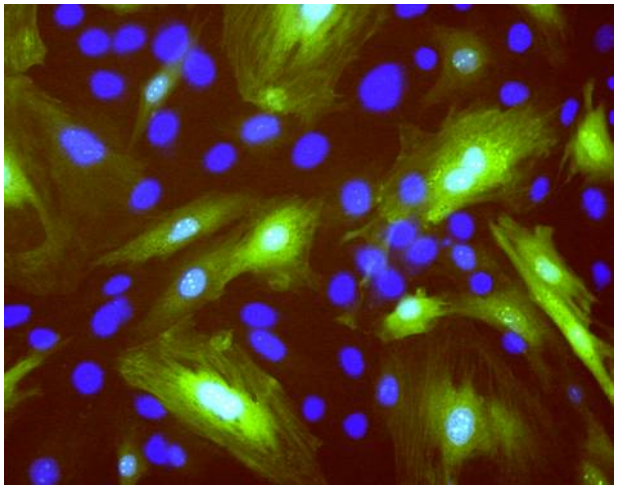
UNIVERSITY OF TROMSØ UIT

FACULTY OF HEALTH SCIENCES
CARDIOVASCULAR RESEARCH GROUP

Cardioprotection

Focus on Gap Junctions, Hemichannels and Mitochondria



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Cover page: Neonatal cardiac myofibroblasts exposed to simulated ischemic buffer. The cells are exposed to Lucifer yellow at the end of the ischemic period, and the nucleus is stained with DAPI. The picture shows the uptake of LY, and indicates that ischemia opens Cx43-hemichannels.

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ABBREVIATIONS

ATP	Adenosine three phosphate
Cx43	Connexin 43
EM	Immune-electron microscopy
ER	Endoplasmic reticulum
Gap 26	Cx43 mimetic peptide
GJ	Gap junction
GJIC	Gap junction intercellular communication
IFM	Interfibrillar mitochondria
IPC	Ischemic preconditioning
K_{ATP}	ATP-sensitive potassium channel
mitoK_{ATP}	mitochondrial ATP-sensitive potassium channel
mitoK_{Ca}	mitochondrial calcium-sensitive potassium channel
MPTP	Mitochondrial permeability transition pore
PAX	Paxilline
Panx1	Pannexin 1
PI3K	Phosphinositide 3 kinase
PKC	Protein kinase C
PKG	Protein kinase G
ROS	Reactive oxygen species
SSM	Subsarcolemmal mitochondria
WB	Western blot
5HD	5-hydroxy decanoic acid
^{10}Panx1	Panx1 mimetic peptide

1. INTRODUCTION

1.1 Ischemia-reperfusion injury

Cardiovascular disease is the leading cause of death worldwide. In Norway, 6,203 men and 7,286 women died of the disease in 2009, with approximately 40% of these deaths due to ischemic heart disease (www.ssb.no/dodsarsak/tab-2010-12-03-01.html). The most successful treatment for acute myocardial infarction is reperfusion by early thrombolytic therapy or primary percutaneous coronary intervention (PCI), although early reperfusion can also induce injury. This phenomenon is termed reperfusion injury, and was first described by Jennings et al. in 1960 [1]. Reperfusion injuries can explain up to 50% of the total infarct size in some animal models, and reperfusion injury may be one explanation as to why patients with myocardial infarction who receive early treatment still can experience reduced ventricular function and elevation of heart specific proteins in blood as a sign of myocardial infarction [2]. Reperfusion might be the cause of four types of cardiac injury: 1) myocardial stunning, 2) no-reflow, 3) reperfusion arrhythmias, and 4) lethal reperfusion injury [2].

Acute coronary artery occlusion results in acute ST-elevation myocardial infarction (STEMI). This event can be modelled in animal hearts by prolonged ligation of a coronary artery followed by reperfusion. The ischemic phase causes a reduced oxygen and nutrition supply, in addition to the accumulation of waste products. The reperfusion phase results in oxidative stress, increased cytosolic and mitochondrial calcium, rapid resolution of intracellular acidosis and inflammation [2]. Cardiac cell death during ischemia-reperfusion injury occurs by necrosis, apoptosis and in association with autophagy [3].

Necrosis is unregulated cell death due to a depletion of cellular ATP, and causes the loss of plasma membrane integrity. Plasma membrane dysfunction results in a lack of sodium control and cell swelling by an increase in osmotic pressure, with swelling in organelles such as mitochondria also being characteristic. Without oxygen, mitochondria can no longer generate a proton gradient across the inner mitochondria membrane, and the mitochondria will gradually lose the proton gradient. The release of cellular contents into extracellular space causes inflammatory response and scar formation. In the clinic, this damage is used as a diagnostic tool by analysing troponin-t release from cardiac cells. If there is a release of troponin-t, this is an indication that the cardiac cells are damaged and there has been a cardiac infarction [2].

An important mechanism that leads to the acceleration of a loss of energy production is the opening of the mitochondrial permeability transition pore (MPTP). MPTP is a non-selective channel in the inner mitochondrial membrane that only allows molecules less that 1.5 KDa to pass through. The molecular composition of the MPTP is still unsolved. Biochemical and pharmacological studies have proposed that the pore consists of a voltage-dependent anion channel (VADC) in the inner mitochondrial membrane, the adenine nucleotide translocase (ANT) in the outer mitochondrial membrane and cyclophilin-D (CypD) in the matrix, although genetic studies have seriously questioned the validity of this theory. The determination of the level of the MPTP opening by use of radiolabelled 2-deoxyglucose indicates that the MPTP remains closed during ischemia and opens within 5-10 min after reperfusion in isolated rat hearts [4]. The opening of the MPTP may be triggered by elevated calcium, oxidative stress, elevated phosphate concentration and adenine nucleotide depletion in the early ischemia-reperfusion period. The opening of the MPTP results in a loss of the electrical potential difference that normally exists across the inner mitochondrial membrane.

As a result, the mitochondria can no longer produce ATP regardless of oxygen presence and results in ATP depletion and mitochondrial swelling, which is an important part of necrosis [1; 5; 6].

Apoptosis is an actively regulated form of cell death that is believed to partly depend on the presence of ATP. Apoptotic cells exhibit cytoplasmic shrinkage, plasma membrane blebbing, nuclear condensation, and later fragmentation of both the cytoplasm and nucleus into membrane-enclosed apoptotic bodies. Apoptosis is mediated by two pathways. The extrinsic pathway utilizes cell surface receptors, whereas the intrinsic pathway involves the mitochondria and endoplasmic reticulum. Both of these pathways lead to caspase activation, and have been shown to be critical in the pathogenesis of ischemia-reperfusion injury [7; 8]. Toxic stimuli such as ischemia-reperfusion injury, oxidative stress and cardiotoxic drugs induce the translocation and integration of the prodeath members of the Bcl2 family (e.g. Bax, Bak) into the outer mitochondrial membrane. These proteins permeabilize the outer mitochondrial membrane to an extent that allows the release of proapoptotic proteins from the intermembrane space, particularly cytochrome C. Cytochrome C binds to the cytosolic protein apaf1 and results in activation of caspase-9 and caspase-3 protease systems [5].

In contrast to necrosis and apoptosis, autophagy is primarily a survival and recycling mechanism. Autophagy maintains the cell itself, but cell organelles such as mitochondria could be lost. Autophagy provides cells with amino acids, fatty acids and energy in times of nutritional deprivation, and is mediated by the formation of a double-membrane vesicle (autophagosome) that surrounds the material to be degraded. The autophagosome fuses with the lysosome to form an autophagolysosome, resulting in a lysosomal degradation of the cargo. Autophagy is induced during both permanent coronary occlusion and ischemia-

reperfusion injury, though the underlying pathways and functional consequences appear to differ. There are data that suggest that during prolonged ischemia autophagy plays a protective role, while during reperfusion autophagy may be a cytotoxic agent, but further investigation is required to achieve a better understanding of this phenomenon [9].

1.2 Cardioprotection

The heart can be protected against ischemia-reperfusion injury with both endogenous activation and pharmacological treatment. Endogenous protection means that the body itself can activate systems/pathways that lead to the protection of an organ. Pharmacological protection means that we can add a compound to the body and that this compound can protect an organ directly or by the activation of protective systems/pathways.

Endogenous cardioprotection was first described by Murry et al. in 1986 [10]. Murry showed that short periods with ischemia and reperfusion before prolonged ischemia protected the heart, and this phenomenon is called ischemic preconditioning (IPC). Since 1986, many labs have been working with IPC, and there is now evidence that IPC is able to activate different pathways that converge on the mitochondria and modulate their function, including their involvement in both necrotic and apoptotic cell death. The cardioprotective capacity of IPC has been demonstrated in the human heart in several clinical presentations of ischemia-reperfusion, including cardiac surgery, preinfarction angina and angioplasty [11-13].

In 2003, Vinten-Johansen's group [14] showed that short periods of ischemia and reperfusion after prolonged ischemia were cardioprotective, and this phenomenon was called ischemic postconditioning (IPOST). Today, there is good evidence that IPOST reduces ischemia-reperfusion injury through activation of the RISK pathway (Fig 1.1 B), which mostly

converges on mitochondria to inhibit the MPTP. From 2003, there have only been a few small clinical trials with IPOST on humans. Staat et al. 2005 [15] reported that IPOST with four episodes of 1 min inflation-deflation of the angioplasty balloon performed within 1 min of reflow in patients with an acutely occluded coronary artery reduced the infarct size by 30-40%.

Pharmacological pre- and postconditioning have been attempted and have shown reduced ischemia-reperfusion injury. From a clinical perspective, pharmacological postconditioning has the greatest potential because of the time point. The use of MPTP inhibitors such as cyclosporine A (CsA) are the most studied in humans. In 2008, Piot et al. [16] tested whether CsA could represent a pharmacological alternative to IPOST in humans. They randomly assigned 58 patients, who presented with acute ST-elevation myocardial injury, to receive either an intravenous bolus of CsA or normal saline immediately before undergoing PCI. They found that CsA attenuated the infarct size by approximately 40%. For the time being, there is no pre- or postconditioning-based therapy that is routinely used in clinical medicine. Today, high potassium and hypothermia are used during open chest heart surgery as cardioprotective strategies. During PCI, there is no cardioprotective strategy in routine use today.

