTP will open during reperfusion [53], and by inhibiting this opening, one will prevent influx of solutes and water, swelling and bursting of the mitochondrial matrix and thus hinder pro-apoptotic factors leaking into the cytosol [21]. Interestingly, Davidson *et al.* have shown that insulin also mediates inhibition of the mPTP through activation of the PI3K/Akt signalling pathway [54]. Still, the mechanism or the link between the prosurvival kinases and the mPTP remains unclear. Various mechanisms are suggested, where insulin mediated phosphorylation and inhibition of GSK3β is described as one of them (Fig. 1-6) [22, 54]. Hence, this serves as a possible explanation for the cardioprotective effect observed with both insulin and GSK3β-inhibitor treatment in the current study.

The exact interaction between GSK3β and the mPTP is not completely understood. However, Das *et al.* suggest a possible association with adenine nucleotide transport through voltage-dependent anion channels (VDAC), which are a component of the mPTP [52]. Their results demonstrate that GSK3β inhibition reduce the adenine nucleotide entry into the mitochondria by dephosphorylation of the mPTP, with likely consequences of lessening both mitochondrial calcium loading during anoxic conditions, as well as reducing endogenous oxygen radical production during reoxygenation/reperfusion [52]. In addition, data from the same study implies that GSK3β-inhibitors cause an increased affinity for the anti-apoptotic protein Bcl-2 to the mPTP, a result supported by Juhaszova *et al.* [22]. Consequently, both reduced calcium loading, reduced oxidative stress and more Bcl-2 binding to the mPTP are described as contributors to the cardioprotective effect of GSK3β-inhibitors [52].

Other theories are also described in the literature aiming to explain the cardioprotective effect of GSK3β-inhibition. One of the downstream targets of GSK3β is the oxygen

regulated Hypoxia-inducible transcription factor 1α (HIF-1α) [55]. This transcription factor is virtually undetectable in cells at normal oxygen levels, but will accumulate at low oxygen levels (hypoxia), resulting in increased transcription of various genes [55]. Active GSK3β is described to down-regulate protein levels of HIF-1α by initiating its degradation through phosphorylation [56]. Schnitzer *et al.* also reports that inhibition of GSK3β by the powerful inhibitor indirubins, reverse HIF1α depletion under prolonged hypoxia/anoxia [57]. As intracellular HIF1α-levels increase, a stimulation of gene-transcription will occur, where one of the regulated genes are coding for the adipocytokine *apelin* [58]. More specifically, Ronkainen *et al.* demonstrated that increased levels of HIF1α induce apelin expression in rat myocardium and in cultured cardiomyocytes [59]. Apelin is reported as cardioprotective by reducing infarct size after myocardial ischemia through increasing the phosphorylation status of Akt and MAPK [60, 61], and interestingly also by delaying the opening of the mPTP [61]. Based on these findings, it seems likely that both HIF1α and apelin might be contributors to the cardioprotective effect of GSK3β-inhibition. In addition, preliminary results from our own group have shown an increase in apelin mRNA at 120 minutes of reperfusion during insulin therapy (unpublished data by Mari Johansen), suggesting a possible role for apelin also in insulin mediated cardioprotection.

4.1.4 The cardioprotective effect of the co-treatment with insulin and GSK3β- inhibitor

The PI3K/Akt signalling pathway, which is activated by insulin, has shown to inhibit GSK3β by phosphorylation. By combining insulin treatment with a pharmacological, direct inhibitor of GSK3β (SB-415286), we hypothesised a synergistic cardioprotective effect compared to the two treatments given alone. A search on PubMed reveals no previous studies where combinational treatment with insulin and GSK3β-inhibitor has been tested. Therefore our study demonstrates a new approach in finding cardioprotective therapy against ischemic-reperfusion injury.

Our results show a significant decrease in infarct size when the co-treatment of insulin and GSK3β-inhibitor were administrated for 15 minutes at the onset of reperfusion to *ex vivo* female rat hearts (Fig. 3-1). Yet, this treatment shows *no additional* reduction in infarct size compared to the single treatments alone. The same results are observed in male rat hearts (unpublished data by A.Wergeland).

The reason why no additional cardioprotective effect is observed with the co-treatment remains uncertain, although a maximum saturation and inhibition of the GSK3β enzyme either by insulin or the direct inhibitor SB-415286 might be a possible explanation. This intensifies the hypothesis that the cardioprotective effect of insulin and the GSK3β-inhibitor is mediated via the same signalling pathway. However, as insulin and the GSK3β-inhibitor are reported to inhibit GSK3β via two distinct mechanisms, through phosphorylation and blocking of ATP binding respectively [62], one could assume that additive inhibition would be possible. Hence, the dose of the GSK3β-inhibitor used in the experiments might have influenced the results, as we have used a lower dose compared to others [22, 52]. Further experiments, including a dose-response study with the GSK3β-inhibitor, are required to clarify this question.

