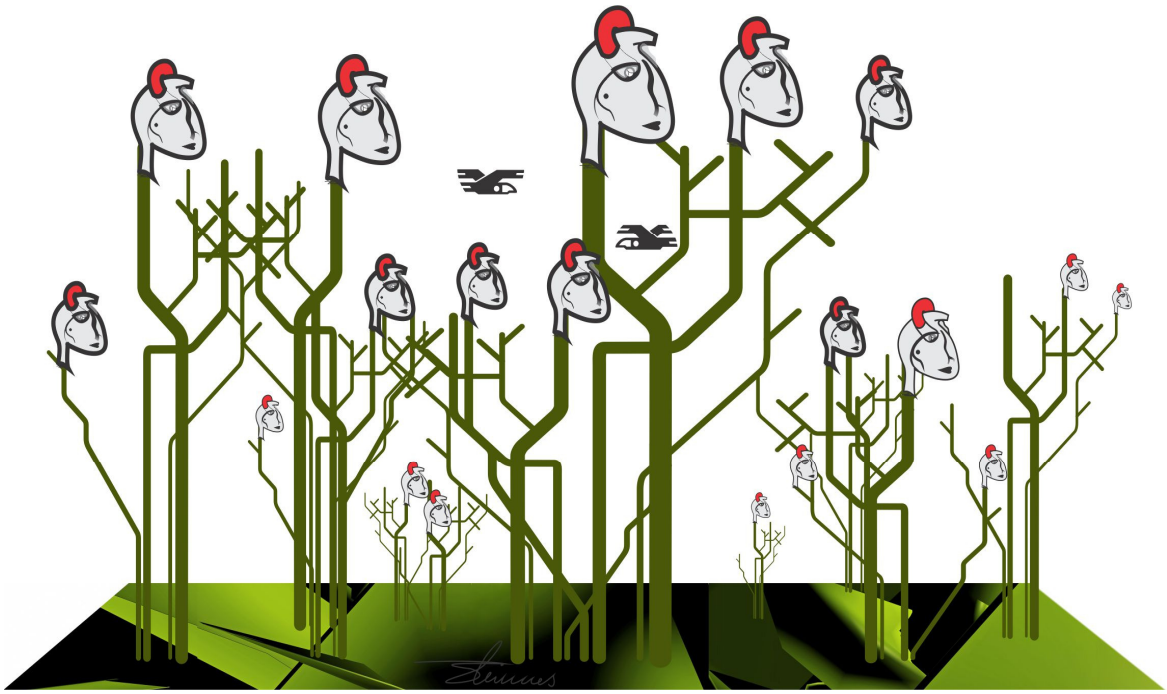
Effects of exogenous hydrogen sulfide administration on cardiac function and reactive oxygen species production

A study in hearts from normal rats and rats with heart hypertrophy or ischemia

by

Paunas Teodora Ioana



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ACKNOLEGEMENTS

This work was carried out at the Cardiovascular Research Group, Institute of Medical Biology, Faculty of Health Sciences, University of Tromsø, Norway.

I would like to thank my supervisor, Professor Kirsti Ytrehus for insightful discussions and guidance throughout the writing of this thesis.

I will forever be grateful to my co-supervisor David Johansen for his endless patience, guidance, and excellent pedagogical skills. Takk for at du tok deg tid til å ta meg med på sykehuset og dele din kunnskap og dine erfaringer med meg.

Thanks to Anne Hafstad and Nils Thomas Songstad, for collaboration during the development of the DHE method.

Thomas Andreasen made an important contribution to this thesis by measuring the hydrogen sulphide.

Karin Akselsen and Knut Steinnes were very helpful with the administrative and technical assistance.

My office colleagues, Neoma Boardman, Wahida Salma and Belal Aljabri deserve my warm thanks for many enjoyable discussions in more or less scientific subjects. I highly appreciate the help of Neoma who took time to proofread through this study.

To Veronika Franekova thanks for all the cookies, flowers and nice words that you always had for me.

In addition I wish to express my gratitude to all the colleagues in the department for their support and their direct or indirect contribution to the thesis.

Finally I want to thank Buzwani for putting up with my frustrations and my imaginary problems, and for making me smile every single day.

ABSTRACT

Coronary heart disease is the leading cause of death worldwide. Infarct size can be limited by interventions used after the ischemic event like the use of thrombolytic therapy or primary percutaneous coronary intervention. Paradoxically, however, the return of blood flow can also result in additional cardiac damage and complications, referred to as reperfusion injury. It has been shown that reperfusion injuries can be decreased by postconditioning- rapid intermittent interruptions of blood flow in the early phase of reperfusion, or post-treatment using various drug therapies which applied during reperfusion can reduce infarct size. H_2S, a gas that is synthesized in mammalian tissue, has been reported to be cardioprotective during ischemia-reperfusion injury. The means by which H_2S is cardioprotective during I/R are believed to be: the opening of the sarcolemmal K_{ATP} channel, the generation of antiapoptotic effects inside the cells as well as a direct antioxidant effect.

Low levels of reactive oxygen species (ROS) are constantly produce within cells and play important roles in cell signaling, cellular homeostasis, differentiation and apoptosis. However an excessive increase in the level of ROS can be harmful and has been proposed to play crucial roles or contribute in the development of various diseases.

The aim of our study was to investigate the effects of H_2S in an acute ischemia-reperfusion model and to determine whether exogenous administration of H_2S in both healthy rats and rats exposed to experimental models of cardiac disease influenced the production of ROS. In order to do this we established a method trough which we were able to measure the presence of ROS in heart tissue samples harvested from normal rats and rats with heart hypertrophy and ischemic heart disease.

ABBREVIATIONS

ACE = angiotensin- converting enzyme

Ang II = angiotensin II

ATP = adenosine triphosphate

BH-4 = tetrahydrobiopterin

CAT = cysteine aminotransferase

CBS = cystathionine β synthetase

CF = coronary flow

-COOH = carboxylic

CSE = cystathionine γ lyase

DHE = dihydroethidium

DMSO = dimethyl sulfoxide

dP/dt = first derivative of pressure over time

eNOS = endothelial nitric oxide synthase

ERK = extracellular-signal regulated kinase

H2O2 = hydrogen peroxide

H2S = hydrogen sulphide

HIT = intensity interval training

HO = hydroxyl radical

HR = heart rate

I/R = ischemia/reperfusion

IU = international units

i.p. = intraperitoneal

JNK = c–jun NH_2-terminal kinase

KHB = Krebs-Henseleits buffer

LV = left ventricle

LVDP = left ventricular developed pressure

PCI = percutaneous coronary intervention

MAPK = mitogen-activated protein kinase

METC = mitochondrial electron transport chain

MIT = moderate intensity interval training

MST = mercaptopyruvate sulfurtransferase

mPTP = mitochondrial permeability transition pore

NADPH = nicotinamide adenine dinucleotide phosphate-oxidase

NaHS = sodium hydrosulfide

NO = nitric oxide

$O2^-$ = superoxide anion

PKC = protein kinase C

RAS = renin-angiotensin system

RISK = reperfusion injury salvage kinase

ROS = reactive oxygen species

SEM = standard error of the mean

TAC = transaortic constriction

UA = uric acid

XO = xanthine oxidase

XOR = oxidoreductase

XDH = xanthine dehydrogenase

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1. INTRODUCTION

Coronary heart disease is the leading cause of death worldwide. Of the 16.7 million deaths from cardiovascular diseases every year, 7.2 million are due to ischemic heart disease. [1]

In early 1980s two modes of therapy were introduced to restore blood flow in occluded artery. One involved pharmacologic dissolution of blood clots with a thrombolytic agent and the other involved mechanical disruption of clots with so-called primary balloon angioplasty. [2]

Early and successful myocardial reperfusion with the use of thrombolytic therapy or primary percutaneous coronary intervention (PCI) is still the most effective strategy for reducing the size of a myocardial infarct and improving the clinical outcome. Paradoxically, however, the return of blood flow can also result in additional cardiac damage and complications, referred to as reperfusion injury.

According to Yellon et al. there are four types of cardiac injury during myocardial reperfusion: myocardial stunning, the no-reflow phenomenon, reperfusion arrhythmias and lethal reperfusion injury. Studies in animal models of acute myocardial infarction suggest that lethal reperfusion injury could account for up to 50 % of the final size of a myocardial infarct in a standardized situation with ischemia- reperfusion, and a number of strategies have been shown to ameliorate lethal reperfusion injury [3].

Reperfusion injury of the myocardium seems to be a complex phenomenon involving several independent factors such as: intracellular calcium overload, inflammation, rapid restoration of physiologic pH and the generation of reactive oxygen species. All of this contributes to the opening of the mitochondrial permeability transition pore (mPTP) and the induction of cardiomyocyte hypercontracture. Trough the opening of the mPTP, the oxidative phosphorylation uncouples and the mitochondrial swells leading to cardiomyocyte death.

Potential causes of injury that developed during reperfusion are difficult to analyze, as these must be clearly differentiated from ischemic causes [4]. The most accurate means of showing the existence of lethal reperfusion injury is to show that the size of a myocardial infarct can be reduced by an intervention used at the beginning of myocardial reperfusion [5].

1.1 POSTCONDITIONING

Postconditioning was first described by Zhao and colleagues in dogs [6], and it was defined as rapid intermittent interruptions of blood flow in the early phase of reperfusion.

The postconditioning algorithm was 30s of reperfusion followed by 30s of coronary occlusion, repeated for three cycles at the onset of reperfusion [7].

Since then the term "postconditioning" was also used to describe various drug therapies which applied during reperfusion can reduce infarct size. This may also be referred as "post-treatment". The mechanism of ischemic postconditioning is not fully understood, but the procedure has been shown to target important mediators of lethal reperfusion injury. It seems that ischemic postconditioning reduces oxidative stress, opens the K_{ATP} channels , improves endothelial function, reduces neutrophil accumulation, decreases intracellular Ca^{2+} overload, delays the restoration of neutral pH, activates the RISK pathway and inhibits the opening of the mitochondrial permeability transition pore through this protecting against lethal reperfusion injury [3].
Zhao ZQ and Vinten-Johansen proposed than when considering postconditioning it is useful to use the concept of triggers, mediators and end effectors such as they are used when talking about preconditioning as similar pathways and signals may be involved in both of the processes [7]. Trigger mechanisms that were shown to play a role in postconditioning are: generation of reactive oxygen species (ROS), induction of pro-inflammatory cytokines, expression of tissue factor, endogenous adenosine, endogenous opioids and nitric oxide (NO). Mediators that might be involved in postconditioning are: the activation of intracellular protein kinase C and of other survival and death kinases, the reduction in intracellular Ca^{2+} overload and the opening of K_{ATP} channels. The end-effectors involved in reperfusion injury seem to be the inhibition of the mPTP.

Although postconditioning using repeated cycles of reperfusion and occlusion was the most investigated strategy, it seems to have different results depending on the animal model that was used, number of cycles and the duration of ischemia and reperfusion in each cycle [8].

Until now two clinical studies have been performed showing the beneficial effects of postconditioning on human hearts. Laskey [9] reported a study of 17 patients undergoing percutaneous coronary intervention for acute myocardial infarction who were randomly assigned

to standard reperfusion therapy or a postconditioning protocol. The postconditioning protocol consisted of two 90 s balloon reinflations with 3–5 min of reperfusion between them. Final ST segment elevation in the postconditioning group (1.60 mV) was less than in the control group (4.0 mV, $P<0.001$) and coronary flow velocity reserve was also improved.

Staat et al. [10] reported a multi-center randomized clinical trial of 37 patients with total coronary artery occlusion undergoing angioplasty/stenting. Patients were assigned to reperfusion with direct stenting alone (control group) or were subjected to a postconditioning protocol following reperfusion by stenting. The postconditioning protocol consisted of 4 cycles of 1-min re-inflation followed by 1min deflation of the angioplasty balloon. Infarct size (area under the creatine kinase curve) was significantly less, and the coronary blood flow achieved was greater in the postconditioned patients.

These results encourage the development of therapeutic approaches to reduce infarct size by specific measures applied during the early phase of reperfusion. Many pharmacological strategies from different pharmacological categories have been studied, some of them having multiple mechanisms of action. Some of this include opioids [11], bradikinin [12], cyclosporine [13], Na(+)/H(+) exchange inhibitors [14,] antioxidants/free radical scavengers [15] , renin-angiotensin system (RAS) antagonists [16], adenosine and adenosine receptor agonists [17] etc.

1.2 HYDROGEN SULFIDE

Hydrogen sulfide, the gas that is believed to be the reason for life extinction on earth 250 million years ago [18], is nowadays seen as an important signaling molecule with essential roles in human biology.

It`s physiological actions were first proposed in 1996 by Abe and Kimura [19] who based their studies on earlier findings which showed that endogenous levels of H_2S are produced in the rat brain [20].

H_2S is synthesized in mammalian tissue through enzymatic and nonenzymatic pathways. The enzymes that are involved in H_2S production are: Cystathionine β synthetase (CBS), Cystathionine γ lyase (CSE), 3 mercaptopyruvate sulfurtransferase (3 MST) and CAT (cysteine aminotransferase).

H_2S can be synthesized in the cell from cysteine, a non-essential amino acid.

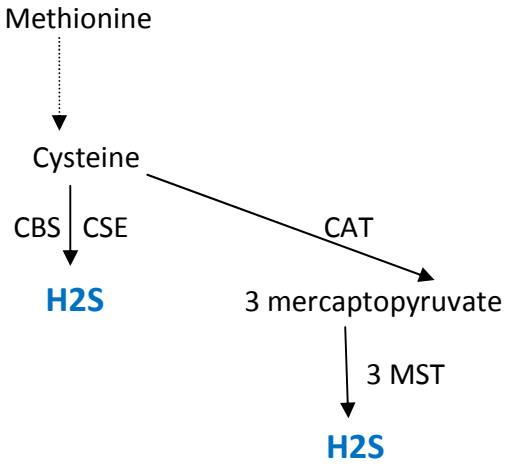


Figure 1.1 The biosynthesis of H_2S in mammalian cells. CSE (Cystathionine γ lyase) and CBS (Cystathionine β synthetase)are cytosolic enzymes and wheres 3 MST (3 mercaptopyruvate sulfurtransferase) is both cytosolic and mytocondrial.

Once synthesized, H_2S can be further oxidized to thiosulfate which will be converted into sulfite and sulfate. H_2S can also be methylated to form methanethiol and dimethyl sulfide or can act as a substrate for rhodanase to form thiocyanate and sulfate:

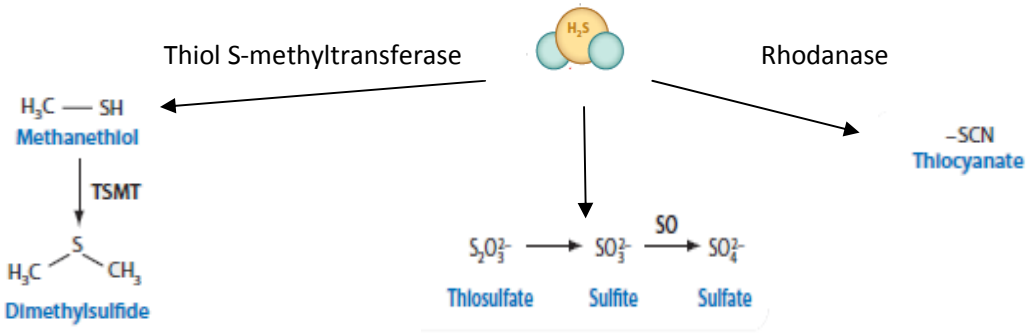


Figure 1.2. The degradation of H2S in mammalian cells (Modified after Ling Li et al. Annu. Rev. of Pharmacol. Toxicol. 2004). NB GSSG -

In the adult rat H_2S generation is highest in the liver and brain, followed by kidney, heart, aorta and small intestine [21].
The physiological concentration of H_2S in blood and various tissues is not really known because different measurement techniques revealed a wide range of concentrations, but recent estimations have placed the human plasma concentration of H_2S in the submicromolar range [22].

Although in most of the studies H_2S has a vasodilatator effect, there are reports that contradict this finding [23] or show no effect on vessel wall [24]. A dual effect of this gas, depended on its concentration, has also been reported [25], [26]. H_2S was reported to mediate vasodilatation trough the opening of K_{ATP} channels in the smooth muscle [25, 27, 28, 29,]. In addition, H_2S was reported to reduce ATP levels in the cell, and by this means to mediate smooth muscle relaxation [30].

