



**OXIDATIVE STRESS AND
ISCHAEMIA-REPERFUSION
OF THE HEART**

A clinical and experimental study

JOEL STARKOPF

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*In memory of
professor Lembit Tähepõld*

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred in the text by their Roman numerals:

- I Starkopf J, Zilmer K, Vihalemm T, Kullisaar T, Zilmer M, Samarütel J. Time course of oxidative stress during open-heart surgery. *Scand J Thorac Cardiovasc Surg*, 29(4): 181–186, 1995.
- II Starkopf J, Tamme K, Zilmer M, Talvik R, Samarütel J. The evidence of oxidative stress in cardiac surgery and septic patients — a comparative study. *Clinica Chimica Acta*, 262: 77–88, 1997.
- III Starkopf J, Bugge E, Ytrehus K. Preischemic bradykinin and ischemic preconditioning in functional and metabolic recovery of globally ischemic rat heart. *Cardiovasc Res*, 33: 63–70, 1997.
- IV Starkopf J, Andreassen T, Bugge E, Ytrehus K. Lipid peroxidation, arachidonic acid and products of the lipoxygenase pathway in ischaemic preconditioning of rat heart. *Cardiovasc Res*, accepted for publication, 1997.
- V Valen G, Starkopf J, Takeshima S, Kullisaar T, Vihalemm T, Kengsepp A-T, Löwbeer C, Vaage J, Zilmer M. Preconditioning with hydrogen peroxide (H₂O₂) or ischemia in H₂O₂-induced cardiac dysfunction. Submitted for publication.

ABBREVIATIONS

+dP/dt	positive derivative of developed pressure
-dP/dt	negative derivative of developed pressure
5HETE	5-hydroxy-eicosatetraenoic acid
AOC	antioxidative capacity
ATP	adenosine triphosphate
Ca-ATPase	calcium adenosine triphosphatase, calcium pump
CAT	catalase
CK	creatine kinase
CP	creatine phosphate
CPB	cardiopulmonary bypass
DAG	1,2-diacylglycerol
DC	diene conjugates
DNA	desoxyribonucleic acid
Fe-TBARS	iron stimulated thiobarbituric acid reactive substances
GSH	reduced glutathione
GSH-Px	glutathione peroxidase
GSSG	oxidized glutathione
HETE	hydroxy-eicosatetraenoic acid
HPETE	hydroxy-peroxy-eicosatetraenoic acid
HPLC	high pressure liquid chromatography
IP ₃	inositol-trisphosphate
LVDP	left ventricular developed pressure
LVEDP	left ventricular end diastolic pressure
LVSP	left ventricular systolic pressure
Na,K-ATPase	sodium, potassium adenosine triphosphatase, sodium-pump
NADH	nicotinamide dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NDGA	nordihydroguaiaretic acid
NO	nitric oxide
NYHA	New York Heart Association
PKC	protein kinase C
PLA ₂	phospholipase A ₂
PUFA	polyunsaturated fatty acid
RBC-GSH	red blood cell glutathione content
ROS	reactive oxygen species
TAS	total antioxidant status
TBARS	thiobarbituric acid reactive substances
TTC	triphenyl-tetrazolium chloride

1. INTRODUCTION

Discovery of oxygen by Joseph Priestley in 18th century fundamented our understanding that oxygen is essential for human life. Prolonged deficit of oxygen, in whatever tissue it would occur, has always harmful consequences. The situation in which oxygen supply to the heart is inadequate to cover tissue oxygen demand is defined as myocardial ischaemia, resulting in some cases with myocardial infarction. The main cause for reduced oxygen supply is impaired coronary blood flow due to stenosis or occlusion of coronary arteries, i.e. coronary artery disease. Modern treatments such as fibrinolytic therapy, percutaneous transluminal angioplasty and coronary artery bypass surgery, have made possible the reopening of occluded coronary arteries and thus the reperfusion of ischaemic myocardium. However, the coronary artery disease has remained one of the major sources of morbidity and mortality in developed countries.

Studies on oxygen biochemistry and reperfusion related events have opened a new, harmful facet of oxygen. Reintroduction of molecular oxygen into ischaemic tissue, a key event in reperfusion, is accompanied with uncontrolled generation of highly toxic oxygen intermediates, reactive oxygen species (ROS). As a result of this, the reperfused tissue is exposed to oxidative stress. ROS have been suggested to contribute to different forms of tissue injury, however, the precise role for oxidative stress in ischaemia-reperfusion of the heart is far from clarified.

2. REVIEW OF THE LITERATURE

2.1. Oxidative stress

What is oxidative stress?

The appearance of oxygen into Earth's atmosphere about 3 million years ago is supposed to have led to the death of many organisms living under anaerobic conditions up to that time. Oxygen, however, provided opportunity for oxidative metabolism, and this gave an enormous metabolic advantage to the aerobic organisms. The combustion of one molecule of glucose under aerobic conditions yields 38 molecules of ATP, which is as much as 19 times more than what is available from anaerobic glycolysis. As a price for this, surviving organisms were obliged to develop antioxidant systems to control the toxicity of oxygen. Toxicity of oxygen results from oxygen's ability to generate reactive, partly reduced intermediates, reactive oxygen species (ROS), notably free radicals. Although controlled ROS production has some importance in the physiology of aerobic organisms, there exists a dynamic balance between the active, pro-oxidant intermediates and antioxidant defence system during normal, healthy state (Kehrer, 1993; Zilmer, 1994; Halliwell, 1996). In certain situations, such a balance becomes disturbed in favour of the pro-oxidants. Thus, oxidative stress could be defined as situation of profound disturbance in the pro-oxidant — antioxidant balance in favour of the former. Prolonged oxidative stress may lead to tissue damage, in whatever site of the organism such a situation developed (Kehrer, 1993; Zilmer, 1994; Halliwell, 1996; Sies, 1997).

Formation of reactive oxygen species (ROS)

Under normal conditions most molecular oxygen in biosystems undergoes tetravalent reduction by efficient intracellular systems, such as the respiratory chain, and H₂O is produced. However, 3–5% escapes this pathway through other routes, and this results in formation of reactive oxygen species (ROS) (Gutteridge, Halliwell, 1994). ROS are atoms, ions, or molecules with one or more electrons in unpaired spin, which highly increases their reactivity. ROS may have either free radical nature (superoxide anion, hydroxyl radical, peroxy and alkoxy radicals) or not (hydrogen peroxide, singlet oxygen, hypochlorous acid) (Kehrer, 1993). All aerobic cells, and therefore also cardiomyocytes and endothelial cells, are capable to produce ROS at certain degree. Numerous intracellular sources of ROS have been identified. These include the mitochondrial electron transport chain, the action of various soluble or membrane-bound enzymes (e.g. xanthine oxidase, lipoxygenase, prostaglandin synthetase), as well as chemical reactions involving transition metals (Kehrer, 1993; Curello *et al.* 1995a).

Consequences of ROS production

From *in vitro* studies it is well demonstrated that ROS are highly cytotoxic agents (Romaschin *et al.* 1990; Ytrehus *et al.* 1991; Kuzuya *et al.* 1993). ROS are able to initiate a lipid peroxidation processes, but also inactivate proteins, to promote DNA injury, and to degrade carbohydrates (Ytrehus *et al.* 1991; Coetzee *et al.* 1994). With respect to cell membrane, peroxidation of lipids cause increase in membrane fluidity. This, in turn, leads to increased permeability and loss of membrane integrity, which results in severe disturbances of cellular homeostasis (Ytrehus *et al.* 1991). ROS promote haemolysis and oxidize lipoproteins in the blood (Das *et al.* 1992; Reaven *et al.* 1996). Overall, under conditions of oxidative stress all types of biomolecules could be targeted by ROS attack.

Protection against ROS

Protection against ROS is offered by the complex and integrated action of antioxidants and scavengers. The scavengers can be divided into enzymatic and non-enzymatic. Intracellularly the enzymatic defence predominates (Halliwell, 1996). The most important scavenging enzymes are probably superoxide dismutases (SOD) which catalyse the dismutation of superoxide to hydrogen peroxide, H_2O_2 (Kehrer, 1993; Halliwell, 1996). H_2O_2 is further enzymatically scavenged by catalase and glutathione peroxidase. The latter enzyme works in tight connection with glutathione (GSH), which is one of the main non-enzymatic intracellular scavengers (Zilmer, 1994). In the extracellular space, the protection of biomolecules against ROS is provided by simultaneous action of non-enzymatic scavengers and antioxidants like ascorbate, α -tocopherol, carotenoids, metal binding proteins transferrin and ceruloplasmin, and also by free sulphhydryl groups in proteins (Halliwell *et al.* 1990; Kehrer, 1993). Independent of their intra- or extracellular presence, the antioxidant system is best characterized by its overwhelming complexity (Zilmer, 1994; Pincemail *et al.* 1996). Due to this, relative lack of one component of this system does not always result in changes in total antioxidant capacity of an organism, and has often minor (patho)physiological consequences. On the contrary, decrease in total antioxidant capacity could deserve specific attention. In this case the balance between pro- and antioxidants is shifted towards the former, and, consequently, oxidative stress and possible tissue damage would be promoted.