Three different cardioprotective protein kinase programmes have been proposed to explain IPC, IPOST and pharmacological treatment: survival pathway, RISK pathway and JAK-STAT pathway [17].

The survival pathway (Fig 1.1 A) was proposed by Downey et al. [18], and has been recruited during the trigger and mediator phase of acute ischemic preconditioning or pharmacological

treatment. After activation of G-protein-coupled receptors by adenosine, bradykinin, opioids, etc., or receptors for peptides such as natriuretic peptides, phosphinositide 3 kinase (PI3K)/AKT is activated with the further downstream activation of NO formation, protein kinase B (PKB) and protein kinase C (PKC) activation. This leads to the activation of the mitoK_{ATP}-channel and reactive oxygen species (ROS) formation, thus resulting in p38 mitogen-activated kinase and PKC activation, as well as inhibiting opening of the MPTP [17; 18].

The reperfusion injury salvage kinase pathway (RISK-pathway) (Fig 1.1 B) was proposed by Yellon and Baxter [19]. This pathway is recruited during early reperfusion with the execution phase of IPC and with IPOST. The activation of sarcolemmal G-protein-coupled receptors, or of receptors for growth factors, results in activation of PI3K/Akt and the extracellular regulated kinase system, with downstream p70 ribosomal protein S6 kinase (p70S6K) and glycogen synthase kinase 3β (GSK-3β) activation and the ultimate inhibition of the MPTP [17; 19].

The JAK-STAT pathway (Fig. 1.1 C) was proposed by Lecour et al. [20] and Boengler et al. [21], and is recruited with IPC, delayed IPC and IPOST. After activation of sarcolemmal glycoprotein 130 (gp130) receptors or tumour necrosis factor-a receptor, the Janus-activated kinase (JAK) signal transducer and the activator of transcription (STAT) pathway are activated with projection to the nucleus and possibly to mitochondria [17; 21].

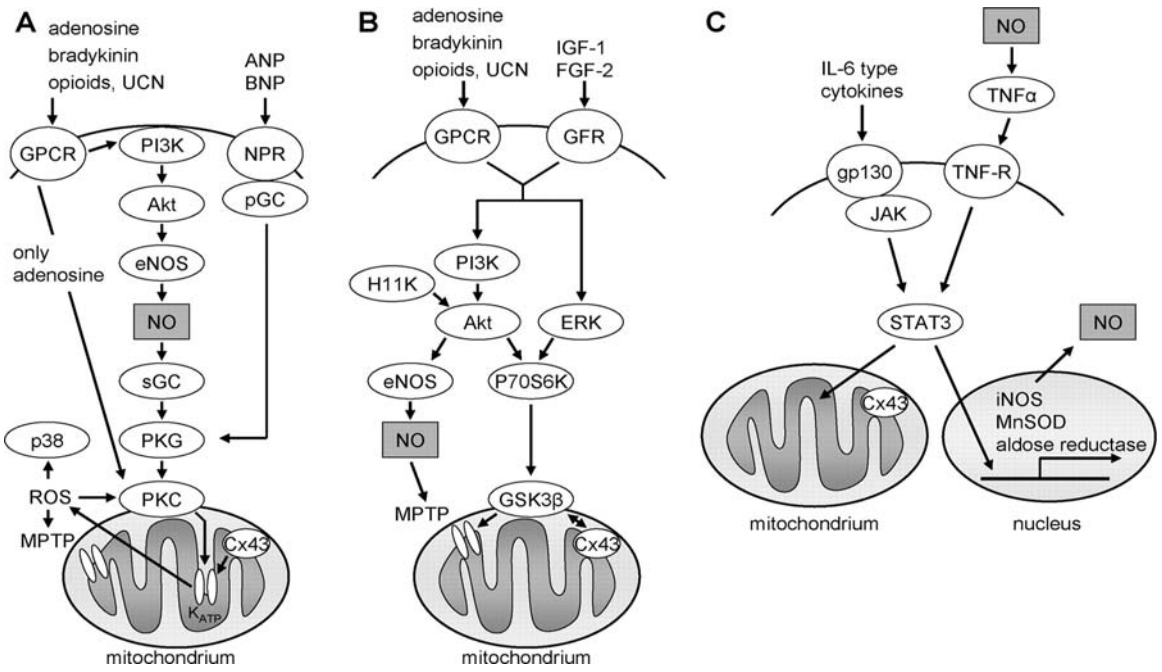

Figure 1.1: Protein kinase activation in cardioprotection, A - Survival pathway; B - RISK pathway, and C - JAK-STAT pathway. Figure is a copy from Heusch et al. [17].

In 2009, Garlid et al. [22] proposed that cardioprotective signals are transmitted to mitochondria by signalosomes, which are vesicular, multimolecular signalling complexes that are assembled in caveolae and deliver signals to the mitochondrial outer membrane (Fig 1.2). They proposed that the receptor-specific signalling platform is assembled in caveolae, and then separates and internalizes as a signalosome. The signalosome migrates via the cytoskeleton to mitochondria, where it binds to receptors on the mitochondrial outer membrane. The terminal kinase of the signalosome phosphorylates its specific receptor, which causes the signal to be transmitted across the mitochondrial outer membrane and intermembrane space to PKCε on the mitochondrial inner membrane. This is followed by the mitoK_{ATP}/mitoK_{Ca}-channel opening and inhibition of the MPTP.

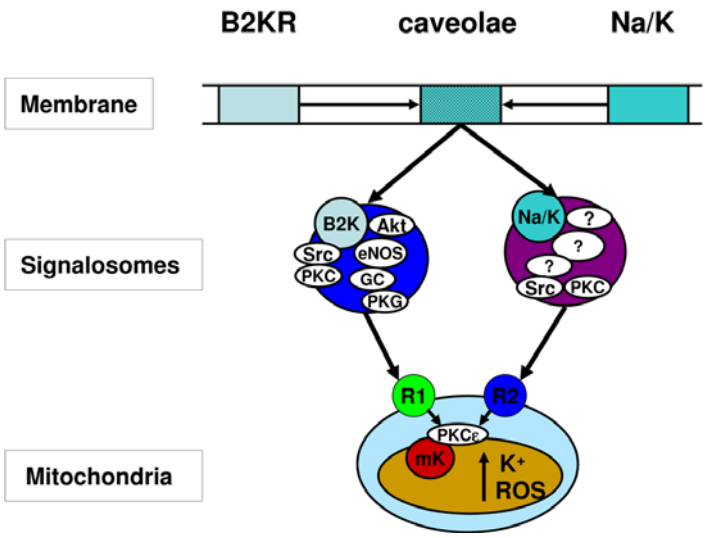

Figure 1.2: Copy from Galid et al.[22], who proposed signal transmission by signalosome to mitochondria.

1.3 Connexin 43

Connexin 43 (Cx43) is the predominant protein forming gap junctions and non-junctional hemichannels in ventricular myocardium and myofibroblasts. The connexins have four transmembrane domains, two extracellular loops and cytoplasmic N-terminal and C-terminal domains. Many, but not all, connexins form channels without any particular selectivity for specific ions [23], and are permeable for small ions and molecules with a molecular weight up to 1,000 Da such as ATP, glucose, glutathione, cAMP and IP3 [24] (Fig 1.3).

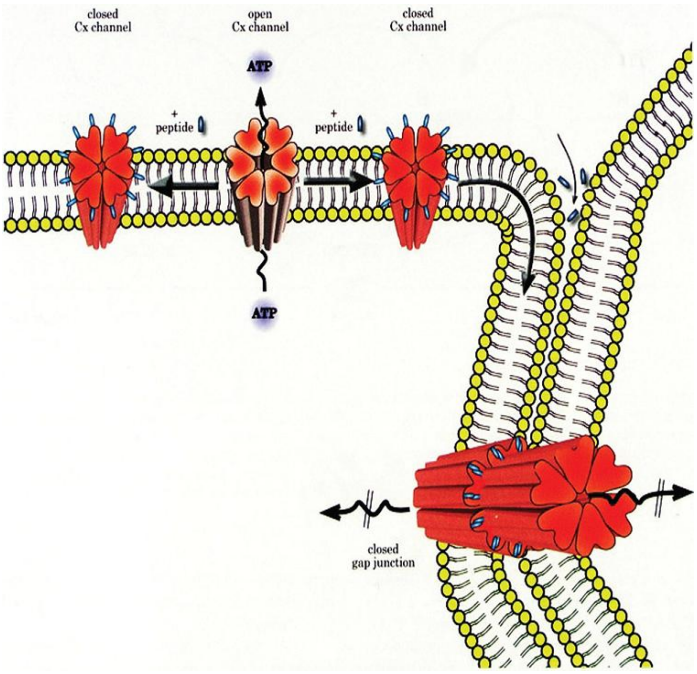

Figure 1.3: Schematical overview of Cx43 as hemichannels and gap junctions. Figure is a copy from Evans et al. [25].

Gap junctions are specialized membrane areas that contain aggregates of intercellular channels connecting the cytoplasm of two adjacent cells. The gap junction channels allow electrical coupling, metabolic cooperation and the coordination of cell activities. Each gap junctional channel is composed of two end-to-end connected hexameric structures called hemichannels or connexons, one from each of the connected cells. These hexameric structures are formed by the connexin proteins, whereas the flux of molecules through the gap junction channels is determined by the chemical and electrical gradients between two connected cells. Moreover, the transfer of molecules between connected cells is also affected by the assembly and degradation of gap junctions [26] that essentially regulate the number of potentially active channels, in addition to their pore size and open probability [27]. Under resting conditions, gap junction channels are in an open state while hemichannels are predominantly in a closed state, and their gating is regulated, among other factors, by the phosphorylation status of the connexins [28].