H_2S has been shown to inhibit ACE activity of endothelial cells and trough this mechanism have the potential to lower blood pressure [31]. Studies that investigated this theory revealed contradictory results. Some studies reported an increase in mean arterial blood pressure after the infusion of H_2S directly into CNS [32], while others reported the opposite [28]. To support the result that H_2S would rather decrees blood pressure is the fact that by suppressing the production of H_2S the blood pressure increases [33].

H_2S has been reported to be cardioprotective during ischemia-reperfusion injury. Studies suggest that H_2S may exert preconditioning and postconditioning actions. H_2S administrated before ischemia limits infarct-size induced by I/R in the heart in a concentration depended manner [24]. Post-treatment with H_2S donors during reperfusion has also been shown to protect the heart against I/R injury [34], although some studies showed no such effect.

The most important means by which H_2S is cardioprotective during I/R is believed to be by opening of the sarcolemal K_{ATP} channel. The opening of the sarcK_{ATP} channel will enhance shortening of the cardiac action potential duration by accelerating phase 3 repolarization. This will inhibit calcium entry into the cell via L-type channels and prevent calcium overload during ischemia and early reperfusion. In addition H_2S has been shown to activate PKC in the heart and consequently lower intracellular calcium [35].

There are studies that suggest that H_2S has also anti-apoptotic roles in the cell during I/R. In one of the studies the role of the pro-apoptotic pathways p38 MAPK and JNK1/2 and administration of NaHS were investigated in an I/R setting [36]. The investigators found that regional myocardial ischemia (25 min) and reperfusion (30 min) increased the phosphorylation of p38MAPK and JNK1/2 and thus activates these pathways, while administration of NaHS significantly attenuated this increase. Another study showed that H_2S induced the activation of ERK1/2 pathway which is believed to play a pro-survival role in the setting of ischemic preconditioning [37].
H_2S has been also shown to preserve the structure and function of mitochondria and therefore protect against ischemic injury [38].

1.3 ROS AND OXIDATIVE STRESS

While O_2 is both essential for life it is also toxic trough the formation of reactive oxygen radicals that are able to damage cellular membranes, proteins and DNA. When O_2 accepts single electrons it forms reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO). Under physiological condition ROS are produced in low concentration and act as a signaling molecule, while in pathological condition ROS contribute to cell death and are involved in the pathogenesis of several important human diseases such as cancer, neurological disease and heart disease. ROS are constantly produced in the cell and under physiological conditions the cells would increase their antioxidant defenses in order to counteract this production. Oxidative stress occurs when the rate of ROS production overbalances the rate of their removal by cellular defense mechanisms.

1.3.1 Free radicals in biology

Radicals are compounds that contain a single electron, usually in an outside orbit. A free radical is a radical capable of independent existence [39]. Most stable molecular species have the electrons in their outer orbit arranged in pairs with opposite spins which make them very stable. Radicals are highly reactive; they initiate chain reactions by extracting an electron from

neighboring molecules in order to complete their own orbits. Oxygen is a biradical; it has two unpaired electrons in separate orbits making it a strong acceptor of electrons. Oxygen free radicals are formed in biology by reduction of molecular oxygen. O_2 reacts slowly by accepting one electron in reactions that require a catalyst (such as a metal-containing enzyme) [39].

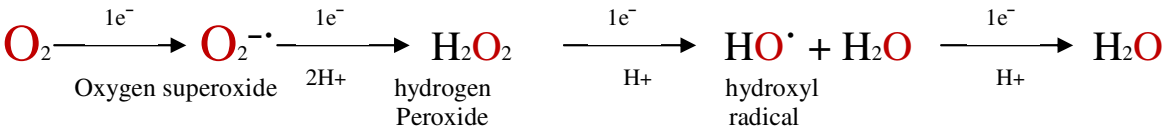


Figure 1.3 Reduction of oxygen by four single-electron steps. O2 can accept four electrons which reduces it to water. When O2 accepts one electron it forms the radical superoxide. If superoxide is reduced by another electron, the two-electron–reduced form of oxygen, peroxide, is produced. This will accept two hydrogens to produce hydrogen peroxide. When hydrogen peroxide accepts an electron it forms hydroxyl radical (HO•) and a hydroxide anion (HO–) that, when combined with hydrogen, produces water.

The superoxide anion although very reactive has limited lipid permeability and cannot diffuse far from the site of origin.

Hydroxyl radical is probably the most powerful ROS reacting quickly with a great number of biomolecules, such as carbohydrates, proteins and DNA.

Hydrogen peroxide is not strictly a radical but is classified as ROS because is an important product in the oxidation of O_2 which can generate the hydroxyl radical.

1.3.2 Sources of Reactive Oxidant Species in the cardiac tissue under physiological conditions

Under basal condition the generation of ROS in the heart is low, but it can increase under pathological conditions.

Under physiological conditions, the main sources of ROS in the heart are: mitochondrial respiration, NADPH oxidase, xantine oxidase and uncoupled NO syntheses (Fig 4):

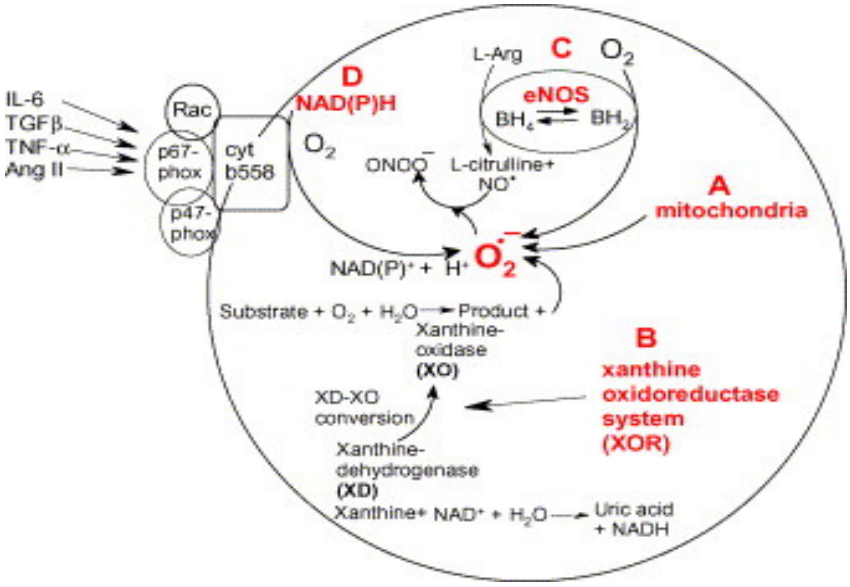


Figure 1.4 Main sources of ROS in the cardiomyocytes. (A) Uncoupling of mitochondrial oxidative phosphorylation. (B) The xanthine-oxidoreductase (XOR) system. (C) Uncoupling of NO• synthesis. (D) Activation of NAD(P)H oxidase system by various mediators. (Modified after Marian V. et al. Int J Biochem Cell Biol. 2007)

Other sources of ROS include cytochrome P450 monooxygenase, cyclooxygenases, lipoxygenases and myeloperoxidases.

1. Under physiological conditions, one of the main ROS sources is **the mitochondria**. During electron transport, approximately 2–5% of electrons escape and react with O_2 resulting in the production of ROS [40].

The main sites of ROS production in the mitochondria is Complex I and Complex III. While complex I seem to be responsible for the O_2^- produced in the heart and lung mitochondria, complex III is responsible for the O_2^- produced in brain under physiological conditions [41]. The exactly site of ROS production in Complex I is not really known, researchers suggesting both FMN and iron-sulfur cluster N1 [42] as electron donors to oxygen. In addition to that it seems that ROS production is also correlated to many different factors such as concentrations of Complex I substrates and products, redox state, proton motive force and pH [43]. The ROS produced from Complex I seem to be released mainly in the matrix and would not escape from intact mitochondria while superoxide produced from Complex III is also released to the extramitochondrial space [44].

2. Another source of ROS generation is **NADPH oxidase**. The NOX family NADPH oxidases are proteins that transfer electrons across biological membranes in order to reduce oxygen to superoxide. The prototype, NOX2 also known as gp91phox and was first described in the intracellular and plasma membrane of the phagocytes in close association with another protein p22. Nowadays six other homologues of the phagocytes NOX2 have been found (NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2) in a variety of cells such as fibroblasts, tumor cells, vascular smooth muscle and cardiac cells and are believed to play crucial roles in a variety of biological process such as cell differentiation, proliferation, survival, senescence and migration [45]. These seven NOX isoforms are now referred to as members of the NOX family.
NOX 2 is the most studied of this isoforms. It consists of 6 transmembrane domains containing two hemes, a –COOH cytoplasmatic domain and a NH_2 cytoplasmatic domain. A number of cytosolic regulatory subunits are required for the activation of it, namely p67phox, p47phox, p40 phox and the GTPase Rac2. Upon activation, these are translocated and assembled with gp91phox and electrons are transferred from NADPH to extracellular or phagosomal oxygen in order to generate superoxide (Fig 1.5).

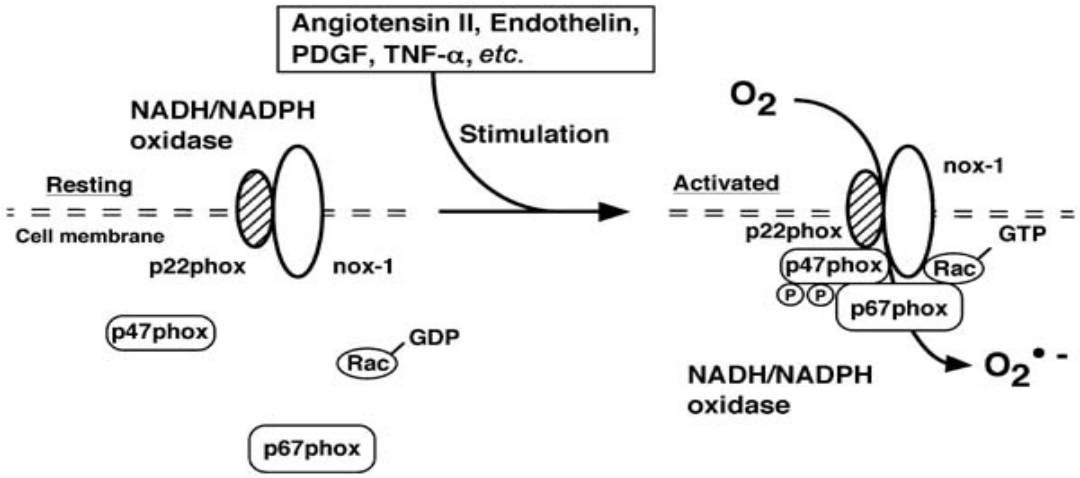


Figure 1.5 Structure and components of NADH/NADPH oxidase. Left panel shows the scheme of the resting state of the components. NADPH oxidase-1 (Nox-1) and p22phox form the electron transfer components of the oxidase, and p47phox and p67phox are cytosolic components that interact with these proteins to modulate its activity. The small G protein Rac also serves a regulatory function. The right panel shows the activated state of NADH/NADPH oxidase induced by agonists such as angiotensin II, endothelin, and PDGF
(Figure and text cited from Kyaw M et al. Acta Pharmacol Sin 2004)

The main NOX isoforms expressed in cardiac tissue are Nox2 and Nox4.
While Nox4 seems to be expressed mainly in the cardiomyocytes during development, Nox2 is the main isoform in the adult cardiomyocytes and was shown to have important roles in redox-sensitive signaling cascades [46].
Recent studies have shown that Nox2 is normally quiescent and is activated by various stimuli important in heart disease such as: angiotensin II, endothelin and cytockines, while Nox4 has constitutive low-level activity [45]. Studies have reported Nox2 to be found predominately on the plasma membrane whereas Nox4 have been reported to be found in the perinuclear endoplasmic reticulum [45, 47] and in the mitochondria [48].

3. Cytosolic **xantine oxidoreductase** (XOR) is a flavoprotein enzyme which has a catalytic role in purine degradation. XOR has to isoenzymes, xanthine oxidase (XO) and xanthine dehydrogenase (XDH). Both of them catalyse the oxidation of hypoxanthine to xanthine and xanthine to UA, which is the end product of purine catabolism in humans. In lower mammals, urate oxidase will metabolizes UA to allantoin, but this enzyme is inactivated in most primates. Under physiological conditions XOR mainly exists as XDH which uses NAD+ for electron transfer resulting in the formation of NADH. In contrast, XO uses O_2 for electron transfer resulting in the formation of superoxide and hydrogen peroxide [49].

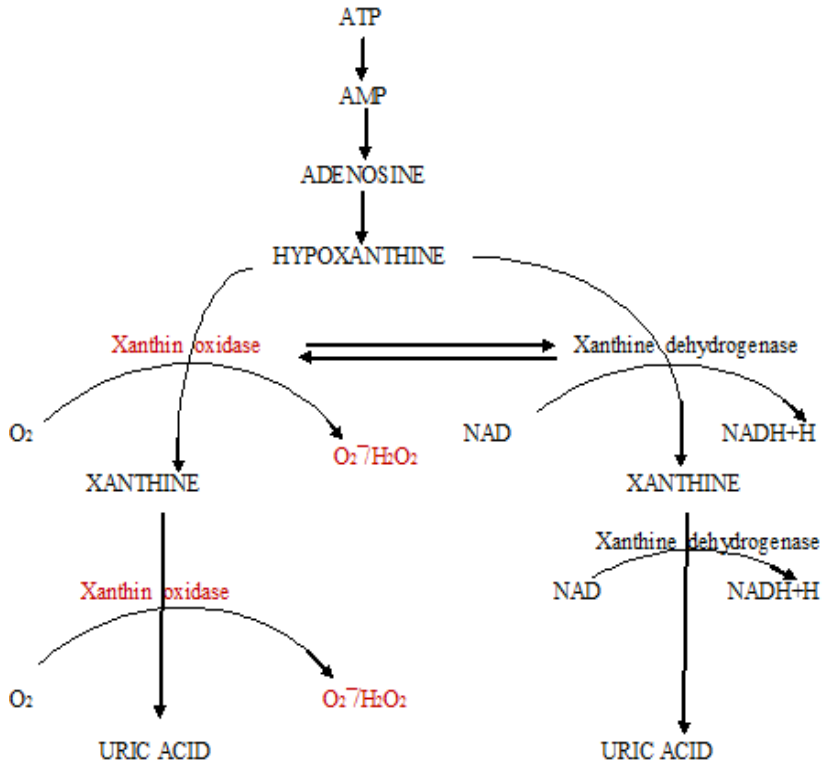


Fig 1.6 Part of the methabolic pathway of purine nucleotide degradation.

Recently it has been proposed that NOX (NOX2) ROS production can participate in oxidative modification of xanthine reductase leading to the formation of xanthine oxidase and thereby promoting further ROS production.

4. Endothelial **NOS** (eNOS) is a cytocrome P450 reductase-like enzyme that catalyses flavin-mediated electron transport from the electron donor NADPH to a prosthetic heme group. This enzyme requires tetrahydrobiopterin (BH-4) bound near this heme group to transfer electrons to guanidine nitrogen of L-arginine to form nitric oxide (NO).
Cardiomyocytes express both neuronal NOS (nNOS) and endothelial NOS (eNOS) which catalyze the production of nitric oxide (NO) from L-arginine. When deprived of their critical cofactor tetrahydrobiopterin or their substrate L-arginine, this will rather produce ROS instead of NO. This is referred as NOS uncoupling [50].

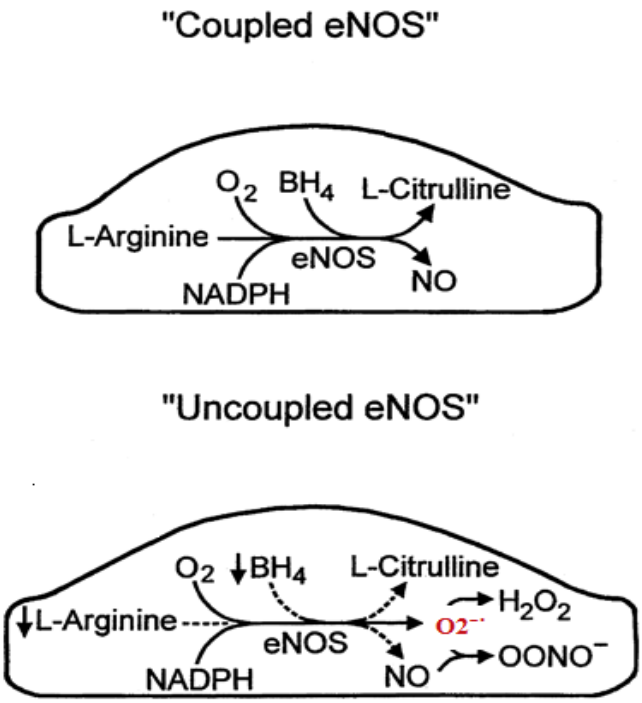


Fig 1.7 "Uncoupling" of nitric oxide (NO) synthesis (Modified after Zvonimir S. Am J Physiol Heart Circ Physiol. 2001)

While low levels of oxygen radicals are constantly produce in cells and play important roles in cell signaling, cellular homeostasis, differentiation and apoptosis, high levels of ROS are harmful and play crucial roles in the development or contribution of various disease.

