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MYOCARDIAL PROTECTION BY HYPEROXIA

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In memory of my father

CONTENTS

ABSTRACT

Oxygen is essential for normal respiration in aerobic organisms, and prolonged deficit of oxygen always has detrimental consequences. However, all aerobic life forms are faced with the threat of oxidation from molecular oxygen. Reactive oxygen intermediates have been suggested to have an essential role in the pathogenesis of ischaemia-reperfusion injury. At high fractions and prolonged exposure, hyperoxia may lead to excessive generation of reactive oxygen intermediates overwhelming the cellular antioxidant defense and inducing oxidative damage. Contrary to these detrimental effects, reactive oxygen intermediates have recently been suggested play a physiological role acting as signal transduction. Theoretically, increased oxygen fractions in inspired air may have beneficial effects.

In the present study, we demonstrate that *in vivo* exposure of rats or mice to increased oxygen fraction induced *ex vivo* functional protection of the heart, and reduced the extent of myocardial necrosis after induced global or regional ischaemia. The obtained cardioprotection was evident when the heart was isolated and perfused immediately afterwards, or when it was perfused 24 hours later. The protection depended on the inspired oxygen fraction and the duration of hyperoxic exposure. The vasomotor response of isolated aortic rings was also modified by hyperoxia. Hyperoxia elicited a systemic low-graded oxidative stress, and caused pulmonary and myocardial nuclear translocation of the transcription factor nuclear factor kappa-B (NFκB). Hyperoxia reduced activation of NFκB during sustained ischaemia and reperfusion, and increased the NFκB cytoplasmatic inhibitory protein IκBα. Administration of NFκB inhibitors during ischaemia and reperfusion improved contractile function and reduced infarct size in hearts from normoxic control animals. These findings demonstrate that hyperoxia elicits myocardial protection through a NFκB-dependent mechanism, and support evidence for a dual role of NFκB in the heart.

In summary, a novel concept of vascular and myocardial protection through exposure of animals to increased oxygen fraction was established in the present work. Hyperoxia may be directly employed in patients for increased endogenous cell defence.

LIST OF ORIGINAL ARTICLES

I **Tähepõld P**, Ruusalepp A, Li G, Vaage J, Starkopf J, Valen G.
Cardioprotection by breathing hyperoxic gas – relation to oxygen concentration and exposure time in rats and mice.
European Journal of Cardiothoracic Surgery; accepted for publication.

II **Tähepõld P**, Valen G, Starkopf J, Kairane C, Zilmer M, Vaage J.
Pretreating rats with hyperoxia attenuates ischemia-reperfusion injury of the heart.
Life Sciences 2001;68:1629-1640.

III **Tähepõld P**, Elfström P, Eha I, Kals J, Taal G, Talonpoika A, Valen G, Vaage J, Starkopf J.
Exposure of rats to hyperoxia enhances relaxation of isolated aortic rings and reduces infarct size of isolated hearts.
Submitted for publication.

IV Li G, Tokuno S, **Tähepõld P**, Vaage J, Löwbeer C, Valen G.
Preconditioning protects the severely atherosclerotic mouse heart.
Annals of Thoracic Surgery 2001;71:1296-1304.

V **Tähepõld P**, Vaage J, Starkopf J, Valen G.
Hyperoxia elicits myocardial protection through a NFκB-dependent mechanism in the rat heart.
Submitted for publication.

ABBREVATIONS

ACh	acetylcholine
AOC	antioxidative capacity
AP-1	activator protein-1
ApoE/LDLr KO	apolipoprotein E / Low density lipoprotein receptor double knockout
CAT	catalase
CD	conjugated dienes
CTnT	cardiac troponin T
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
GSH-Px	glutathione peroxidase
GSH-Red	glutathione reductase
GSSG	oxidized glutathione
HSP	heat shock protein
iNOS	inducible nitric oxide synthase
IPC	ischaemic preconditioning
I/R	ischaemia-reperfusion
K_{ATP}	ATP-dependent potassium channel
LOOH	lipid hydroperoxides
LPO	lipid peroxides
MAPK	mitogen activated protein kinases
NFκB	nuclear factor kappa-B
NO	nitric oxide
NOS	nitric oxide synthase
PDTC	pyrrolidine dithiocarbamate
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
Phe	phenylephrine
PKC	protein kinase C
ROI	reactive oxygen intermediates
SNP	sodium nitroprusside
SOD	superoxide dismutase
TTC	triphenyl tetrazolium chloride

INTRODUCTION

Clinical background

Ischaemic heart disease is a major cause of morbidity and mortality in the developed countries, although the incidence varies among populations and areas of the world. Atherosclerosis causes coronary artery stenosis, and rupture of an atherosclerotic plaque may trigger the formation of a thrombotic occlusion [Davies and Thomas, 1985]. Inadequate blood-flow and oxygen supply leads to myocardial ischaemia with subsequent tissue injury and cell death. The major goal of treatment of ischaemic heart disease is therefore to restore blood-flow to unperfused areas by means of thrombolytic agents, percutaneous transluminal coronary angioplasty, or surgical revascularization of ischaemic myocardium. However, the acute restoration of blood-flow to ischaemic myocardium may be injurious. Reintroduction of molecular oxygen into ischaemic tissue may be accompanied by uncontrolled generation of reactive oxygen intermediates (ROI) and an injurious oxidative stress.

The oxygen paradox and oxygen toxicity

Oxygen is essential for life in aerobic organisms. Concomitantly, oxygen is a dangerous agent, and all aerobic life forms are faced with the threat of cellular damage from molecular oxygen [Stogner and Payne, 1992; Kazzaz *et al.*, 1996]. This basic dilemma is known as the "oxygen paradox" [Davies, 1995]. In spite of this, oxygen therapy at higher fractions than ambient concentration has to be used in the care of critically ill patients to maintain organ viability. The beneficial effects of increased inspired oxygen fraction include improved oxygen delivery to tissues, and decreased incidence of infections associated with surgical procedures [Greif *et al.*, 2000]. Kotani *et al.* [2000] have also shown that exposure to 100% O_2 preserves antimicrobial function in alveolar macrophages after surgery. Although the benefits of oxygen therapy have been known for many years, its potential toxicity has been recognized only in the last two decades. However, the toxic length of exposure, as well as the toxic level of oxygen fraction is still debated [Capellier *et al.*, 1999].

Organ damage from hyperoxia depends on both the oxygen fraction in inspired air, and the pressure of oxygen during exposure [Jenkinson, 1993]. The primary target for increased oxygen fraction are the lungs, as the lungs are exposed to the highest partial pressure of inspired oxygen. Therefore, the lungs are most severely damaged by prolonged hyperoxia. Other organs injured by

hyperoxia in humans include the eye and central nervous system [Stogner and Payne, 1992]. Injury to other human organs by hyperoxia has not been systematically studied.

In animals, 24-72 hours of hyperoxic exposure (100% O_2) does not cause pathologic changes [Jenkinson, 1988]. Exposure of rats to a sublethal fraction of oxygen (85% O_2) for five to seven days protects the animals from the subsequent usually lethal oxygen fraction of 100% [Crapo *et al.*, 1980]. This protective effect correlates with the induction of increased antioxidant enzyme activity [Jenkinson *et al.*, 1983; Jamieson *et al.*, 1986].

Dose-response and duration-response studies in humans have shown that administering of 100% O_2 in the absence of pulmonary pathology is safe for up to 12 hours [Winter and Smith, 1972; Clark, 1988]. However, oxygenation of patients with 100% O_2 during induction of general anaesthesia has been shown to increase the formation of atelectasis within few minutes [Rothen *et al.*, 1995; Reber *et al.*, 1996]. Although critically ill patients often have to be ventilated with high inspiratory oxygen fraction until pulmonary function recovers at least partially, the goal is to achieve optimal oxygenation with the lowest possible oxygen fraction [Tobin, 2001].