Western Blot results demonstrate a similar degree of Akt phosphorylation in female rat heart treated with either insulin or insulin+GSK3β-inhibitor (Fig. 3-4). As insulin is the factor that mediates phosphorylation of Akt, and the direct GSK3β-inhibitor acts downstream, this result is expected.

4.1.5 Cardioprotection in female versus male rat hearts

The prevalence and incidence of ischemic heart disease (IHD) at all ages are higher in males than in females. However, due to the larger female population beyond 75 years, the absolute number of elderly women with IHD overcomes the number of males [5]. Compared to men, women with all types of IHD are older, have more vascular risk factors and co-morbidities and thereby represent a high risk group [5]. It is also reported that females with acute coronary syndromes have more atypical symptoms [5] and appear to be relatively better protected from sudden death outside hospital [6]. All this has resulted in an increased focus on women and cardiovascular disease in the setting of medical research. Based on this, one of the main aims of this study was to investigate whether various reperfusion treatments conferred different response in female compared to male rat hearts. Nevertheless, treatments with insulin, GSK3β-inhibitor or the combination of the two, demonstrates no sex differences in terms of cardioprotection, when comparing my results with parallel data from male rats collected by A.Wergeland (unpublished, 2009).

Certain diseases, diet, smoking and genetics are all known risk factors of cardiovascular disease [5], however estrogen has by some researchers been outlined as an important contributor to the higher protection against cardiovascular disease observed in women [4, 63]. The rats used in this study were between 11-14 weeks, and as the age of puberty in female rats are between the 6^{th} and 11^{th} week [64], we can expect normal estrogen levels in our experimental animals. The female sex hormone is reported as cardioprotective both through rapid and long-term effects on blood vessel walls [4], as well as activating the prosurvival PI3K/Akt signalling pathway following acute ischemia [63, 65]. Whether these effects contribute to the reduced infarcts seen in our experiments is uncertain, as we have performed experiments on *ex vivo* isolated rat hearts. The KHB-buffer used to perfuse our hearts, did not contain estrogen, hence the hearts are not exposed to the same hormone concentrations as in an *in vivo* situation. Yet, the overall role of estrogen in cardioprotection remains to be fully confirmed, as results from clinical trials demonstrated no cardioprotective effect of hormone replacement therapy on postmenopausal women [66].

4.2 Methodological considerations

4.2.1 The isolated e*x vivo* Langendorff perfused rat heart

4.2.1.1 Anaesthesia

In this study the rats were given Pentobarbital (i.p.) which is a widely used anestheticum. As for all anaesthetics, a potential harm is its influence on experimental parameters, like hemodynamic recordings and infarct size. Pentobarbital is shown to cause an increase in vascular resistance and a reduced cardiac output in rats [67]. In our *ex vivo* model system these effects are probably not relevant, as the vascular resistance is more dependent on the perfusion pressure from the system. Additionally, the heart is cannulated through the aorta, making measurements of the cardiac output impossible. Yet, most importantly Pentobarbital is demonstrated not to influence infarct size or react with TTC staining [46].

4.2.1.2 The Langendorff heart model

The isolated Langendorff heart perfusion method was first described more than hundred years ago [68], and is still a widely used method in basic cardiac research, due to its

simplicity, high reproducibility and relatively low cost. With the ability to study both biochemical, physiological, morphological and pharmacological factors, a large amount of information can be gained in a relatively short time. Temperature and perfusion pressure of the system can be easily controlled, as well as the ion-, substrate- and drug content of the perfusion medium. As the heart is completely isolated, confounding effects like circulating neurohormonal factors and the influence from sympathetic and vagal stimulation are avoided, exclusively allowing the investigation of the cardiac response to our interventions [45]. Simultaneously, this can also be considered as a disadvantage with the heart not being exposed to the same environment as in an *in vivo* situation.

Krebs Henseleit buffer, used as perfusion fluid in this experiment, has the same ionic content as ordinary blood, and contains glucose as energy source. Under normal physiological conditions (*in vivo)* most of the ATP is produced by oxidation of fatty acids. However, dissolution of fatty acids and albumin are difficult to perform in an aqueous solution, and is the main reason why glucose is used as primary energy source in this model [45]. Absence of erythrocytes gives a reduced oxygen carrying capacity and leads to a higher coronary flow rate compared to *in vivo* situations. Even so, the isolated Langendorff perfusion method provides an excellent test- bed for pharmacological dose- response studies as well as the assessment of arrhythmias, ischemia- and reperfusion induced injury [45].