1.3.3 Defense against ROS toxicity

Because utilization of O_2 can lead to the formation of toxic compounds, organisms have developed antioxidant defenses in order to detoxify ROS.

Our defense against oxygen toxicity is: antioxidant defense enzymes, dietary and endogenous antioxidants, cellular compartmentation and repair of damaged cellular compounds.

The antioxidant enzymes react with ROS to convert them into nontoxic compounds.

Dietary antioxidants such as vitamin E and flavonoids and endogenous antioxidants like urate can terminate free radical chain reactions. Defense trough compartmentation means that the site where usually ROS are produced is separated from the rest of the cell. For example many of the enzymes that produce hydrogen peroxide are in the peroxisomes with a high content of antioxidant enzymes.

1.3.4. ROS and cardiovascular disease

1.3.4.1 Cardiac hypertrophy

Cardiac hypertrophy can broadly be divided into pathological or physiological hypertrophy. The heart responds to chronic pressure or volume overload by a significant increase in cardiomyocytes size which contributes to increased chamber mass and wall thickness. Studies have demonstrated that physiological and pathological hypertrophies have distinct structural and molecular bases.

Although the stimuli and signaling pathways involved in the induction of pathological and physiological cardiac hypertrophy are different [51] these pathways may sometimes overlap. The physiological stimuli like exercise and pregnancy lead to the production of IGF I [52] which will activate the IGF1-PI3K (p110α)-Akt pathway. In contrast pathological stimuli will rather lead to increased levels of Ang II, catecholamines and ET-1 which in turn will stimulate PI3K(p110γ), mitogen activated protein kinases (MAPKs), protein kinase C and calcineurin pathways.

The structural and molecular profile of physiological and pathological cardiac hypertrophy is also distinct [51]. Whereas physiological hypertrophy is characterized by normal organization of cardiac structure and normal or enhanced cardiac function, pathological hypertrophy is associated with fibrosis, cell death and cardiac dysfunction.

Studies have shown that ROS can modulate many signaling pathways known to be involved in cardiomyocyte hypertrophy, such as ERK1/2, JNK, p38MAPK, Akt, PKCs, and NF-κB [53]. The main source of ROS production in cardiac hypertrophy seems to be NADPH oxidases [54]. The role of various different NOX isoforms in cardiac hypertrophy depends on the stimulus. NOX2 seems to be involved in Angiotensin II-dependent cardiac hypertrophy [55]. On the other hand pressure overload-induced cardiac hypertrophy does not require NOX2, but might possibly involve NOX4 [56].

Another source of ROS in cardiac hypertrophy seems to be the uncoupled nitric oxide synthase [57]. A recent study proposed that the first production site of ROS in Ang II mediated hypertrophy is NADPH oxidase, but these ROS will increase mitochondrial ROS production, in an amplifying manner [58].

1.3.4.2 Ischemia – reperfusion injury

Lethal reperfusion injury of the myocardium seems to be a complex phenomenon involving several independent factors such as: intracellular Ca_2+ overload, inflammation, mitochondrial damage, altered NO production with an altered vascular reactivity and the formation of oxygen radicals. It seems that the free radical formation theory is of special importance because ROS can be a potential trigger for most of the other events that take place during reperfusion [59].

A number of mechanisms have been proposed to cause oxygen radical generation in reperfused myocardium. These include: the enzyme xanthine oxidase, mitochondrial oxidation, activation of NADPH oxidase, uncoupled NO synthase, cyclooxygenase and lipoxygenase.

It has long been demonstrated that an important source of ROS production in reperfusion is the enzyme xanthine oxidase.

As explained previously XOR has to isoenzymes, xanthine oxidase (XO) and xanthine dehydrogenase (XDH). In healthy tissue XDH is the predominant form. XDH uses NAD as the electron acceptor for the oxidation of hypoxantine to xanthine. Studied showed that in the ischemic tissue the XO is the predominant form [60]. This may be as a result of increased cytosolic calcium which will activate Ca_2+ - dependent proteases which convert the dehydrogenase form to oxidase form by proteolysis. XO reacts with molecular oxygen and forms superoxide and hydrogen peroxide.

It seems that during ischemia the substrates, hypoxantine and xanthine accumulate because of an increased ATP degradation. This in addition to the high concentration of XO and the return of oxygen during reperfusion will lead to the formation of superoxide and hydrogen peroxide. Despite numerous studies that support this theory, there have been also negative reports so that the role of XOR in I/R injury remains controversial [61]. Importantly, no treatment attempts have been introduced based on the proposed negative role of xanthine oxidase.

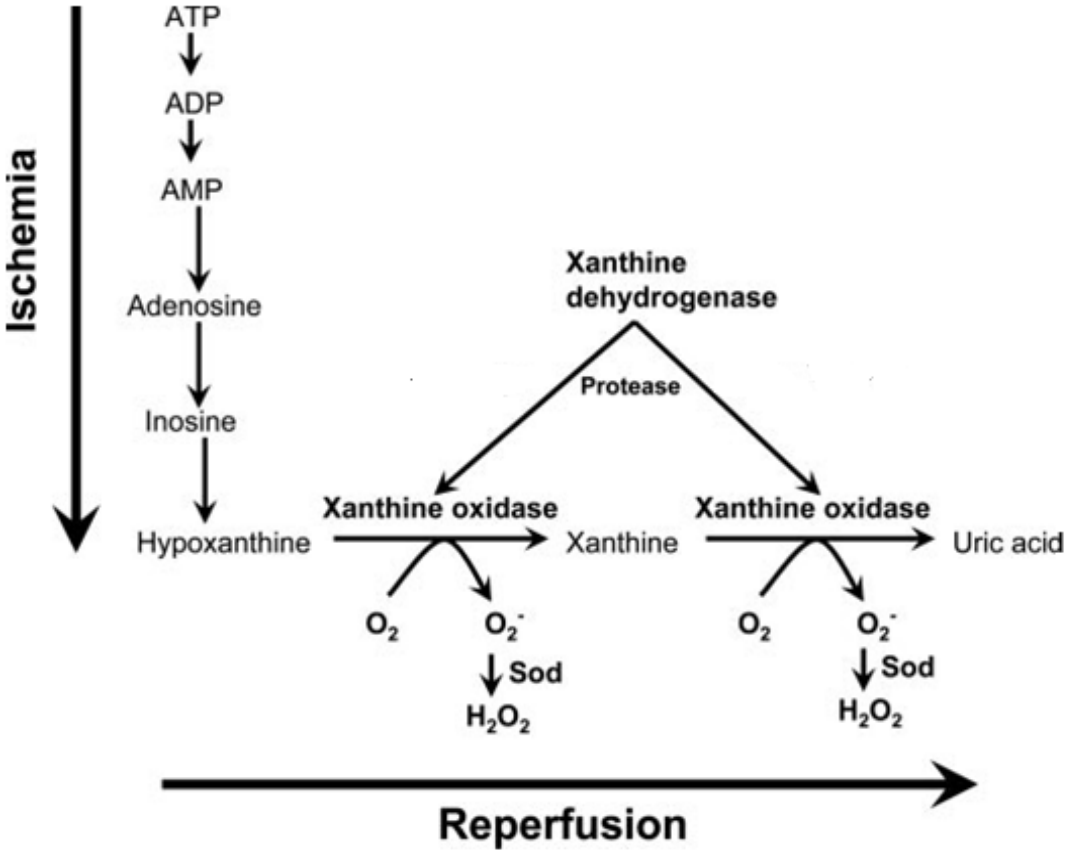


Fig 1.8 Ischemia-reperfusion injury hypothesis (Modified after Pacher et al. Pharmacol Rev. 2006)

Mitochondria seem to be another important source of ROS during reperfusion injury. Although scientists are still debating the exact source of ROS production in the mitochondrial electron transport chain (METC) (se previous paragraphs) and whereas the most ROS production occurs during ischemia, or during reperfusion, there is a general acceptance that ischemia and reperfusion can result in significant mitochondrial dysfunction in electron flow and ROS generation. Since ROS are highly reactive and short lived species, and it is believed that their effect should be greatest in immediate vicinity surrounding their place of production it is possible that mitochondrial membrane constituents, including the complexes of the respiratory chain and phospholipids constituents, could be the major target of ROS attack [62].

In addition to faulty generation of ROS from METC another factor that contributes to mitochondrial dysfunction and further generation of ROS seems to be the mitochondrial permeability transition pore (PTP) opening [62].

PTP is a membrane protein complex located in the inner mitochondrial membrane.
During ischemia a decreased in O_2 supply results in a decreased ATP generation. If the levels of ATP decrease to the level that the Na+/K+-ATPase is affected, the levels of intracellular Na+ will increase, leading to cellular swelling, and increased intracellular Ca_2+ levels. The decrease in ATP and increase in Ca_2+ seems to open the mitochondrial PTP resulting in further inhibition of the oxidative phosphorylation. In addition, long-lasting PTP opening leads to matrix swelling and disruption of the outer mitochondrial membrane. The last, causes release of proapoptotic molecules, leading to cell death via both caspase-dependent and caspase-independent mechanisms [63].

Other enzymatic systems that might play a role in the production of ROS during ischemia-reperfusion injury are NADPH oxidase, uncoupled NOS, COX and lipoxygenase.
The role of NADPH oxidases in IR is still controversial. Although some studies are reporting results that may be in favor of a role of NADPH in IR, other studies found no such function. NOX2 is present in human cardiomyocytes and is upregulated after myocardial infarction [64]. Coronary arteries from patients with coronary heart disease show increased expression of NOX2 and increased ROS generation [65]. A recent study that included patients with chronic granulomatous disease (CGD) showed that in these patients brachial artery endothelial function were preserved after IR, in contrast with healthy individuals [66]. Patients with CGD have mutations in genes coding for NADPH oxidase subunits that cause almost complete disruption in oxidase activity in neutrophils.
On the other hand in mouse models deficient in NOX2 and p47phox there is no decrease in infarct size compared with controls [67].
Uncoupled NO Synthase has also been indicated as a source of ROS in ischemia-reperfusion injury. Uncoupling of eNOS contribute to ROS generation when deficient of L-arginine or BH-4. BH-4 may be oxidized by post-ischemic oxidants and though it`s level decreased [59].
The product of reaction between NO and superoxide generated by uncoupled NOS can oxidize BH-4 which may lead to further eNOS uncoupling.
ROS generated during ischemia-reperfusion mediates signaling cascades that lead to apoptosis. Although cardiomyocytes express defense mechanisms against ROS, this antioxidant defenses are overwhelmed after ischemia and reperfusion. A large number of preclinical studies have

shown that free radical scavengers or antioxidants have favorable effect on reperfusion injury by reducing myocardial infarct size, although the reproducibility of these studies is low. When the end point was myocardial function and stunning of the myocardium, antioxidant have been shown to be protective in a large number of studies. This studies used a short period of total ischemia (10-20 minutes), suggesting that ROS produced in the first minutes of reperfusion are involved in myocardial stunning in a situation not involving cell death [68].
Clinical studies have failed to show improved outcome after administration of various antioxidants in primary or secondary prevention.

2. AIM

1) To investigate the effects of H2S in an acute ischemia-reperfusion model
2) To develop and establish a laboratory technique in order to measure ROS presence in the heart, in different cardiac-disease models
3) To determine whether exogenous administration of H2S in healthy rats and different models of cardiac disease influences the production of ROS

3. MATERIALS AND METHODS

3.1. H_2S ADMINISTRATION IN AN ACUTE ISCHEMIA-REPERFUSION MODEL

To test the effect of H_2S in an acute heart disease model, we used the Langendorff retrograde perfusion technique where hearts were perfused with Krebs-Henseleits perfusion buffer (KHB) subjected to 30 minutes of ischemia and reperfused with or without H_2S added to the Krebs-Henseleits perfusion buffer (Fig.3). Sodium hydrosulfide (NaHS) was purchased from Sigma Chemical. The end point of this substudy was infarct size.

Animals

Female Wistar rats weighing 185–210 g were used. Rats were anesthetized and anticoagulated with a mixture of sodium pentobarbital (100 mg/kg) and 300 IU heparin sodium i.p.
The study conforms to the Guidelines on Accommodation and Care of Laboratory Animals (by the European Convention for the protection of vertebrate animals) and was approved by the Norwegian Committee on Ethics in Animal Experimentation.

Perfusion technique

Hearts were rapidly removed from the animal and after a brief stay in ice-cold perfusion medium they were cannulated by the aorta and retrograde perfusion (80 mmHg) in a standard Langendorff retrograde perfusion system was initiated. The perfusion medium (Krebs-Henseleits buffer (KHB)) contained (mM): NaCl 118.5; $NaCO_3$ 24.8; d-Glucose 11; KCl 4.7; $MgSO4 \cdot 7H_2O$ 1.2; KH_2PO4 1.2; $CaCl_2 \cdot 2H_2O$ 2.25 . The perfusate was equilibrated with 95% O_2 and 5% CO_2 (temp. 37°C) to obtain a pH of 7.35-7.40 and was filtered (5 µM Millipore filter) before use. A fluid filled latex balloon connected to a pressure transducer was introduced into the left ventricle (LV) so as to record hemodynamic parameters such as LV diastolic and systolic pressure, LV developed pressure, dP/dt and heart rate. The coronary flow (CF) was measured manually by timed collections of effluate and myocardial temperature was thermostatically controlled by inserting a temperature probe into the right atrium. Before the experimental protocol was initiated, the isolated hearts were allowed to stabilize at 37°C for 25 min.

The perfusion system consisted of two parallel lines (one for the Krebs–Henseleit buffer alone and one for the H_2S containing buffer) and clamps that allowed for changing between the two perfusates according to the experimental protocol. Global no-flow ischemia was induced by clamping the main perfusion line.

Experimental Protocol

After a stabilization period of 25 minutes, hearts were subjected to global no-flow ischemia for 45 minutes followed by reperfusion with KHB or 1 µM H_2S solution (Fig.3). The H_2S solution was delivered from the onset of reperfusion and continuously for a period of 15 minutes.

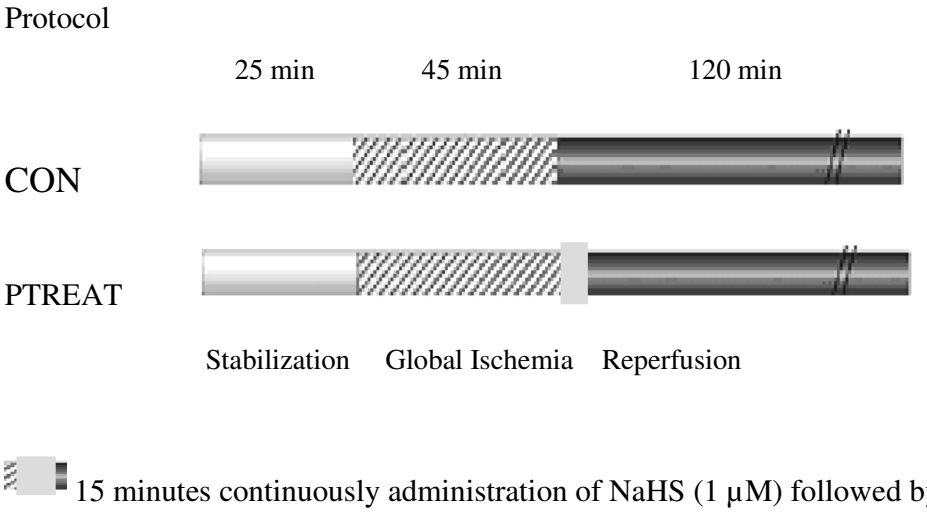


Fig 3.1 Protocol for perfusion

Control group (CON) (n=7), no intervention either before or after global ischemia. Post-treated (Ptreat) (n=11), NaHS (1µM) was given respectively at the onset of reperfusion for 15 minutes followed by reperfusion with K–H buffer for 120 min.