Oxidative stress and diseases

Up today, an increased level of ROS has been suggested in more than 100 pathophysiological conditions (Lunec, 1990; Kehrer, 1993; Zilmer, 1994). With respect to cardiovascular disorders it is widely believed that they contribute to the aetiology of a variety of conditions including reperfusion-induced arrhythmias, postischaemic reperfusion injury, myocardial stunning, as well as inflammation, atherosclerosis and even anthracycline drug-induced toxicity

(Naylor, 1991; Opie, 1991; Ceriana, 1992; Hudson, 1994; Reaven *et al.* 1996). ROS are also implicated in the aetiology of diseases which range from general inflammatory responses to retinopathia and cancer (Lunec, 1990; Kehrer, 1993). Such wide list of diseases arises the question if these different disorders could have partly common in their pathophysiology. Therefore, it is important to note that although ROS may initiate a series of damaging biochemical events, they are not necessarily directly responsible for the final tissue dysfunction.

Does ROS have always harmful effects?

It is reasonable to believe that if ROS are continuously generated in the organism they may participate in various processes in normal state of health. Indeed, ROS play also an important physiological role. Killing of bacteria by granulocytes and macrophages is mediated by the “oxidative burst” from oxygen radical producing enzyme system named NADPH oxidase, located in the membrane of these cells (Chambers *et al.* 1987; Redl *et al.* 1993). The regulation of blood pressure is to a great extent carried out by nitric oxide, and this compound (known also as reactive nitrogen species — RNS) has been demonstrated to have free radical properties (Freeman, 1994; Maulik *et al.* 1995). ROS are believed to be implicated in “programmed cell death” — apoptosis (Thompson, 1995), a process which has deserved a great attention during the last decade. More recently, a role for ROS as important intracellular signalling molecules, regulating several cellular processes, has been proposed (Schilling *et al.* 1992; Maulik *et al.* 1995). The activity of several intracellular enzymes, for example, is supposed to be controlled to some extent by ROS (Meij *et al.* 1994). From this point of view, it is essential to recognize, that the role of the antioxidant systems in the organism is not to restrain but to control the ROS mediated reactions.

2.2. The heart. Ischaemia-reperfusion injury and oxidative stress

What is ischaemia?

The term “ischaemia” was first proposed by Rudolf Virchow in 19th century and is conventionally defined as lack of blood in a tissue (Virchow, 1858). In modern heart research, myocardial ischaemia could be defined in two, slightly different ways. Biochemically ischaemia is defined as a condition in which coronary blood flow is inadequate to maintain a steady state metabolism. Physiologically ischaemia is a condition in which coronary flow is inadequate to permit the heart to perform at a level, sufficient to support the body over its full physiological range of activity (Hearse, 1994).

The nature of the ischaemic process in the heart

Immediately following the onset of ischaemia (i.e. within a few seconds or a few beats in severe ischaemia) there is a precipitous decline of contractile activity. Although the contractile failure is substantial and rapid, it is not complete and mechanical activity may persist for some time (Katz, 1973; Sonneblick *et al.* 1976; Hearse, 1987).

On the cellular level, the lack of oxygen supply leads to an almost immediate cease in aerobic metabolism (Hearse, 1987; Opie, 1991; Opie, 1992b). At this stage, anaerobic glycolysis with formation of lactate will be the main source for ATP formation. The continuous myocardial energy demand is not, however, sufficiently covered, and cellular ATP level falls rapidly (Opie, 1992b). The increased levels of protons, lactate, and other metabolic products resulting from the reduced wash-out will inhibit anaerobic glycolysis, and this leads to further decline in ATP levels. As a result of energy shortage, efficiency of cellular membrane ion-pumps (Na,K-ATPase, Ca-ATPase) is reduced, and cellular accumulation of sodium, chloride and water occurs. In later stage, this is followed by increase in intracellular calcium concentration (Nayler, 1991; Elliott *et al.* 1992; Altschuld, 1996). Once severe calcium overload takes place, a further series of events will follow, including hypercontracture, inhibition of mitochondrial metabolism, progressive paralysis of ion pumps, and formation of intracellular oedema. All these events and activation of degrading lysosomal enzymes predispose to sarcolemmal rupture, the actual phenomenon that clearly signals irreversible cell death (Opie, 1993; Ytrehus *et al.* 1993).

The exact point in the cascade, where reversible injury overcomes into irreversible injury, is not known, even despite of extensive research during the last decades. A number of ways have been discovered for how to protect the heart against ischaemic injury, and these include hypothermia, cardioplegic arrest, pre-ischaemic administration of Ca-antagonists or beta-blockers, and most recently, ischaemic preconditioning. However, whatever manipulation is employed with the purposes of reducing ischaemic injury, it can only delay but not disrupt the chain of events in ischaemia. In other words, the ultimate way to avoid ischaemic damage would be early reperfusion.

Two facets of reperfusion

Despite this obvious observation, that early reperfusion is the only essential way for cells to survive from ischaemic injury, there has been concern that the act of reperfusion itself might have some deleterious effects on the myocardium. The potential for reperfusion-induced injury was first recognized in 1970s when hearts that failed immediately after bypass operation were studied (Bulkley *et al.* 1977). During same years Hearse and co-workers studied the effects of anoxia on isolated rat hearts, and found that reoxygenation resulted in ultrastructural damage to the myocardium. The phenomenon was called "oxygen paradox" (Hearse *et al.* 1978). The concept of reperfusion injury was fully

developed in 1983 when Rozenkrantz and Buckberg defined it as “those metabolic, functional, and structural consequences of restoring coronary arterial flow that can be avoided or reversed by modification of the conditions of reperfusion” (Rosenkranz *et al.* 1983).

The role of ROS at reperfusion

Intrinsic to the concept of reperfusion injury is the concept that certain harmful substances, formed at reperfusion, may cause cell damage. ROS, including free radicals, have been implicated as a main cause of this damage (Opie, 1992b; Hearse, 1992b; Ferrari *et al.* 1993). Experimental studies have demonstrated that reperfusion of ischaemic or hypoxic hearts results in an explosive release of ROS. Most convincing evidence, supporting ROS production at reperfusion comes from Bolli's group, who have used spin-trap and electron-paramagnetic resonance spectroscopy for direct detection of ROS in a model of regional ischaemia in open chest dogs. They found that reflow after 15 minutes coronary occlusion resulted in dramatic increase of ROS production, peaking at 2 to 4 minutes of reperfusion (Bolli *et al.* 1989). After the initial burst, production of ROS abated but did not cease, persisting up to 3 hours after onset of reperfusion. Similar findings have been demonstrated also in isolated rat hearts (Hearse, 1992b). Later studies demonstrated that postischaemic myocardial dysfunction is correlated with the extent of ROS production, which further stresses the critical role of ROS in reperfusion injury (Opie, 1992a). However, the precise role for ROS, produced at reperfusion, remains unresolved.

Most of the studies have suggested that peroxidation of polyunsaturated fatty acids (PUFAs) contained in cellular membranes is a likely mechanism of reperfusion-induced injury (van Bilsen *et al.* 1989; Romaschin *et al.* 1990; Ytrehus *et al.* 1991; Massey *et al.* 1993). Damage to membrane lipids would impair membrane selectivity and interfere with function of various cellular organelles, resulting in disturbances of transmembrane ion balance and thereby leading to ultimate injury. Romaschin *et al.* observed increased myocardial concentration of hydroxy conjugated dienes, products of PUFAs oxidation, during and after 45 minutes period of global normothermic ischaemia in open chest dogs (Romaschin *et al.* 1987). Importantly, the tissue examined after reperfusion was dysfunctional but not necrotic, thus representing stunned myocardium. It has been reported that in patients undergoing cardioplegic arrest during coronary artery bypass surgery, there was myocardial release of conjugated dienes in the coronary blood at 3 and 60 minutes of reperfusion, which was associated with decrease in the myocardial concentration of the antioxidant, α -tocopherol (Wechsler *et al.* 1993). However, evidence for lipid peroxidation due to ROS generation at reperfusion after shorter ischaemia (10 to 15 minutes of duration) is somewhat lacking.