4.2.1.3 Infarct size measurements

Infarct size is the major end point in the present study, thus validation of methodological issues concerning infarct size determination, is important. To compare infarct size between the experimental hearts, variations of the ischemic risk zones (area at risk) should be minimized, as larger risk zones could cause larger infarctions. Due to small variations in the anatomy of the coronary vasculature in the rat, it is possible to overcome this, and as presented in Figure 3-2, no significant difference in risk area is found between our various experimental groups. Hence, we can conclude that the infarct reducing effects of the treatments is a consequence of the therapy itself, rather than variations in the size of area at risk.

A histochemical method based on triphenyl tetrazolium chloride (TTC)-staining was used to separate viable from non-viable myocardial tissue. As TTC reacts with dehydrogenase enzymes and cofactors present in viable cells, a deep red colour develops. Non-viable cells lack these enzymes, and dead myocardium will therefore remain unstained, appearing grey/white. Drawbacks with the TTC staining method is its lacking ability to stain scattered cell damage, as well as its potential interference with pharmacological agents [46]. Although reaction between TTC and the agents used in this study can not be excluded, the short period of administration (15 minutes) followed by a wash-out period of 2 hours (reperfusion), reduce the probability of such interference.

4.2.1.4 Hemodynamic variables

Hemodynamic parameters, such as heart rate (HR), left ventricular systolic (SP), diastolic (DP) and developed pressure (LVDP) were measured by inserting a compliant balloon connected to a pressure transducer, into the left ventricle. Great care must be taken while inserting the balloon, as heart tissue easily can be damaged. Although the Working Heart preparation is a more suitable method for evaluating functional recovery (such as pump function) [45], our measurements of developed pressure still function as a useful validation of our experimental protocol. Figure 3-3 illustrates this by a clear reduction in cardiac function at the onset of regional ischemia, confirmed by a decrease in both LVDP and coronary flow (CF); while appropriate reperfusion is confirmed by an increase of the same variables. Furthermore, no significant differences are found in either LVDP, CF or HR between the four experimental groups at the given timepoints, hence we can deduce that the observed differences in infarct size is not due to hemodynamic variations.

4.2.2 Immunoblotting

Immunoblotting comprises the isolation and homogenisation of heart tissue, protein estimation and finally Western Blotting. Many steps are involved in these processes, making the number of potential sources of error high. First of all, the isolation of cardiac tissue is a critical point, as phosphorylation/dephosphorylation of proteins can easily be influenced. Our protein of interest (p-Akt) can easily dephosphorylate at high temperatures, thus rapid freezing is essential. Inaccuracy during this step could be an explanation to our somewhat varying Western Blot results observed in the present study, i.e. the reduced Akt

phosphorylation in $Insulin_{BL}$ hearts as compared to previous studies [14]. Also, temperature control during the tissue homogenisation is important to prevent protein dephosphorylation, and the samples were therefore kept on ice. Still, as disintegration of the tissue by the Precellys was not possible to perform in a cold environment, we can not exclude this as a potential error.

Main sources of errors in the Western Blot protocol are unequal loading of proteins onto the gel, inadequate transfer of proteins from the gel to the PVDF membrane, as well as issues concerning detection of the proteins. As loading control, levels of total Akt should have been analysed to verify equal amounts of protein in each lane; however, due to time limitations this step was not performed. During this study, a lot of time has been used in optimizing each step of the protocol, especially in regard to the transfer process and determination of antibody concentrations. Although we finally gained clear Western Blots with distinct bands, the strength of the bands is not all as expected, compared to previous results in our lab. Consequently, it is more likely to believe that methodological issues previous to the Western Blotting, such as temperature dependant dephosphorylation of proteins, might have influenced our result.

4.3 Conclusions

The main objectives in the present study was to investigate whether treatment with insulin, the GSK3β-inhibitor SB-415286 or the combination of the two, administrated for 15 minutes at the onset of myocardial-ischemic reperfusion, could reduce infarct size in the isolated *ex vivo* female rat heart. Furthermore, the phosphorylation status of the prosurvival kinase Akt was examined in the treated hearts.

1. Treatments with insulin, GSK3β-inhibitor (SB-415286) or the combination of the two, administrated for 15 minutes at the onset of ischemic reperfusion, significantly reduce infarct size in the *ex vivo* isolated female rat heart.

2. No synergistic cardioprotective effect, in terms of reducing infarct size, is observed with co-treatment of insulin and the GSK3β-inhibitor SB-415286, compared to the two treatments alone.