Reactive oxygen intermediates and oxidative stress

Biochemical theory attributes oxygen toxicity to the formation of free radicals [Stogner and Payne, 1992; Kazzaz *et al.*, 1996; Davies, 2000; Loiseaux-Meunier *et al.*, 2001]. A free radical is defined as "any species capable of independent existence that contains one or more unpaired electrons" occupying an atomic orbital by itself. Most of the important free radicals in human biology are derived from oxygen. These highly reactive species can cause cellular damage by oxidizing lipids [Janssen *et al.*, 1993], carbohydrates, proteins [Ischiropoulos and al-Mehdi, 1995] and nucleic acids [Ueda and Shah, 1992]. Under normal physiologic conditions (21% O_2 at 1 atm pressure), about 1 to 5% of O_2 undergoes univalent reduction, producing the superoxide radical (O_2^-) and derivates, hydrogen peroxide, and hydroxyl radical [Deneke and Fanburg, 1980; Capellier *et al.*, 1999]. Accumulation of reactive oxygen intermediates (ROI) is effectively neutralized by endogenous antioxidants [Halliwell and Gutteridge, 1989; Fridovich, 1995; Kazzaz *et al.*, 1996]. The antioxidants can be devided into enzymatic and non-enzymatic. Intracellularly, the enzymatic defence predominates [Halliwell, 1996]. Important scavenging enzymes are superoxide dimutases, which catalyse the dismutation of superoxide to hydrogen peroxide [Kehrer, 1993]. Hydrogen peroxide is further enzymatically scavenged by catalase, and glutathione peroxidase. The latter works in connection with glutathione, one of the main non-

enzymatic intracellular scavengers [Zilmer *et al.*, 1994]. However, at high oxygen fractions and prolonged exposure, ROI are generated at a rate overwhelming the antioxidant capacity of the cells, and may induce oxidative damage [Mustafa and Tierney, 1978; Kazzaz *et al.*, 1996; Kleen and Messmer, 1999; Loiseaux-Meunier *et al.*, 2001]. Oxidative stress may be defined as follows: "A disturbance in the pro-oxidant/antioxidant system in favour of the former may be denoted as an oxidative stress" [Sies, 1985]. Oxidative stress is an unavoidable consequence of life in oxygen-rich atmosphere, and can result from increased exposure to oxidants (oxygen), or from decreased antioxidant capacity, or even from both problems occurring simultaneously [Cadenas, 1989]. Prolonged oxidative stress may induce tissue damage, no matter in what site of the organism it developed [Halliwell, 1996].

Antioxidant protection

The antioxidant defense systems of the cell include renewable enzymes and compounds that allow existance in a hostile oxygen environment. Additionally, there exists damage removal and repair systems for oxidized proteins [Pacifici and Davies, 1991; Grune *et al.*, 1997], membrane lipids [Van Kuijk *et al.*, 1987; Pacifici and Davies, 1991], and DNA [Demple and Halbrook, 1983; Wang *et al.*, 1998]. Genes encoding antioxidant enzymes and repair systems respond to changing levels of oxidative stress. A battery of 30-40 genes are known to be "turned on" in a rapid and highly coordinated fashion in response to oxidation. Synthesized proteins include mitochondrial manganese-superoxide dismutase, mitogen activated protein kinases, and 32-kDa heme oxygenase, which all have been suggested as tissue protective enzymes [Fornace *et al.*, 1989]. This rapid genetic response enables cells to survive oxidant exposures that would normally be lethal. Oxidative stress causes a wide spectrum of genetic, metabolic, and cellular responses. Only the most extreme outcome, necrosis, involves direct cell destructions, while most oxidative stress conditions will modulate gene expression, may stimulate cell growth, or cause a protective temporary growth-arrest and transient adaptive response [Wiese *et al.*, 1995; Davies, 1999]. Even the apoptotic response seen at high oxidant exposures appears to protect surrounding cells and tissues [Davies, 2000].

Ischaemia-reperfusion injury - role for ROI

Although reoxygenation is the only way for myocardial cells to survive an ischaemic insult, reperfusion itself may possess deleterious effects, referred to as reperfusion injury [Braunwald

and Kloner, 1985]. Reperfusion injury includes a series of events such as reperfusion arrhythmias, microvascular damage, reversible myocardial dysfunction referred to as "stunning", and irreversible injury as cell death, which all may occur separately or in combination with each other [Braunwald and Kloner, 1985; Vaage and Valen, 1993; Valen and Vaage, 1993]. The exact mechanisms underlying reperfusion injury are unclear. In general, ischaemia-reperfusion (I/R) injury is an inflammatory-like reaction with generation of ROI, calcium overload [Dhalla *et al.*, 1996;], mitochondrial damage [Ferrari, 1996], and production of proinflammatory mediators such as cytokines, activation of leukocytes, and release of various vasoactive substances [Reikeras and Ytrehus, 1992; Vaage and Valen, 1993; Valen and Vaage, 1993].

Several studies have proposed an essential role of ROI in the pathogenesis of myocardial I/R injury [Garlich *et al.*, 1987; Bolli *et al.*, 1989; Grech *et al.*, 1996; Griendling and Alexander, 1997]. During reperfusion, molecular oxygen reintroduced into the ischaemic myocardium augments the production of ROI, resulting in oxidative damage to essential cellular components [Zweier *et al.*, 1987; Ytrehus and Hegstad, 1991; Hearse and Bolli, 1992; Valen and Vaage, 1993]. Thus, enhancing the antioxidant activities of the myocardium may be useful to prevent I/R injury. It has been demonstrated that inhibition of lipid peroxidation induced by exogenous ROI, or by ischaemia and reperfusion, attenuated functional and biochemical injury of the isolated rat heart [Nagy *et al.*, 1996; Nagy *et al.*, 1998]. Recent studies using a genetically engineered animal model have directly shown the importance of antioxidative enzymes in protecting the myocardium against I/R injury [Chen *et al.*, 1998; Woo *et al.*, 1998]. It has been suggested that ROI themselves may trigger an increase in antioxidant enzyme activity, and promote tolerance to ischaemia in the myocardium. The studies by Lai *et al.* [1996] and Zhou *et al.* [1996] strongly support the suggestion that oxidative stimulus by ROI to enhance antioxidant defense may offer a promising method against I/R injury of the heart.

Ischaemic preconditioning

Brief episodes of nonlethal ischaemia protects the myocardium against subsequent I/R injury of the heart [Murry *et al.*, 1986]. This phenomenon has been termed as ischaemic preconditioning (IPC), and has since then been documented in all species tested, including humans. IPC is by far the most powerful form of cardioprotection available. The preconditioning phenomenon is potentially of great clinical interest. However, the ultimate aim for therapy of patients with unstable angina or imminent myocardial infarction, or in cardiac surgery with cardioplegic arrest involves pharmacological exploitation of the underlying mechanisms. The mechanisms

underlying IPC are not fully clarified, and it is currently not possible to exploit its cardioprotective effects clinically.

IPC consists of an early phase (classic preconditioning), which develops within minutes and affords protection up to 3-4 hours after the preconditioning episode [Murry *et al.*, 1986; Van Winkle *et al.*, 1991], and a late phase (delayed preconditioning or "second window of protection"), which is manifest 24-72 hours after ischaemic stimulus [Kuzuya *et al.*, 1993; Marber *et al.*, 1993]. IPC has also been found to limit the extent of myocardial infarction when the duration of sustained ischaemic insult is up to 90 minutes [Yellon *et al.*, 1998]. Przyklenk and colleagues [1993] demonstrated that a brief coronary artery occlusion adapted the myocardium to ischaemia outside the perfusion region of occluded vessel. From their findings, the hypothesis of remote preconditioning originated, where short episodes of ischaemia and reperfusion in other organs protect the heart against I/R injury.

The preconditioning response can be induced by several stimuli, such as ischaemia and reperfusion, hypoxia, oxidative stress, heat stress, and lipopolysaccharides [Marber *et al.*, 1993; Baxter *et al.*, 1994; Cohen *et al.*, 1995; Tritto *et al.*, 1997; Rowland *et al.*, 1997]. Common for all these stimuli is that they induce a low-graded inflammation, and that they are not applicable in humans.There is some evidence that preconditioning may occur naturally in patients suffering from angina before onset of myocardial infaction [Kloner *et al.*, 1995; Ottani *et al.*, 1995; Tamura *et al.*, 1997]. Unstable angina may cause short episodes of spontaneous ischaemia due to intermittent hypoperfusion.