There might be a different regulation of Cx43 hemichannels and full gap junction channels during ischemia. Whereas metabolic inhibition opens Cx43 hemichannels [29], ischemia induces the closure of gap junction channels, both when determined electrophysiologically [30] or by transfer of gap junction-permeable dyes [31; 32]. An increased cytosolic Ca^{2+} concentration, reduced ATP concentration, changes in the phosphorylation of Cx43 and acidification, all occurring during ischemia-reperfusion injury, close gap junction channels [33], whereas a decrease in extracellular Ca^{2+} concentration or changes in phosphorylation have been described to open Cx43 hemichannels [28]. It is possible that environmental conditions differentially modulate the response of Cx43 to ischemic conditions, although no clear explanation for such differences is available.

Recent findings may indicate that Cx43 is important in ischemic preconditioning, which is demonstrated by a failure to protect Cx43-deficent (Cx43+/-) animals [34]. Also, hearts from animals with heart failure and hearts from old animals were resistant to IPC and showed decreased level of Cx43. Boengler et al. [35] showed that Cx43 was localized in the inner mitochondrial membrane of cardiac subsarcolemmal mitochondria (SSM). They found an increased amount of Cx43 in SSM after IPC, and they also suggested that mitochondrial Cx43 forms a hemichannel-like structure in the inner mitochondrial membrane that functions as a positive regulator of the mitoK_{ATP}-channel during IPC [35; 36]. If present in the mitochondrial inner membranes, Cx43 hemichannels should normally be closed to inhibit mitochondrial uncoupling and loss of electrochemical gradients in a similar way as for MPTP. Under basal conditions, most Cx43 protein is found at the intercalated disks, whereas only 4% is localized in mitochondria and 8% in the cytosol. The rapid increase in mitochondrial Cx43

content in response to IPC indicates that it is due to changes in the intracellular kinetics of Cx43, rather than to increased protein synthesis [37].

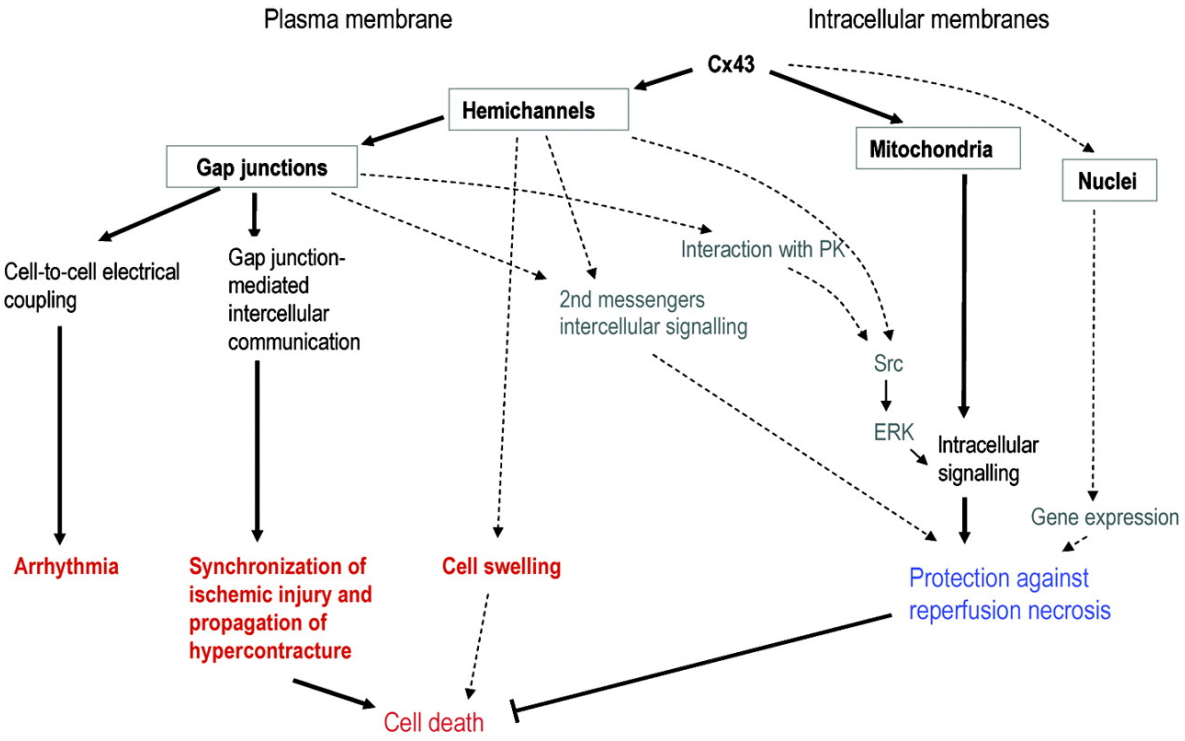

Figure 1.3: Scheme proposed by Ruiz-Meana et al. summarizing the potential roles of Cx43 in the pathophysiology of ischemia-reperfusion injury [37].

1.4 Pannexin 1

In 2003, the mammalian pannexin gen family was cloned for the first time. It is homologous to the invertebrate gap junction protein, innexin [38]. Although there are structural similarities between connexins and pannexins, they appear to be two evolutionary distinct families [39]. In humans and other mammals, there are three pannexins: 1, 2 and 3, and Pannexin 1 (Panx1) is the most studied pannexin. Pannexins have the same membrane topology as connexions: four transmembrane domains, two extracellular loops, a cytoplasmic loop and cytosolic N-and C-termini [40]. Panx1 forms hemichannels in a number of cell types, and can be opened by ischemia-like conditions in pyramidal neurons [41]or by purinergic receptor stimulation in red

blood cells [42]. Panx1 only rarely forms gap junctions, and all reported cases have been in selective overexpression systems. Panx1 is widely expressed in the body [43], making it a potential candidate for hemichannel activities found in different cells and organs. Panx1 has also been found in ER and seems to form intracellular calcium-leak channels [44]. Vessey et al. [45; 46] have suggested that a brief period of ischemia-reperfusion induces the formation of pannexin-1/P2X7 channels, which then promotes the release of multiple cardioprotectants that mediate the protection of IPC and ischemic postconditioning. Karpuk et al. [47] revealed that Panx1 mimetic peptide ^{10}Panx1 can block Panx1 hemichannels during inflammation in astrocytes.

1.5 Hydrogen sulfide

Hydrogen sulfide (H_2S) is commonly recognized as a gas with an odour of rotten eggs. The toxicology of the gas has been known for many decades, and the physiological role was first proposed as late as in 1989 [48]. Today, hydrogen sulfide is termed as a gasotransmitter, together with nitric oxide (NO) and carbon monoxide (CO) (Fig 1.4). Gasotransmitters are lipid soluble, endogenously produced and freely permeate the plasma membrane.

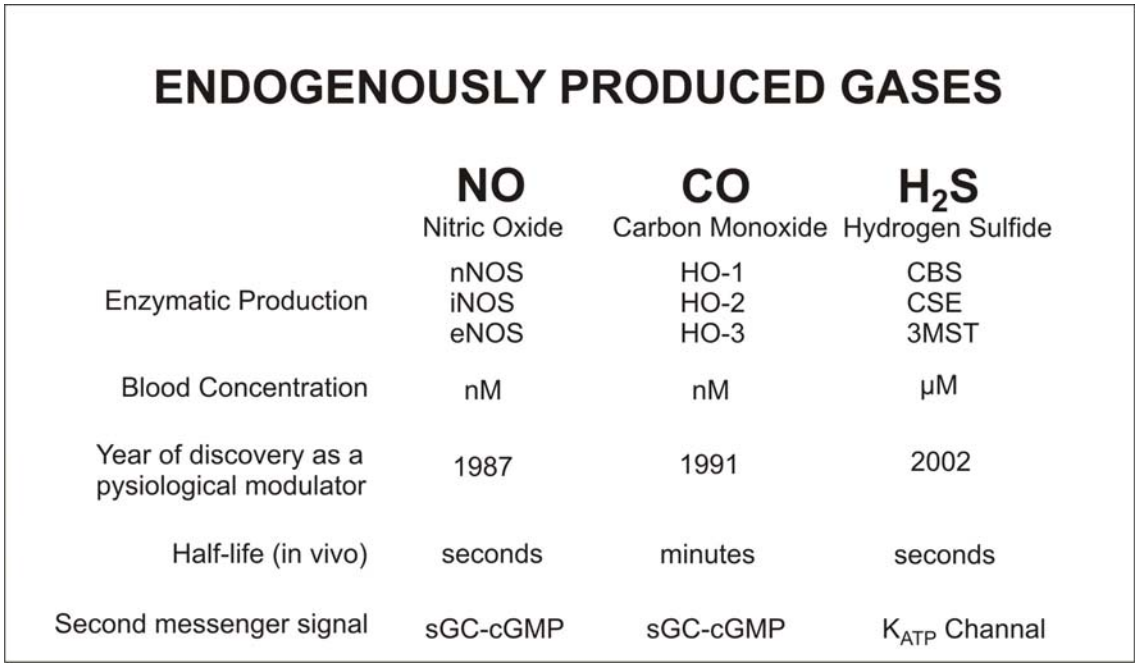

Figure 1.4: Overview over endogenously produced gases in the cardiovascular system. Figure is modified from Calvert et al. [49] .

Hydrogen sulfide is synthesized in mammalian tissues via endogenous enzymes and by nonenzymatic pathways. There is strong evidence that the cardiovascular system is an endogenous source of hydrogen sulfide generation. This evidence includes both the expression of hydrogen sulfide generating enzymes and the detection of hydrogen sulfide within these tissues [49]. There are three important enzymes that catalyze hydrogen sulfide production: cystathionine b-synthase (CBS), cystathionine g-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) see Fig. 1.5. Hydrogen sulfide is detected in many tissues and in blood. In some reports, concentrations up to 160 µM in the brain and 100 µM in blood are quoted, although the concentrations differ quite a bit from lab to lab [50]. There is still no gold-standard method for measuring hydrogen sulfide, and the reported hydrogen sulfide concentration in similar biological samples differs.