3. No difference in infarct size is discovered between female and male rats when given the various treatments described above (experimental data collected in male rat hearts by A.Wergeland).

4. Preliminary data suggest that phosphorylation of the prosurvival kinase Akt is involved in insulin mediated cardioprotection in the female rat heart. However, its phosphorylation status is not affected by treatment with GSK3β-inhibitor.

4.4 Future perspectives

4.4.1 Further studies

The present study opens for many further study approaches. First of all, a dose response study with the GSK3β-inhibitor SB-415286 should be performed to investigate whether a more pronounced degree of cardioprotection could be obtained with higher doses. In addition, our data demonstrating the cardioprotective effect of insulin, GSK3β-inhibitor, and the combination of the two, should be verified through *in vivo* experiments, to evaluate potential harmful and toxic effects.

As already mentioned, our Western Blot results are preliminary, hence several experiments assessing the phosphorylation status of Akt should be added to draw final conclusions on the effect of the various treatments. Additionally, phosphorylation of GSK3β and other proteins involved in alternative signalling pathways (i.e. p70S6K, BAD, mTOR) should be investigated, to get a deeper understanding of the signalling pathways mediated by insulin and the GSK3β-inhibitor. Another approach to increase our understanding of downstream mechanisms involved in this cardioprotection, is to explore the effect our treatments exert on the mPTP by investigating pore opening and closure, and also investigate protein levels of HIF1α and apelin.

To further investigate the potential sex difference in response to myocardial ischemia, an *in vivo* model system would be suitable, as the animals then will be exposed to normal circulating hormone concentrations, such as estrogen. In addition, comparative studies of post- and pre- menopausal females would be interesting to more specifically address the effect of estrogen in cardioprotection.

4.4.2 Clinical relevance

Despite an improved management of patients with acute myocardial infarction (AMI) during the last decades, there is still a need for novel therapeutic strategies aiming to protect the myocardium from ischemic reperfusion induced injury. An advantage of the cardioprotective therapies used in this study is their ability to exert effect even when administrated at the

immediate onset of reperfusion. Insulin is a well characterised hormone where both beneficial- and potential side-effects are known. Interestingly, signalling pathways involved in insulin mediated cardioprotection might suffer from impairment by common co-morbidities in AMI- patients, such as diabetes [69] and post-infarct remodelling [70]. Thus, the use of insulin in a clinical setting might be limited. As for the GSK3β-inhibitor SB-415286, its chemical, physical and toxicological properties have not been fully investigated; and further testing is therefore needed. Nevertheless, an advantage of this compound is the ability to directly inhibit GSK3β without interfering with upstream pathways [71], and by this diminish the problem with concurrent diseases, such as diabetes. Even though chronic inhibition of GSK3β is reported to potentially increase the risk of cancer development [72], it is unlikely to believe that a single injection of a GSK3β inhibitor would amount to this risk. Thus, GSK3β-inhibiton serves as a promising route for reducing myocardial damage in AMI patients.

Appendix A

Buffers and solutions for Langendorff Perfusion of Rat Heart

- **Krebs- Heinslet Buffer for perfusion of rat hearts**

Concentration	g/5L	
118 mM	34.48 g	NaCl
25 mM	10.5 g	$NaHCO_3$
11 mM	9.9 g	d- Glucose
4.7 mM	1.75 g	KCl
1.22 mM	1.5 g	$MgSO_4(7H_2O)$
1.21 mM	0.825 g	KH_2PO_4
2.4 mM	1.76 g	$CaCl_2$

- Stored at 4°C

- $CaCl_2$ dissolved in ddH_2O prior to buffer to avoid precipitation

- **Phosphate buffer (0.2 M)**

  Solution 1:

  27.6 g $NaH_2PO_4*2H_2O$ in 1 L ddH_2O

  Solution 2:

  71.6 g $Na_2HPO_4*12H_2O$ in 1 L ddH_2O

  - Mix 2 parts solution 1 + 8 parts solution 2
  - pH = 7.4
  - For tissue staining: 1% triphenyl- tetrazolium- chloride dissolved in buffer

- **Formalin**

  1L Formaldehyde

  40 g NaH_2PO_4

  65 g $Na_2HPO_4*12H_2O$

  ddH_2O to 10 L

Buffers and solutions for tissue homogenization and Western Blot

- **Lysis Buffer**

20 mM	Tris- HCl
0.5 mM	EGTA
1%	Triton
300 mM	Sucrose
2 mM	EDTA
1x	Complete Mini EDTA- free Protease Inhibitor Tablet (1 tablet dissolved in 10 mL ddH_2O = 1x)