Mechanisms of ischaemic preconditioning

The mechanisms underlying IPC appear to differ between species, and no universal key factor has so far been targeted. It has been suggested that ROI, nitric oxide, bradykinin, catecholamines, opioids, and/or adenosine, all of which may be released during preconditioning, play roles in triggering this phenomenon [Baxter *et al.*, 1994; Downey and Cohen, 1995; Goto *et al.*, 1995; Baxter, 1997; Bolli *et al.*, 1997; Tritto *et al.*, 1997]. Trigger substances may stimulate membrane receptors linked to protein kinases via G-proteins. The G-proteins induce phosphorylations of subsequent protein kinases, of which protein kinase C, tyrosine kinase, and mitogen-activated protein kinases are currently in focus [Ytrehus *et al.*, 1994; Downey and Cohen, 1995; Yellon *et al.*, 1998]. In turn, this may lead to activation of transcription factors [Barnes and Adcock, 1997; Valen *et al.*, 2001]. Transcription factors may trigger gene programs generating cytoprotective

enzymes as mediators of protection. Nitric oxide synthase, antioxidants, and heat shock proteins (HSP) of the 27 and 70 kDa families have been suggested as possible mediators of preconditioning [Marber *et al.*, 1993; Zhou *et al.*, 1996; Bolli *et al.*, 1997; Dana *et al.*, 2000]. Receptor-mediated signalling mechanisms may also involve sarcolemmal or mitochondrial ATP-dependent potassium channels [Yellon *et al.*, 1998]. However, it has been suggested that opening of ATP-dependent potassium channels are not end-effectors of preconditioning, but rather their opening generates ROI, which may trigger the preconditioning state [Pain *et al.*, 2000].

The hypothesis for the delayed phase of IPC implies synthesis and/or posttranslational modifications of cytoprotective genes. Increased activity of antioxidative enzymes [Kuzuya *et al.*, 1993; Zhou *et al.*, 1996], elevation of the myocardial content of HSP [Marber *et al.*, 1993], and upregulation of nitric oxide synthase [Takano *et al.*, 1998] have been indicated as effectors of protection. Valen *et al.* [2000] have demonstrated that HSP72, and endothelial nitric oxide synthase (eNOS), cardioprotective proteins associated with preconditioning response, are increased in cardiac tissue of the patients with unstable angina, while the transcription factor nuclear factor kappa-B (NFκB), is increased in hearts of unstable patients with recent symptoms as well.

Reactive oxygen intermediates in preconditioning

A role for ROI in preconditioning was first suggested by Murry *et al.* [1988], who demonstrated that administration of oxygen radical scavengers during reperfusion could block the beneficial effect of IPC on infarct size. These authors suggested that reperfusion after the short ischaemic insult results in the generation of low amounts of ROI, insufficient to induce cell injury, but sufficient to induce preconditioning. It has also been suggested that the beneficial effects of IPC can be prevented by administration of antioxidants during the preconditioning episode, as well as during reperfusion after a sustained ischaemic insult [Osada *et al.*, 1994]. Furthermore, exposure of isolated hearts to non-toxic doses of ROI – in the absence of ischaemia – may reproduce the beneficial effects of IPC [Tritto *et al.*, 1997]. It has also been demonstrated that low doses of exogenous ROI protect myocardial contractile function against subsequent injury [Ytrehus *et al.*, 1995; Hegstad *et al.*, 1997; Valen *et al.*, 1998].

Role of NFκB in ischaemic preconditioning and ischaemia-reperfusion

ROI are suggested as activating agents of NFκB [Schreck *et al.*, 1991; Blackwell and Christman, 1997]. NFκB has been shown to have a privotal role in the regulation of many genes involved in immune and inflammatory responses [Sha, 1998], and may have a key role in both the pathophysiology of IPC and of I/R injury. Li et al. [1997] have demonstrated that NFκB is activated also by normobaric hyperoxia. NFκB seems to play essential role in both early (classic) [Maulik *et al.*, 1998; Morgan *et al.*, 1999] and delayed [Xuan *et al.*, 1999] IPC. Under normal physiological conditions, NFκB is held in the cytoplasm in an inactive form by the inhibitory protein IκB. In cellular stress IκB is phosphorylated by specific kinases, and released from the NFκB homo- or heterodimer [Blackwell and Christman, 1997; Valen *et al.*, 2001]. NFκB translocates to the cell nucleus, where it binds to promoter or promoter/enhancer regions, and gene transcription is induced [Bowie and O'Neill, 2000]. NFκB regulates a battery of genes with proinflammatory effects: leukocyte adhesion molecules, cytokines, and chemokines [Baeuerle and Baltimore, 1996; Blackwell and Christman, 1997; Gumina *et al.*, 1997; Valen *et al.*, 2001]. However, in addition NFκB regulates genes potentially associated with tissue repair and protection, such as inducible nitric oxide synthase (iNOS), inducible cyclooxygenase, and manganese superoxide dismutase [Barnes and Adcock, 1997; Xu *et al.*, 1999]. NFκB is activated during the preconditioning, and pharmacological inhibition of NFκB abolishes the beneficial effects of IPC in both classic and delayed models [Maulik *et al.*, 1998; Xuan *et al.*, 1999; Morgan *et al.*, 1999]. However, activation of NFκB during reperfusion after sustained ischaemia appears to be detrimental. Inhibition of NFκB-activation during ischaemia and reperfusion through employing a NFκB oligo decoy reduced infarct size and improved myocardial contractile function [Morishita *et al.*, 1997; Sawa *et al.*, 1997]. Thus, the role of NFκB in myocardial ischaemia and reperfusion seems to be dual, as evidence is provided for both a cardioprotective role in IPC, and a detrimental role during sustained ischaemia and reperfusion.

Why hyperoxia?

Hearts stimulated *in vivo* with transient ischaemia, hyperthermia, or inflammatory mediators have increased myocardial antioxidant enzyme activity and tolerance to subsequent I/R injury [Brown *et al.*, 1990; Currie and Tanguay, 1991; Hoshida *et al.*, 1993]. The molecular basis of the adaptation process still remains unclear. However, a growing body of evidence suggests that ROI

generated during the stimuli trigger an increase in antioxidant enzyme activity and promote ischaemic tolerance in myocardial cells [Lai *et al.*, 1996; Zhou *et al.*, 1996]. Thus, pretreatment with an oxidative stimulus to enhance the antioxidant defense may offer protection against I/R injury.

Hyperoxia generates ROI and induces lipid peroxidation in cell cultures and isolated organs [Ahotupa *et al.*, 1992; Gille and Joenje, 1992; Van Klaveren *et al.*, 1998]. Although prolonged exposure to high oxygen fractions is injurious, a short exposure inducing a low-graded oxidative stress may potentially elicit a preconditioning-like response for myocardial protection.

One way to induce the preconditioning response would possibly be to let experimental animals and/or patients breathe supraphysiologic concentrations of oxygen for a limited period of time. If this does provide protection, it is an easy and inexpensive way to elicit the preconditioning response in clinical practice, regardless of mechanisms of action.

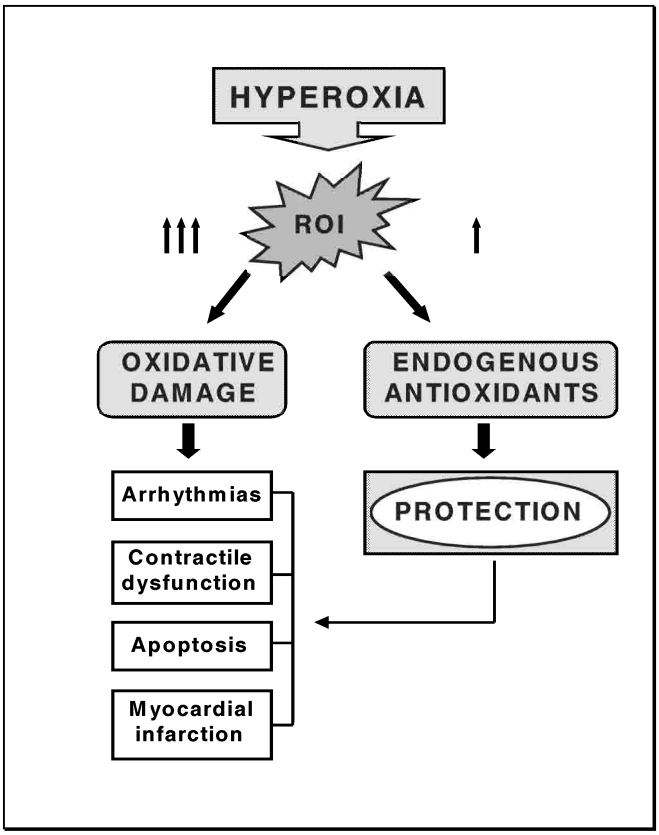

Figure 1. *Schematic diagram showing pathophysiologic implications of reactive oxygen intermediates (ROI) and endogenous antioxidants in hyperoxia-induced oxidative stress.*

AIMS

The main objective of the present thesis was to establish a model of myocardial protection against ischaemia-reperfusion injury by subjecting animals *in vivo* to increased oxygen fractions in inspired air. Furthermore, the application areas and possible mechanisms underlying protection were studied.