Another theory to explain possible ROS mediated injury is that radical-mediated changes in proteins play prominent role in reperfusion induced injury

(Hearse, 1992a). Oxidative damage of membrane proteins, which are responsible for controlling ionic movement, may be ultimately responsible for the rapid ionic disturbances (cellular calcium overload in particular) that characterise reperfusion. The activity of many enzymes and proteins (e.g. pyruvate kinase, adenyl and guanyl cyclase, protein kinases and hexokinase) is controlled by the redox state of one or more thiol groups at the active centre (Meij *et al.* 1994). Also, several ion translocating proteins (sarcolemmal Na/Ca exchange, Na/K-ATPase and Ca-ATPase) are thought to be thiol-regulated (Tani, 1990; Coetzee *et al.* 1994). Oxidation of thiol groups by ROS can lead to activation or inactivation of these proteins and thereby greatly affect the cellular ionic homeostasis. From this point of view, the role of intracellular glutathione deserves specific attention. Under normal conditions, the protein SH groups are mostly kept in reduced state, and this is guaranteed by the high intracellular concentrations of reduced glutathione (GSH), and the low concentration of oxidized glutathione (GSSG) (Kehrer, 1993; Opie, 1992b). During oxidative stress at reperfusion, the GSH/GSSG ratio is changed (Ceconi *et al.* 1988; Ferrari *et al.* 1993; Curello *et al.* 1995b). If the GSH decreases to critical level the extent of protein SH-group oxidation would be expected to increase, and the activity of numerous enzymes and proteins is altered (Opie, 1992a). In addition, it has been reported that higher levels of GSSG *per se* may be toxic for the cells (Henning *et al.*, 1996).

Thus, the question whether it is oxidative damage to lipids or to proteins, which is crucial in reperfusion injury, remains far from clarified. There is some evidence indicating that it could depend on the severity of the ischaemic insult. For the less severe and acute consequences of ischaemia-reperfusion (e.g. reperfusion arrhythmias, myocardial stunning) protein oxidation and subsequent disturbances in cellular ionic homeostasis could be critical. Uncontrolled peroxidation of membrane lipids, in turn, could be implicated in disruption of sarcolemmal membranes and ultimate cell death, presumably occurring in both lethal reperfusion injury and in accelerated necrosis due to reperfusion.

Pathophysiological manifestations of ischaemia-reperfusion

The consequences of ischaemia-reperfusion are tightly connected to the duration of the ischaemic period. Thus, reperfusion after short lasting ischaemia (5 to 10 minutes) may even increase the tolerance of the heart to a subsequent ischaemic insult, phenomenon described as *ischaemic preconditioning* (Lawson *et al.* 1993, see next chapter). Opposite, prolonged ischaemia (with or without reperfusion) causes irreversible changes, with the development of cell death and necrosis or *myocardial infarction*. Four types of injury have been related to the reperfusion component in the ischaemia-reperfusion (Hearse, 1992b; Opie, 1992b; Kloner, 1993). These include *reperfusion arrhythmias*, *vascular reperfusion injury*, *myocardial stunning*, and *lethal reperfusion injury*. Reperfusion arrhythmias include ventricular arrhythmias such as ventricular tachycardia and

fibrillation that occur within seconds to minutes of reperfusion after brief periods (5 to 15 minutes) of ischaemia. Vascular reperfusion injury refers to the progressive damage to the vasculature, resulting in an expanding zone of no reflow and deterioration of coronary flow reserve during the phase of reperfusion. Lethal reperfusion injury is defined as myocardial cell death due to reperfusion. Myocardial cells, reversibly injured at the end of a period of ischaemia, become irreversibly injured by reperfusion itself. This form of reperfusion injury remains controversial, with some investigators attributing significant amounts of myocardial cell death to it and others attributing little or no myocyte death on reperfusion.

Myocardial stunning

Myocardial stunning is defined as prolonged (lasting hours, days, and even weeks) but completely reversible contractile dysfunction of the heart encountered during reperfusion after a short period of ischaemia (Opie, 1992b; Hearse, 1992a; Jeroudi *et al.* 1994). There is substantial evidence suggesting that myocardial stunning occurs in humans, including in patients after cardiopulmonary bypass, during percutaneous transluminal coronary angioplasty and also after thrombolysis (Bolli *et al.* 1992; Kloner, 1993). Thus, in certain conditions this phenomenon could have important clinical implications.

This state of temporary contractile dysfunction is not accompanied by ultrastructural damage, severe electrophysiological disturbances or persistent energy depletion (Bolli, 1992; Jeroudi *et al.* 1994). The stunned heart, importantly, responds to inotropic stimuli and therefore has contractile (and metabolic) reserve. A likely cause of the contractile dysfunction is sustained increase in cytosolic Ca^{2+} , which desensitizes the myofibrils to Ca^{2+} (Nayler, 1991; Opie, 1991; Hearse, 1992a). The raised cytosolic Ca^{2+} is a consequence of altered sarcolemmal or sarcoplasmic reticulum function, which, in turn, is suggested to be caused by ROS mediated injury at reperfusion. Evidence supporting the role of ROS in myocardial stunning is compelling. First, exogenously administered ROS produces measurable contractile dysfunction similar to stunned heart (Ytrehus *et al.* 1987; Steare *et al.* 1994; Skjelbakken *et al.* 1996). Second, infused antioxidants have improved contractile function upon reperfusion after reversible ischaemia. R. Bolli *et al.* have demonstrated that intracoronary infusion of mercaptopropionyl glycine, when administered just 1 minute before reperfusion, improved left ventricular wall thickening in a canine model of 15 minutes of ischaemia and reperfusion (Bolli *et al.* 1989). Ferrari and co-workers have administered another antioxidant, N-acetylcysteine into the pulmonary artery of patients undergoing coronary artery bypass surgery, and found that such pretreatment reduced the release of oxidized glutathione into coronary sinus as well as resulted in more rapid postoperative recovery of cardiac function (Ferrari *et al.* 1991). Finally, the direct de-

monstration of ROS at the onset of reperfusion strongly supports the role of ROS in myocardial stunning (Garlick *et al.* 1987; Bolli *et al.* 1989).

Oxidative stress, open heart surgery and cardiopulmonary bypass

With respect to modern cardiac surgery four main components are recognized to contribute to formation of perioperative myocardial injury (Wechsler *et al.* 1993). Any myocardial damage after cardiac surgery is thought to be equally related to myocardial ischaemia, reperfusion, cardiopulmonary bypass (CPB), and, perhaps most importantly, to technical skills of the surgeons. Myocardial ischaemia-reperfusion injury, as discussed above, is shown to be associated with ROS generation and oxidative stress. Cardiopulmonary bypass, an essential component of most cardiac operations, has, however, harmful side-effects. In early 1990s, Kirklin formulated the hypothesis of the “whole body inflammatory response” stating that the deleterious effects of CPB were secondary to the exposure of blood to abnormal surfaces in the bypass circuit (Kirklin, 1989). This response is characterized by activation of coagulation, fibrinolysis, complement and the kallikrein system, all of which are now recognized as mediators of the disseminated intravascular post-pump syndrome (Allen *et al.* 1995; Boyle, Jr. *et al.* 1997). Further work has identified the presence of circulating pro-inflammatory cytokines, also observed in the systemic inflammatory response syndrome associated with shock and sepsis (Carrico *et al.* 1986; Beal *et al.* 1994; Thompson *et al.* 1996). The end result of the humoral cascading that is initiated by CPB includes widespread endothelial cell activation, which, in turn, likely results in the diffuse expression of leukocyte adhesion molecules on the surface of vascular endothelial cells (Hachida *et al.* 1995; Levy, 1996). Once adherent to the endothelium, neutrophils release cytotoxic proteases and ROS, which are supposed to be responsible for much of the end-organ damage seen after cardiac surgery (Mair *et al.* 1995; Menasche, 1995; Boyle, Jr. *et al.* 1997). Thus, oxidative stress may have a versatile role in perioperative injury of the heart. Oxidative stress during cardiac surgery might also promote injury of other organs like lungs, liver, and kidneys. In the present thesis the time-course and extent of oxidative stress in patients undergoing open heart surgery has been assessed in order to characterize this phenomenon.

2.3. Myocardial protection by ischaemic preconditioning: a new role for ischaemia — reperfusion

What is ischaemic preconditioning?