- **Running Buffer**

BupH™ Tris (100mM) - HEPES (100mM) - SDS (3mM) Running Buffer dissolved in

500 ml ddH_2O

- **Transfer Buffer**

100 ml	10x Tris/ Glycine Buffer
700 ml	Distilled H_2O
200 ml	Methanol

- Stored at 4°C

- **Tris Buffered Saline- Tween (TBS-T) – 1x**

  50 ml 20xTBS (320g NaCl + 96.8g Tris, ddH_2O to 2 L. pH adjusted to 7.6)

  950 ml ddH_2O

  1 ml Tween

Appendix B

Calibration of pressure transducer and Power Lab

Before the heart could be mounted onto the Langendorff perfusion system it was necessary to remove all air bubbles in the reservoirs and rubber tubes of the Langendorff system, to prevent formation of air- emboli in the heart. In addition, air bubbles had to be removed from the water filled balloon, the water filled plastic catheter connecting the balloon to the pressure transducer, and from the transducer itself as air could influence the compliance in the system. During the experiments, as pressure is applied to the water filled balloon by the contracting heart, the water column in the catheter moves, causing a physical deformation of a thin membrane placed inside the transducer. This membrane movement will produce a change in electrical resistance proportional to the applied pressure, causing an electrical signal (mV) which is passed on to the Power Lab. Conversion of the electrical signal (mV) to the actual pressure (mmHg) is done by the Power Lab and is expressed graphically on the computer.

Calibration of the transducer was carried out every day before starting an experiment, by applying two known pressures to the transducer: 0 mmHg (atmospheric pressure) and 200 mmHg (applied by a sphygmomanometer). The pressures were registered in the Power Lab and the expressed graphs were adjusted according to the calibrated results (Fig. B-1).

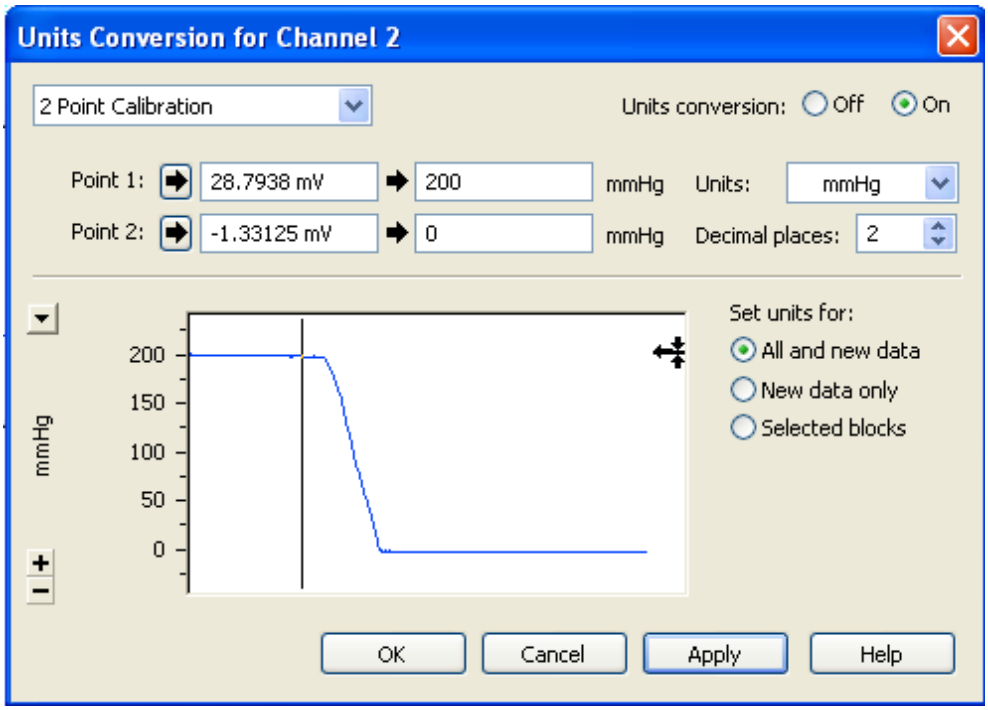

Figure B-1: ***Calibration of the pressure transducer.*** To ensure stabile pressure measurements from day to day, the transducer was calibrated prior to the experiments by applying two known pressures (0 mmHg and 200mmHg) to the system. The pressure transducer converts pressures into an analogue electrical signal (mV) which again is converted to a pressure (mmHg) by the Power Lab. The graph illustrates this last conversion, where, in this case, the graph is adjusted against the pressures of 0 and 200 mmHg thereby ensuring adequate calibration.