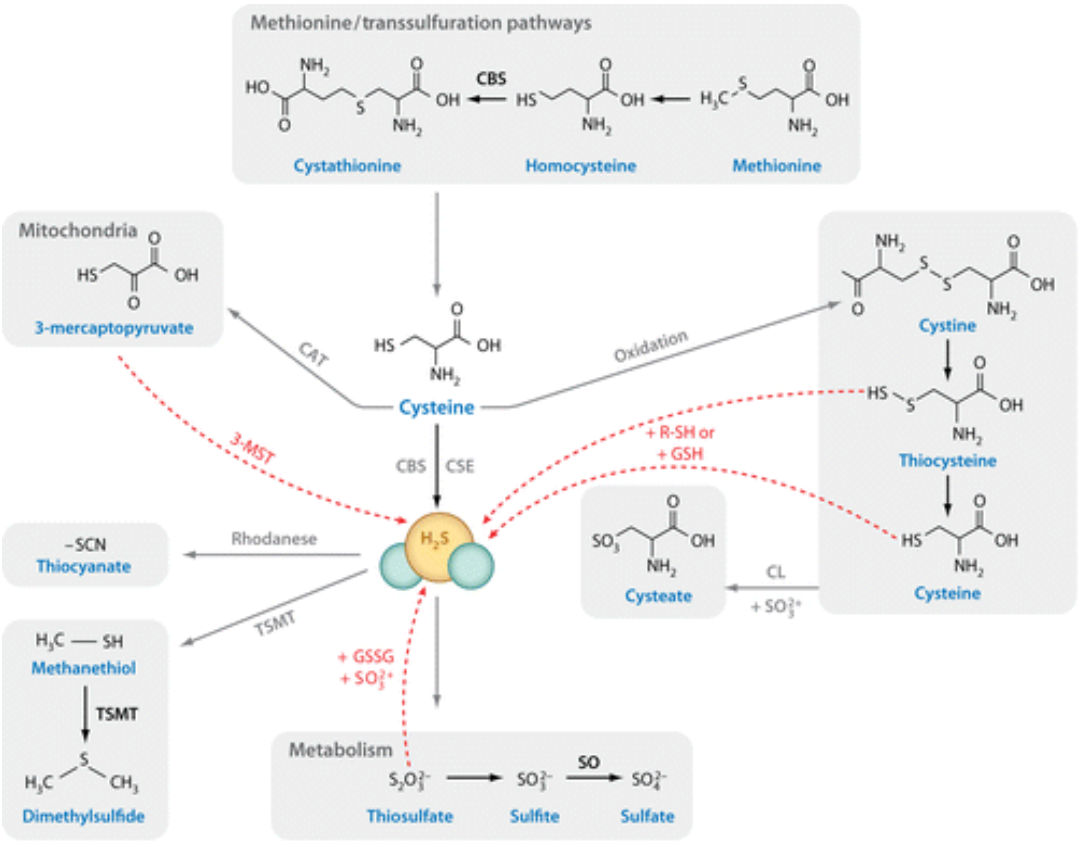

Figure 1.5: Enzymatic production of hydrogen sulfide [50].

2. AIMS OF THE STUDY

The main aim of this thesis was to examine gap junctions, hemichannels and mitochondria during ischemia-reperfusion injury, and to try and use the knowledge obtained to develop new strategies for cardioprotection.

The first sub-aim was to examine gap junction intercellular communication and hemichannel permeability during simulated ischemia, as well as to investigate changes in the phosphorylation of Cx43 during simulated ischemia and the importance of Cx43-hemichannels in cell death. We also wanted to examine whether the channel protein pannexin 1 protein was expressed in the heart, and if so, to demonstrate the subcellular localization.

The second sub-aim was to test treatments to reduce ischemia-reperfusion injury, which is related to gap junctions and hemichannels. We aimed to investigate the underlying mechanisms and importance of mitochondria. Pretreatment with heptanol as an unspecific gap junction uncoupler, Gap 26 mimetic peptide as a specific Cx43-hemichannel blocker in early ischemia-reperfusion and the new gasotransmitter hydrogen sulfide were all used as treatments.

3. METHODOLOGICAL CONSIDERATIONS

3.1 Isolated retrograde perfused rat hearts

Oskar Langendorff was the first to perfuse an isolated heart in 1895 [51]. For the last 40-50 years, isolated perfused hearts have been used on a large scale in cardiovascular research, with the method now established in many cardiovascular labs [1; 10; 17: 18; 19; 22; 37; 52; 53]. By using this experimental model, we can examine the heart itself. The heart is not affected by changes in blood composition and neurotransmitters, in addition we are able to control the pre- and afterload. The advantage of the technique is also that it is easy to perfuse many hearts within a few days, and to test new hypotheses in a short period of time. All hearts are exposed to the same buffer, perfusion pressure, temperature and concentration of various chemicals. In the present thesis, the main end point was infarct/risk ratio, although the coronary flow, heart rate, left ventricular pressure and temperature were also measured. There are some technical challenges with the model when using ischemic injury as an end point. It is very important to get the heart to the perfusion rig quickly; if not, the heart can be accidentally exposed to ischemic preconditioning and the infarct size can be low. Regional ischemia is made by a suture and a snare, and it is important that this suture covers the coronary artery and that the snare is tightened enough so that ischemia is complete. It is important to take into consideration that this is an ex vivo model, and not an in vivo model, when conclusions are drawn. Chemical compounds used in the buffer may have changed their properties in blood, so to confirm findings in isolated perfused hearts, in vivo experiments have to be performed before treatment is adapted in a preclinical setting.

3.2 Cell cultures

Fibroblasts are important cells in the heart, with the heart containing more fibroblasts than cardiac myocytes by number, but not by volume. Sixty-seventy percent of the cells in the human heart are cardiac fibroblasts, which are a key source of the components of the extracellular matrix that regulate the structure of the heart and hence the mechanical, chemical and electrical signals between the cellular and non-cellular components. Neonatal rat cardiac myofibroblasts have been used in this thesis, though caution should be taken in extrapolating results from these developmentally immature cells to those of the adult cardiac fibroblasts [54].

Gap junction intercellular communication was investigated by the microinjection of Lucifer yellow. Lucifer yellow is a non-toxic tracer that diffuses through gap junctions but does not cross the cell membrane. A limitation of microinjection is that only a few cells can be microinjected, and not at the same time. Thus, the technique is not convenient when a large number of cells need to be simultaneously monitored for intercellular communication. Scrape loading is another technique if there is a need for investigating a large number of cells [55], but we have not used this method in this thesis.

Hemichannel permeability was also examined by the use of Lucifer yellow, which was added in the buffer/medium 2 min before the end of the experiment. Lucifer yellow diffused into cells if the hemichannels were opened, although a limitation of this method is that the cells lost the connection to the cell culture dish if they were exposed for too long a time to simulated ischemia or to very high concentrations of the oxygen scavenger, dithionite. Consequently, this may make it difficult to calculate the exact relationship between numbers of cells with open or closed hemichannels (underestimation).

3.3 Viability

The measurement of infarct size in perfused rat hearts was performed at the end of the reperfusion period (120 min after ischemia). The heart was then perfused with 0.5-1 ml of 0.5% Evans Blue to stain the non-ischemic tissue, and then frozen for 12-24 hours. Two mm transverse sections were incubated in 1% triphenyltetrazolium chloride (TTC) in PBS at 37 degree for 15-20 min. The non-infarcted tissue was stained red, while the necrotic tissue remained unstained.

Cell viability in cell cultures was measured by use of a viability kit from Promega. This kit contain MTS (3-(4,5 dimethylthiazol-2-ly)- 5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium). The kit demands an accurate control of the number of cells in the dish, as the change in colour may be too high or too low (outside detection limit) if not.

Both of these methods are based on TTC/MTS being enzymatically reduced to a formazan product with colour (red or yellow) by dehydrogenases, which are most abundant in mitochondria. The staining intensity correlates with the number and functional activity of mitochondria [56]. Furthermore, these methods do not give an answer with respect to the type of cell death, apoptosis, necrosis or autophagy.

3.4 Antibodies as analytical tools

In this thesis, we have used antibodies against Cx43, Panx1, GSK-3β and AKT. Antibodies have been used in Western blot, immune-electron microscopy and immunofluorescence microscopy, and are made to recognize specific proteins or part of proteins (epitopes). The quality of antibodies differs. In some cases, the producers have incomplete information about

the sequence or specificity. Antibodies may bind to other proteins with similarities in a sequence to the protein we examined, which may lead to difficulty in analysing the results. We have attempted to reduce this problem by using more than one antibody against each protein and with the use of positive and negative controls in our experiments.

Western blot was used to study protein expression and changes in the phosphorylation of proteins, and was performed on extracts from isolated hearts, cells in culture and isolated mitochondria. The phosphorylation status is quickly lost during the preparation of cells and tissue, so to examine phosphorylation demands the use of protease inhibitors, and when working with isolated hearts there is a need for quick cooling by use of liquid nitrogen and freeze clamping to avoid dephosphorylation. This method is a semi-quantitative amplification reaction since it is based on chemiluminescence reaction coupled to the binding of an antibody.

Immune-electron microscopy was performed on samples from isolated hearts, with both a formaldehyde fixation and high-pressure freezing technique being used. Formaldehyde fixation was used to fixate samples for Cx 43 detection. Panx1 detection did not work in formaldehyde fixation, most likely because formaldehyde blocks Panx1 antibodies from attaching to the Panx1 protein; thus, we had to use high-pressure freezing fixation to detect Panx1. Tissues were ultra cryosliced and the antibody was used on 70 nm thick slices. The secondary antibody was gold particles (5nM), and we used negative controls without primary antibodies to check the sensitivity for gold particles. All primary antibodies were tested in a dose-dependent manner and we chose the lowest concentration for detection.