1. To investigate the basic physiology of the hyperoxia model in rats and mice; importance of the inspired oxygen fraction, duration of hyperoxic exposure, and immediate versus delayed protection was studied on heart function and infarct size.

2. To study whether hyperoxia induced a systemic oxidative stress, and attempt to determine whether HSP72, eNOS, or cardiac antioxidants may mediate the protection.

3. To evaluate the effect of hyperoxia on vasomotor function and study if hyperoxia protects against regional myocardial ischaemia.

4. To explore whether hearts of animals with severe atherosclerosis could be adapted to ischaemia by hyperoxia, and compare the hyperoxic protection with the effects of classic IPC.

5. To study the possible role of NFκB in myocardial protection by hyperoxia.

METHODOLOGICAL CONSIDERATIONS

The isolated, retrogradely perfused heart model

This model was first described more than a hundred years ago [Langendorff, 1895], and is still widely utilized in research of heart physiology. The model of isolated heart perfusion is well established, it is easy to control and standardize, and it is technically easy to perform. It allows excellent evaluation of function, and it is reproducible. In order to evaluate myocardial contractile function and coronary flow, we employed the retrograde perfusion model as modified Langendorff model. The modification is a balloon inserted into the left ventricle for isovolumetric recordings of left ventricular systolic (LVSP) and end-diastolic (LVEDP) pressures, while left ventricular developed pressure (LVDP) was calculated as LVDP=LVSP-LVEDP. Although Langendorff-perfusion provides a good assessment of heart performance, it is a non-working model. The latter is at least partially compensated by the intraventricular balloon, which fills an otherwise empty left ventricle. Hearts were isolated from anaesthetized rats or mice, and perfused retrogradely with Krebs-Henseleit buffer at a constant pressure of 70 mmHg for rat hearts and 55 mmHg for mouse hearts. The Krebs-Henseleit buffer does not contain any cells, therefore lacking the influence of circulating blood cells and components. The perfusate buffer is kept at 37 °C, constantly oxygenated and it maintains physiological pH. Coronary flow (CF) was measured by timed collections of coronary effluent. Heart rate (HR) and arrhythmias were evaluated from the pressure curves in rat heart, and by the computer in mice. The maximal and negative value of the first derivative of pressure (dP/dt max and negative dP/dt) and rate pressure-product (RPP=HR × LVSP, left ventricular systolic pressure) were evaluated in mouse hearts and calculated by a computer system (PCLAB, Astra Hässle AB, Mölndal, Sweden).

Animal models

In the present studies, rats and mice have been employed as experimental animals. The particular disadvantage of the mouse is its size, which challenges the technology and dexity of physiology research. However, the mouse genome is well characterized, and a large range of genetically engineered animals are easily available. The advantage of gene targeted animals is that they can be designed to overexpress or to have a deletion for a specific gene of interest. This is considered superior to pharmacological approaches where drugs may influence clear-cut results by unspecific side effects. At the same time, animals deficient for a substance may compensate by overexpressing other substances.

The apolipoprotein E and low density lipoprotein receptor double knockout (ApoE/LDLr KO) mice were fed an atherogenic diet (21% fat, 0.15% cholesterol) for 7-9 months to speed up the process of atherosclerosis. The mice were severely atherosclerotic at the time of Langendorff-perfusion. Three to four months old wild type animals fed on chow served as controls. The latter is a limitation of the study as age and diet matching would have been appropriate. This was not performed due to limitations in animal housing.

In vitro vessel reactivity

To investigate the effect of pretreatment with hyperoxia on vasomotor response, the isometric tension of rat thoracic aortic rings was recorded in the classic organ bath model. After anaesthesia, rings of 2 mm length from the descending thoracic aorta were isolated and mounted onto two thin stainless steel holders connected to a force transducer which measures the isometric tension. *In vitro* dose-response to phenylephrine (Phe), prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$), and endothelin-1 was tested to evaluate contraction, while acetylcholine (ACh) and sodium nitroprusside (SNP) were tested to evaluate relaxation of the aortic rings precontracted with Phe. A limitation to this study is that the reactivity of the thoracic aorta may be different from peripheral arteries, including coronary arteries, as the aorta has less reserve for contraction and dilatation compared to peripheral vessels.

Determination of infarct size and cardiac troponin T

To evaluate the extent of myocardial necrosis at the end of reperfusion, we employed triphenyl tetrazolium chloride (TTC)-staining. Initially TTC was delivered to the hearts by intracoronary perfusion (Paper I and IV). Retrospectively, this may not be an optimal model as ischaemic contraction may influence postischaemic coronary perfusion, or coronary stenosis in atherosclerotic animals may cause uneven distribution of TTC solution. We changed the method to collecting hearts, freezing, sectioning, and thereafter TTC-staining. TTC-staining is based on separating viable cells from nonviable. In the viable myocardium, TTC is reduced to red pigment by intracellular dehydrogenases, while in nonviable areas reducing equivalents are lost, and consequently unstained tissue can be considered as infarcted. The images were digitized, and infarct size measured semiautomatically by software (Adobe Photoshop version 4.0). However, this model lacks the ability to detect scattered cell damage, and light microscopic evaluation of tissue might have been a more reliable technique. Demarcation of necrotic area requires several days, and it has been reported that TTC staining after a few hours of reperfusion and microscopic examination after several days of reperfusion gave corresponding results [Ytrehus

and Downey, 1993]. In our experimental protocol hearts were reperfused for a hour. Nevertheless, we have continuously observed clear-cut differences in infarct size between hearts from animals pretreated with hyperoxia and compared to heart from normoxic control animals.
Coronary effluent for cardiac troponin T evaluation was sampled immediately before global ischaemia and after 30 minutes of reperfusion (Paper IV). The cardiac troponin T release correlated with the extent of myocardial necrosis, and was omitted from further studies.
Substantial increase of LVEDP during reperfusion after induced global ischaemia may influence postischaemic CF and thus interfere with the development of myocardial infarction. Therefore, we have employed in addition regional ischaemia model, where no large ischaemic contracture occurs.

Microscopic analysis of coronary artery disease

The extension of coronary atherosclerosis was evaluated in hearts from ApoE/LDLr KO mice fed an atherogenic diet for 6, 7, or 8 months. Three sections were obtained from each heart: aortic root, middle part of the heart, and the apex area of the heart. Consecutive sections were stained with Oil Red-O. Counterstaining was performed with hematoxylin. The slides were mounted with Kaiser's glycerol gelatin, and cryosections observed microscopically.

Biochemical markers for oxidative stress

Direct measurement of ROI is methodologically very complicated. Similar to many other studies we have used measurement of biological molecules damaged by ROI (lipid peroxidation products) and/or parameters of antioxidant defense as biochemical indices of oxidative stress.
Oxidative stress markers were evaluated both in serum and myocardial samples. The whole blood was sampled *via* intracardiac puncture after median sternotomy. After centrifugation the serum was stored at –80 °C until analysis. Hearts were freeze-clamped in liquid nitrogen after removal of blood by brief perfusion with Langendorff-apparatus. In serum samples the level of conjugated dienes, lipid hydroperoxides, and antioxidant capacity was assessed, while myocardial tissue was analysed for lipid peroxidation markers (lipid peroxides, lipid hydroperoxides, conjugated dienes), oxidized and total glutathione, and antioxidant enzymes.
Conjugated dienes were evaluated spectrophotometrically according to Recknagel and Glende [1984] with modifications previously described by us [Starkopf *et al.*, 1995], while lipid hydroperoxides were measured by a kit from Kamiya Biomedical Company, Seattle, WA, USA and quantified calorimetrically. Both of these parameters characterize the initial steps in chain-reaction of lipid peroxidation.