Measurement of infarct size

At the end of the experiments all hearts were weighed and frozen overnight. The following day the hearts were sectioned into 2-mm-thick slices and stained using triphenyltetrazolium chloride (1%) in phosphate buffer (pH 7.4) at 37°C for 15 min. Non-infarcted tissue was stained red while necrotic tissue remained unstained. Slices were then fixed in 4% formaldehyde solution to enhance the contrast between stained viable tissue and unstained necrotic tissue. Slices were then compressed to a uniform 2 mm thickness by placing them between two glass plates separated by a 2 mm spacer. Following this, all hearts were scanned and the pictures further analyzed using ImageJ.

3.2. PILOT SUDY OF DHE STAINING TECHNIQUE AND ROS QUANTIFICATION

To measure the presence of ROS in the heart we tested a method that uses dihydroethidium (DHE), a fluorescent dye to detect superoxide production. Dihydroethidium or hydroethidine is a cell-permeable compound that has been used for the detection and imaging of ROS in several studies [69]. Upon entering the cells DHE interacts with O_2^- to form oxyethidium which in turn interacts with nucleic acids to emit a bright red color detectable by fluorescent microscope.

A pilot study was performed in order to confirm the reproducibility of this method when used in fresh frozen heart samples. The aim of the pilot study was also to establish a protocol suitable for our laboratory in order to use DHE for ROS detection in cardiac tissue harvested from *in vivo* experimental animal studies.

3.2.1. Material and methods

3.2.1.1. Tissue samples

Hearts from female Wistar rats were perfused as previous described, for 45 minutes with Krebs-Henseleit buffer.

3.2.1.2. Freezing of the samples

After 45 minutes of perfusion, the hearts were removed from the Langendorff system, the atriums and right ventricle were removed, and left ventricle was divided into 4 pieces which were frozen trough 'snap freezing' technique in liquid nitrogen.

Material

- Fresh tissue
- Container with liquid nitrogen
- 2-methylbutane (isopentane)
- Metal beaker
- Marking pen
- Aluminum foil
- Base molds
- O.C.T. tissue compound (Tissue-Tech)
- Long forceps

Procedure

A drop of O.C.T. was added into bottom of a mould and the specimen was added into O.C.T. in the mould. The specimen was then completely covered with O.C.T., avoiding air bubbles.

A metal beaker containing isopentane was lowered into the liquid nitrogen until opaque drops appeared in the isopentane and the solution becomes misty indicating that the isopentane was near the freezing point (−160 °C). The metal beaker was pulled out from the liquid nitrogen and the mould containing the tissue was plunged into the isopentane and allowed to freeze until O.C.T. is white. The metal beaker with the mould inside is then lowered again into the liquid nitrogen for 10 second then pulled out.

The mould was taken out with forceps, covered with a marked piece of aluminum foil and stored into liquid nitrogen or at -70 degrees until further use.

3.2.1.3.Microtomy

A Leitz 1720 digital Cryomicrotome was used for sectioning of frozen heart samples and thus there was no need for parafine or plastic embedding of the samples.

On the day of microtomy, the mould containing the tissue is taken from liquid nitrogen with forceps. After 15 seconds, the specimen is pressed out from the back side of the mould and is then placed on a layer of O.C.T. embedding medium on top of a pre-cooled cryostat chuck. More O.C.T. was applied around the tissue to give it extra support if necessary. The chuck was then placed into the metal beaker containing isopentane previously cooled in liquid nitrogen, until the O.C.T. becomes white in color and indicated that it was adequately frozen. Following this, the metal beaker containing the chuck was again immersed into liquid nitrogen for 10 seconds prior to being placed in the interior of the cryostat at –24°C for 30 minutes and then mounted on the microtome.

The specimen was cut in 20 µm sections. The sections were mounted on glass slides (2 sections per glass) and 15 µl of the prepared solution of DHE was topically added on each section and then cover-slipped. The sections were then incubated at 37°C in a humidified chamber for 30 minutes.

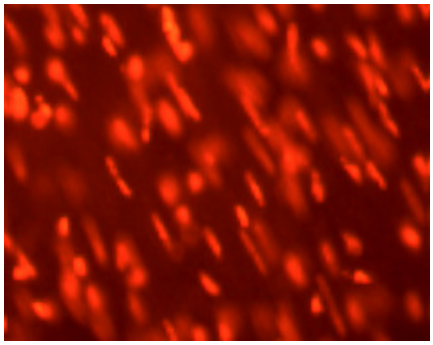
3.2.1.4.Microscopy & Analyzing

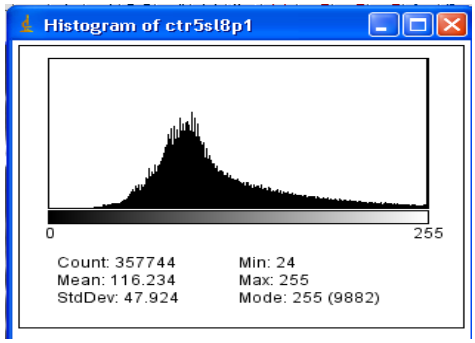
The sections were examined by fluorescence microscopy and images were acquired at 25X magnification using the Leica Application Suite program. Different filters were tested in order to optimize quantification of DHE florescence. Based on this, fluorescence was detected in further experiments using a 585-nm long-pass filter.

In order to have good quality pictures, the setting of the camera must be manually selected via Leica Application Suite program. Different values for each setting (***Exposure, Saturation, Gain, Gamma***) were tested in pilot experiments and the pictures analyzed by ImageJ. The pictures that were taken with different settings were converted into RGB images. The RED channel was used and the Pixel Intensity Histogram analyzed. We were looking for a histogram that would not show over- or under exposure, meaning that the range of pixels will be between 0 and 255 but with a small amount or no pixels gathered at the end values.

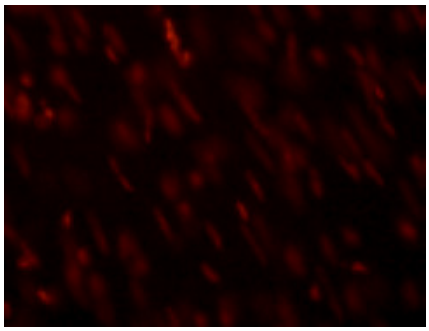
Below are some examples of pictures and their histograms. The pictures are taken from the same slice, with different settings.

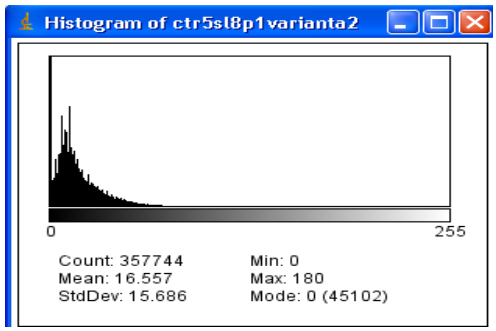
This histogram shows that some of the pixels have high intensity values and might be overexposed:



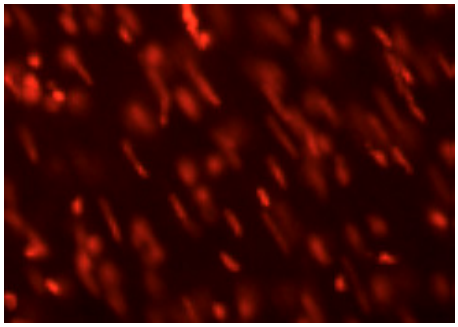


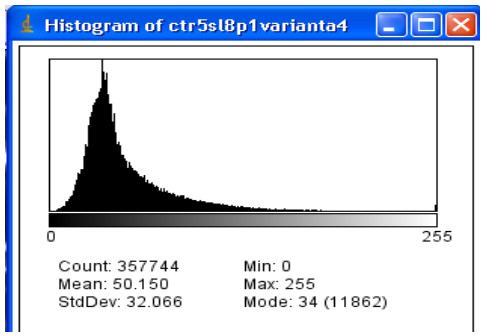
In this histogram lots of the pixels have rather low intensity values and might be underexposed:





In this histogram, the bottom and top half of the intensity values is used by only a few pixels:





As this final histogram did not show over- or underexposure in addition to a good quality of picture, we selected these settings for the camera for the remaining experiments:

Exposure= 675; Saturation= 0.65; Gain= 0.8 ; Gamma= 1.4

All images were analyzed using Image J and average intensity obtained for each image.

3.2.1.5. DHE staining

In order to find the right DHE concentration and to test the stability of the DHE solution, a series of experiments were done.

3.2.1.5.1. DHE concentration

Three different concentrations of DHE in DMSO diluted with PBS were tasted (10, 12.5 and 15 μM) and the minimal concentration (12.5 μM) that was able to color the sample in order to be detectable by fluorescent microscope was used. For each concentration, the solution was prepared in a dark microtube (capacity 1.5ml) and placed in a recipient surrounded by ice. The solution was vortexed for 20 seconds before each use.

3.2.1.5.2. Stability of the DHE solution

In order to check the stability of DHE solution with time, we colored sections coming from the same heart with DHE solution that was prepared and left in a dark vial for 1 to 4 hours (Fig. 3.2).The tissue sections came from the same heart so that any difference observed between the sections would not be due to other external factors such as age, gender, diet etc. The specimen was mounted into the microtome and cut in 20 μm sections. Between 2 and 4 sections were mounted per glass slide with a maximum time difference of 5 minutes between each glass slide. Thus, within 30 minutes 6 glass slides, each containing 2 to 3 sections were colored with DHE solution (in a dark room due to light sensitivity) and incubated in the dark, heated chamber (37°C). After 30 minutes incubation for each glass, the sections were examined in the microscope. Each glass was examined on the microscope for a maximum time period of 5 minutes. This procedure was repeated four times for the same tissue specimen. The experiments were designed so that one person could work alone, performing each step in sequence.

Results

There was a decrease in fluorescence intensity after 60 minutes which was followed by more stable values after 180 min. However, as the heart specimen was left in the microtome (at -20 degrees) during this experiment (respectively 4 hours), we could not be sure that the decrease in fluorescence intensity is due to the DHE instability or due a 'loss of ROS' from the tissue. Therefore ROS 'stability' at -20 degrees was tasted in a later set of experiments.

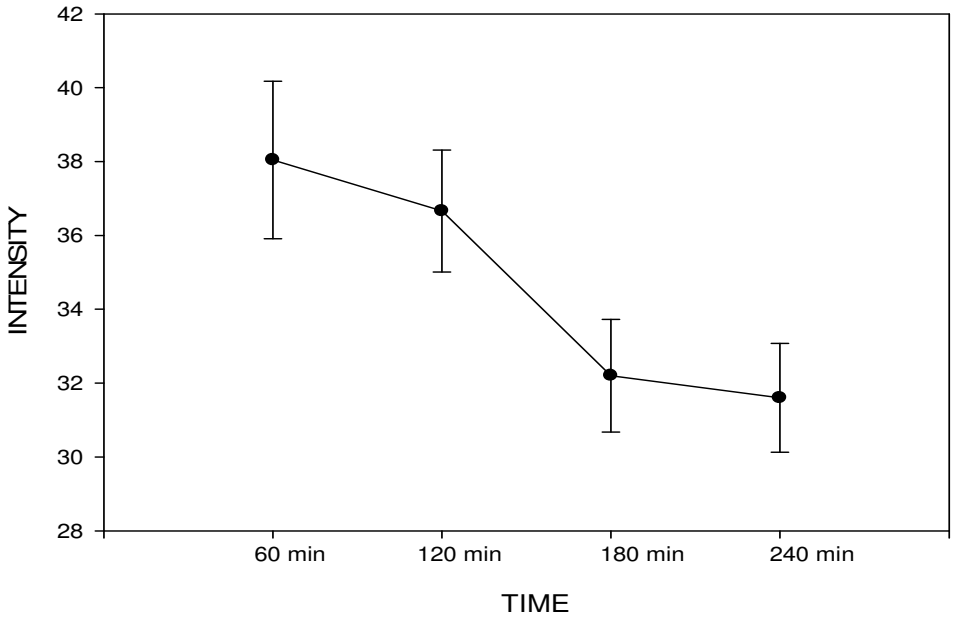


Figure 3.2. DHE fluorescence intensity of sections from the same specimen when the DHE solution was used at minute 60, 120, 180 and 240 after preparation of the DHE solution.

As future experiments to be included in the present thesis regarding H_2S would require comparison of the DHE intensity between different groups, we proceeded to validate experiment comparing 4 different specimens at a time. The tissue sections came from the same heart so that any difference observed between the sections would not be due to other external factors such as age, gender, diet etc.

Four different specimens (A,B,C,D) coming from the same heart were mounted on 4 different cryostat chucks and left in the microtome at -20 degrees for 20 minutes before they were first sliced. Each of the 4 specimens was sliced with a 7 minute time difference between them (a total of 28 minutes for the four of them) prior to the addition of DHE as described above. The procedure was repeated 3 times changing the order of the specimens. The time and order of the 3 cycles is shown in Table 3.1.

Time (min)	0	75	150
Time point	1	2	3
Order	A, B, C, D	A, B, D, C	C, B, A, D

Table 3.1. Time and order of the experiments. The 4 specimens coming from the same heart are named with alphabetical letters from A to D. Time denotes time from when DHE was prepared.

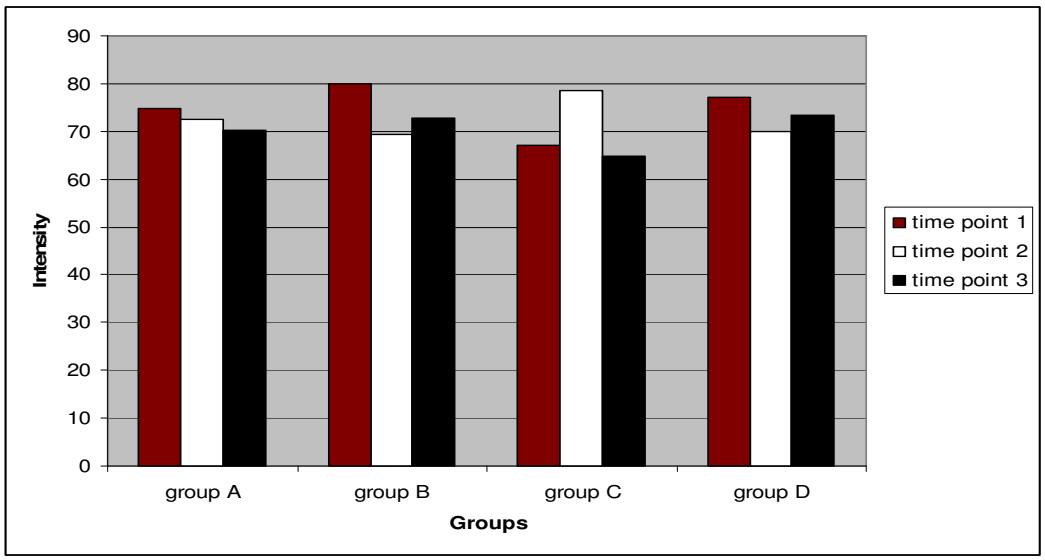


Figure 3.3. DHE fluorescence intensity in the four samples from the same heart measured at different time points.