3.5 Chemical compounds as experimental tools

Pharmacological treatment and the use of blockers against enzymes, receptors and channels demonstrate the same challenge as for antibodies. Heptanol is an unspecific gap junction uncoupler, but may also affect other channels and pumps. Heptanol is a long-chain alcohol that causes closing of the gap junction channels by decreasing the fluidity of cholesterol-rich domains in the membrane. Gap26 mimetic peptide is a more specific blocker, and is derived from the first extracellular loop sequence of Cx 43. This short mimetic peptide reversibly inhibits gap junction and Cx43-hemichannels (Anaspec).[10]Panx1 is also a mimetic peptide, but this peptide blocks Panx1-hemichannels (Anaspec). However, there are indications that even these small mimetic peptides may not be specific [57].

Hydrogen sulfide is a very difficult substance to handle because it is a gasotransmitter and its half-life is very short (only seconds in plasma). As a result, it has been proven to be difficult to measure the concentration of this gas in a buffer or serum. In this thesis, we have used sodium hydrosulfide (NaHS #161527 Sigma-Aldrich) as an exogenous hydrogen sulfide donor. Hydrogen sulfide is generated by the spontaneous dissociation of the hydrogen sulfide donor NaHS in aqueous solution according to the equations:

$$NaHS \rightarrow Na^+ + HS^-$$
$$2HS^- \leftrightarrow H_2S + S^{2-}$$
$$HS^- + H^+ \leftrightarrow H_2S$$

This compound contains approximately 28-32% water according to the supplier, though the water content may increase when the cap is opened. When the NaSH is added to the buffer, some hydrogen sulfide will always diffuse to the air and the concentration of hydrogen sulfide is reduced over time (see Discussion). A good donor for the exogenous delivery of hydrogen sulfide is still lacking. We therefore developed a method to measure hydrogen sulfide in the buffer: To measure hydrogen sulfide in the buffer, we used a modification of the method of

W-J Cai et al. [58]. Briefly, 1.0 ml of the sample to be measured (buffer) was added to 0.125 ml 1% zinc acetate in tightly sealed Eppendorf tubes. The resulting precipitate was dried under 2 millibar of vacuum using a Speed Vac SC-210A concentrator (Thermo Scientific), followed by the addition of 0.375 ml water. Next, 0.067 ml of 20mM N,N-dimethyl-phenylenediamine dihydrochloride in 7.2 M HCl was added, which was followed by the addition of 0.067 ml of 30mM FeCl3 in 1.2mM HCl. After 30 min of incubation in the dark, the absorbance of the resulting Methylene blue solution was measured at 670 nm against a calibration curve of NaHS (0.1-2.5 mM).

Dithionite is a chemical compound which is a strong reducing agent and acts as an oxygen scavenger and rapidly reduces oxygen content in an oxygen-containing buffer. In a given culture medium one can not exclude that also other components in the buffer will be reduced. There are also indications that dithionite may cause reduction of electron transfer proteins directly in the mitochondria [59].

Glibenclamide, 5HD and paxilline have all been used to block various potassium channels. Glibenclamide is a sulfonylurea derivate that was originally developed in 1966 as an antidiabetic drug. It is known to block both sarcolemmal and mitochondrial ATP-dependent potassium channels in pancreatic beta cells, but this effect was also shown in cardiac cells [60]. 5HD is a natural lipid in milk that is proposed to be a specific blocker for mitoK_{ATP}-channels [61], although one report claims that 5HD also blocks sarcolemmal K_{ATP}-channels [62]. Paxilline is an indole alkaloid produced by Penicillium paxilli, and is known to block mitoK_{Ca}-channels [63].

3.6 Mitochondrial isolation and respiration

The method used for mitochondria isolation and the measurement of respiration is described in Paper 2. Mitochondrial isolation and the fraction of mitochondria into SSM and IFM have also been used in Paper 4, though the purity of the mitochondria fraction could be a problem. We have tried to verify the purity of our mitochondrial fraction by using antibodies against NaK-ATPase and VDAC. Cx43 was found in the mitochondria, but only in SSM fraction and not in the IFM fraction. Immune-electron microscopic pictures of the heart have showed Cx43 detection both in SSM and IFM. The standard protocol for separating SSM and IFM is that the IFM fraction is added trypsin. Our observations that Cx43 is only detected in SSM and not IFM lead us to the hypothesis that maybe trypsin itself removes Cx43 in the IFM fraction. By adding trypsin to the SSM fraction there is no longer detection of Cx43 (Figure 3.6).

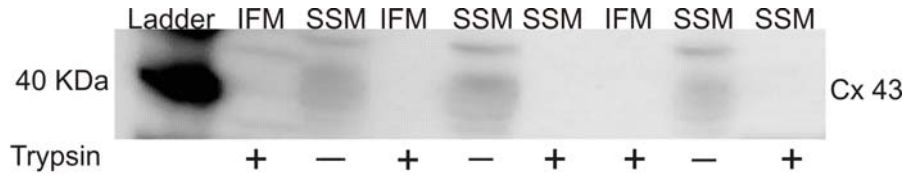

Figure 3.6: Western blot showing that Cx43 is not detected if trypsin is added to the buffer.

3.7 Time to the MPTP opening in isolated myocytes

Zorov et al. 2000 and Juhaszova et al. 2004 have described this technique [64; 65]. Isolated myocytes are loaded with tetramethylrhodamine (TMRM), and because of the positive charge of TMRM, it will accumulate in the mitochondria. The mitochondria are then exposed to laser light, and the TMRM molecules generate ROS. The time to the MPTP opening is calculated as the average time from laser exposure until the selected array of mitochondria have lost their membrane potential. By using this technique we can simulate ischemia-reperfusion, such as

conditions in the mitochondria, and examine the effect of pharmacological interventions on a mitochondrial level. Findings should be confirmed in an intact organ (heart) or animal using an endpoint-like infarct after ischemia-reperfusion in order to draw conclusions about cardioprotection.

4. LIST OF PAPERS

1. David Johansen, Véronique Cruciani, Rune Sundset, Kirsti Ytrehus, Svein-Ole Mikalsen
Ischemia induces closure of gap junctional channels and opening of hemichannels in heart-derived cells and tissue. *Cellular Physiology and Biochemistry. 2011 ;28(1):103-14.*

2. David Johansen, Kirsti Ytrehus, Gary Baxter
Exogenous hydrogen sulfide (H2S) protects against regional myocardial ischemia-reperfusion injury--Evidence for a role of K ATP channels. *Basic Res Cardiol. 2006 Jan;101(1):53-60.*

3. David Johansen, Espen Sanden, Martin Hagve, Xi Chu, Rune Sundset, Kirsti Ytrehus
Heptanol triggers cardioprotection via mitochondrial mechanisms and mitochondrial potassium channel opening in rat hearts. *Acta Physiol. 2011 Apr;2011(4):435-44.*

4. David Johansen, Espen Sanden, Svein-Ole Mikalsen, Kirsti Ytrehus
Pannexin 1, a new channel protein in cardiac mitochondria? *Manuscript.*

5. SUMMARY OF RESULTS

Gap junction intercellular communication and hemichannel permeability are affected by ischemia. In Paper 1, GJIC and hemichannel permeability were examined in rat neonatal cardiac fibroblast when exposed to simulated ischemia. Figure 5.1 A shows that GJIC is rapidly reduced (15-30 min) when the cells are exposed to simulated ischemia, while Figure 5.1 B shows that hemichannel opening occurs later (90-120 min) and that hemichannel opening correlates with reduced cell viability (Figure 5.1 C).

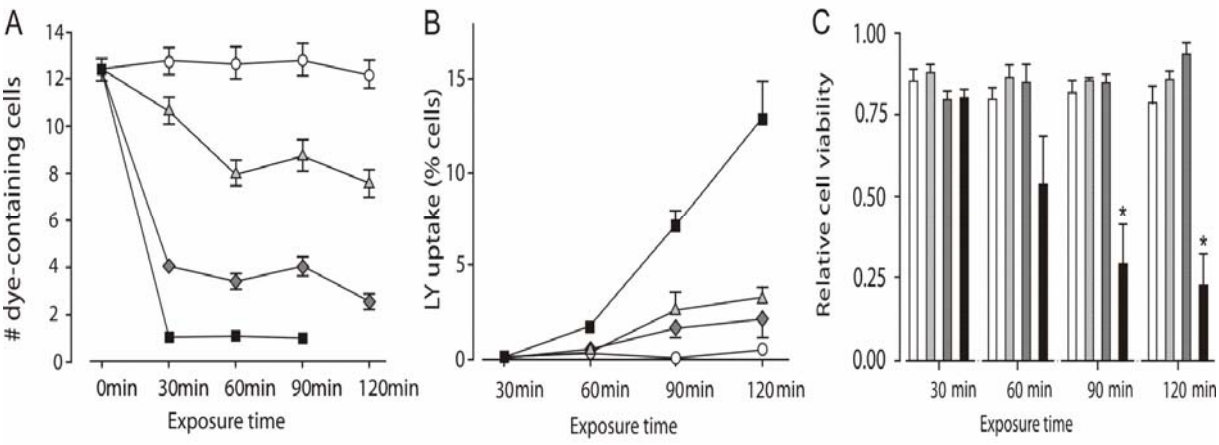

Figure 5.1: A) Number of dye-containing cells after microinjection of Lucifer yellow, B) Opening of hemichannels calculated as percent Lucifer yellow containing cells when LY was added in the buffer 2 min before the end of the experiment, C) Cell viability when cells were exposed to simulated ischemia. Modified HEPES buffer without dithionite (open bar), with 0.25 mM dithionite (light grey bar), 0.75 mM (dark grey bar) and with 2.5 mM (black bar).