Ischaemic preconditioning has been defined as the phenomenon in which sublethal episode(s) of ischaemia followed by reperfusion results in increased tolerance to a later, potentially lethal episode of ischaemia (Walker *et al.*

1992). This powerful form of endogenous adaptation was first described by Murry and co-authors in 1986. They reported that in dogs the amount of infarction resulting from 40 minutes of coronary occlusion could be markedly reduced if the hearts are first preconditioned with several cycles of transient (5 minutes) coronary occlusions (Murry *et al.* 1986). Since then the phenomenon has been described without exceptions in every species studied (Parratt, 1994; Cohen *et al.* 1996). Experimental studies have demonstrated that isolated human cardiomyocytes as well as human atrial strips could be preconditioned (Ikonomidis *et al.* 1994; Walker *et al.* 1995). In clinical setting, the preconditioning with intermittent cross-clamping has been shown to preserve myocardial level of high energy phosphates in patients undergoing coronary bypass surgery (Yellon *et al.* 1993). Recently, Leesar *et al.* have reported that the pattern of EKG ST-segment elevations during repeated balloon occlusions in coronary angioplasty in humans can be modified by adenosine (Leesar *et al.* 1997). Finally, the evidence for preconditioning in humans is supported by data from retrospective analysis of TIMI 4 (phase 4 of the Thrombolysis in Myocardial Infarction trial). The analysis demonstrated that patients with angina in the days preceding their infarction had better in-hospital course and survival than those in whom the pain of infarction was the first symptom (Kloner *et al.* 1995b).

Ischaemic preconditioning has been proposed to protect against almost all consequences of ischaemic injury. It is demonstrated to preserve myocardial energy stores during ischaemia (Miyamae *et al.* 1993), attenuate ionic disturbances during ischaemia (Steenbergen *et al.* 1993), protect against Ca^{2+} paradox injury (Ashraf *et al.* 1994), reduce the incidence of reperfusion arrhythmias (Vegh *et al.* 1992; Miura *et al.* 1995), improve postischaemic functional recovery (Cave *et al.* 1993; Sun *et al.* 1995), and reduce the extent of myocardial cell necrosis (Ytrehus *et al.* 1994; Liu *et al.* 1994).

The potential pharmacological and clinical importance of this adaptive phenomenon is that if we understood the cellular mechanisms involved in this endogenous form of protection, the mechanisms could be exploited for therapeutic gain (Parratt, 1994; Jenkins *et al.* 1995).

The preconditioning protocol

According to recent knowledge, there appears to exist a certain threshold for stimulation which must be achieved by the preconditioning protocol in order to protect the heart (Cohen *et al.* 1996). In the rabbit (Van Winkle *et al.* 1991; Miura *et al.* 1992), and dog (Li *et al.* 1990), a single 5-minutes period of ischaemia followed by 5 or 10 minutes reperfusion is sufficient to put the heart into preconditioned state. Two 2-minutes occlusions, however, do not elicit protection, indicating a sharp ischaemic threshold. In man 90-second coronary occlusion during the course of angioplasty may be sufficient to precondition the heart (Inoue *et al.* 1996). Multiple cycles of ischaemia may, but not constantly, offer more protection than a single cycle (Parratt, 1994; Miura, 1996), indi-

cating all-or-none type of protection on the cellular level. Finally, it has been suggested that other factors than brief episodes of total coronary occlusion can induce adaptive responses similar to ischaemic preconditioning. Partial coronary artery stenosis, exposure of heart to hypoxia, stretch, rapid pacing, (endogenous) catecholamines, sympathomimetic stimulation, etc. can result in preconditioning-like phenomena (Lasley *et al.* 1993; Koning *et al.* 1994; Kloner *et al.* 1995a; Meldrum *et al.* 1996; Ravingerova, 1996).

Another important determinant for the extent of the protection is the time of reperfusion between preconditioning and prolonged ischaemia. Surprisingly, the window of protection is rather short. Reports vary, but in most models the protection wears off after about an hour (Miura, 1996; Cohen *et al.* 1996). After 24 hours of reperfusion, however, protection occurs again and lasts for some hours (Yamashita *et al.* 1994; Sun *et al.* 1995). This form of preconditioning is called "late window of protection" and was first described in 1994 (Baxter *et al.* 1994). The present overview will be concentrated only on mechanisms of acute or early protection.

Cellular mechanisms of preconditioning

Over the past few years extensive research has been carried out to clarify the cellular mechanisms of ischaemic preconditioning, one of the most powerful means known to protect the heart against ischaemia-reperfusion injury.

The early studies eliminated opening of collaterals (Murry *et al.* 1986; Li *et al.* 1990), induction of antioxidants (Turrens *et al.* 1992), and changes in mitochondrial function (Steenbergen *et al.* 1993) as explanations to the protection.

Today, the hypothesis most widely accepted is that preconditioning is triggered by stimulation of membrane-bound receptors by agents released during the short preconditioning ischaemia (Cohen *et al.* 1996; Wainwright, Sun, 1996). During ischaemia, numerous agonists are released by the myocardium, including adenosine (Dorheim *et al.* 1990), catecholamines (Ardell *et al.* 1996), angiotensin II (Noda *et al.* 1993), bradykinin (Linz *et al.* 1996; Baumgarten *et al.* 1993), and endothelin (Wang *et al.* 1995). In rabbit heart it has been shown that stimulation of adenosine A₁-receptors may be the initial signalling mechanism, as blockade of these receptors abolished the protection (Hoshida *et al.* 1994) and stimulation of these receptors mimicked it (Miura, 1996). In rat heart, however, blockade of adenosine receptors do not prevent preconditioning (Cave *et al.* 1993; Miura *et al.* 1995; Bugge *et al.* 1995).

The intracellular signalling pathway downstream from receptor activation leading to protection is not completely understood. Mounting evidence suggests that in rabbits stimulation of adenosine receptors elicit protection by activating protein kinase C (PKC) (Mitchell *et al.* 1995; Miura, 1996; Cohen *et al.* 1996). PKC inhibitors have been shown to abort the protection from ischaemic preconditioning protocol in rabbit (Ytrehus *et al.* 1994; Armstrong *et al.* 1995), rat (Hu *et al.* 1995; Bugge *et al.* 1995), and dog (Kitakaze *et al.* 1994) hearts,

and, more importantly, human myocytes (Cohen *et al.* 1996). The PKC hypothesis further broadens the pharmacological possibilities because there are many PKC-coupled receptors on the cardiomyocyte. Receptors for angiotensin II (Busse *et al.* 1993), α_1 -adrenergic agonists (Mitchell *et al.* 1995), bradykinin (Tippmer *et al.* 1994), and endothelin (Bugge *et al.* 1996a) have been shown to activate PKC. Activation of all of these receptors have been shown to fully mimic preconditioning's protection in ischaemic myocardium (Tang *et al.* 1994; Mitchell *et al.* 1995; Bugge *et al.* 1996a; Ravingerova, 1996). In the rabbit, along with adenosine, bradykinin is suggested to be one of major players triggering preconditioning (Goto *et al.* 1995; Wall *et al.* 1994). In other species the proportions between various possible triggers may differ. For example, adenosine is clearly not a trigger in rat, but evidence shows that it may be a trigger in man (Cohen *et al.* 1996; Leesar *et al.* 1997). The role of bradykinin in ischaemic preconditioning in the rat heart has been investigated in paper III of the present work.

The link between the membrane receptors and PKC is suggested to be provided by activation of phospholipase C through specific G-proteins (Fu *et al.* 1993a; Hu *et al.* 1995; Cohen *et al.* 1996). Phospholipase C degrades phosphatidylinositol to 1,2diacyl-glycerol (DAG) and inositol trisphosphate (Lewis *et al.* 1993). The latter releases calcium from sarcoplasmic reticulum, while DAG is an important stimulatory cofactor for PKC (Ytrehus *et al.* 1994; Cohen *et al.* 1996). Recently, evidence that phospholipase D could be involved in activation of PKC has been reported (Cohen *et al.* 1996). Most recent studies have shown that protein kinases other than PKC — tyrosine kinase and MAP kinases — might be participate in mechanisms preconditioning (Das *et al.* 1996; Valhaus *et al.* 1997). Murphy et co-workers have suggested the role for phospholipase A₂ derived lipid second messengers (Murphy *et al.* 1995). This hypothesis has also been tested in present thesis (paper IV).

The ultimate action of protein kinase C leading to protection is not definitively determined. The PKC is expected to phosphorylate a protein, and that protein may be end-effector itself, or it may be another kinase that will then continue the intracellular signalling process until the end-effector (or effectors) are finally phosphorylated. The identity of the end-effector is not yet known, but the ATP-sensitive potassium channel (K_{ATP}) has been repeatedly proposed (Parratt *et al.* 1994; Liu *et al.* 1996; Grover, 1996). Several studies have demonstrated that K_{ATP} -channel openers (drugs like cromkalim, pinacidil and nicorandil) can trigger preconditioning (Yao *et al.* 1994; Menasche *et al.* 1995; Mizumura *et al.* 1995; Hearse, 1995; Pignac *et al.* 1996). Until now, the results from studies with K_{ATP} -channel blockers have been somewhat conflicting. Glibenclamide, a K_{ATP} -channel blocker, abolished the preconditioning in dogs (Mei *et al.* 1995) and pigs (Schulz *et al.* 1993). Results from rabbit hearts were conflicting and seemed dependent on the anaesthetic regimen used (Toombs *et al.* 1993; Miura, 1996). Munch-Ellingsen *et al.* showed, however, that high

dose glibenclamide blocked preconditioning in buffer perfused rabbit heart (Munch-Ellingsen *et al.* 1996). First reports from rat hearts were negative, but recently Bugge *et al.* reported, that a new specific K_{ATP} -channel blocker, 5-hydroxydecanoate, blocked the preconditioning against infarction in isolated rat heart model (Bugge *et al.* 1997). The proposed mechanism for protection by K_{ATP} opening, and thereby the mechanism of preconditioning, is shortening of action potential duration, the consequence of which is reduced calcium overload and decreased ATP consumption (Hearse, 1995; Grover, 1996). In addition to the K_{ATP} theory, it has been proposed, that changes in the cytoskeleton makes the cells more resistant to osmotic swelling, and have a crucial role in protection by preconditioning (Cohen *et al.* 1996). In summary, the end-effectors for preconditioning remains to be elucidated. Whatever end-effector would be identified, it would simplify application of preconditioning in clinical situations.