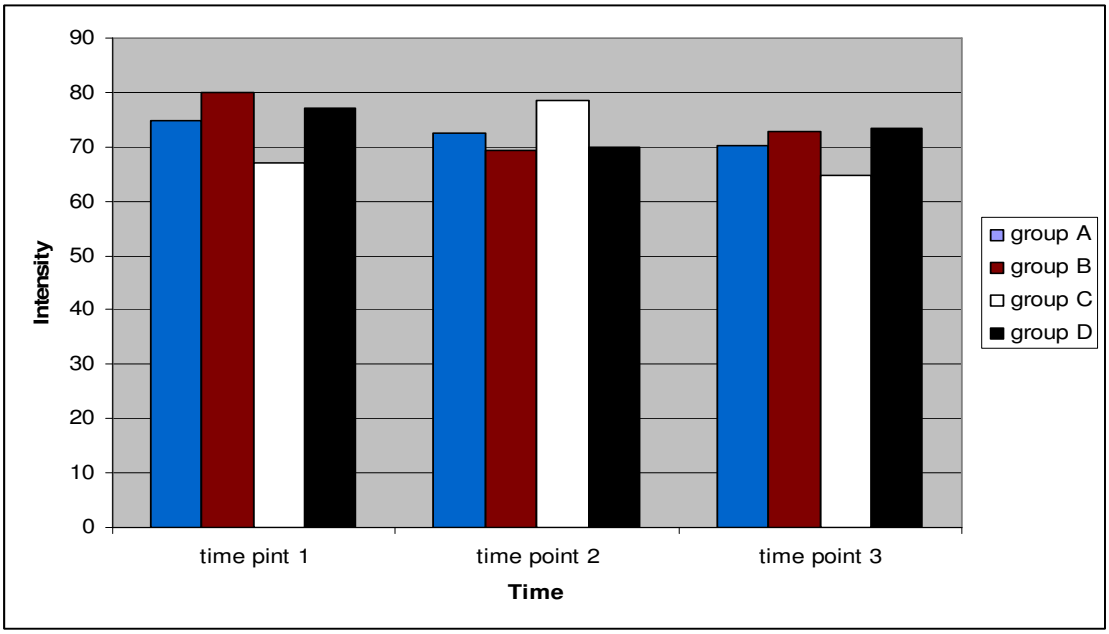


Figure 3.4. DHE fluorescence intensity presented as a function of different time points in the four groups. Data are the same as the figure 3.3. Each bar (group) represents the average result of 5 slices mounted on the same glass

Results

The specimens were compared using Two-Way ANOVA test. No statistical difference between the intensity of the DHE in the specimens at the same time point or at different time points were found (Fig 3.3 and 3.4). These results suggest that we can compare different groups (coming from different hearts) at different time points (no longer that 150 minutes after preparation of DHE) and the changes in fluorescence intensity wont be due DHE instability but rather to a different ROS production in the tissue (if that would be the case).

3.2.1.5.3. ROS stability at -20°C

Based on the first set of experiments we were unable to conclude whether the decrease in fluorescence intensity was due DHE instability or that ROS levels in the tissue decreased with time. In order to validate the stability of ROS in the samples over time, we performed a new set of experiences. Slices from one specimen were colored with DHE and analyzed using fluorescent microscope (day I). The specimen was then left in the microtome for 24 hours and a new set of slices were taken, colored and analyzed (day II). The new set of slices (day II) ware then colored with two types of DHE, one prepared the same day (solution II) and another prepared a day before (solution I).

Results

There was a statistically significant difference (T-test, $p<0.05$) in the fluorescence intensity between the specimens taken the first day and the ones taken after 24 hours, in both the specimens stained with newly prepared DHE solution or the one that was prepared the day before (Fig. 3.5). This demonstrates not only the loss of stability of the DHE solution with time but also a decreased presence of ROS with time.

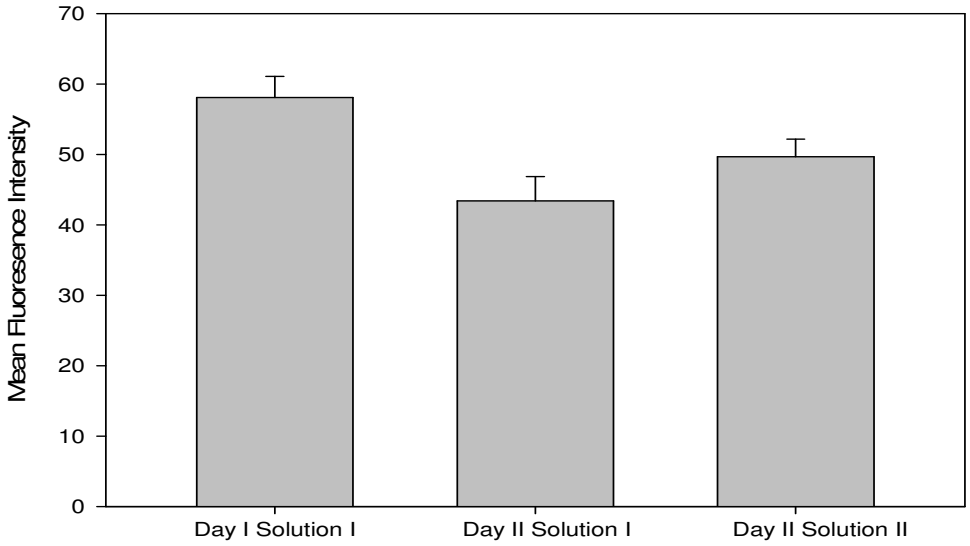


Figure 3.5. Comparison of DHE fluorescence intensity of specimens left in the microtome for 24 hours and colored with different solutions of DHE.

Conclusions

We concluded that DHE is not stable with time so when possible, groups that will be compared should be tasted at the same time point using the same DHE solution.

Because DHE is light sensitive, the solution (12.5 µM) should be prepared and added on the tissue slices in a dark room. In our future experiments DHE solution was prepared 1 hour before each of the experiments and left in a dark tube surrounded by ice. The heart specimens were cut in 15µm or 20 µm sections and mounted on glass slides (2 to 5 sections per glass) and 15 µl of the prepared solution of DHE was topically added on each section and then cover-slipped. The sections were then incubated at 37°C in a humidified chamber for 30 minutes. Because sometimes the group that is colored first can have increased fluorescence intensity, every time we did a new experiment we changed the order of coloring the groups. The sections were examined by fluorescence microscopy and images were acquired at 25X magnification using the Leica Application Suite program. We took approximately 25-30 pictures per heart. The pictures were then analyzed by ImageJ.

3.3. ROS MEASUREMENTS IN TWO MODELS OF CARDIAC DISEASE

To test the feasibility of the DHE staining in groups where ROS production was believed to be different, we measured ROS presence from heart samples taken from animals with different models of heart disease where an increased ROS generation has demonstrated to contribute to the patho-physiological development of these diseases.

3.3.1. ROS measurements in hearts from diet-induced obese mice

Heart samples were examined from diet-induced obese mice that were or were not subjected to high intensity interval training (HIT) or moderate intensity interval training (MIT) for 8 weeks following the administration of a specialized diet. These samples were compared to a control group that received standard chow for the entire period.

3.3.2. ROS measurements in hearts from pregnant rats subjected to transthoracic aorta banding

In these experiments 4 different groups were tasted:

Tac (n=8) - Rats that were subjected to transthoracic aortic banding

Sham (n=8) - Rats that underwent surgery without binding of the aorta

Pregnant Tac (n=8) - Pregnant rats that were subjected to transthoracic aortic banding

Pregnant Sham (n=8) - Pregnant rats that underwent surgery without binding of the aorta

3.4. SHORT TERM ADMINISTRATION OF H2S IN LANGENDORF PERFUSED HEARTS

Animals

Female Wistar rats (n=15) weighing 185–210 g were were anesthetized with a mixture of sodium pentobarbital (100 mg/kg) and 300 IU heparin sodium i.p. and the hearts were rapidly excised and mounted in the Langendorff perfusion system. After a stabilization period of 15 minutes, the hearts were perfused for 30 minutes with one of the following solution: KHB, KHB-AgII, KHB-H2S, where KHB denotes Krebs-Henseleits buffer.

The experimental protocol is illustrated in Figure 3.6.

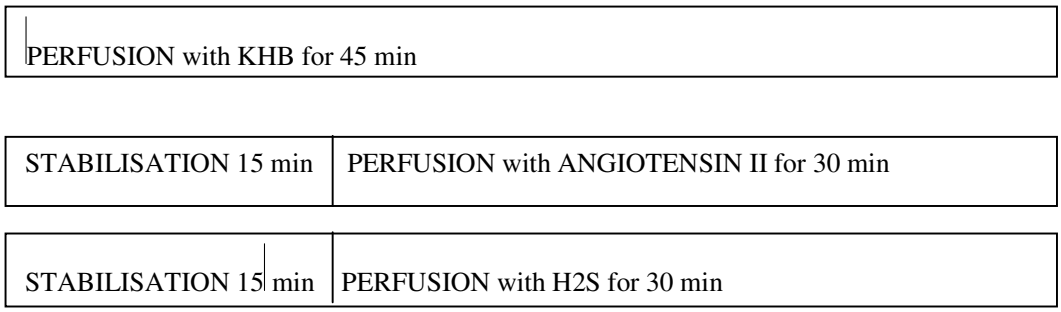


Figure 3.6. Experimental protocol
Control group (n=5): Hearts were perfused throughout with normal KHB
H2S group (n=5): Hearts were perfused 15 min with KHB for stabilization and then with H2S (1µM)
Ag II group (n=5): Hearts were perfused 15 min with KHB for stabilization and then with Angiotensin II (0,1 µM)

3.5. LONG TERM ADMINISTRATION OF H_2S TO HEALTHY RATS

In order to investigate the effects of long term administration of H_2S in healthy rats, sodium hydrosulfide (a H_2S donor) at a concentration of 50µM and 500 µM was added in the drinking water for 7 days. The end point of these experiments ware ROS production measured in the cardiac tissue.

Animals

The study conforms to the Guidelines on Accommodation and Care of Laboratory Animals (by the European Convention for the protection of vertebrate animals), and was approved by the Norwegian Committee on Ethics in Animal Experimentation.
Female Wistar rats (Charles River, Germany)(n=18) were acclimatized for 5 days in housing conditions, maintained at a 21°C temperature in air filtered and light controlled (12 hours light cycle) room. The rats were feed standard commercial pellets and water ad libitum.

Rats were divided into three groups:

Control (n=6): rats that received normal drinking water.

Low dose H_2S (n=6): rats that received H_2S in drinking water at a concentration of 50 µM.

High dose H_2S (n=6): rats that received H_2S in drinking water at a concentration of 500 µM.

3.6. LONG TERM ADMINISTRATION OF H2S IN AN EXPERIMENTAL MODEL OF CARDIAC HYPERTROPHY

In order to investigate the effects of long term administration of H_2S in a chronic cardiac disease model, we used rats that were subjected to transthoracic aortic banding and developed cardiac hypertrophy. These rats received H_2S in drinking water for 14 days (500 µM). Following this, echocardiography was performed and blood pressure was measured.

The end point of this experiment was: blood pressure, heart weight and ROS production in the cardiac tissue as measured by DHE staining.

Animals

The study conforms to the Guidelines on Accommodation and Care of Laboratory Animals (by the European Convention for the protection of vertebrate animals), and was approved by the Norwegian Committee on Ethics in Animal Experimentation.
Female Wistar rats (n=24) that weighed between 193 – 273 grams (at surgery) were randomized to either banding of the ascending aorta or sham surgery. After surgery rats were housed in separate cages and received H_2S (500µM) in their drinking water for 14 days. The rats were divided into 4 groups:

Tac (n=6): rats that were subjected to transthoracic aortic banding

Sham (n=6): rats that underwent surgery without transthoracic aortic banding

H_2S Sham (n=6): rats that underwent surgery without transthoracic aortic banding and received Sodium hydrosulfide in drinking water

H_2S Tac (n=6): rats that were subjected to transthoracic aortic banding and received Sodium hydrosulfide in drinking water

3.7. MEASURMENT OF HYDROGEN SULPHIDE IN PERFUSION BUFFER AND DRINKING WATER

In order to measure the final concentration of H2S in the buffer we used a modification of the method described by W-J Cai et al [69]. Briefly 1.0 ml of buffer added to 0.125 ml 1% zinc acetate in tightly sealed Eppendorff tubes. The resulting precipitate was dried under 2 millibar of vacuum using a Speed Vac SC-210A concentrator (Thermo Scientific) followed by addition of 0.375 ml water. Then 0.067 ml 20mM N,N-dimethyl-phenylenediamine dihydrochloride in 7.2 M HCl was added. This was followed by addition of 0.067 ml 30mM FeCl3 in 1.2mM HCl. After 30 min 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)

STATISTICS

Results are given as mean ± SEM if not otherwise stated. Sigmaplot statistical software was used to analyze the data.

4. RESULTS

4.1. H_2S ADMINISTRATION IN AN ACUTE ISCHEMIA-REPERFUSION MODEL

LVDP, left ventricular developed pressure (mmHg); HR, heart rate (beats/min); CF, coronary flow (ml/min) were measured during the perfusion with H_2S;

Exclusion criteria

Hearts were excluded from further study if they failed to produce a left ventricular systolic pressure greater than 60 mmHg during stabilization, or they had a coronary flow rate outside the range of 6-20 ml at the end of the stabilization period. Hearts that were not successfully mounted and perfused within the first 5 minutes following removal were also excluded.

Coronary flow

There were no statistically significant differences in the coronary flow between the two groups.

TIME (min)	CORONARY FLOW (ml/min)	
	CTR	H2S
15	15,0± 1,8	15,1± 0,7
20	14,7± 1,8	14,8± 0,7
25	14,7± 1,8	14,7± 0,7
75	10,7± 1,9	10,0± 0,7
80	11,3± 1,8	10,5± 0,8
85	11,3± 1,6	10,7± 0,7
90	11,8± 1,7	10,4± 0,8
95	11,6± 1,9	10,5± 0,8
100	11,3± 1,9	10,2± 0,8
130	10,0± 1,6	9,1± 0,7
160	8,3 ±1,5	7,9± 0,6
190	7,2 ±1,3	7,0± 0,5

Table 4.1. Coronary flow (ml/min, mean±SEM)

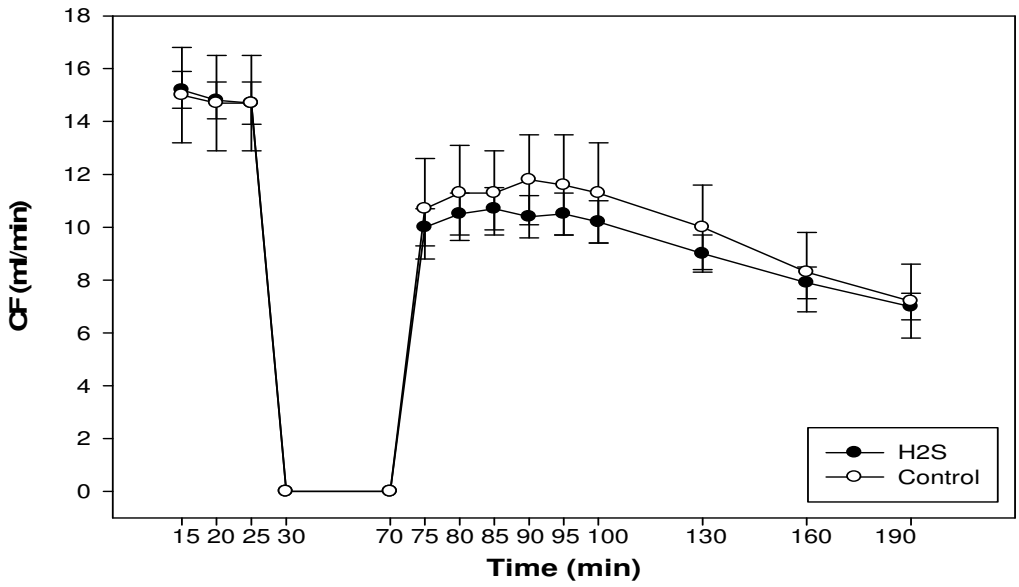


Fig. 4.8. Coronary flow at baseline, during ischemia, and reperfusion.

Heart rate

Time (min)	Heart rate (beats/min, mean ± SEM)	
	Control	H2S
15	228,3± 14,1	265,5± 15,4
20	238,3± 16,5	262,1± 14,0
25	240,9± 21,2	266,6± 13,9
75	172,2± 16,7	129,9± 19,9
80	144,5± 39,2	136,0± 26,0
85	182,7± 39,9	196,4± 25,9
90	188,7± 35,3	217,1± 16,9
95	201,1± 21,3	239,7± 16,3
100	213,4± 46,5	238,0± 17,4
130	211,5± 28,7	223,7± 18,7
160	185,1± 26,9	228,6± 19,0
190	168,5± 32,9	227,8± 15,5

Table 4.2. Heart rate at baseline (mean±SEM)

No statistically significant differences were found in baseline heart rate values (t-test)

There was no statistical significant difference between the groups upon reperfusion. The percent recovery was comparable between groups (76% for H_2S and 82% for controls).