A reduction in GJIC was not correlated to cell viability, but there was a good correlation to changes in the Cx43 phosphorylation pattern (Figure 5.2). GJIC was reduced significantly after 30 minutes, and as Figure 5.2 II and III shows, there is a shift from phosphorylated Cx43 after 30 minutes to non-phosphorylated Cx43, as well as a reduction in total Cx43 (Figure 5.2 I).

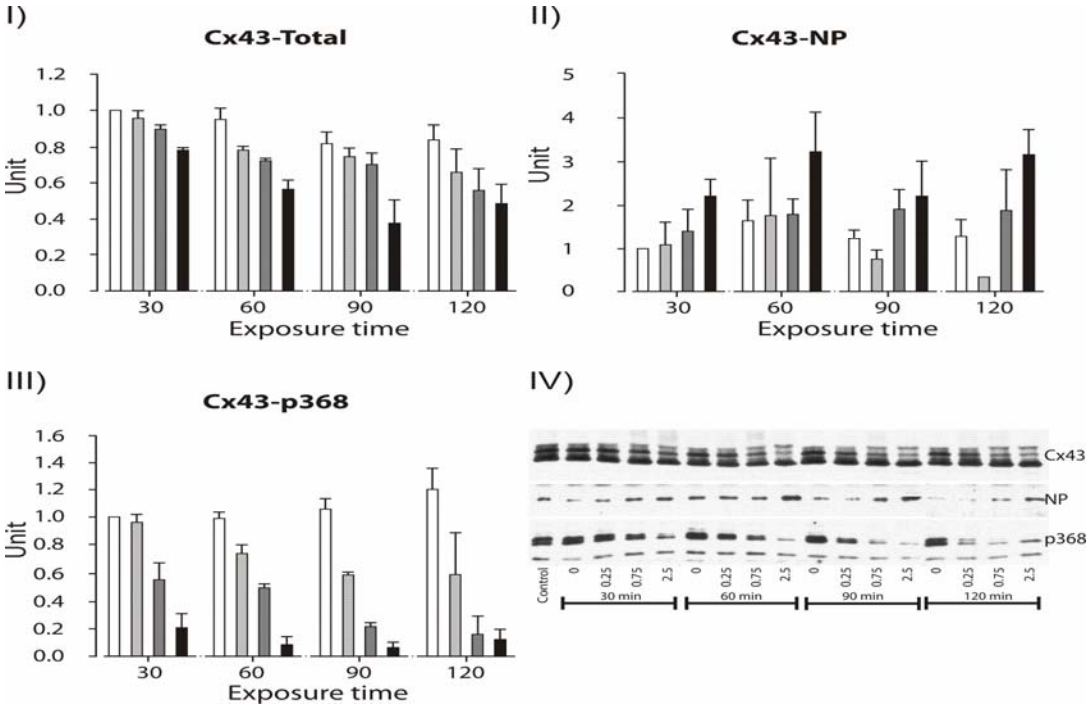

Figure 5.2: The cells were exposed to a modified HEPES buffer and dithionite as indicated (0 mM to 2.5 mM dithionite). Modified HEPES buffer without dithionite (open bar) n=4, with 0.25 mM (light grey bar) n=2, 0.75 mM (dark grey bar) n=4 and with 2.5 mM dithionite (black bar) n=4. Results are shown as densitometric quantification and are normalized to 30 min modified HEPES buffer with 0 mM dithionite; I) Western blot with antibody that recognizes all phosphovariants of Cx43; II) WB with antibody that recognizes only non-phosphorylated Cx43; III) WB with antibody that recognizes only phosphorylated Cx43, and IV) shows representative Western blot.

These results documented that Cx43 phosphorylation is important in the regulation of GJIC and that hemichannel permeability is associated with a loss of cell viability. In addition, recent publications have indicated that Cx43 is also localized in mitochondria. We have confirmed these findings with both WB and immune-electron microscopy (Figure 5.3). These findings led to the hypotheses that Pannexin 1 (another channel protein) could be present in the mitochondria. Paper 4 shows for the first time that Panx1 is localized in the mitochondria in rat heart cells, and that Panx1 is found in both the SSM and IFM fraction. There was no

detectable Panx1 in neonatal rat cardiac myofibroblasts tested by WB, although there was also significant expression at the gen level (mRNA) in these cells.

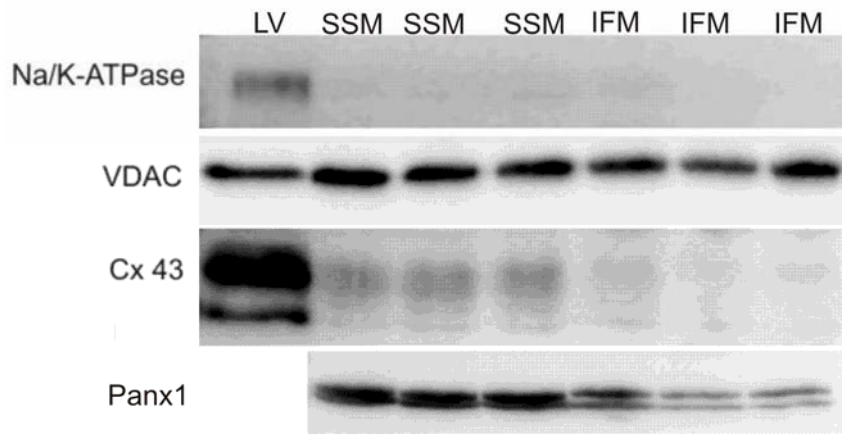

Figure 5.3: Representative Western blots showing Cx43 in left ventricle (LV) and in mitochondria, especially in SSM. Panx1 are found in both mitochondria fractions, but in higher concentrations of Panx1 in SSM compared to IFM. Na/K-ATPase and VDAC were tested to confirm the purity of the mitochondrial fractions.

Against the background that GJIC is rapidly reduced during simulated ischemia and that ischemic preconditioning is very cardioprotective, we tested whether cyclic pretreatment with the gap junction uncoupler Heptanol was cardioprotective. We used isolated perfused rat hearts, and the treatment was 10 min of 2 mM Heptanol and 5 min of reperfusion three times before prolonged regional ischemia and 2 hours of reperfusion. The proposed cardioprotection was confirmed with a 58% reduction in infarct/risk volume (Figure 5.4). Heptanol also significantly increased the time to the MPTP opening in TMRM-loaded cardiac myocytes when tested using the line scan mode of the confocal microscope. The increased phosphorylation of AKT and GSK-3β as well as the opening of mitoK_{ATP}- and mitoK_{Ca}-channels were proposed as the underlying mechanism for this cardioprotection, and since we were able to demonstrate reduced mitochondrial respiration, the mechanism triggering

cardioprotection was most likely related to mitochondrial function and not to the cell to cell coupling.

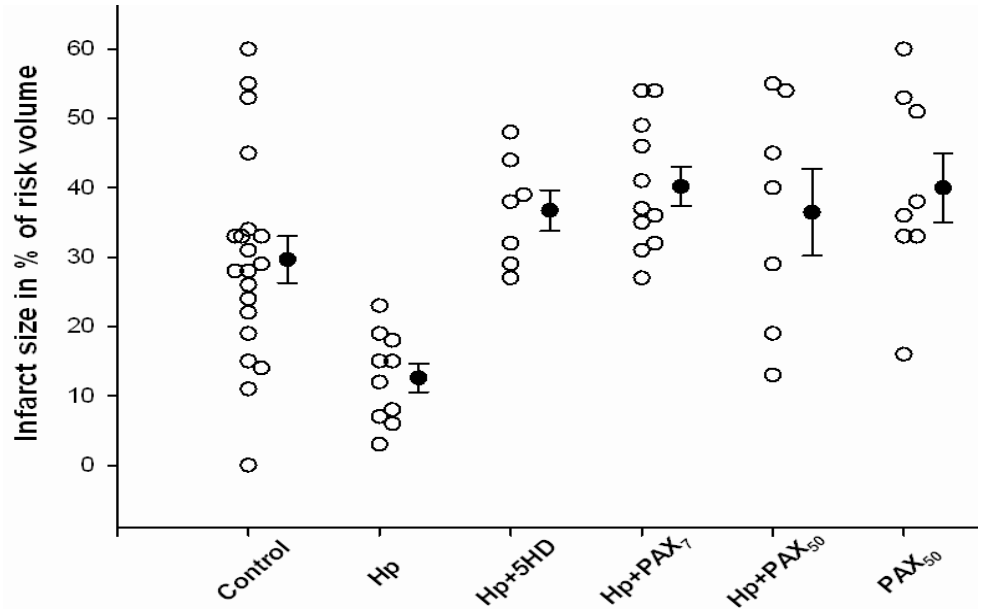

Figure 5.4: Isolated perfused rat hearts with 30 minutes of regional ischemia and 2 hours of reperfusion. Each open circle represents one heart, and a filled circle with error bars represents means ± SEM.

The opening of the mitoK_{ATP}-channels is known to be cardioprotective, and as we showed with heptanol, the blocking of the mitoK_{ATP}-channels abolished the cardioprotection given by heptanol. In 2005, there were reports which indicated that the new gasotransmitter hydrogen sulfide causes the relaxation of smooth muscle of different types and that glibenclamide (K_{ATP} blocker) abolishes this effect. Glibenclamide is also an effective blocker of IPC. Against this background, we tested whether the new gasotransmitter hydrogen sulfide was cardioprotective, and found that with a specific concentration (1 μM), the infarct/risk volume was reduced by 50% and that this effect was abolished by blocking both K_{ATP}-channels, and most importantly, mitoK_{ATP}-channels (Figure 5.5). This was the first study to demonstrate that hydrogen sulfide was a cardioprotective agent and that the mechanism was through the mitoK_{ATP}-channels.