References

1. Sans, S., et al., *The burden of cardiovascular diseases mortality in Europe: Task Force of the European Society of Cardiology on Cardiovascular Mortality and Morbidity Statistics in Europe.* Eur Heart J, 1997. **18**(8): p. 1231-1248.
2. *Statistics Norway, Dødsårsaker 2006. [cited; Available from: http://www.ssb.no/dodsarsak/.* 2008.
3. Opie, L.H., *Heart Physiology, From Cell to Circulation*. Fourth ed. 2004: Lippincott Williams & Wilkins.
4. Mendelsohn, M.E. and R.H. Karas, *The protective effects of estrogen on the cardiovascular system.* N Engl J Med, 1999. **340**(23): p. 1801-11.
5. Andreotti, F. and N. Marchese, *Women and coronary disease.* Heart, 2008. **94**(1): p. 108-16.
6. Tunstall-Pedoe, H., et al., *Sex differences in myocardial infarction and coronary deaths in the Scottish MONICA population of Glasgow 1985 to 1991. Presentation, diagnosis, treatment, and 28-day case fatality of 3991 events in men and 1551 events in women.* Circulation, 1996. **93**(11): p. 1981-92.
7. Opie, L.H., *Myocardial ischemia--metabolic pathways and implications of increased glycolysis.* Cardiovasc Drugs Ther, 1990. **4 Suppl 4**: p. 777-90.
8. Jennings, R.B. and K.A. Reimer, *Lethal myocardial ischemic injury.* Am J Pathol, 1981. **102**(2): p. 241-55.
9. Braunwald, E. and R.A. Kloner, *Myocardial reperfusion: a double-edged sword?* J Clin Invest, 1985. **76**(5): p. 1713-9.
10. Yellon, D.M. and D.J. Hausenloy, *Myocardial reperfusion injury.* N Engl J Med, 2007. **357**(11): p. 1121-35.
11. Ito, H., *No-reflow phenomenon and prognosis in patients with acute myocardial infarction.* Nat Clin Pract Cardiovasc Med, 2006. **3**(9): p. 499-506.
12. Mocanu, M.M., G.F. Baxter, and D.M. Yellon, *Caspase inhibition and limitation of myocardial infarct size: protection against lethal reperfusion injury.* Br J Pharmacol, 2000. **130**(2): p. 197-200.
13. Hausenloy, D.J. and D.M. Yellon, *New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway.* Cardiovasc Res, 2004. **61**(3): p. 448-60.
14. Jonassen, A.K., et al., *Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling.* Circ Res, 2001. **89**(12): p. 1191-8.
15. Yellon, D.M. and G.F. Baxter, *Reperfusion injury revisited: is there a role for growth factor signaling in limiting lethal reperfusion injury?* Trends Cardiovasc Med, 1999. **9**(8): p. 245-9.

16. Jonassen, A.K., et al., *Glucose-insulin-potassium reduces infarct size when administered during reperfusion.* Cardiovasc Drugs Ther, 2000. **14**(6): p. 615-23.
17. Jonassen, A.K., O.D. Mjos, and M.N. Sack, *p70s6 kinase is a functional target of insulin activated Akt cell-survival signaling.* Biochem Biophys Res Commun, 2004. **315**(1): p. 160-5.
18. Zweier, J.L., *Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury.* J Biol Chem, 1988. **263**(3): p. 1353-7.
19. Steenbergen, C., et al., *Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat heart.* Circ Res, 1990. **66**(1): p. 135-46.
20. Lemasters, J.J., et al., *The pH paradox in ischemia-reperfusion injury to cardiac myocytes.* EXS, 1996. **76**: p. 99-114.
21. Hausenloy, D.J. and D.M. Yellon, *The mitochondrial permeability transition pore: its fundamental role in mediating cell death during ischaemia and reperfusion.* J Mol Cell Cardiol, 2003. **35**(4): p. 339-41.
22. Juhaszova, M., et al., *Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore.* J Clin Invest, 2004. **113**(11): p. 1535-49.
23. Gross, E.R., A.K. Hsu, and G.J. Gross, *Delayed cardioprotection afforded by the glycogen synthase kinase 3 inhibitor SB-216763 occurs via a KATP- and MPTP-dependent mechanism at reperfusion.* Am J Physiol Heart Circ Physiol, 2008. **294**(3): p. H1497-500.
24. Lockshin, R.A. and Z. Zakeri, *Apoptosis, autophagy, and more.* Int J Biochem Cell Biol, 2004. **36**(12): p. 2405-19.
25. Opie, L.H., *Heart Physiology: From Cell To Circulation p.560*. 4th ed. 2004: Lippincott Williams & Wilkins.
26. Gottlieb, R.A., et al., *Reperfusion injury induces apoptosis in rabbit cardiomyocytes.* J Clin Invest, 1994. **94**(4): p. 1621-8.
27. Jonassen, A.K., et al., *Insulin administered at reoxygenation exerts a cardioprotective effect in myocytes by a possible anti-apoptotic mechanism.* J Mol Cell Cardiol, 2000. **32**(5): p. 757-64.
28. Hausenloy, D.J., A. Tsang, and D.M. Yellon, *The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning.* Trends Cardiovasc Med, 2005. **15**(2): p. 69-75.
29. Tsang, A., et al., *Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway.* Circ Res, 2004. **95**(3): p. 230-2.
30. Piper, H.M., Y. Abdallah, and C. Schafer, *The first minutes of reperfusion: a window of opportunity for cardioprotection.* Cardiovasc Res, 2004. **61**(3): p. 365-71.
31. Srivastava, A.K., *Use of pharmacological agents in elucidating the mechanism of insulin action.* Trends Pharmacol Sci, 1998. **19**(6): p. 205-9.