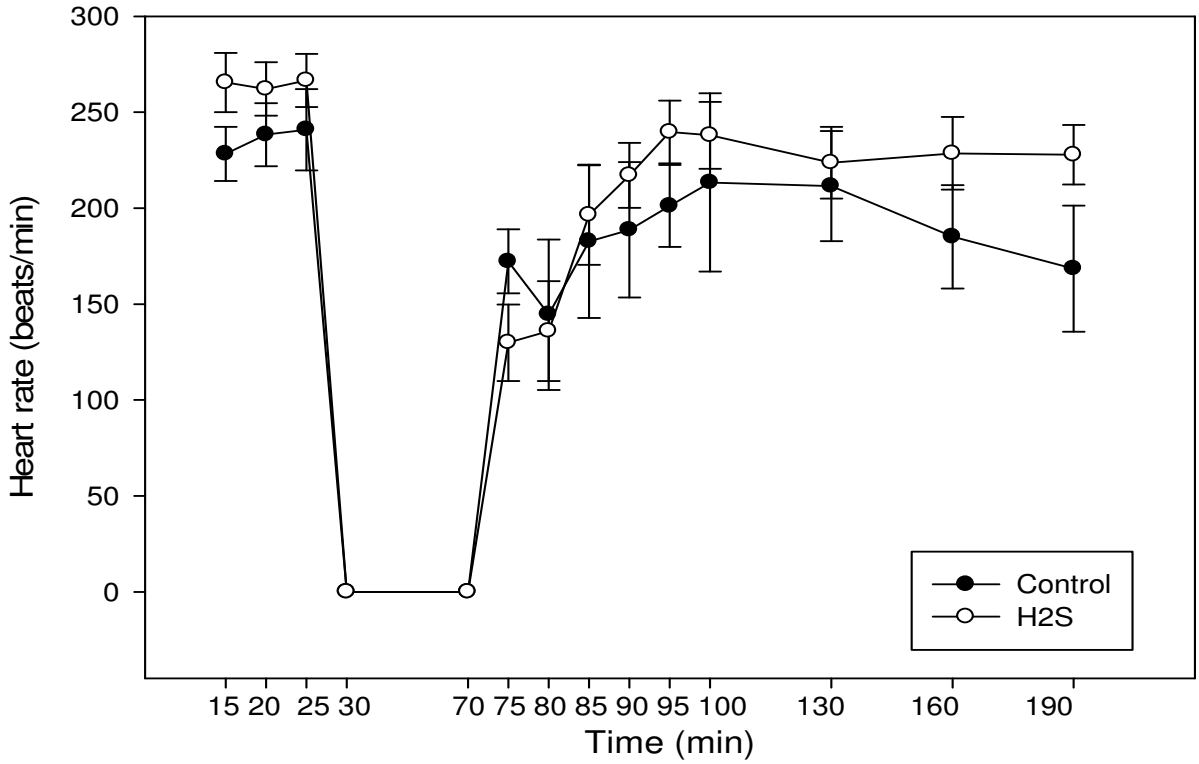


Fig. 4.9. Heart rate at baseline, during ischemia and reperfusion.

LV Function

Left ventricular systolic pressure

There was no statistical significant difference between the groups, at baseline or during reperfusion.

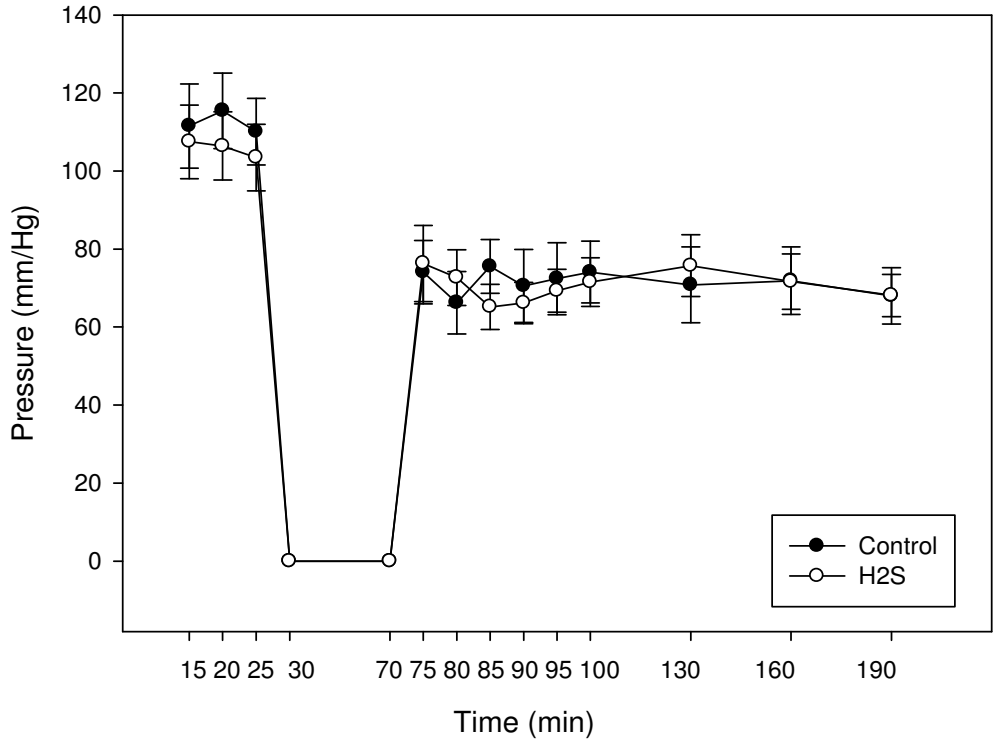


Figure 4.10. Left ventricle systolic pressure

Left ventricular developed pressure

The baseline values of LVDP were comparable between groups. LVDP was reduced during regional ischemia in both groups. At the end of reperfusion, was no statistical significant difference between the groups (t-test).

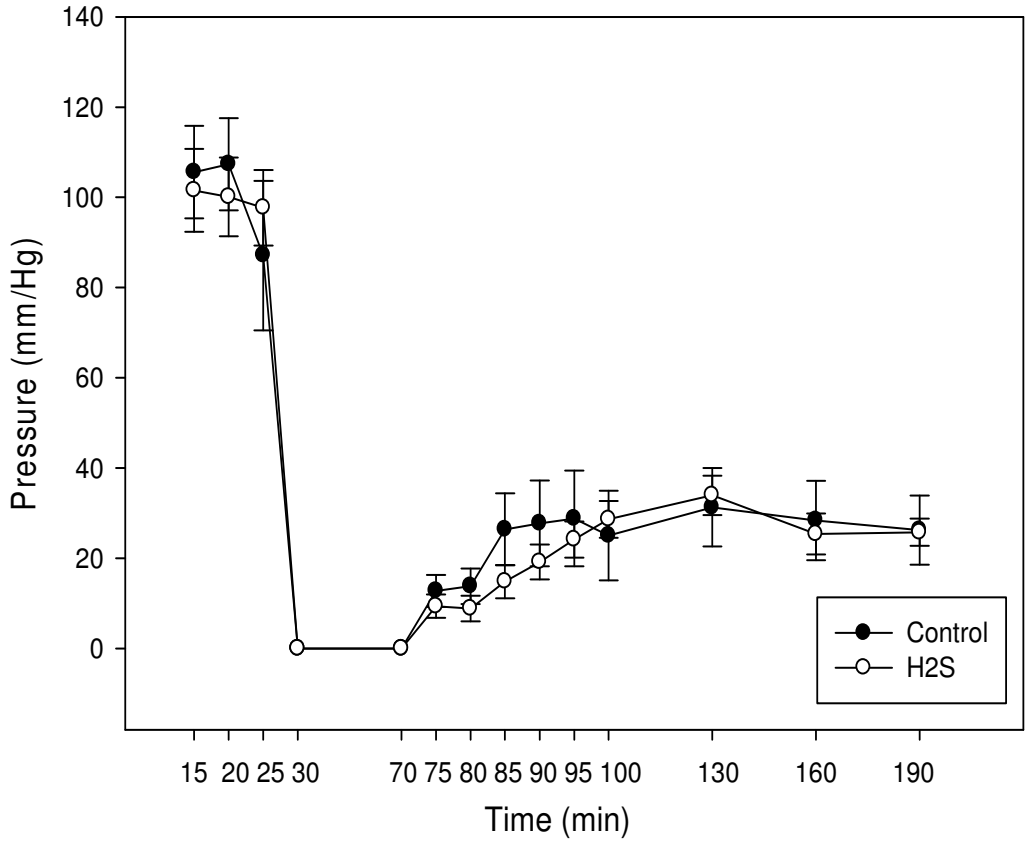


Figure 4.11. Left ventricle developed pressure.

Left ventricular diastolic pressure

There ware no statistical difference in left ventricle diastolic pressure between groups, neither at baseline nor during reperfusion. The LV diastolic pressure increased rapidly during ischemia however, no functional recovery was observed in neither of the two groups during reperfusion.

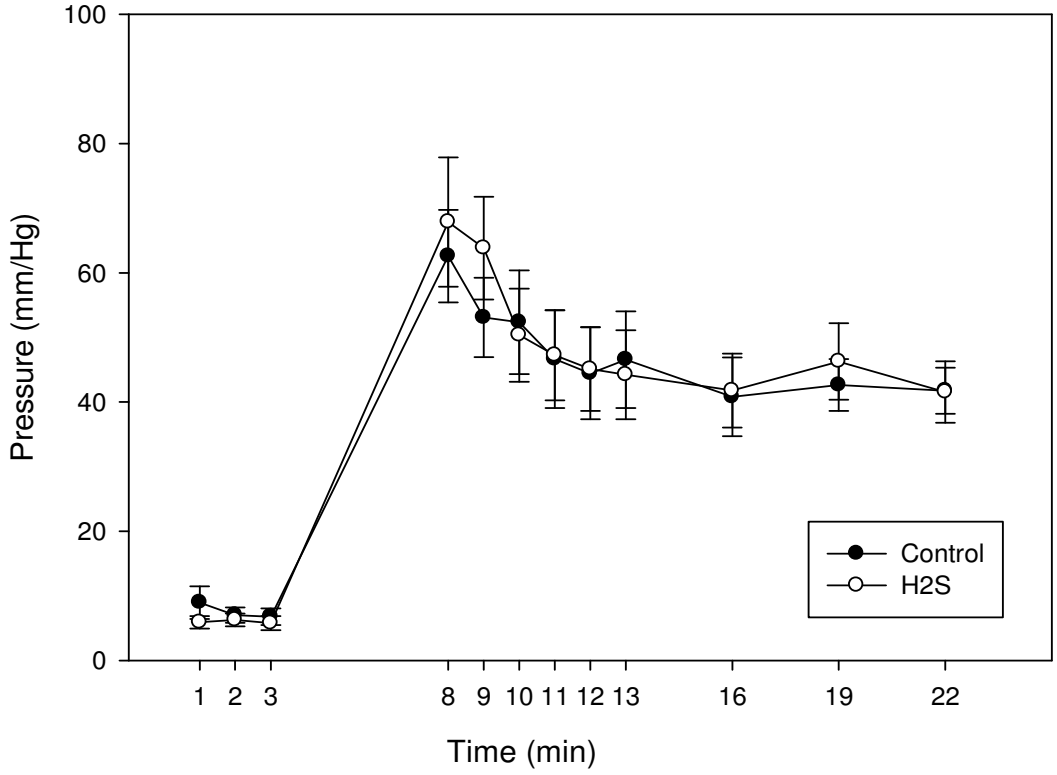


Figure 4.12. Left ventricular diastolic pressure.

Infarct size

Infarct size was 25.09 ±6.29 and 26.81± 6.09 in untreated hearts (controls) and H_2S treated hearts respectively. There were no statistically significant differences in the infarct size between the groups. Infarct size expressed as percent of the total ventricle volume is presented in Fig. 4.7:

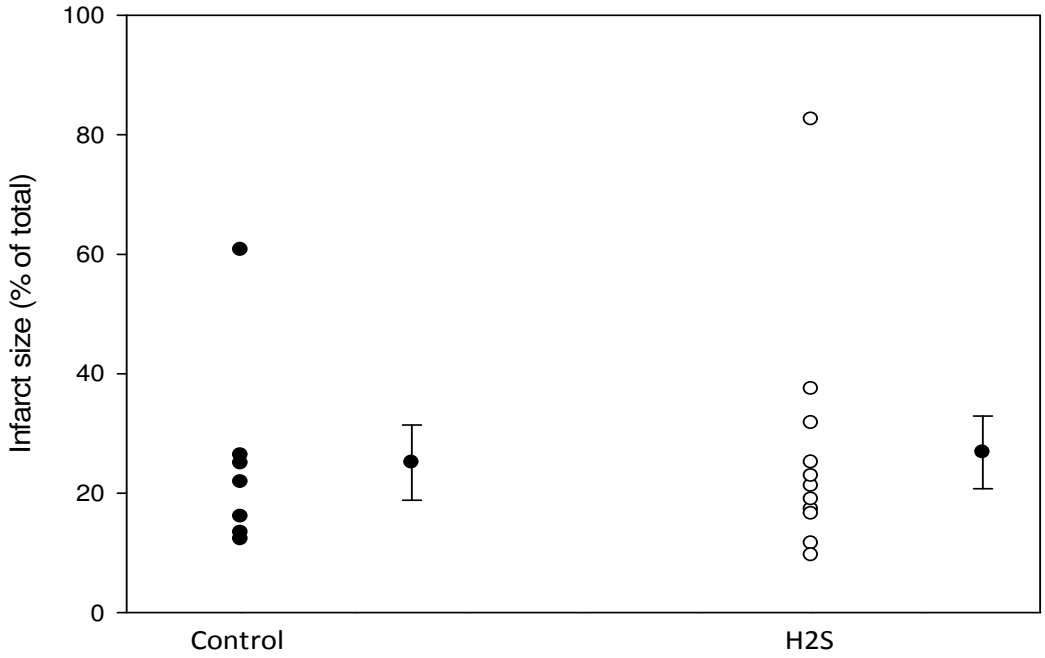
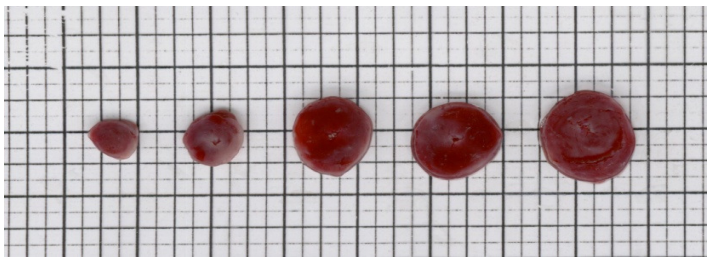


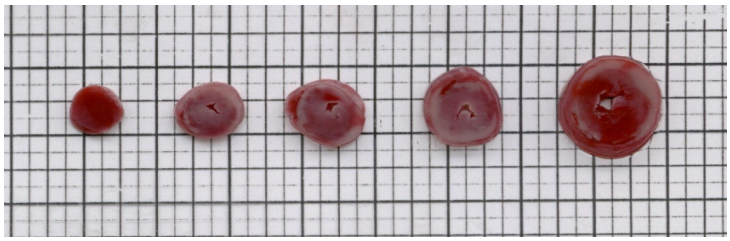
Fig. 4.7 Infarct size in control (n=7) and post-treated group (n=11). Infarct size is expressed as percent of the total volume. Each circle represent one heart, and filled circles with error bars represent mean of groups ± SEM.

Photograph of heart slices. Infarct areas are visualized by tetrazolium staining.

Control



H_2S



4.2. ROS MEASURMENTS IN TWO MODELS OF CARDIAC DISEASE

4.2.1. ROS measurement in the hearts from diet-induced obese mice

There was a significant increase in the presence of ROS between the control group and the diet induced obese mice (one way Anova with Holm-Sidak, P = 0.016).