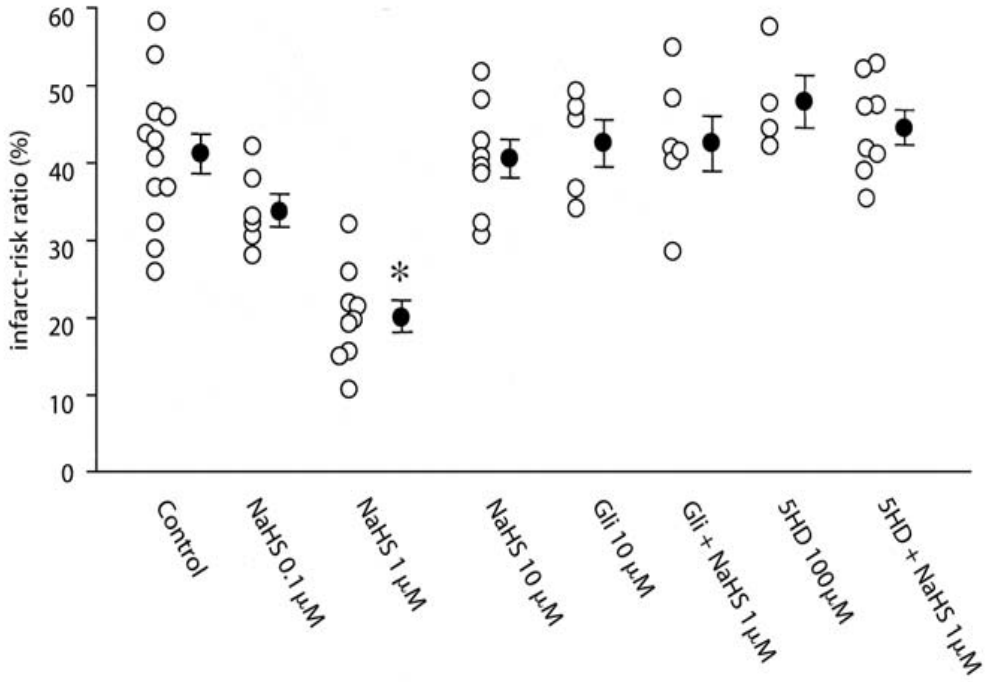

Figure 5.5: Isolated perfused rat hearts with 30 minutes of regional ischemia and 2 hours of reperfusion. Each open circle represents one heart, and a filled circle with error bars represents means ± SEM.

We have also attempted to use exogenous hydrogen sulfide as post-treatment and postconditioning, but have failed to show cardioprotection (data not shown). The new method developed for analysing hydrogen sulfide indicates that the concentration of hydrogen sulfide is rapidly reduced by time and that gassing the buffer with CO_2/O_2 increases the evaporation of gas, thereby reducing the concentration in the buffer. When we were using hydrogen sulfide after ischemia the buffer with hydrogen sulfide had been bubbled for approximately 1 hour and the concentration of hydrogen sulfide was very low. This could be the explanation for why we do not get cardioprotection when we tried to give hydrogen sulfide after the ischemic period. Consequently, we cannot conclude that treatment with hydrogen sulfide lasted for 50 minutes, but we must conclude that we have shown the effect of treatment only a

few minutes before ischemia, and that the phenomenon we observed was a precondition effect.

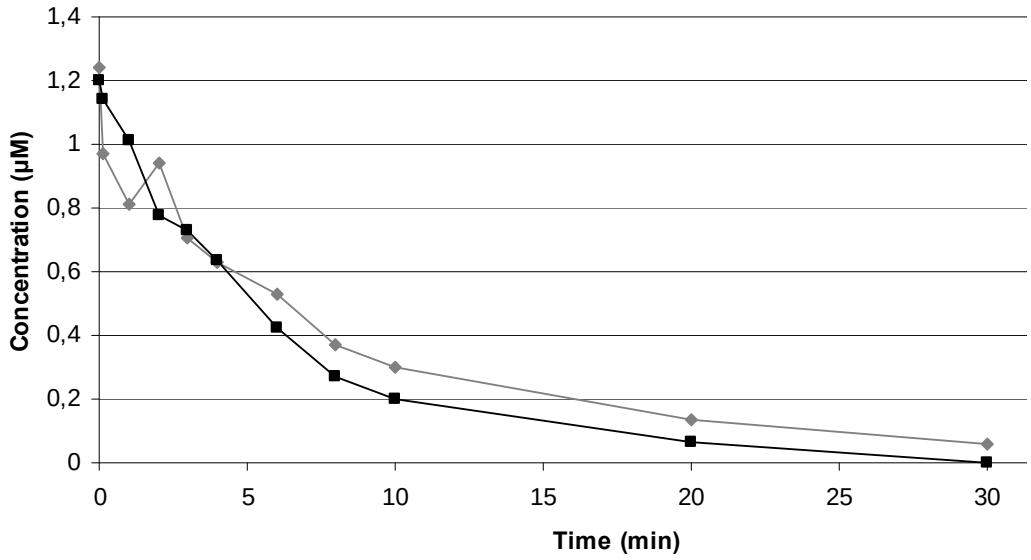

Figure 5.6: Results from two separate experiments in which we measured the concentration of hydrogen sulfide over time in KHB during bubbling with $CO_2/0_2$.

After a prolonged time (90-120 min) exposure to simulated ischemia, neonatal heart fibroblasts opened hemichannels and cell viability was therefore reduced. By adding heptanol or Gap26, the opening of hemichannels was abolished or decreased in these cells. We then hypothesized that by keeping hemichannels closed during ischemia-reperfusion, the infarct size could be reduced. We used isolated perfused rat hearts and added Gap26 or ^{10}Panx1 to the 2 latest min of regional ischemia and 10 min into reperfusion. By adding Gap26 the infarct/risk volume was reduced by 60%, although ^{10}Panx1 did not change the infarct/risk volume. These results show that inhibiting the opening of Cx43-hemichannels during early ischemia-reperfusion injury reduces cell death.

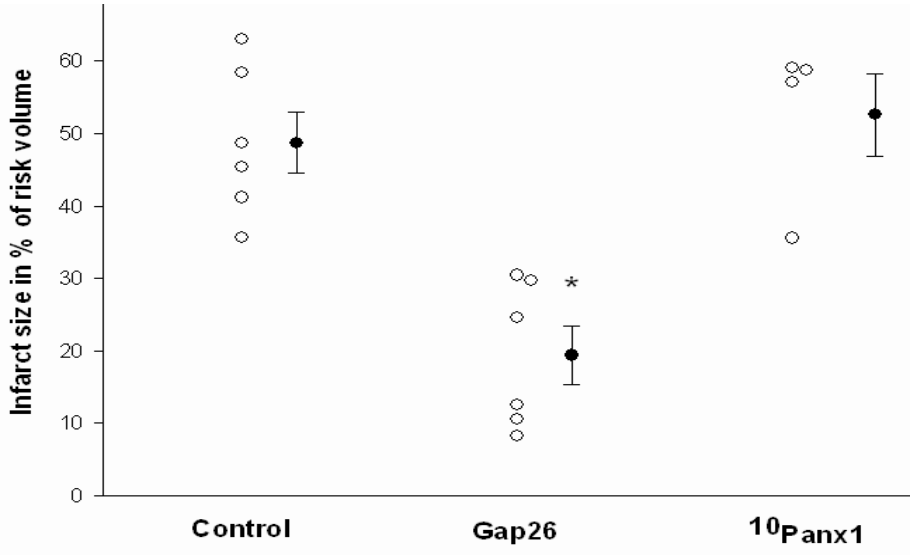

Figure 5.7: Isolated perfused rat hearts with 30 minutes of regional ischemia and 2 hours of reperfusion. Each open circle represents one heart, and a filled circle with error bars represents means ± SEM.

6. GENERAL DISCUSSION

The present thesis has displayed different routes to cardioprotection, with a focus on gap junction, hemichannels and mitochondria. GJIC and hemichannel permeability have been examined under simulated ischemia. GJIC is quickly reduced during simulated ischemia, while hemichannels open during prolonged ischemia. Cx43 exhibited a rapid dephosphorylation during early ischemia and a gradual loss of Cx43 during prolonged ischemia. Cx43 has been revealed in mitochondria in addition to sarcolemma. For the first time, Panx1 has been demonstrated to be localized to cardiac mitochondria. Pretreatment with the gap junction uncoupler heptanol was shown to be cardioprotective, as well as to inhibit mitochondrial respiration. The possible mechanisms of protection are therefore a direct effect of heptanol on mitochondria, or that heptanol by transient blocking gap junctions initiates phosphorylation of AKT and GSK-3β and the further opening of mitoK_{ATP}- and mitoK_{Ca}-channels. The hydrogen sulfide treatment was cardioprotective in a dose-dependent manner and the opening of the mitoK_{ATP}-channel was proposed as the mechanism. Inhibiting the opening of Cx43 hemichannels was also cardioprotective when added in the early reperfusion period, which shows the importance of hemichannels during ischemia-reperfusion injury. This thesis therefore indicates that mitochondrial mechanisms and hemichannels are both crucial for cardioprotection.