In myocardial tissue we measured also the end-products of chain-reaction, malondialdehydes, by a commercially available kit from Oxis International Inc., Portland, USA. In the text, they are referred to as lipid peroxides.

For full characterization of oxidative status the parameters of antioxidant defense were also evaluated. Total antioxidant status, superoxide dismutase all isoenzymes, and glutathione peroxidase activity were measured by the commercially available kits from Randox Laboratories Ltd., Ardmore, United Kingdom, while catalase activity was assessed by the method described by Goth [1991]. Glutathione reductase activity was evaluated by colorimetric assay from Oxis International Inc., Portland, USA, and tissue content of oxidized glutathione and total glutathione were measured according to Bhat and coworkers [1991]. The limitation of the study is that the direct evidence of ROI production cannot be given, even despite of the complex measurements listed above.

Immunoblotting

Possible hyperoxia-induced changes of eNOS, HSP72, and the NFκB inhibitor IκBα were evaluated by immunoblotting. This method provides high specificity of the detected protein signal, and permits quantitative analysis. Cardiac proteins were extracted, fractionated, and electrophoresed on 10% SDS polyacrylamide gels. After transfer to a nitrocellulose membrane, the proteins were detected by specific antibodies and visualized using an alkaline phosphatase conjugate substrate kit. The density of the bands were scanned and measured to quantify differences between groups.

Electrophoretic mobility shift assay (EMSA)

EMSA provides a sensitive method for detection of DNA or RNA binding proteins. It is an established approach for monitoring transcription factors. Activation of NFκB and activating protein 1 in the myocardium was determined by this method. Briefly, nuclear protein extract (16 μg) was incubated with 50,000 cpm of ^{32}P-labeled NFκB probe containing and κB binding site 5'-AGT TGA GGG GAC TTT CCC AGG C-3' or the AP-1 binding site 5' CGC TTG ATG AGT CAG CCC GGA A. Subsequently, DNA-protein complexes were electrophoresed on a 4% polyacrylamide gels. Dried gels were analyzed by autoradiography. For supershift analysis nuclear protein extracts were incubated with specific anti-p50 or anti-c-jun antibodies (Santa Cruz Biotechnology) for 15 minutes prior to the addition of radiolabeled probe. For competition analysis, unlabelled probe in 100-fold excess was added prior to radiolabeled probe.

Statistical methods

Functional data are expressed as mean±standard error of the mean. In Paper I functional data in the table are expressed as mean±standard deviation. Differences in functional recovery were tested by using two-way analysis of variance (ANOVA) with repeated measures on one factor, taking treatment as an independent factor, and time as dependent factor. The treatment-time interaction in ANOVA refers to the statistical test of whether mean profiles for one group are the same as for the other groups. In the case of significant interaction, simple effects, i.e. effects of one factor holding another factor fixed, were examined. Planned comparisons between the groups across factor time were then performed. The p-values were thereafter corrected according to the Bonferroni procedure. Normal probability plots were performed to confirm that the underlying model assumptions were met by the data. In Paper II and IV post hoc tests were applied to verify or falsify apparent differences in comparison between groups.

Comparisons of infarct size were perfomed by either t-test for independent samples, or by one-way ANOVA followed by post hoc test. These tests are mathematically interchangeable.

Differences in biochemical markers of oxidative stress, optical densities of EMSA bands, and vessel reactivity were tested by one-way ANOVA. When significant p-values were calculated, intergroup comparisons were performed with post hoc test. $P<.05$ was considered statistically significant.

SUMMARY OF RESULTS

Pretreatment with hyperoxia - relation to inspired oxygen concentration and duration of hyperoxic exposure in myocardial protection (Paper I)

Exposure of rats or mice *in vivo* to increased oxygen fraction in inspiratory gas induced functional protection of the Langendorff-perfused heart, and reduced the extent of myocardial necrosis after induced global ischaemia and reperfusion. Due to potentially toxic effects of hyperoxia, it is clinically more advantageous and acceptable to utilize as low as possible inspired oxygen fraction, and as short as possible exposure to higher oxygen concentrations as in ambient air. A dose-dependent relationship was found to exist between hyperoxia-induced myocardial protection and inspired oxygen fraction in rats, with a fraction of ≥95% of O_2 as the most efficient. This O_2 concentration was therefore selected for following studies. Furthermore, the protection induced by ≥95% O_2 was dependent on the duration of hyperoxic exposure, indicating a bell-shaped response curve of the exposure duration in both rats and mice. Rats and mice required different treatment times for effect, in rats 60 minutes of hyperoxia was optimal, while 30 minutes of exposure gave optimal protection in mice. In rats, cardioprotection induced by pretreatment with hyperoxia was both immediate, when the sustained ischaemia was induced immediately after pretreatment, and delayed, with sustained ischaemia 24 hours later. However, in mice functional protection could be induced in the immediate model only.

Hyperoxia and oxidative stress. Effect of hyperoxia on activation of HSP72 and eNOS (Paper II)

Exposure of rats to short-term hyperoxia (≥95% O_2) elicited a systemic low-graded oxidative stress, evident as increased serum levels of conjugated dienes and reduced serum antioxidative capacity. Hyperoxia induced both early and delayed functional protection of the isolated rat heart, and reduced infarct size. The systemic oxidative stress induced neither lipid peroxidation, nor largely influenced the activity of antioxidative enzymes in the myocardium. We found no increase of oxidative stress markers neither immediately or 24 hours after 60 minutes exposure to ≥95% O_2. A small increase of glutathione reductase activation does not explain the protection. Other possible mediators of cardioprotection by IPC, HSP of the 70 kDa family and eNOS, were not influenced by exposure to increased oxygen fractions in the delayed model.

Effect of hyperoxia on vessel reactivity. Cardioprotection by hyperoxia after regional ischaemia of the heart (Paper III)

Exposing rats to hyperoxia modified vasomotor response of isolated aortic rings. Hyperoxia *in vivo* increased the relaxation of aortic rings to ACh and SNP, and delayed contraction to Phe. The effect was more evident when the vessels were harvested immediately rather than 24 hours after hyperoxic exposure. The effect of hyperoxia on isolated heart function was also investigated in a regional ischaemia model where no large ischaemic contracture occurs, which potentially might interfere with the development of infarction. In hearts isolated immediately after pretreatment with hyperoxia and subjected to regional ischaemia and reperfusion, infarct size was still profoundly reduced and postischaemic ventricular contractile function improved.

Protection of hearts of severely atherosclerotic mice (Paper IV)

The development and consequences of atherosclerosis in animals have previously been studied in cholesterol-fed rabbits, monkeys, and rats. A limitation of these models is that animals do not develop fibrofatty lesions as compared to humans. The development of recombinant DNA technology has created new animal models of atherosclerosis. One of them is the apolipoprotein E and low density lipoprotein receptor double knockout (ApoE/LDLr KO) mouse, which develops atherosclerosis more similar to humans. The degree of atherosclerosis in ApoE/LDLr KO mice was verified by sectioning hearts, and staining with oil Red-O. The atherosclerotic lesions in the hearts of these animals after 6-8 months on an atherogenic diet were widespread, with fibrofatty lesions and lipid deposits in the thoracic aorta and aortic root, and in the proximal coronary arteries. Atherosclerotic lesions in distal coronary arteries could be observed in the middle and apex regions of the heart. Isolated hearts from animals with severe atherosclerosis had more pronounced ventricular dysfunction during reperfusion after induced global ischaemia than hearts of animals with normal vessels. A larger extent of myocardial necrosis accompanied by higher cardiac troponin T release was also observed compared to hearts of animals with healthy vessels. Classic IPC improved postischaemic ventricular contractile function, and reduced myocardial necrosis in both atherosclerotic and normal mouse hearts. Exposure of animals with severe atherosclerosis to hyperoxia prior to heart excision and perfusion mimicked the beneficial effects of IPC, and was as potent.