32. Taniguchi, C.M., B. Emanuelli, and C.R. Kahn, *Critical nodes in signalling pathways: insights into insulin action.* Nat Rev Mol Cell Biol, 2006. **7**(2): p. 85-96.
33. Jonassen, A.K., *Changing the concept of GIK (Glucose-Insulin-Potassium) in Cardiac Protection*, in *Department of Medical Physiology, Institute of Medical Biology*. 2002, University of Tromsø.
34. Woodgett, J.R., *Molecular cloning and expression of glycogen synthase kinase-3/factor A.* EMBO J, 1990. **9**(8): p. 2431-8.
35. Plyte, S.E., et al., *Glycogen synthase kinase-3: functions in oncogenesis and development.* Biochim Biophys Acta, 1992. **1114**(2-3): p. 147-62.
36. Hoeflich, K.P., et al., *Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation.* Nature, 2000. **406**(6791): p. 86-90.
37. Grimes, C.A. and R.S. Jope, *The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling.* Prog Neurobiol, 2001. **65**(4): p. 391-426.
38. Cross, D.A., et al., *Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B.* Nature, 1995. **378**(6559): p. 785-9.
39. Pap, M. and G.M. Cooper, *Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway.* J Biol Chem, 1998. **273**(32): p. 19929-32.
40. Mozaffari, M.S. and S.W. Schaffer, *Effect of pressure overload on cardioprotection of mitochondrial KATP channels and GSK-3beta: interaction with the MPT pore.* Am J Hypertens, 2008. **21**(5): p. 570-5.
41. Sutherland, C., I.A. Leighton, and P. Cohen, *Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling.* Biochem J, 1993. **296 (Pt 1)**: p. 15-9.
42. Gross, E.R., A.K. Hsu, and G.J. Gross, *Opioid-induced cardioprotection occurs via glycogen synthase kinase beta inhibition during reperfusion in intact rat hearts.* Circ Res, 2004. **94**(7): p. 960-6.
43. Nishihara, M., et al., *Erythropoietin affords additional cardioprotection to preconditioned hearts by enhanced phosphorylation of glycogen synthase kinase-3 beta.* Am J Physiol Heart Circ Physiol, 2006. **291**(2): p. H748-55.
44. Kamada, N., et al., *Cardioprotective effects of propofol in isolated ischemia-reperfused guinea pig hearts: role of KATP channels and GSK-3beta.* Can J Anaesth, 2008. **55**(9): p. 595-605.
45. Sutherland, F.J. and D.J. Hearse, *The isolated blood and perfusion fluid perfused heart.* Pharmacol Res, 2000. **41**(6): p. 613-27.
46. Ytrehus, K., et al., *Rat and rabbit heart infarction: effects of anesthesia, perfusate, risk zone, and method of infarct sizing.* Am J Physiol, 1994. **267**(6 Pt 2): p. H2383-90.
47. Yu, Q.J., et al., *Insulin inhibits beta-adrenergic action in ischemic/reperfused heart: a novel mechanism of insulin in cardioprotection.* Apoptosis, 2008. **13**(2): p. 305-17.
48. Russell, R.R., 3rd, et al., *Additive effects of hyperinsulinemia and ischemia on myocardial GLUT1 and GLUT4 translocation in vivo.* Circulation, 1998. **98**(20): p. 2180-6.