6.1 Gap junction channels and cardioprotection

Sundset et al. 2004 [32] showed that ischemic preconditioning improved cell survival in cardiac myofibroblasts and proposed that the initiating mechanism was that the IPC reduced GJIC before prolonged ischemia, and then that GJIC was improved immediately after the prolonged ischemic period, thereby resulting in improved cell survival. The present results

with the opening and closing of the gap junction channels, with heptanol before ischemia-reperfusion (Paper 3), support Sundset's finding that changes in GJIC before prolonged ischemia may be cardioprotective. Nevertheless, the change in mitochondrial metabolism with heptanol indicates that there is also a direct effect on mitochondria of heptanol. The reduction in the mitochondrial metabolism we observed may indicate that heptanol reduces F_0F_1ATPsyntase since State 3 is reduced by heptanol, but there are no changes in State 4. This enzyme is responsible for the majority of cellular production of ATP from ADP and Pi. It is located in the inner mitochondrial membrane and using the proton gradient for the energy transfer converting ADP to ATP. It is been known also that the ATPase under some conditions can function in reverse mode using ATP to maintain the proton gradient [66]. Contessi et al. [66] have shown that a mitoK_{ATP} opener such as diazoxide enhances F_0F_1ATP synthase inhibition during ischemia, and that this mechanism is important for cardioprotection. Moreover, our results with heptanol support this finding. Saltman et al. 2005 [67] have also shown that a pretreatment with heptanol is cardioprotective. There is good evidence that IPC activates AKT and GSK-3β, which further inhibits the opening of the MPTP [52; 65; 68]. A pretreatment with heptanol also acts through this pathway, and also increases the time to the opening of the MPTP. Based on this, it is possible that changes in mitochondrial function result in AKT and GSK-3β, which further inhibits the opening of the MPTP. The data could hence indicate that there is a two-way communication between the cytosol and the mitochondria, which is important in cardioprotection. Studies using the mitoK_{ATP} opener diazoxide support this view [69], and Juhaszova et al. [70] proposed in 2009 that mitoK_{ATP} channels opens during protection triggers and further activate PKC which give a positive-feedback or memory loop back to mitoK_{ATP} channels. This memory loop increases the activation of GSK-3β and further inhibition of MPTP.

6.2 Hemichannels and cardioprotection

Shintani-Ishida et al. [71] showed that cardiomyocytes undergo a transient opening of Cx43 hemichannels during simulated ischemia and that inhibition of the opening of these hemichannels was cardioprotective. In our study (Paper 1), we used cardiac myofibroblasts and observed an opening of the Cx43 hemichannels, though not in a transient way. We observed a good correlation between opening the hemichannels and reduced cell viability. One explanation for the difference may be that neonatal cardiomyocytes and neonatal myofibroblasts have a different regulation of the hemichannels during simulated ischemia. Shintani-Ishida et al. [71] showed an increased cell viability when they inhibited Cx43 hemichannels with Gap26. Hawat et al. [72] used isolated perfused rat hearts and added Gap26 during ischemia and reperfusion, and observed a significant reduction in the infarct/risk ratio. These observations were supported by our finding in Paper 1, but in contrast to the above mentioned studies we showed that just a few minutes with Gap26 at reperfusion was cardioprotective. Gap 26 and ^{10}Panx1 are newer compounds than, e.g. heptanol and are more specific, but these compounds have also been criticized for not being specific enough [57]. Our result shows that the critical period for the opening of the hemichannels is in the early reperfusion period. Hawat et al. 2011 (abstract) have also demonstrated that Gap26 is cardioprotective in an in vivo rat model with regional ischemia.

6.3 Mitochondrial connexin and pannexin

There is evidence that Cx43 is also present in the mitochondrial membrane, and is important for cardioprotection [35; 73; 74]. We have shown that Cx43 is present in mitochondria, although only in the SSM fraction of isolated mitochondria although gold immune EM demonstrated labelling also in interfibrillar mitochondria. It is very important to examine

SSM and IFM separately because recent reports have indicated that SSM can be preconditioned, but not IFM (Presentation ISRH Haifa 2011 Ruiz-Meana). Both Boengler [35] and our group (Paper 3) have shown that only SSM contains Cx43, and that this may be the reason way IFM cannot be preconditioned. It should be noted that the difference between these two mitochondrial fractions has to be addressed within the perspective of the use of different isolation protocols to obtain the two fractions. We have showed (figure 3.6) that the uses of trypsin removes Cx43, and indirectly that the use of trypsin onto IFM would make a false negative answer. However, this finding also adds to the good evidence for a link between mitochondrial Cx43 and preconditioning.

Vessey et al. [45; 46] have indicated that Panx1 channels are important, both during ischemic preconditioning and postconditioning in isolated rat hearts. They have also revealed by Western blot that Panx1 is expressed in the heart. They have shown that the blocking of Panx1 channels with carbenoxolone and other unspecific blockers abolish pre- and postconditioning. The present results (Paper 4) show that Panx1 is expressed in rat heart, but that the subcellular localization is in the mitochondria and not in the sarcolemma under normal conditioning. This is in contrast to what has been proposed for astrocytes, in which studies might indicate localization in or close to the cell membrane. The blockers Vessey et al. [45; 46] used may affect other channels, especially Cx43 gap junction channels or Cx43 hemichannels that are important during pre- and postconditioning. Another explanation may be that Panx1 changes localization during different stimuli such as ischemia or pharmacological treatment. In Paper 1, we showed that ^{10}Panx1 did not close hemichannels during simulated ischemia in cell cultures, and that the infarct/risk ratio in the rat heart was not changed by adding ^{10}Panx1 in the early ischemia-reperfusion period. The physiological

and pathophysiological function of Panx1 in heart and cardiac mitochondria is still not clear, and needs further examination.

6.4 Hydrogen sulfide

Paper 2 in this thesis provided the first evidence that exogenous hydrogen sulfide can limit infarct size, and that this protection was dependent on the mitoK_{ATP}-channel [53]. The present thesis indicates that H_2S can insert a preconditioning like effect. Later studies have shown cardioprotection by both exogenous and endogenous hydrogen sulfide, both in vitro and in vivo [49; 75; 76]. Recent studies suggest that hydrogen sulfide may be important during IPOST as well as IPC [75; 76; 77; 78].

7. MAIN CONCLUSION

Simulated ischemia rapidly reduced GJIC, thereafter leading to the opening of Cx43 hemichannels. GJIC was associated with a shift from phosphorylated Cx43 to non-phosphorylated Cx43, and the hemichannel opening was associated with reduced cell viability.

Cx43 is present in the mitochondrial, and heptanol increases the time to the opening of the MPTP. Panx1 is also present in the mitochondria in the heart, but not in the sarcolemma The blocking of Panx1 channels in heart-derived cells, or in the heart, does not changes hemichannel permeability or infarct size when added in the early ischemia-reperfusion period.

A pretreatment with heptanol is cardioprotective, with the mechanism relying on the phosphorylation of AKT/GSK-3β and on the opening of the mitochondrial potassium

channels. Exogenous hydrogen sulfide is cardioprotective in a dose-dependent manner and the mechanism also relies on the opening of the mitochondrial potassium channels. Inhibition of the Cx43 hemichannel opening during the early ischemia-reperfusion is cardioprotective, and may be a future way to reduce ischemia-reperfusion injury in patients.

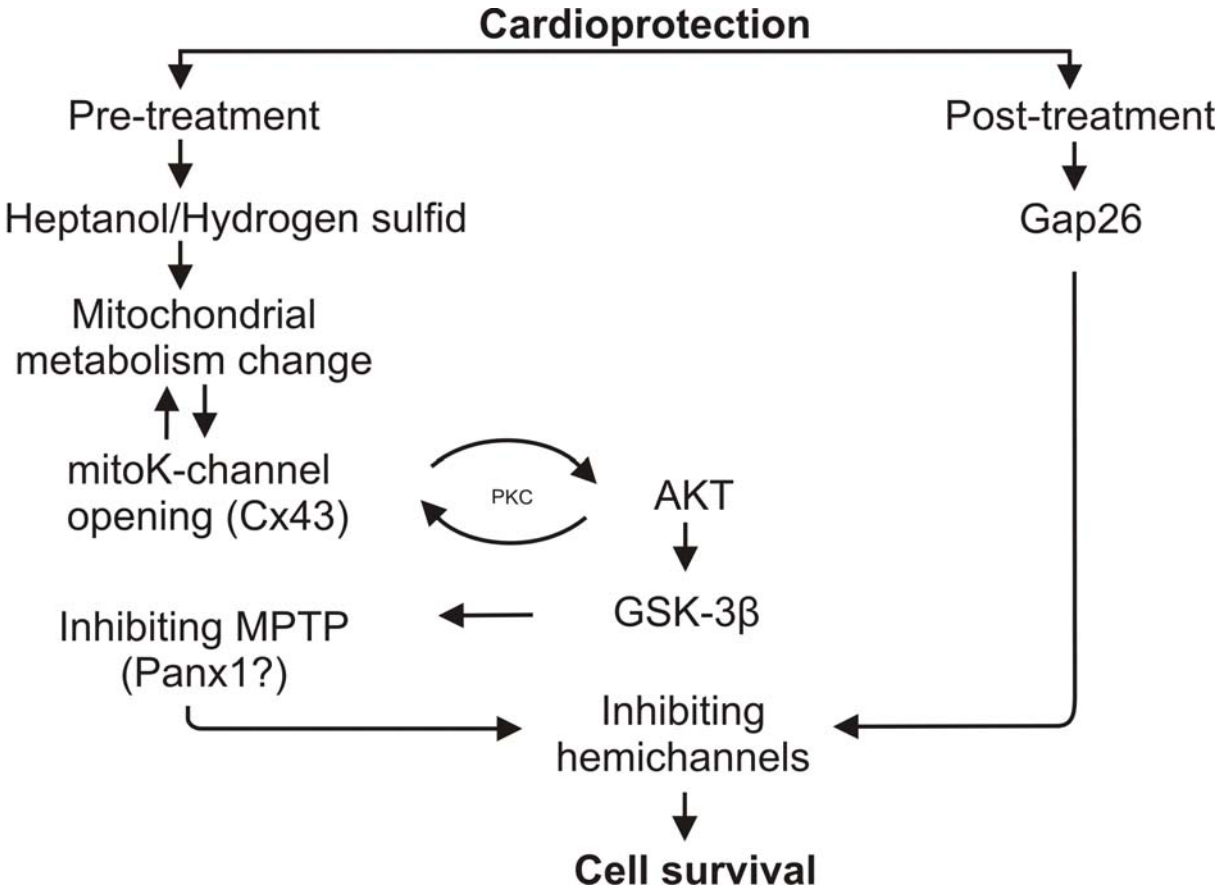

Figure 7.1: Schematical summary of potential pathways for cardioprotection. See page 4 for abbreviations.

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Paper 1

Paper 2

Paper 3

Paper 4

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