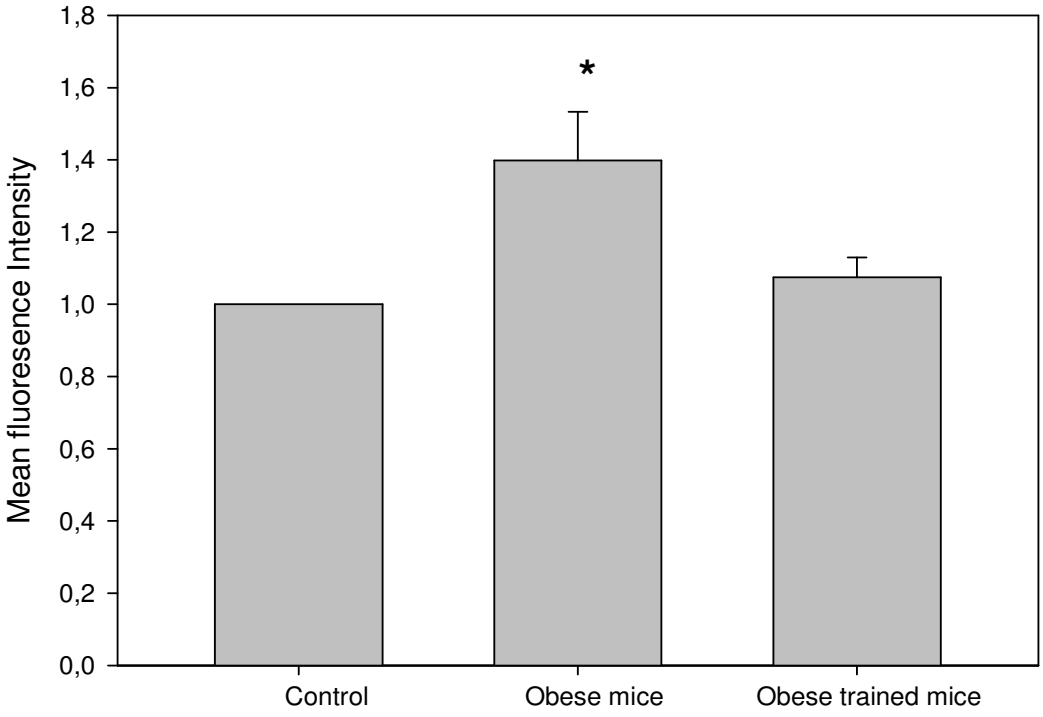


Fig. 4.13 ROS production measured by dihydroethidium (DHE) staining. Results are means±SEM. (n=6 hearts per group)

4.2.2. ROS measurements in the hearts from pregnant rats that were subjected to transthoracic aorta banding

No significant statistical difference ware found between the four groups (one way ANOVA with post Kruskal-Wallis test). The mean fluorescence intensities normalized to sham were 1.005± 0.03 in Tac group, 1.078± 0.09 in Pregnant Sham group and 1.09± 0.044 in Pregnant Tac

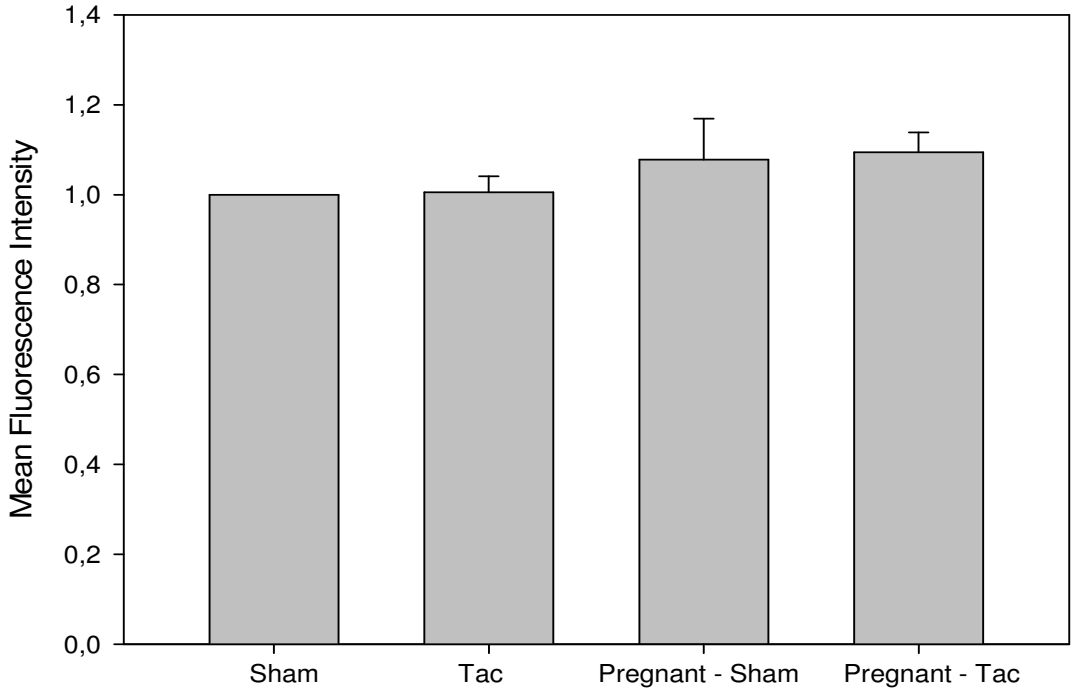
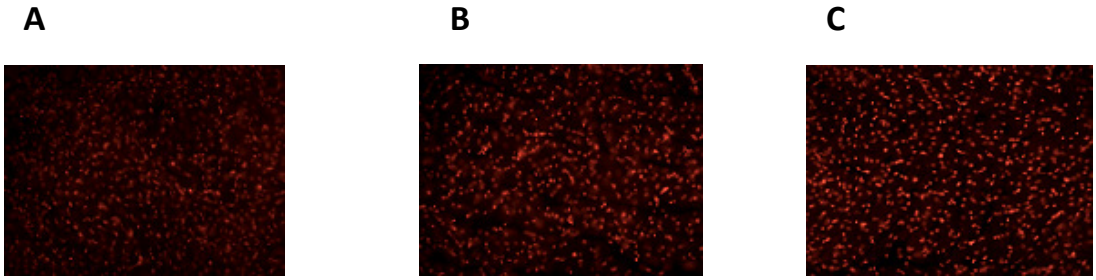


Fig. 4.14. ROS production measured by dihydroethidium (DHE) staining. Results are means±SEM (n=6 hearts per group)

4.3. SHORT TERM ADMINISTRATION OF H_2S IN LANGENDORF PERFUSED HEARTS

No significant statistical difference in the presence of ROS were found between rats that were perfused with KHB buffer and the ones perfused with H_2S solution. However, ROS levels were increased in samples from rats perfused with Angiotensin II as compared to controls. ($p<0.05$)(One way Anova with Tukey´s post test)



Representative photomicrographs of dihydroethidium staining in frozen sections of the left ventricle from control rats (A) and rats treated with H2S (B) and Ag II (C)

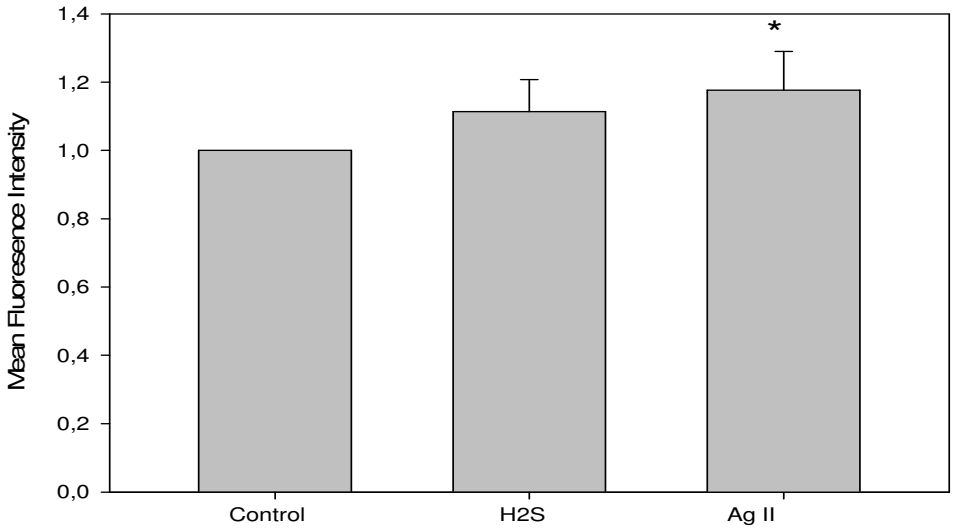
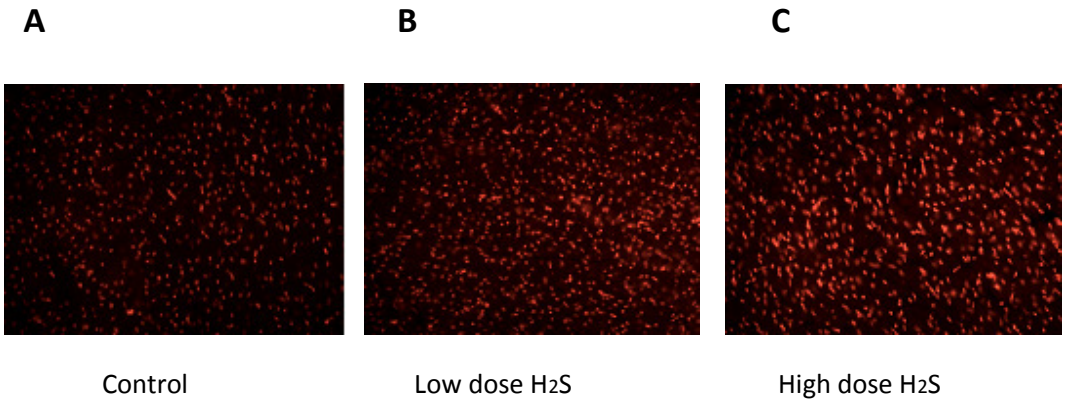


Fig. 4.15. ROS production measured by dihydroethidium (DHE) staining. Results are means±SEM (n=5 hearts per group)

4.4. LONG TERM ADMINISTRATION OF H2S TO HEALTHY HEARTS

After 7 days of H_2S administration, rats were sacrificed and the left ventricle divided into 4 pieces which were frozen as previous described. ROS measurement was performed as described in Materials and Methods (page 36). For each sample an average of 15 pictures were taken and analyzed. ROS levels were increased in cardiac tissue from rats that received H_2S in a concentration of 500μM as compared to controls (One way Anova followed by Holm-Sidak method).



In situ detection of superoxide in the control and H_2S groups. Fluorescence of microscopic sections of cardiac tissue labeled with the oxidative dye dihydroethidium (red fluorescence when oxidized to ethidium bromide by superoxide). (A) Weak superoxide signals were detected in the cardiac tissue of the control rats. (B, C) More intense production of superoxide was observed in the cardiac tissue of the rats that received NaHS.

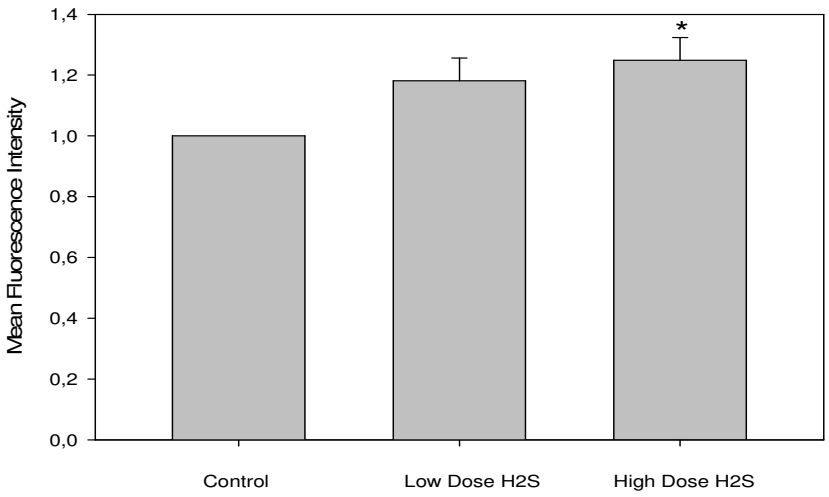


Fig. 4.16 ROS production measured by dihydroethidium (DHE) staining. Values are mean± S.E.M. of six specimens in each group.

4.5. LONG TERM ADMINISTRATION OF H_2S IN A MODEL OF CARDIAC HYPERTROPHY

Rats were assessed postoperatively for hemodynamic functions and hypertrophic responses. Heart weight in TAC animals was significantly higher: 0.876 ± 0.042 g compared to 0.673 ± 0.016 g in Sham hearts. As a consequence of the banding systolic pressure in the left ventricle of the hearts subjected to TAC was 195.0 ±8.0 mmHg compared to 128.2±5.8 mmHg in sham. Heart rate was however comparable, 425.8±10.3 in TAC animals and 407.8±11.9 beats per min in sham animals.

There were no signs of heart failure. No significant changes could be detected in sham heart treated with H_2S compared to sham alone or TAC H_2S treated hearts compared to TAC with respect to these parameters.

After 14 days of H_2S administration, rats were sacrificed and the left ventricle divided into 4 pieces which were frozen as previous described and stored at -70 degrees. ROS measurement was performed as described earlier. For each sample an average of 15 pictures were taken and analyzed. There ware no statistically significant difference between the groups (fig 4.17).

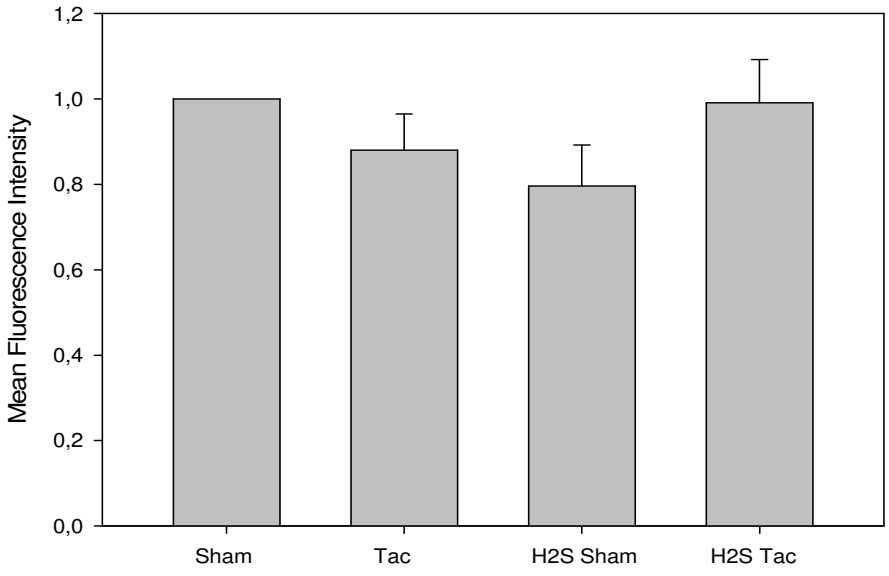


Fig. 4.17 ROS production measured by dihydroethidium (DHE) staining. Values are mean± S.E.M.

4.6. MEASURMENT OF H_2S IN PERFUSION BUFFER AND DRINKING WATER

In order to test the stability of the H_2S a method developed during the course of this thesis work was used. Buffer with H_2S was gassed with 5% CO_2 in oxygen using the same procedures as when performing the perfusion experiments. The concentration of hydrogen sulphide in the buffer decreased from the first minutes of gassing of the perfusate. After 20 minutes of gassing the concentration of the H_2S decreased to a level that was below the detection limit of the test (Fig. 4.1.8)

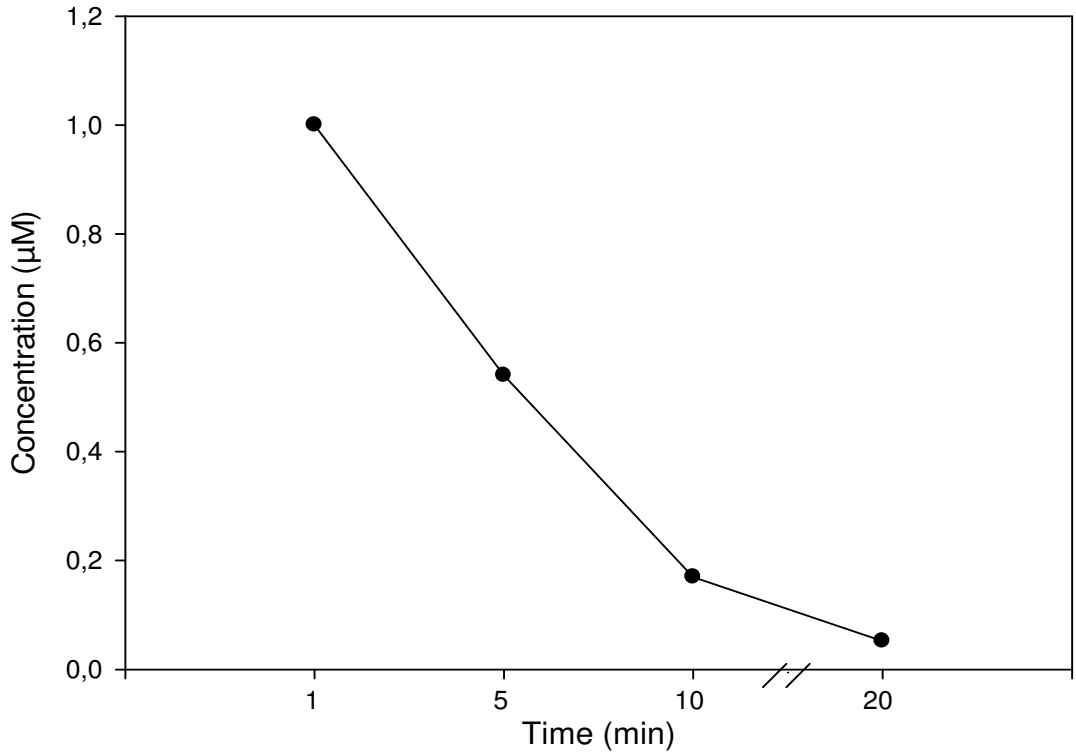


Fig. 4.1.8 Measurement of H_2S in the perfusion buffer. Hydrogen sulphide concentration decreases with time. After 20 minutes the concentration of the H_2S decreased to a level that was below the detection limit of the test.