Role of NFκB in hyperoxia-induced myocardial protection (Paper V)

Translocation of NFκB was detected in nuclear extracts in hearts from hyperoxic animals. In the lungs sampled serially after different durations of hyperoxic exposure NFκB was activated after two minutes of exposure, and thereafter gradually decreased during 60 minutes of exposure. In the heart activation of NFκB could be seen after 2-5 minutes of exposure, was thereafter reduced, and increased again at the end of 60 minutes of hyperoxia. Pretreatment with the NFκB inhibitors SN50 and pyrrolidine dithiocarbamate (PDTC) prior to exposure to hyperoxia abolished the functional and infarct-limiting protection of hyperoxia. NFκB translocation after hyperoxia was reduced when SN50 was utilized compared to hyperoxia alone, indicating that SN50 inhibited NFκB activation. Hyperoxia reduced NFκB activation in the heart during sustained ischaemia and reperfusion. This might be due to hyperoxia increasing cardiac contents of the NFκB inhibitor IκBα as evaluated by immunoblotting. Administration of SN50 or PDTC during ischaemia and reperfusion to the isolated hearts from normoxic control animals improved postischaemic contractile function and coronary flow, and reduced infarct size. The results demonstrate that hyperoxia elicits myocardial protection through a NFκB-dependent mechanism, and support evidence for a dual role of NFκB in I/R of the heart.

DISCUSSION

The main finding of the present thesis is that exposure of the heart donor to increased oxygen fractions mimicks the beneficial effects of IPC on I/R injury in the isolated, perfused heart from rats and mice. Pretreatment with hyperoxia reduced the incidence of severe reperfusion arrhythmias, improved postischaemic ventricular functional recovery, and reduced the extent of myocardial necrosis. Hyperoxic pretreatment induced functional and infarct-limiting protection also in the hearts with coronary atherosclerosis. Furthermore, exposing rats to hyperoxic conditions modified the vasomotor response of isolated thoracic aortic rings. The underlying mechanisms involve oxidative stress and NFκB activation.

A. THE MODEL

Myocardial protection – ischaemic preconditioning and pretreatment with hyperoxia

In the strictest sense, the end-point of IPC is the delay of lethal injury reducing infarct size [Murry *et al.*, 1986]. However, the evolution of necrosis by IPC is delayed but not prevented [Yellon *et al.*, 1998]. Preconditioning will limit infarct size during temporary coronary occlusion but not during sustained occlusion, and the reduction of infarct size is lost when sustained ischaemia is extended to three hours [Murry *et al.*, 1986]. Like classic IPC, pretreatment of animals with hyperoxia profoundly reduced the extent of myocardial necrosis in both global and regional ischaemia models (Paper I-V).

Reduction of the incidence of reperfusion arrhythmias by IPC has been primarly described in rats *in vivo* [Shiki and Hearse, 1987; Hagar *et al.*, 1991]. Lawson *et al.* [1993] demonstrated preconditioning-induced reduction of postischaemic arrhythmias in isolated blood-perfused rat heart. Preconditioning reduced reperfusion arrhythmias also in globally ischaemic buffer-perfused rat hearts [Takeshima *et al.*, 1997], and as a delayed effect, 24 hours after IPC [Yamashita *et al.*, 1998]. In Paper II we demonstrate that exposing rats to increased inspired oxygen fraction reduced the incidence of severe reperfusion arrhythmias in both immediate and delayed models. However, it is considered that the rat is not a representative model to study I/R arrhythmias as the action potential characteristics of the rat ventricle, and their changes during ischaemia, are different from larger mammals [Dekker, 1998]. In mice we have not found effects of either IPC (Paper IV), or hyperoxia (Paper I) on reperfusion arrhythmias.

It has been demonstrated that IPC improves ventricular contractile function of the isolated blood-perfused [Kolocassides *et al.*, 1996] and the buffer-perfused [Asimakis *et al.*, 1992; Valen *et al.*, 1996; Starkopf *et al.*, 1997; Starkopf *et al.*, 1998] rat heart after sustained global ischaemia. It is currently controversial whether IPC has a direct effect against stunning, or whether improved function is secondary to reduced/delayed necrosis. Probably, improvement of postischaemic function is secondary to the delay of myocardial damage, as infarct size and enzyme leakage have been shown to correlate with the enhancement of functional recovery [Cohen *et al.*, 1991; Jenkins *et al.*, 1995]. We have demonstrated that pretreating animals with hyperoxia induced functional protection and improved coronary flow after ischaemia-reperfusion (Paper I-V).

Protection of the heart with atherosclerotic vessels

Although IPC is a powerful mode of myocardial protection in experimental models, results from clinical studies remain controversial [Zeeuw *et al.*, 1999]. An explanation for that may be that the patients have atherosclerosis, while experimental studies have mostly employed animals with healthy vessels. An extensive research has recently been introduced to study preconditioning in the mouse heart, as genetically engineered mice provide an excellent tool to study underlying mechanisms of any mode of myocardial protection. The beneficial effect of classic IPC on reperfusion arrhythmias [Sakamoto *et al.*, 1999], and postischaemic contractile function [Sumeray and Yellon, 1998] have been also demonstrated in murine heart with normal vessels, while infarct size in these hearts has been shown to be reduced in both the early [Guo *et al.*, 1998; Xi *et al.*, 1998; Miller and Van Winkle, 1999] and late [Guo *et al.*, 1998; Guo *et al.*, 1999] phase of IPC. In Paper IV we demonstrate that subjecting ApoE/LDLr KO mice with severe atherosclerosis to either classic IPC or to increased inspired oxygen fraction induced functional protection and reduced infarct size in the isolated heart after global ischaemia. Hearts of mice with severe atherosclerosis had depressed left ventricular performance during reperfusion compared to mice with healthy vessels, and the functional and infarct-limiting effect of IPC was more evident in hearts from ApoE/LDLr KO mice as compared to healthy animals. A possible mechanism for reduction of postischaemic contractile function in hearts of atherosclerotic mice may be decreased production of nitric oxide, as hearts from ApoE/LDLr KO mice perfused with the nitric oxide donor S-nitrose-N-acetylpenicillamine (SNAP) showed improved function during reperfusion and reduced infarct size [Tokuno *et al.*, 2001]. The fact that hyperoxia protected also these hearts is clinically promising.

Effect of hyperoxia on vessel reactivity

The preconditioning phenomenon has been demonstrated to exist in all organs tested. Several studies have shown that the vasculature can be preconditioned against loss of vasomotor function following local I/R injury [Giannella *et al.*, 1997; Maczewski and Beresewicz, 1998; Merkus *et al.*, 2000]. In Paper III, *in vivo* hyperoxia increased the relaxation of *ex vivo* aortic rings to ACh and SNP, indicating that both endothelium-dependent and independent mechanisms are involved. Pretreatment with hyperoxia also delayed contraction to phenylephrine, indicating α_1 receptor involvement. However, the effect was more evident when the vessels were harvested immediately compared to 24 hours after exposure to increased oxygen fraction. Improved relaxation and delayed contraction of isolated aortic rings may *in vivo* represent improvement of blood flow and oxygen supply. Similar changes in vessel reactivity may also occur in coronary arteries after hyperoxic pretreatment and may theoretically be involved in the beneficial effect of hyperoxia on postischaemic coronary perfusion (Paper I and V). However, in hearts of ApoE/LDLr KO animals CF has been shown to be initially reduced as compared to hearts from healthy animals, and improved only in the presence of nitric oxide donor SNAP during reperfusion [Tokuno *et al.*, 2001]. In aortic rings from mice with severe atherosclerosis and *in vivo* spontaneous ischaemic events relaxation to ACh was unchanged [Tokuno *et al.*, 2002], indicating that in hearts with atherosclerotic vessels hyperoxia-induced protection might involve other mechanisms than protection against loss of vasomotor function. The exact pathway of hyperoxia-induced modification of vasomotor response still remain to be elucidated.