3. AIMS OF THE STUDY

The main purpose of the present work was to investigate the relations between ischaemia-reperfusion and oxidative stress. In the clinical setting, oxidative stress was studied in patients undergoing open heart surgery. The mechanisms whereby shortlasting ischaemia-reperfusion, i.e. ischaemic preconditioning, protects against ischaemia-reperfusion injury were examined in experimental conditions using the isolated buffer-perfused rat heart model. The specific aims were as follows:

1. To evaluate the time course of oxidative stress in open heart surgery;
2. To characterize the extent of oxidative stress associated with open-heart surgery;
3. To investigate whether bradykinin is involved in ischaemic preconditioning of the rat heart, and whether preischaemic stimulation of bradykinin receptors can protect against ischaemia-reperfusion injury;
4. To investigate the relationship between the extent of ischaemic cell death, contractile dysfunction and myocardial energy status after ischaemia-reperfusion with either ischaemic preconditioning or bradykinin pretreatment as protection;
5. To study the role of oxidative stress, cellular lipid alterations and lipoxygenase derived arachidonic acid metabolites in the protective effect of ischaemic preconditioning against infarction in the rat heart;
6. To investigate if moderate oxidative stress itself can be protective in ROS mediated myocardial dysfunction.

4. PART I: OXIDATIVE STRESS IN OPEN HEART SURGERY (PAPER I-II)

The assessment of oxidative stress in open heart surgery was performed by measurements of three separate markers of lipid peroxidation and blood antioxidant status in patients undergoing elective heart valve surgery (paper I). In paper II, the oxidative stress occurring in cardiac surgery patients was further compared to oxidative stress in septic patients suffering from systemic inflammatory response syndrome. Sepsis has been described as a classical and undoubted example of the systemic inflammatory response syndrome, and is known to be associated with oxidative stress. Therefore, comparison of oxidative stress markers measured by the same laboratory could be a reliable way for assessment of extent of oxidative stress in cardiac surgery patients.

4.1. Patients and methods

4.1.1. Patients

14 adult patients (9 males, 5 females, mean age of 48.7, range 30–75 years) scheduled for elective open heart surgery at Department of Cardiac Surgery, Tartu University Hospital, in time period from September 1993 to April 1994, were studied. The study was approved by the Ethics Committee and all patients gave informed consent to blood sampling during the operative procedure. Among the patients four had preoperative heart failure of NYHA class IV, nine NYHA III, and one NYHA II. The surgical procedure consisted of one valve surgery in 8 cases, and two valve correction in 6 cases. Anaesthesia for surgery was provided according to the routine of the department, using fentanyl, N₂O and halothane as the main anaesthetics. For cardiopulmonary bypass (CPB) standard roller pumps (Gambro) were used to apply pulsatile flow at 2.4 l/min/m². The mean arterial pressure was maintained at 60 mmHg. For extracorporeal blood oxygenation a bubble oxygenator (Jostra, for eleven patients) or a membrane oxygenator (Compactflo, Dideco, for three patients) was used. Systemic hypothermia up to 24.6±0.5°C (mean±SEM) was employed for organ protection during CPB, while myocardial preservation was provided by antegrade cold crystalloid cardioplegia (St. Thomas Hospital solution II at 4°C) repeated every 20 minutes. All operations were performed by the same surgical and anaesthetic team. The mean aortic cross clamp time was 84.9±8.9 minutes, CPB time 119.0±10.6 minutes, and operating time 251.9±15.1 minutes.

Blood sampling. To evaluate the time-course of oxidative stress the blood was sampled at the following time-points: 1 hour before operation, 15 minutes

after induction of anaesthesia, 15 minutes after the start of CPB, 5 minutes after release of aortic cross clamp, 15 minutes after cessation of CPB and in the morning of the first postoperative day. The blood was drawn from the arterial line into plastic syringes, serum was then separated by centrifugation and kept in plastic tubes at -4°C until analysis.

The changes in oxidative stress markers of cardiac surgery patients were compared to respective changes occurring in septic patients suffering from systemic inflammatory response syndrome. For these purposes assessment of oxidative stress markers in 10 septic patients, admitted to the Department of Intensive Care, Tartu University Hospital, was performed. From these patients eight had soft tissue infection of different body regions, while two patients had diffuse peritonitis. All patients required general surgery aimed at removal of the source of infection and included: fasciotomies, amputation of an infected limb or resection of the small intestine with drainage of the peritoneal cavity. All were emergency operations performed by different surgeons of the hospital. Endotracheal anaesthesia was performed according to the routine of the hospital, using fentanyl, N_2O and halothane as the main anaesthetics.

Blood sampling. From septic patients the blood samples were obtained from *v. cubitalis* 1 hour before surgery, 15 minutes after surgery, and 18 hours after surgery. The sampling protocol was designed in order to match as much as possible that used in cardiac surgery patients.

4.1.2. Laboratory analyses

Markers of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured spectrophotometrically by method compiled on the ground of Ohkawa method and our own experiments (Ohkawa *et al.* 1979; Zilmer *et al.* 1994).

Conjugated dienes (CD) were measured spectrophotometrically by a method compiled on the ground of the method described by Recknagel, Glende and our own experiments (Recknagel *et al.* 1984; Zilmer *et al.* 1994).

For more complex characterization of serum lipid status with respect to peroxidation measurements of Fe-stimulated TBARS (Fe-TBARS) were performed. This parameter reflects the peroxidative capacity of lipids not yet peroxidized at the time of blood sampling. In this assay the samples were treated with Fe^{2+} (FeSO_4) as pro-oxidant at 37°C for 30 minutes and thereafter TBARS were measured according to the method described above.

Markers of antioxidant status

To assess serum antioxidative capacity (AOC), the ability of the sample to inhibit linolenic acid peroxidation *in vitro* was evaluated (Zilmer *et al.* 1994). Linolenic acid peroxidation products were measured via TBARS determination.

For calculation of glutathione content in red blood cells (RBC-GSH), blood was drawn into heparinized tubes, centrifuged, erythrocytes separated and RBC-GSH was measured according to the method described by Ellman (Ellmann, 1959).

To correct the results for hemodilution, protein content in samples was determined by the method of Lowry *et al.* (Lowry, 1951). Data from lipid peroxidation analyses are therefore presented as per gram protein. All measurements of oxidative stress markers were performed in triplicate within 4 hours after blood sampling. Means were calculated and used for statistical analysis.

4.1.3. Statistical methods

Values are presented as mean±standard error of the mean. One-way analysis of variance was used to test the time-dependent changes in oxidative stress markers. Student's t-test (with Bonferroni correction in case of multiple comparisons) was applied to determine significant differences ($p \leq 0.05$) between or within groups.

4.2. Results

Clinical outcome of cardiac surgery patients

The anaesthetic and surgical procedures of the heart operations were uncomplicated in all cases. During the study period there was no mortality and no patients had infectious complications. The mean duration of postoperative mechanical ventilation of the lungs was 18 hours. Dopamine infusion (2–4 µg/kg/min) and intravenous digoxin were given as postoperative routine, and in three cases dobutamine and/or epinephrine were used for additional inotropic support. In 4 patients increase in serum level of bilirubin (>25 mmol/L) and in 2 patients raised serum urea and creatinine levels (>10 mmol/L and >100 mmol/L, respectively) developed postoperatively without any other symptoms of hepatic or renal failure.