49. Schomig, A., et al., *Release of endogenous catecholamines in the ischemic myocardium of the rat. Part A: Locally mediated release.* Circ Res, 1984. **55**(5): p. 689-701.
50. Jeremy, R.W., et al., *The functional recovery of post-ischemic myocardium requires glycolysis during early reperfusion.* J Mol Cell Cardiol, 1993. **25**(3): p. 261-76.
51. Camper-Kirby, D., et al., *Myocardial Akt activation and gender: increased nuclear activity in females versus males.* Circ Res, 2001. **88**(10): p. 1020-7.
52. Das, S., et al., *Glycogen synthase kinase 3 inhibition slows mitochondrial adenine nucleotide transport and regulates voltage-dependent anion channel phosphorylation.* Circ Res, 2008. **103**(9): p. 983-91.
53. Xi, J.K., et al., *Cardioprotection against reperfusion injury: updated mechanisms and strategies.* Sheng Li Xue Bao, 2007. **59**(5): p. 553-61.
54. Davidson, S.M., et al., *Signalling via the reperfusion injury signalling kinase (RISK) pathway links closure of the mitochondrial permeability transition pore to cardioprotection.* Int J Biochem Cell Biol, 2006. **38**(3): p. 414-9.
55. Mottet, D., et al., *Regulation of hypoxia-inducible factor-1alpha protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3beta pathway in HepG2 cells.* J Biol Chem, 2003. **278**(33): p. 31277-85.
56. Flugel, D., et al., *Glycogen synthase kinase 3 phosphorylates hypoxia-inducible factor 1alpha and mediates its destabilization in a VHL-independent manner.* Mol Cell Biol, 2007. **27**(9): p. 3253-65.
57. Schnitzer, S.E., et al., *Inhibition of GSK3[beta] by indirubins restores HIF-1[alpha] accumulation under prolonged periods of hypoxia/anoxia.* FEBS Letters, 2005. **579**(2): p. 529-533.
58. Glassford, A.J., et al., *HIF-1 regulates hypoxia- and insulin-induced expression of apelin in adipocytes.* Am J Physiol Endocrinol Metab, 2007. **293**(6): p. E1590-6.
59. Ronkainen, V.P., et al., *Hypoxia inducible factor regulates the cardiac expression and secretion of apelin.* FASEB J, 2007. **21**(8): p. 1821-30.
60. Smith, C.C., et al., *Temporal changes in myocardial salvage kinases during reperfusion following ischemia: studies involving the cardioprotective adipocytokine apelin.* Cardiovasc Drugs Ther, 2007. **21**(6): p. 409-14.
61. Simpkin, J.C., et al., *Apelin-13 and apelin-36 exhibit direct cardioprotective activity against ischemia-reperfusion injury.* Basic Res Cardiol, 2007. **102**(6): p. 518-28.
62. MacAulay, K., et al., *Use of lithium and SB-415286 to explore the role of glycogen synthase kinase-3 in the regulation of glucose transport and glycogen synthase.* Eur J Biochem, 2003. **270**(18): p. 3829-38.
63. Wang, M., et al., *Estrogen receptor {beta} mediates increased activation of PI3K/Akt signaling and improved myocardial function in female hearts following acute ischemia.* Am J Physiol Regul Integr Comp Physiol, 2009. **296**(4): p. R972-8.
64. Waynforth, H.B., *Expirimental and Surgical Technique in the Rat*. 2nd ed. 1992: Elsevier Academic Press.

65. Sovershaev, M.A., et al., *Preconditioning by 17beta-estradiol in isolated rat heart depends on PI3-K/PKB pathway, PKC, and ROS.* Am J Physiol Heart Circ Physiol, 2006. **291**(4): p. H1554-62.
66. Rossouw, J.E., et al., *Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial.* JAMA, 2002. **288**(3): p. 321-33.
67. Skolleborg, K.C., et al., *Distribution of cardiac output during pentobarbital versus midazolam/fentanyl/fluanisone anaesthesia in the rat.* Lab Anim, 1990. **24**(3): p. 221-7.
68. Langendorff, O., *Untersuchungen am uberlebenden Saugethierherzen.* Pflugers Archives fur die Gesamte Physiologie des Menschen and der Tiere 1885: p. 291-332.
69. Tsang, A., et al., *Preconditioning the diabetic heart: the importance of Akt phosphorylation.* Diabetes, 2005. **54**(8): p. 2360-4.
70. Miki, T., et al., *Cardioprotective mechanism of ischemic preconditioning is impaired by postinfarct ventricular remodeling through angiotensin II type 1 receptor activation.* Circulation, 2000. **102**(4): p. 458-63.
71. Coghlan, M.P., et al., *Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription.* Chem Biol, 2000. **7**(10): p. 793-803.
72. Jope, R.S., C.J. Yuskaitis, and E. Beurel, *Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics.* Neurochem Res, 2007. **32**(4-5): p. 577-95.