Clinical perspectives

Studies in isolated human ventricular myocytes [Ikonomidis *et al.*, 1994; Arstall *et al.*, 1998] and in isolated atrial trabeculae obtained during surgery [Walker *et al.*, 1995] suggest that preconditioning can be induced *in vitro* in human tissue. Furthermore, in similar *in vitro* models it has been demonstrated that the mechanisms of protection in human tissue are also close to those observed in different animal species, including adenosine as a trigger [Liang, 1996; Ikonomidis *et al.*, 1997], protein kinase C as an intermediate intracellular messenger [Speechly-Dick *et al.*, 1995; Ikonomidis *et al.*, 1997], and the ATP-dependent potassium channel as a potential end-effector [Speechly-Dick *et al.*, 1995; Liang, 1996]. However, ethical considerations restrict experimental work to be carried out on the human heart, and IPC as a mode of myocardial protection has not become generally accepted in clinical practice. Thereby the evidence from experimental studies remain mostly indirect, although evidences have been provided that IPC protects also the human

heart against postischaemic contractile dysfunction [Wu *et al.*, 2000; Wu *et al.*, 2001], and irreversible myocyte injury [Jenkins *et al.*, 1997]. From clinical perspectives, the benefits of natural occurrence of IPC have been demonstrated in follow-up studies suggesting that in patients with preinfarction angina, long-term survival is improved as compared to patients asymptomatic before onset of myocardial infarction [Ishihara *et al.*, 1997; Kloner *et al.*, 1998].

By delaying the development of myocardial necrosis, preconditioning prolongs the time-span during which restoration of blood-flow to ischaemic myocardium can be effectively instituted. Reperfusion of the myocardium will still remain the most effective method to limit infarct size and determine prognosis. The use of pharmacological agents to induce preconditioning-like response and mimick beneficial effect would be desirable. However, the timing of administration of such agents would be a critical limiting factor, as well as the duration of the protection afforded. The model of inducing a preconditioning-like response by increasing the inspired oxygen fraction is attractive in its simplicity and may easily be clinically applicable. Hyperoxia will also influence and potentially "precondition" other organs, thus inducing a "whole body preconditioning." This may have a relevance not only in myocardial protection, but be important in all major surgical procedures by providing increased endogenous cell defense. In fact, increased oxygen fraction in ispired air has been shown to have some important beneficial clinical effects [Greif *et al.*, 1999; Greif *et al.*, 2000; Kotani *et al.*, 2000; Goll *et al.*, 2001].

B. THE MECHANISMS

The potential pathways of hyperoxia-induced myocardial protection is presented in Figure 2.

eNOS and HSP72 as potential mediators of hyperoxia-induced myocardial protection

An important regulator of endothelial functions and vascular tone is nitric oxide, which is synthesized in a reaction catalyzed by nitric oxide synthase, a family of enzymes with three isoforms, neuronal nitric oxide (nNOS), eNOS, and iNOS [Nathan, 1992]. eNOS is present constitutively in the heart [Balligand and Cannon, 1997]. In addition to vasodilatory effect, evidence suggest that nitric oxide may also have antiarrhythmic, antithrombotic, and possible positive inotropic effect [Parrat, 1993; Yan *et al.*, 1996; Bolli *et al.*, 1997]. Experimental studies suggest that nitric oxide has cardioprotective actions. However, overproduction of nitric oxide may be associated also with peroxynitrate formation. Although previously thought to be a predominantly constitutive enzyme, eNOS turns out to be rapidly inducible by a range of stimuli

[Förstermann *et al.*, 1998]. eNOS is also induced by IPC [Xuan *et al.*, 2000], and mediates delayed adenosin 1 receptor-triggered preconditioning [Bell *et al.*, 2002]. Thus, increased expression of eNOS in the heart may be beneficial.

Other possible mediators, in particular of the delayed phase of protection, are HSP of the 70 kDa family consisting of the inducible HSP (HSP72) and the constitutive HSP (HSP73) [Marber *et al.*, 1993]. It has been demonstrated that oxidative stress directly increases HSP [Kukreja *et al.*, 1994]. HSP have cardioprotective properties, acting as molecular "chaperons" in removal of misfolded proteins and in re-establishment of normal protein synthesis [Latchman, 2001]. Induction of HSP by hyperthermia or IPC reduces infarct size after I/R injury [Donnelly *et al.*, 1992; Marber *et al.*, 1993], decreases leakage of lactate dehydrogenase [Tekin *et al.*, 2001], and enhances postischaemic ventricular contractile function [Currie *et al.*, 1988; Amrani *et al.*, 1993; Tekin *et al.*, 2001]. Furthermore, transgenic mice overexpressing HSP70 [Marber *et al.*, 1995; Hutter *et al.*, 1996;], and rats transfected *in vivo* with HSP70 [Suzuki *et al.*, 1997] have improved heart function when subjected to ischaemia and reperfusion. However, induction of HSP70 in the myocardium is not consistently associated with myocardial protection [Tanaka *et al.*, 1994; Saganek *et al.*, 1997; Xi *et al.*, 1998; Kukreja *et al.*, 1999].

Valen *et al* [2000] have demonstrated that both eNOS and HSP72 are increased in hearts of patients with unstable angina, who appear to have a preconditioning-like state prior to myocardial infarction [Ottani *et al.*, 1995; Kloner *et al.*, 1995].

The time span between pretreatment of animals with hyperoxia might have been too short to induce protein synthesis. However, cardiac contents of HSP72 or eNOS evaluated by immunoblotting of tissue harvested immediately or 24 hours after exposure to increased oxygen fractions were not increased. Thus, neither HSP72 nor eNOS appear to be mediators of the hyperoxia-induced protective response.