Oxidative stress markers

The perioperative changes in lipid peroxidation markers, thiobarbituric acid reactive substances and diene conjugates, in two groups of patients are illustrated by Figure 1 and Figure 2. Cardiopulmonary bypass employed for open

heart surgery caused a significant increase in arterial TBARS already after 15 minutes (20.82 ± 1.89 nmol/g protein). The highest level was reached 5 minutes after release of the aortic cross clamp (38.55 ± 3.42 nmol/g protein). 15 minutes after the cessation of CPB TBARS remained elevated (34.83 ± 3.20 nmol/g protein), but on the morning of the first postoperative day it was almost back to the preoperative level (10.11 ± 2.73 nmol/g protein). The alterations in serum diene conjugates showed similar tendencies, however, no significant changes were detected. The cardiac surgery patients had significantly lower preoperative level of lipid peroxidation when compared to severely ill septic patients undergoing general surgery. However, the cardiopulmonary bypass and reperfusion of ischaemic cardioplegic heart increased the serum TBARS and DCs to the level found in systemic inflammation as seen in severe sepsis.

Simultaneous with the increase in lipid peroxidation, the antioxidant capacity of the serum of patients undergoing open heart surgery was attenuated (Figure 3). Thus, the ability of blood serum to inhibit linolenic acid peroxidation *in vitro*, was significantly decreased 15 minutes after the start of CPB, and remained suppressed at all points assessed. The lowest level was detected 5 minutes after release of the aortic cross clamp ($18.1 \pm 1.5\%$ compared to $34.9 \pm 0.8\%$ before operation). In the morning of the first post-operative day AOC was still significantly decreased and stayed at the level detected in septic patients ($28.1 \pm 1.6\%$ in cardiac surgery and $27.6 \pm 1.5\%$ in septic patients, respectively). The pattern of AOC decrease in cardiac surgery patients correlated with the changes in TBARS and DC, $r=0.30$ ($p<0.001$) and $r=0.46$ ($p<0.02$), respectively.

The cardiac surgery patients had significantly smaller preoperative red blood cells glutathione levels compared to septic patients (Figure 4). In both groups, however, this parameter was not further altered by surgery.

Serum Fe-TBARS were detected only in cardiac surgery patients, and no significant perioperative changes in this parameter were found.

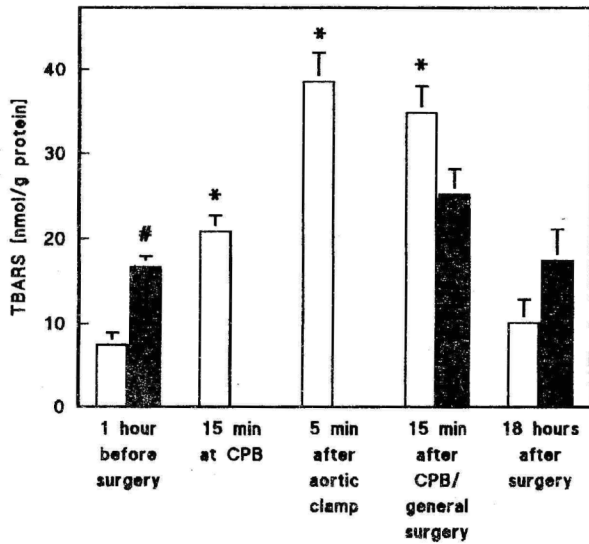


Figure 1. Perioperative dynamics of thiobarbituric acid reactive substances (TBARS) in cardiac surgery patients (open bars, n=14) and septic patients undergoing general surgery (solid bars, n=10). Mean±SEM, * p≤0.05 compared to preoperative value within respective group, # p≤0.05 between groups.

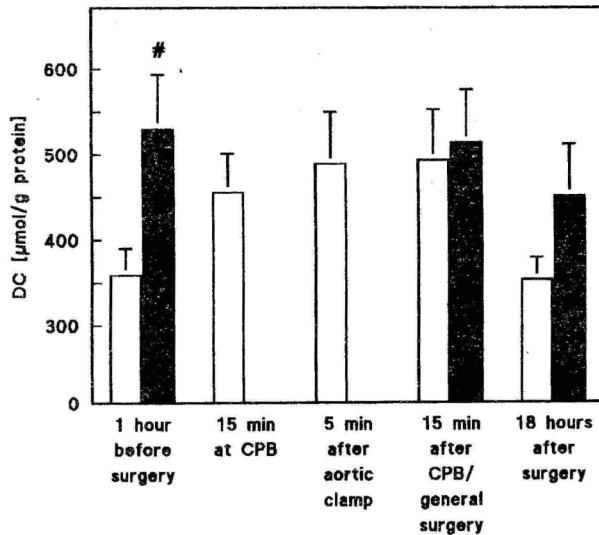


Figure 2. Perioperative dynamics of diene conjugates (DC) in cardiac surgery patients (open bars, n=14) and septic patients undergoing general surgery (solid bars, n=10). Mean±SEM, * p≤0.05 compared to preoperative value within respective group, # p≤0.05 between groups.

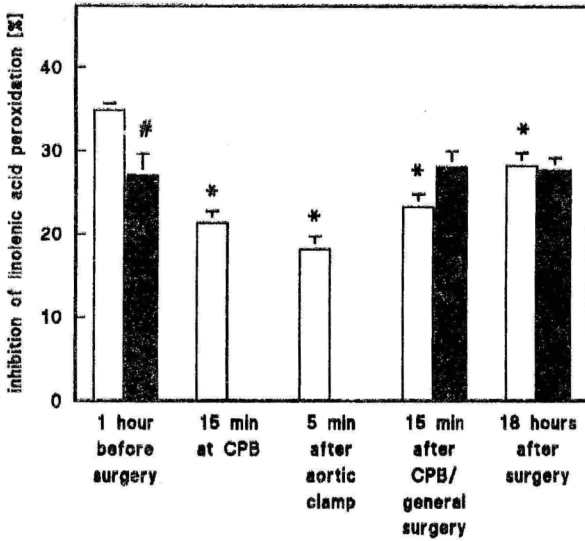


Figure 3. Perioperative dynamics of serum antioxidative capacity (AOC) in cardiac surgery patients (open bars, n=14) and septic patients undergoing general surgery (solid bars, n=10). Mean±SEM, * p≤0.05 compared to preoperative value within respective group, # p≤0.05 between groups.

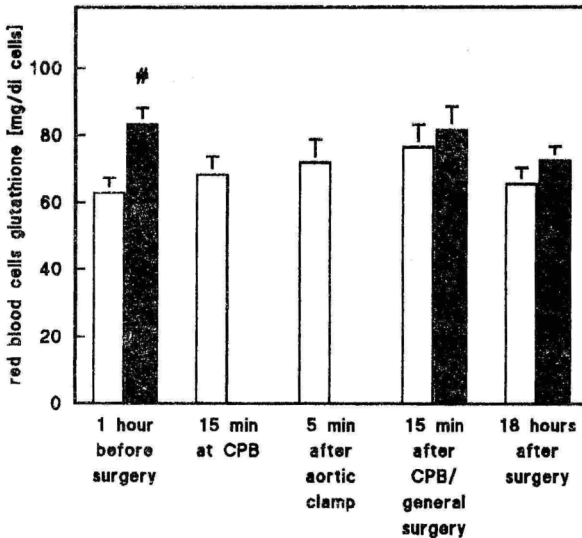


Figure 4. Perioperative dynamics of red blood cells glutathione content (RBC-GSH) in cardiac surgery patients (open bars, n=14) and septic patients undergoing general surgery (solid bars, n=10). Mean±SEM, * p≤0.05 compared to preoperative value within respective group, # p≤0.05 between groups.

4.3. Discussion

The main findings of our clinical study — increased serum level of lipid peroxidation products and decreased antioxidant capacity — clearly demonstrate the evidence of oxidative stress in patients undergoing open heart surgery. The changes in oxidative stress markers were clearly time-dependent: significant alterations appeared already 15 minutes after the start of cardiopulmonary bypass, peaked 5 minutes after the release of aortic cross clamp, and all parameters, except AOC, returned to almost preoperative levels in the morning of the first postoperative day.

With respect to lipid peroxidation, our observations are generally in accordance with earlier studies, in which marked increase of either TBARS or DC during coronary bypass surgery is demonstrated (Davies *et al.* 1993; Lazzarino *et al.* 1994). However, whereas we found a three-fold increase in serum TBARS already 15 minutes after the start of CPB, Davies *et al.* (Davies *et al.* 1993) have reported increase in lipid peroxidation products only after release of the aortic cross clamp. Differences in oxygenator types (bubble versus membrane oxygenator) could be one possible explanation for this discrepancy. Moreover, it is not known with certainty how the degree of systemic hypothermia influences free radical reactions. Hypothermia might decrease free radical reactions, but circulatory dysfunction as a result of the hypothermia might promote ROS. In their study Davies *et al.* have kept the temperature during CPB substantially higher than in our study (32–34°C versus 24.4±0.5°C). In another study, using systemic hypothermia 28–30°C, increase in arterial blood malondialdehydes was found to occur already after 20 minutes of CPB (Valen *et al.* 1994).