The concentration of hydrogen sulphide in the drinking water that the rats received was also measured. There was a marked decrease in concentration with time.

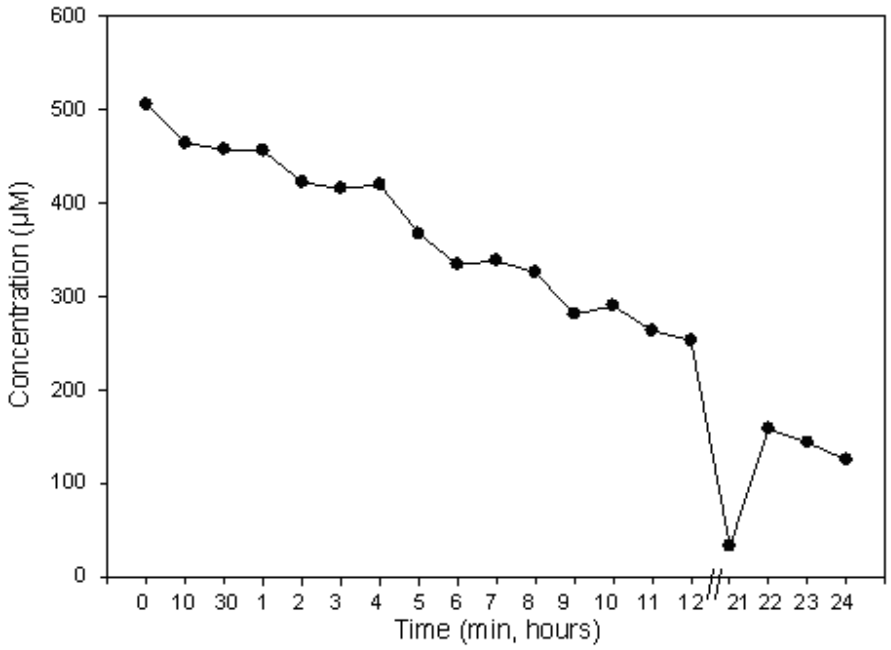


Fig. 4.1.9 Measurement of H_2S in the drinking water. Hydrogen sulphide concentration decreases with time. The first numbers denotes minutes (0, 10, 30) and the following ones, hours.

There is a marked decrease in concentration at hour 21. This can be explained by the fact that the bottles were left overnight and when we started measuring again, we measured the concentration from the first drops of water which was in contact with the air for a long time.

5. DISCUSSION

5. H_2S ADMINISTRATION IN AN ACUTE ISCHEMIA-REPERFUSION MODEL

Ischemic postconditioning by intermittent interruptions of blood flow in the early phase of reperfusion has been shown to reduce myocardial injury not only in animal studies but also in clinical trials [9, 10] however the application of ischemic postconditioning can be difficult to implement in clinical practice.

Although a large number of patients undergo angioplasty for acute myocardial infarction, there are also patients that still receive thrombolysis as a treatment to induce reperfusion. Therefore these patients are not able to benefit from ischemic postconditioning that may be realized during angioplasty.

In these cases pharmacological postconditioning would be a better option. The term "pharmacological" postconditioning is used to describe a diverse array of pharmacological agents which have been demonstrated in experimental studies to reduce myocardial infarct size when given during reperfusion. Agents such as inhalational anesthetics [70], nitric oxide [71], adenosine [72], insulin [73], erythropoietin [74] have been demonstrated to be cardioprotective when given at reperfusion.

These agents have been demonstrated to activate the RISK pathway, a pathway composed of "reperfusion injury survival kinases" such as PI3-K and MAP kinase ERK 1/2 which will inhibit the mitochondrial transition permeability pore (mPTP).

H_2S is produced in the cardiovascular system from L- cysteine by the enzyme Cystathionine gamma-lyase. Studies have shown that H_2S regulates vascular tone by opening K_{ATP} channels. Johansen *et al*. [24] have demonstrated that H_2S given before, during and at the beginning of reperfusion is protective against I/R injury by opening of the K_{ATP} channels and the protection is concentration dependent. Since this discovery, there have been many studies which have tried different protocols of H_2S administration prior to ischemia and have demonstrated a protective effect. Whether H_2S given during reperfusion protects the heart against I/R injury has been investigated in a limited number of studies [34, 75]. One of the studies used regional ischemia

and showed that post-treatment with six episodes of a 10-s infusion of NaHS or 2 min continuous NaHS infusion (100 μM) improved the cardiodynamic performance, and reduced myocardial infarct size.

The other study used global ischemia and showed that NaHS (1μM) given at the onset of reperfusion in 4 cycles of 15 seconds is protective against I/R injury. In contradiction with these findings, Osipov *et al.* [20] reported that bolus treatment with NaHS at reperfusion in a porcine model had no infarct-limiting effect, although infusion with the donor throughout ischemia and reperfusion was protective. Elsey et al. also have reported no effect of NaHS (1-50μM) given during reperfusion in rat hearts, although these data was not published [76].

In the present study we used NaHS, a donor of H_2S. NaHS dissociates to Na+ and HS– in solution and HS– associates with H+ and produces H_2S. Since the concentration of 1 μM has been previously shown to be protective given before ischemia, we decided to try this concentration.

In the present study NaHS given at the onset of reperfusion failed to limit infarct size. The reason for this could be related to the concentration dependent cardioprotective effect, the loss of H_2S from the buffer or the failure of H_2S to mobilize the RISK pathway or other cellular pathways involved in postconditioning. We measured the concentration of H_2S in the perfusion buffer and saw a decrease in H_2S concentration after just 5 minutes of perfusion with the H_2S supplemented buffer. The concentration decreased after 20 minutes to a concentration of 0.01 μM. This is one tenth of the concentration that we initially had in the buffer. Given the result of this substudy we concluded that our results could be explained by the fact that the H_2S concentration was low and that this could be below the levels that confer cardioprotection.

5.1. PILOT SUDY OF DHE STAINING TECHNIQUE AND ROS QUANTIFICATION

Numerous methods are available for ROS detection and they all have different downsides and advantages. The ideal assay for ROS detection should be sensitive enough to ensure that measurements are within the linear range of the assay and well above the limits of detection, it should be specific for one subtype of ROS, at least in physiological/pathophysiological concentrations and it should be applicable to a wide variety of experimental conditions and comparable between these applications. [77]

Some of the methods used to measure ROS in cardiovascular system are: Cytocrome C reduction, Chemiluminescence- based techniques, Electron Spin Resonance, and detection of intercellular superoxide with dihydroethidium.

Dihydroethidium, a cell-permeable compound, enters the cells and interacts with O_2^- to form oxyethidium which interacts with nucleic acids to emit a bright red color detectable by fluorescent microscope.

The strengths of this method are that it can be performed with equipment commonly available in research departments, it can be used to detect intracellular O_2^-, and the result can be correlated with other methods.

Because DHE is unstable with time, it is preferable that the tissue belonging to different groups is compared at approximately the same time point using the same supply of DHE solution.

For each of our experiments a new solution of DHE was prepared 1 hour before the samples were colored. Because a new solution of DHE was prepared for each experiment and there was variability among the controls, we needed to normalize samples to the arithmetic mean of control. In order to test the feasibility of the method, we measured ROS presence in different models of heart disease. The measurements of ROS in the diet-induced mice yielded similar result to other studies [78, 79] which measured ROS in obese and/or insulin resistant mice. This was a confirmatory indication that the method and the protocol chosen works.

Methodological considerations

There are some downfalls that should be taken into consideration when using this technique in order to detect ROS.

The settings of the camera were chosen manually based on a number of pictures taken from different tissue samples. These tissue samples came from hearts that were perfused with Krebs-Henseleit buffer. If a certain intervention would increase the amount of ROS in the rat's heart to a great extent, it might be overseen by the program because it would be overexposed.

For every heart we took approximately 25 pictures and we had approximately of 5 hearts in each group. The large number of photos for each group would decrease the chance for statistical errors. Although we took a large number of pictures for each heart/group, there was some variability in the fluorescence intensity within the same group.

When taking pictures from a slice, we excluded the margins (there are usually more intense than the rest of the slice), and the vessels (endothelial cells showed increase intensity compared with myocytes). Since under some conditions the vessel wall could be an important source of ROS this could be a factor leading to a false conclusion of no the lack of increase in ROS.

5.2. SHORT TERM ADMINISTRATION OF H_2S IN LANGENDORF PERFUSED HEARTS

A number of studies have demonstrated the cardioprotective effect of H_2S in I/R injury. It has been shown that this protective effect involves the K_{ATP} channel, but other mechanisms have also been proposed to be involved. This include: an anti-inflammatory effect by inhibiting leukocyte adherence to the epithelium [80], the inhibition of p38MAPK [81], mitochondrial preservation [82], inhibition of myocardial contractility [83] and decrease in ROS formation [84].

It has also been suggested that H_2S reduces oxidative stress through a direct mechanism, by the scavenging of ROS and through an indirect mechanism by the upregulating of antioxidant defences. Geng *et al.* [85] reported that exogenous administrated hydrogen sulphide protects the myocytes by directly scavenging oxygen-free radicals and reducing the accumulation of lipid peroxidations. Kimura and colleagues [86] demonstrated that H_2S protects neurons from cell death and oxidative stress by increasing the levels of the antioxidant glutathione.

In order to test the hypothesis that H_2S reduces ROS production in the cardiovascular tissue, we measured ROS presence in hearts that were perfused with H_2S.

As a positive control we used hearts that were perfused with Angiotensin II. In physiological conditions Ang II plays an important role in regulating blood pressure and fluid homeostasis. In pathological conditions Ang II contributes to altered vascular tone, endothelial dysfunction, structural remodeling, and vascular inflammation and thus is involved in the pathogenesis of hypertension, atherosclerosis, cardiac hypertrophy, vasculitis, and diabetes. Growing evidence indicates that Ang II induces its pleiotropic vascular effects partly through generation of reactive oxygen species (ROS) [87] and studies have shown the involvement of Ang II in increased ROS production [88]. In acordance with these studies, we also have found an increased ROS production in the hearts that were perfused with Ang II.

In the present substudy there were no difference between the hearts that were perfused with H_2S and the controls. This result may suggests that exogenous H_2S administrated in a Langerdoff perfused heart, outside a pathological condition doesn't play a role in modulating tissue levels of ROS. Another explanation may come from the fact that H_2S evaporates when bubbled with O_2/CO_2, resulting in a fall in its concentration in the perfusion buffer to below a level that is not

cardioprotective. In order to test this theory we proceeded to measure the concentration of H_2S in the perfusion buffer and saw a marked decrease in the concentration of H_2S after 20 minutes of perfusion. Based on this we concluded that the low concentration of H_2S was the main reason for not achieving cardioprotection. In the present substudy we investigated if exogenous H_2S influences the production of ROS in a Langerdorff perfused heart that had no associated pathology. An interesting future experiment would be to investigate the role of exogenous H_2S administration in a Langerdoff perfusion model that has a associated pathology that would lead to increase formation of ROS (for example in hearts coming from diabetic rats) or to perfuse the hearts with a combination of Ag II and H_2S.

5.3. LONG TERM ADMINISTRATION OF H2S TO HEALTHY HEARTS

Although several studies have investigated the role of H_2S in rats with different cardiac pathologies, there are up to date no studies that investigate the role of H_2S in healthy rat hearts. The previous substudy showed that acute exogenous H_2S administration in healthy rats did not change ROS levels, however chronic administration of high dose NaHS (500 µM) in drinking water increased ROS production in cardiac tissue. A low dose of NaHS (50 µM) had no effect on ROS production.

Even though H_2S can be detected in blood and other tissue, there is no agreement on a good method that can give a precise measurement of free H_2S concentration. This problem arises from the fact that H_2S can react with a number of different species (*e.g.* superoxide radical, hydrogen peroxide, peroxynitrite, etc.) thus making the measurement of free H_2S difficult. The most used method relies on trapping H_2S with a metal followed by acidification and reaction with a dye (DMPD to form methylene blue) which is then measured spectophotometrically. Through this method concentration of H_2S of about 50 to 100 µM were reported in the rat and human plasma. The limitation of this method is that it measures not only free H_2S but also other species such as hydrosulfide anion and sulfide. Some scientists consider that these concentrations are too high to be consistent with life, and put the plasma concentration of H_2S in the submicromolar range. [22]

When NaHS is dissolved in saline, about one-third of H_2S exists as undissociated gas and the remaining two-thirds as HS^- anion. By administrating NaHS in drinking water is hard to predict how much of it would be absorbed and how much the concentration of H_2S in the plasma would be changed. Our attempt to measure plasma H_2S failed to give accurate information since our method was not sensitive enough to measure low levels of H_2S.

5.4. LONG TERM ADMINISTRATION OF H2S IN AN EXPERIMENTAL MODEL OF CARDIAC HYPERTROPHY

In this substudy we investigated the role of exogenous administration of NaHS (500μM) in a modelheart hypertrophy. Previous studies have suggested a role of ROS in the development of cardiac hypertrophy. Although this mechanism is not fully understood, it seems that ROS produced by NADPH oxidase are able to modulate signaling pathways known to be involved in cardiomyocyte hypertrophy, such as ERK1/2, JNK, p38MAPK, Akt, PKCs, and NF-κB [53]. In the present study we used pressure-overload induced hypertrophy by transthoracic aorta banding but did not find a higher production of ROS in the hearts of these rats compared with the controls. Compared with other studies [89, 90], our model of hypertrophy involved just 14 days of pressure overload by TAC. In such a short period the rats may have developed a compensatory hypertrophy which would have not been characterized by negative changes, such as an increase in ROS production, but rather positive ones. There was also no statistical difference between the sham, hypertrophic hearts and hearts from rats that received NaHS in drinking water. A reason for this result is that the NaHS in the water might have evaporated before the animals drank it. In order to test this hypothesis we proceed to measuring the H_2S concentration in the water bottles and saw marked decrees in concentration with time. While we could see a significant difference between healthy rats that received H_2S (500 μM) in drinking water and the control group (see RESULTS- 4.4. LONG TERM ADMINISTRATION OF H_2S TO HEALTHY HEARTS), we could not find such a difference between Sham and H_2S-Sham (500 μM). The difference between this to types of experiments is that in the latter the rats were subjected to transthoracic surgery. This could have increased the level of stress and in the same time these rats could have drank less water that they would normally would. These rats also received pain medication, which might have influenced the production and measurement of ROS. Another explanation that we should take into consideration is that exogenous administration of H_2S doesn't have a cardioprotective effect. It has recently been proposed that a marked decrease in the amount of H_2S will influence the cardiovascular system rather that increased levels of H_2S. Yang et al. showed that mice lacking CSE display pronounced hypertension and diminished endothelium-dependent vasorelaxation [33]. An interesting prospective will be to use animals that are not able to produce H_2S rather that trying to increase the level of H_2S in the body.

CONCLUSION

In this study we investigated the cardioprotective role of short time H_2S administration in an acute ischemia- reperfusion model and the influence of long term administration of H_2S in a chronic cardiac disease on ROS production. In order to do this we established a method trough which we are able to measure ROS presence in tissue sampled from in vivo experimental animal studies. Although the method is widely used in laboratories around the world, putting it into practice with our own equipment represented a challenge that we finally managed to overcome. This method represents a simple, accurate and cheep way for estimating intracellular reactive oxygen species and can also easily be used by other groups in our institute.

Our attempt to investigate the proposed beneficial role of H_2S in an acute ischemia-reperfusion model failed to yield any cardioprotection, probably because of the low concentration of H_2S that we had in the buffer.

To our knowledge we are the first laboratory to investigate the role of exogenous administration of H_2S on ROS production in healthy rat hearts. We found an increase in ROS formation at a concentration of 500 μM in the drinking water. Surprisingly in the cardiac hypertrophy model we did not find any difference between control and treatment groups.

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