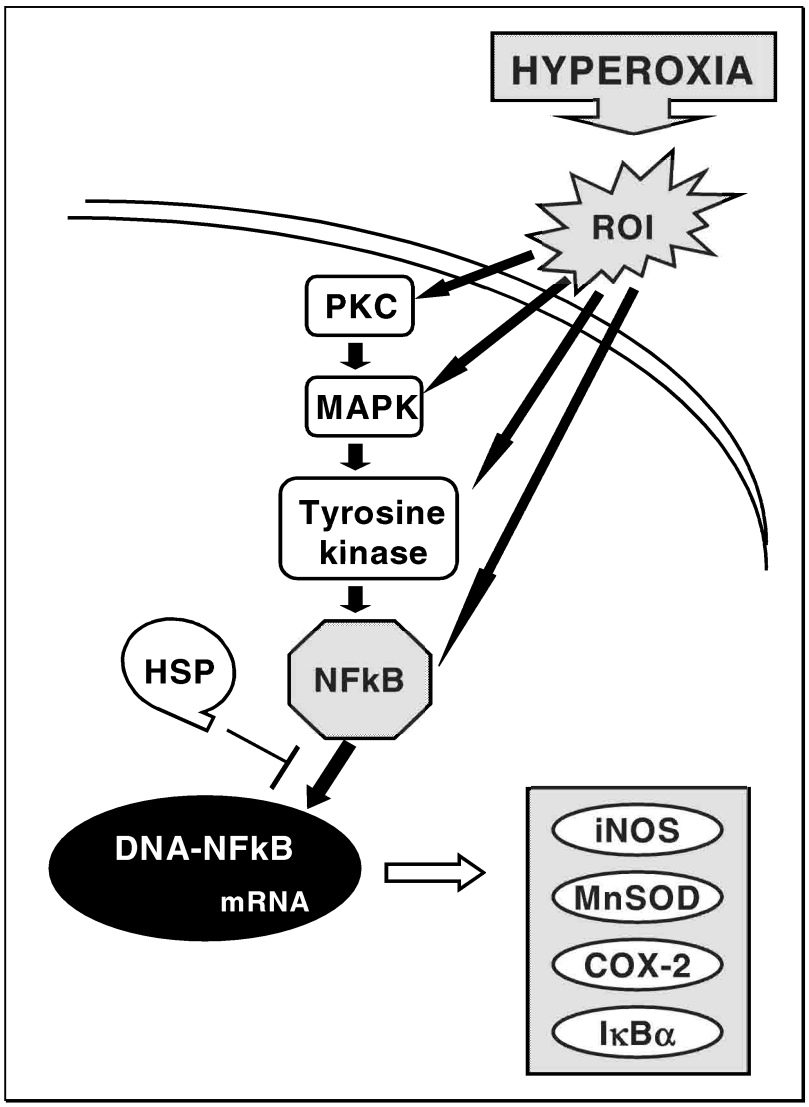

Figure 2. *Schematic presentation of hypothesis in cellular pathways for hyperoxia-induced myocardial protection. Exposure to increased oxygen fractions (HYPEROXIA) elicits induction of reactive oxygen intermediates (ROI), which activate signal transduction cascade involving protein kinase C (PKC), mitogen activated protein kinases (MAPK), and tyrosine kinases. In turn of that transcription factor nuclear factor kappa-B (NFκB) is transactivated, and gene transcription induced. Potentially tissue protective genes regulated by NFκB include inducible nitric oxide synthase (iNOS), manganese superoxide dismutase (MnSOD), and inducible cyclooxygenase (COX-2). Activation of NFκB by hyperoxia may upregulate IκBα, and thereby reduce inflammation during sustained ischaemia by inhibiting NFκB activation. Heat shock proteins (HSP), suggested as mediators of ischaemic preconditioning, may prevent translocation of NFκB to the cell nucleus by binding NFκB in the cytosol.*

Is oxidative stress involved?

Increased serum levels of conjugated dienes and reduced antioxidative capacity demonstrate that systemic oxidative stress occured after *in vivo* exposure to increased oxygen fractions (Paper II). The changes in oxidative stress markers were time-dependent, with significant alterations in conjugated dienes after one-hour of exposure, but normalized after three hours. At that time point serum antioxidative capacity was significantly reduced. This is in accordance with studies showing that normobaric hyperoxia generates ROI and induces lipid peroxidation in both cell cultures and organs [Gille and Joenje, 1992; Ahotupa *et al.*, 1992]. The benefit from systemic oxidative stress induced by hyperoxia could have been an increase in myocardial antioxidant activity, which has been a suggested end-effector of IPC [Zhou *et al.*, 1996]. The systemic oxidative stress, however, neither induced lipid peroxidation nor to a large extent influenced activity of antioxidative enzymes in the myocardial tissue. After sixty minutes of hyperoxia only reduced glutathione activity was increased in the myocardium. This activity might have been upregulated in response to temporarily increased oxidized glutathione in the conditions of systemic oxidative stress. As a result, oxidized glutathione might have been rapidly transformed back to reduced form, and no differences in the oxidized/reduced glutathione ratio could be detected at that time. The time of hyperoxic exposure, in this case 1 and 3 hours, may have been too short to induce the activation of enzymes, as longer time of hyperoxia has been indicated to induce an increase in antioxidant defense [Stock *et al.*, 1990; Ho *et al.*, 1996; Jornot and Junod, 1997; Van Golde *et al.*, 1998].

Evidence for oxidative stress in the present study is demonstrated by indirect measurements. To measure ROI directly is complicated. Therefore, the origin of ROI induced by increased inspiratory oxygen fraction remains unknown. We showed that although oxidative stress is part of the hyperoxia response, antioxidants are not organ effectors of protection.

A role for NFκB in hyperoxia-induced myocardial protection

NFκB is activated by hyperoxia in cultured human alveolar epithelial cells [Li *et al.*, 1997]. Hyperoxia induces oxidative stress in cell cultures [Gille and Joenje, 1992], and exogenous ROI can mediate the activation of NFκB [Schreck *et al.*, 1991]. Furthermore, antioxidants can prevent NFκB activation induced not only by ROI, but also by stimuli such as inflammatory cytokines, mitogens, and certain drugs [Schreck *et al.*, 1992]. In Paper V we demonstrate that after exposure to increased oxygen fractions NFκB and activator protein 1 were activated in the myocardial tissue, and inhibition of NFκB activation by two different pharmacological agents, SN50 and

PDTC, abolished the beneficial effects of hyperoxia. NFκB becomes activated in a time-dependent manner starting in the lungs, and rapidly followed by activation in the hearts during hyperoxic exposure. In the lungs the activation gradually decreased, while in the heart it followed a biphasic pattern with a second peak after 20-60 minutes of exposure. NFκB is activated also by IPC, and this activation is associated with the beneficial effcts of IPC. Maulik *et al.* [1998] demonstrated that IPC of isolated rat heart induced translocation of NFκB, and SN50 inhibited the achieved cardioprotection. Similarly, Morgan *et al.* [1999] inhibited preconditioning in rabbits by employing the NFκB-inhibitor ProDTC. Xuan *et al.* [1999] showed that even the delayed cardioprotection by IPC was abolished by pharmacological inhibition of NFκB.

NFκB and AP-1 are activated also during prolonged ischaemia and reperfusion of the heart [Chandrasekar and Freeman, 1997; Li *et al.*, 1999], and appear to play a detrimental role as its inhibition with non-sense decoy oligos improves functional recovery and reduces infarct size [Morishita *et al.*, 1997; Sawa *et al.*, 1997]. These results are supported by our findings of a reduction of myocardial necrosis, and imrovement of postischaemic contractile function when the inhibitors were administered during ischaemia and reperfusion.

The beneficial effects of NFκB activation by hyperoxia or IPC could be related to upregulation of the rapidly inducible IκBα, thereby reducing inflammation during sustained ischaemia by inhibiting NFκB activation [Lawrence *et al.*, 2001]. Hearts from animals exposed to hyperoxia had less NFκB activation during Langendorff-perfusion, induced global ischaemia and reperfusion than hearts from normoxic control animals. Furthermore, the NFκB inhibitor IκBα, which is transcriptionally induced by NFκB activation, was downregulated in hyperoxic hearts during sustained ischaemia, indicating partly an antiinflammatory effect of NFκB activation during adaptation to ischaemia. The beneficial effect of hyperoxia, however, could also be due to transcription of a NFκB-regulated, beneficial gene [Valen *et al.*, 2001].

CONCLUSIONS

1. Exposure of donor animals (rats and mice) to short-term increased oxygen fraction in inspired gas reduced the incidence of severe reperfusion arrhythmias, induced functional protection, and limited the extent of myocardial necrosis in isolated hearts after sustained global or regional ischaemia. The obtained cardioprotection was dependent on oxygen concentration and duration of exposure. Pretreatment with hyperoxia induced both early and delayed myocardial protection, but this response was species-dependent.

2. Exposure of rats to increased inspiratory oxygen fraction elicited a systemic low-graded oxidative stress. Hyperoxia neither induced lipid peroxidation, nor significantly influence the activity of antioxidative enzymes in the myocardium. Other potential mediators of protection, HSP72 and eNOS, were not influenced by exposure to hyperoxia.

3. Pretreatment with hyperoxia modified the vasomotor response of isolated aortic rings Hyperoxia *in vivo* increased the *in vitro* relaxation of aortic rings to ACh and SNP, and delayed the contraction to Phe. However, the effect was more evident when the vessels were harvested immediately rather than 24 hours after exposure to increased inspiratory oxygen fraction.

4. Isolated hearts from animals with severe atherosclerosis (ApoE/LDLr KO mice) had more pronounced postischaemic ventricular dysfunction, and larger extent of myocardial necrosis compared to hearts from healthy animals. Classic IPC improved heart function and reduced infarct size in hearts from ApoE/LDLr NO animals. Exposing atherosclerotic animals to hyperoxia mimicked the beneficial effects of IPC.

5. Exposure to short-term hyperoxia protects heart function and preserves cell viability through a NFκB-dependent mechanism. Hyperoxia activated pulmonary and myocardial NFκB. Pretreatment with NFκB-inhibitors prior to hyperoxia abolished the functional and infarct-limiting protection of hyperoxia. Hyperoxia reduced NFκB activation during sustained ischaemia and reperfusion, and increased cytoplasmatic inhibitory IκBα. Administration of NFκB-inhibitors during ischaemia and reperfusion to the hearts from normoxic animals improved heart function and reduced infarct size. These results support evidence for a dual role of NFκB in the heart – beneficial during adaptation and detrimental during sustained ischaemia and reperfusion.

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