In the present study the serum diene conjugates were not significantly changed, but tended to increase. This discrepancy between TBARS and DC may be partly explained by accumulation of lipid peroxidation end-products (i.e. malondialdehydes which could be measured by TBARS assay) in response to the high-rate peroxidation processes. Although wide variability between the single values of DC should also be noted, the hypothesis of oxidative stress could be further supported by the finding of a negative correlation between changes in DC and AOC. Measurements of Fe-TBARS were performed for further characterization of serum lipid status as they reflect the peroxidative potential of lipids not yet peroxidized at the time of blood sampling. Although no significant changes were found in this parameter, the tendency of Fe-TBARS to decrease during CPB (Table II, paper I) may reflect consumption of substrates (polyunsaturated fatty acids) in response to the high-rate peroxidation processes.

In contrast to lipid peroxidation markers, very little data are available on antioxidant status in patients undergoing cardiac surgery. In human plasma, the

integrated action of antioxidants and scavengers like ascorbate, α -tocopherol, carotenoids, proteins (transferrin, ceruloplasmin, proteins with free sulphhydryl groups), etc. protect lipid particles in the blood as well as the endothelium against free radicals attack (Lunec, 1990; Halliwell *et al.* 1990; Halliwell, 1996). In some earlier studies, decrease in plasma levels of α -tocopherol during CPB has been demonstrated (Cavarocchi *et al.* 1986; Coghlan *et al.* 1993). In our opinion, however, it is difficult to choose one single agent from the complex of antioxidants, which could fully reflect the changes in whole blood antioxidant status. Therefore we have assessed serum total antioxidative capacity during surgery. In the present study, the hemodilution, accompanied with CPB, could be in part responsible for the remarkable reduction in serum antioxidative capacity in cardiac surgery patients. On the other hand, our results reflect the actual capacity of serum antioxidative defence, and thus, increased susceptibility to oxidative stress during CPB is demonstrated. Furthermore, most likely due to consumption of antioxidant reserves, AOC was still depressed in the morning of the first postoperative day. This finding — insufficient capacity of the physiological defence systems in the early postoperative period — may influence the clinical outcome after cardiac surgery.

With respect to red blood cell glutathione content we found slight, though not statistically significant increase during cardiac surgery. When compared to septic patients, cardiac surgery patients had significantly lower preoperative level of red blood cell glutathione. 15 minutes after CPB, however, it was nearly similar to the level of glutathione found in septic patients after general surgery. Since the method we used for measuring glutathione content is based on detection of thiol groups after precipitation of proteins, our results principally reflect changes in reduced glutathione. Therefore, it might be speculated that increase in glutathione reductase activity occurred as an adaptive response to blood injury during CPB, and, consequently, the erythrocyte-glutathione pool was increased as an adaptive response to oxidative stress during either CPB or septic illness.

In the present study, evidence for oxidative stress is demonstrated by indirect measurements, i.e. by complex assessment of serum lipid peroxidation markers and antioxidant status. To measure free radicals directly is complicated and difficult in clinical setting. Thus the origin of ROS is not known in the present study. Ischaemia-reperfusion of the heart and lungs could be one of the events leading to the generation of ROS. Peripheral tissue hypoperfusion during hypothermic extracorporeal circulation can also lead to reperfusion induced ROS generation and subsequent stimulation of lipid peroxidation in liver, kidneys and pancreas. In addition, during CPB several inflammatory mediators are activated and a generalized inflammatory response occurs (Boyle, Jr. *et al.* 1997; Das *et al.* 1992). As a result the neutrophils are activated, which leads to a burst of ROS from these cells. Similar events occur in sepsis which is known

as a classical example of systemic inflammatory response syndrome (Deitch, 1992). Therefore, to characterize the extent of oxidative stress, demonstrated to occur during CPB, we performed comparable analysis of OS markers in septic patients. As a result, we found that the free radical load during CPB temporarily reached the same level as in critically ill septic patients. In the case of uncomplicated perioperative course, however, signs of oxidative stress spontaneously abates. The data also support the view that a systemic inflammatory response syndrome caused by cardiopulmonary bypass is an important promoting factor for oxidative stress in open heart surgery.

The oxidative stress demonstrated to occur during open heart surgery is suggested to contribute to the formation of diffuse tissue damage in heart, lung, and other organs (Sellke *et al.* 1993; Valen *et al.* 1993; Mair *et al.* 1995; Suzuki *et al.* 1997). In particular, postischaemic depression of myocardial function (“myocardial stunning”), in the pathogenesis of which the role of oxidative stress is well documented, but also impaired pulmonary function, may have critical influence on early postoperative recovery (Menasche *et al.* 1989; Tortolani *et al.* 1993; Jeroudi *et al.* 1994; Menasche, 1995). Therefore, preventive antioxidant therapy could be useful, especially in more complicated cases of surgery. The aim of such therapy, however, should not necessarily be complete blockage of ROS related processes, but only to control, or limit, formation of ROS. The background for this suggestion is that we have found experimentally that moderate oxidative stress might have an important role in mobilizing the endogenous defence mechanism of the heart (see Part II of the present thesis).

Methodological considerations

Although it has been suggested that ROS contribute to many pathological states, their exact role is difficult to quantify. Free radical activity has been indirectly assessed in most of the studies, by measurement of damaged biomolecules, especially of lipids. Some authors have suggested the detection of malondialdehydes by HPLC technique, which is relatively expensive and time consuming, as most specific method for evaluation of peroxidized lipid (Ceconi *et al.* 1991; Draper *et al.* 1993). The spectrophotometric assay of TBARS for malondialdehydes assessment is known to have non-specific properties, including formation of adducts with sugars, nucleic acids and proteins (Ceconi *et al.* 1991; Pincemail *et al.* 1996). To avoid such possible errors we introduced several methodological modifications such as heating samples up to 80°C instead of boiling them, addition of antioxidant immediately after drawing the sample, and acidification of the reaction medium for more efficient extraction of TBARS. In addition, we also believe, that evaluation of one single parameter is not sufficient for detection of oxidative stress, and therefore we have introduced a set of methods for simultaneous assessment of lipid peroxidation and antioxidant status which is suitable and easy to use in clinical studies.

5. PART II: ISCHAEMIC PRECONDITIONING AND OXIDATIVE STRESS (PAPERS III–V)

In this part of the thesis, different aspects of ischaemic preconditioning and ischaemia-reperfusion injury were investigated on the experimental model of isolated buffer-perfused rat heart.

In paper III, the role of bradykinin in ischaemic preconditioning was studied. For these purposes the preconditioned hearts were treated with the specific bradykinin B₂-receptor antagonist, HOE 140 (Hock *et al.*, 1991), prior to 30 minutes of global ischaemia. Additional groups of hearts were subjected to short preischaemic infusion of bradykinin alone or in combination with HOE 140, to evaluate protective properties of bradykinin independent of ischaemic preconditioning. To study the relationship between the extent of cell death and contractile dysfunction, the experiments with global ischaemia, in which contractile function could be assessed, were supplemented with a model of regional ischaemia, in which the extent of cell death (i.e. infarct size) could be measured. In separate experiments, hearts were freeze-clamped at the end of ischaemia, at the end reperfusion, as well as at the end of the preischaemic exposure to either bradykinin or ischaemic preconditioning, and myocardial content of high energy phosphates, glycogen, lactate and glucose were measured.

In paper IV, the role of oxidative stress, lipid alterations, and lipoxygenase derived arachidonic acid metabolites in ischaemic preconditioning was studied. For these purposes arachidonic acid, oleic acid, linoleic acid, and docosahexaenoic acid were measured in the free fatty acid fraction and in the polar lipid fraction of myocardial lipid extracts. Measurements of hydroxy conjugated dienes of polyunsaturated fatty acids in free fatty acid and phospholipid fractions were performed using the HPLC technique to separate unchanged and peroxidated polyunsaturated fatty acids. To further clarify the role of lipoxygenase metabolites of arachidonic acid in ischaemic preconditioning, we have in a separate set of experiments used nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, to block preconditioning against infarction in an isolated rat heart model of regional ischaemia.

In paper V, oxidative stress as potentially protective intervention was investigated. Isolated buffer-perfused rat hearts were subjected to either a low dose of hydrogen peroxide or ischaemic preconditioning prior to exposure to a higher dose of hydrogen peroxide. To assess the severity of heart injury functional parameters and myocardial release of troponin-T were evaluated, while tissue contents of lipid peroxidation products and antioxidative parameters were measured to study the role of oxidative stress in protection.

5.1. Methods

5.1.1. Experimental model

Perfusion procedure

The procedure of isolated rat heart perfusion is described in detail in papers III–V. Briefly, the hearts were rapidly excised from anaesthetized male Wistar rats, placed in ice-cold buffer, then mounted via aorta on a Langendorff perfusion system within 60 seconds, and perfused retrogradely with oxygenated Krebs-Henseleit buffer at 37°C. A water filled latex balloon, connected to a pressure transducer and coupled to a Gould recorder, was inserted into the left ventricle through an incision in the left atrium to record heart rate, left ventricular systolic pressure (LVSP) and end diastolic pressure (LVEDP), positive (+dP/dt) and negative (-dP/dt) first derivatives of left ventricular pressure. Left ventricular developed pressure (LVDP) was calculated as the difference between LVSP and LVEDP, and coronary flow was measured by timed collections of effluent. Global ischaemia was instituted by clamping the inflow tract above the aortic cannula. In the series of experiments with regional ischaemia, a 3–0 silk thread was passed around the main branch of the left coronary artery, and the ends were threaded through a small vinyl tube to form a snare. Regional ischaemia was achieved by pulling the snare. Ischaemia was confirmed by a substantial fall in both left ventricular developed pressure and coronary flow. In paper V a model of cardiac injury induced by exogenous ROS (140 μM H_2O_2) was used. This model was aimed to mimic reversible postischaemic contractile dysfunction of the heart (myocardial stunning) (Skjelbakken *et al.*, 1996). The drugs used were administered either by switching to a separate perfusion reservoir or by an infusion pump into an infusion port directly above the aortic cannula.

Experimental protocol

To investigate the protective mechanisms of ischaemic preconditioning, we have chosen an ischaemic insult of 30 minutes duration as we wanted to study the effect of preconditioning on ischaemic cell death. Thus, we found that occlusion of the left coronary artery for 30 minutes results in infarction of approximately 30–40% of the risk zone, and this is an appropriate extent of injury for studies investigating cardioprotective interventions. In the studies of ischaemic injury (paper III, IV) we have used one cycle of 5 minutes ischaemia + 5 minutes reperfusion to precondition the hearts, while two cycles of 2 minutes of ischaemia + 5 minutes reperfusion were used to protect the hearts against injury caused by exogenous ROS (paper V). Both these protocols of preconditioning were found to be protective against ischaemic injury in isolated rat heart. The exact protocol for drug administrations is described in detail in the respective papers.

5.1.2. Assessment of ischaemic injury

Global injury

To assess the extent of cell necrosis in hearts exposed to global ischaemia, release of creatine kinase (CK) to coronary effluent was measured spectrophotometrically (Rosalski, 1967) at room temperature and expressed as IU released during 30 min of reperfusion (IU/30 min/heart).

In experiments where myocardial injury was induced by exogenous ROS, the release Troponin T to the coronary effluent as marker of irreversible injury was measured by enzyme-linked immunosorbent assay (Muller-Bardorff *et al.* 1997).

Regional ischaemia

In hearts, subjected to regional ischaemia, infarct size was measured by triphenyl-tetrazoliumchloride (TTC) staining technique described in details previously (Ytrehus *et al.* 1993; Bugge *et al.* 1995). Risk zone (i.e. the area of left ventricle suffering from ischaemia during occlusion of the main branch of the left coronary artery) was determined by fluorescent particles and infarct size was expressed as percentage of risk zone infarcted.

5.1.3. Laboratory analyses

Tissue content of adenosine triphosphate (ATP), creatine phosphate (CP), glycogen, lactate and glucose

For metabolic assays, the hearts were freeze-clamped and stored in liquid nitrogen. Adenosine triphosphate (ATP) and creatine phosphate (CP) were measured by HPLC technique as described by Sellevold *et al.* (Sellevold *et al.* 1986).

For tissue glycogen and glucose detection an enzymatic method based on hexokinase and glucose-6-phosphate dehydrogenase was used (Passonneau *et al.* 1974).

Myocardial content of lactate was measured spectrofluorometrically according to Passonneau (Passonneau, 1974).

Assays for fatty acids and peroxidized lipids

The ventricular part of the freeze-clamped hearts was homogenised under liquid nitrogen, and approximately 250 mg of the tissue extracted according to Folch *et al.* (Folch *et al.* 1957). Following extraction, the lipids were dissolved in chloroform and applied to hexane-conditioned solid phase extraction silica column (Bond Elut, Varian) using the method of Kaluzny *et al.* (Kaluzny *et al.* 1985) with modifications to separate phospholipids and free fatty acids.

The phospholipid fraction was evaporated to dryness under nitrogen, redissolved in a small amount of diethyl ether, and then treated with phospholipase A₂ from *Naja Mozambique*. Released free fatty acids were extracted with diethylether, evaporated to dryness and redissolved in chloroform/methanol 1:2.

Lipid peroxidation was detected by assessment of monohydroxy-fatty acid conjugated dienes in both the nonesterified lipid fraction as well as in fatty acids released after enzymatic hydrolysis. For these purpose the fatty acid samples were chromatographed by reverse phase HPLC in order to separate the different components of the sample; i.e. monohydroxy fatty acids were eluted after 15–30 minutes whereas unchanged fatty acids were eluted later. Conjugated dienes were detected at 235 nm using a variable UV-detector (Model 481, Waters). Quantification was based on integration of all peaks corresponding to the retention-time interval for monohydroxy isomers of linolenic, arachidonic, and docosahexaenoic acid. 5-HETE was used as a standard, and the levels were expressed in units/mg dry wt based on the absorbance produced by 1 pmol of 5-HETE in the HPLC system.

Quantitative analysis of fatty acids present in samples from the non-esterified fatty acid fraction and the fatty acids released from the 2-position of phospholipids was performed by gas chromatography. Total amount of polyunsaturated fatty acids (PUFA) was calculated as the sum of measured concentrations of linoleic acid, arachidonic acid, and docosahexaenoic acid.

Other oxidative stress markers

In the study investigating the effect of H₂O₂ on cardiac function, the hearts were freeze-clamped in liquid nitrogen and tissue reduced glutathione content (GSH), glutathione peroxidase (GSH-Px), total antioxidant status (TAS), catalase (CAT), superoxide dismutase (SOD), and antioxidant capacity (AOC) were measured. Lipid peroxidation was evaluated by tissue contents of thiobarbituric acid reactive substances (TBARS).

TBARS, AOC and GSH were measured as described in the methods section in the first part of the thesis.

Total antioxidant status, superoxide dismutase 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 (Goth, 1991).

5.1.4. Statistical methods

Results are expressed as mean±standard error of the mean (SEM). In papers III and IV, one way analysis of variance was performed and Tukey's test was applied to identify significant differences ($p \leq 0.05$) between groups, while paired t-test was used for within-group analyses to test for drug effects on functional parameters prior to ischaemia. In paper V, a Mann Whitney test was employed to compare differences between groups, and a Wilcoxon signed rank test for differences ($p \leq 0.05$) within groups.

5.2. Results

5.2.1. Preischaemic bradykinin and ischaemic preconditioning

Functional recovery

Global ischaemia. The time course of left ventricular pressures of the control, preconditioned (IP) and bradykinin (BK) treated hearts is illustrated by the Figure 5. The graphs show the time course of the recorded parameter and are produced by calculating the means of recordings at different timepoints from the single experiments. The graph therefore show the average time course of an experiment in each group. Compared to the control group, hearts in both IP and BK groups had an earlier onset of ischaemic contracture. During the reperfusion period, however, the increase in end diastolic pressure was substantially lower in preconditioned hearts compared to controls, which resulted in a significantly higher developed pressure at 30 minutes of reperfusion in this group. Hearts receiving bradykinin for 10 minutes before ischaemia expressed a similar improvement in postischaemic functional recovery (Table 1). The effect of ischaemic preconditioning on postischaemic contractile dysfunction was not significantly influenced by bradykinin B₂-receptor antagonist HOE 140. In contrast, HOE 140 completely abolished the protective effect of bradykinin on functional recovery of ischaemic myocardium (Table 1).

Regional ischaemia. Coronary flow and left ventricular developed pressure during regional ischaemia in the control, preconditioned (IP) and bradykinin (BK) treated hearts are shown in Table 2. Occlusion of the coronary artery caused a substantial and similar fall in coronary flow and developed pressure in all three groups, and there was no significant difference in recovery of function at 120 minutes of reperfusion (Table 2). The baseline values for heart rate and end diastolic pressure were also not different between the groups.

Irreversible cell injury

Global ischaemia. There were no significant differences in release of creatine kinase between the groups subjected to global ischaemia. In the control group the release was 16.8 ± 2.5 IU/30 min/heart, whereas a release of 19.0 ± 2.0 IU was found in preconditioned and 16.5 ± 2.0 in bradykinin-treated hearts, respectively.

Regional ischaemia. Both ischaemic preconditioning and bradykinin treatment, when applied before 30 minutes of regional ischaemia, significantly reduced infarct size (Figure 6). Risk zone volumes in the three groups were not statistically different, the combined mean value was 357 ± 31 mm³.

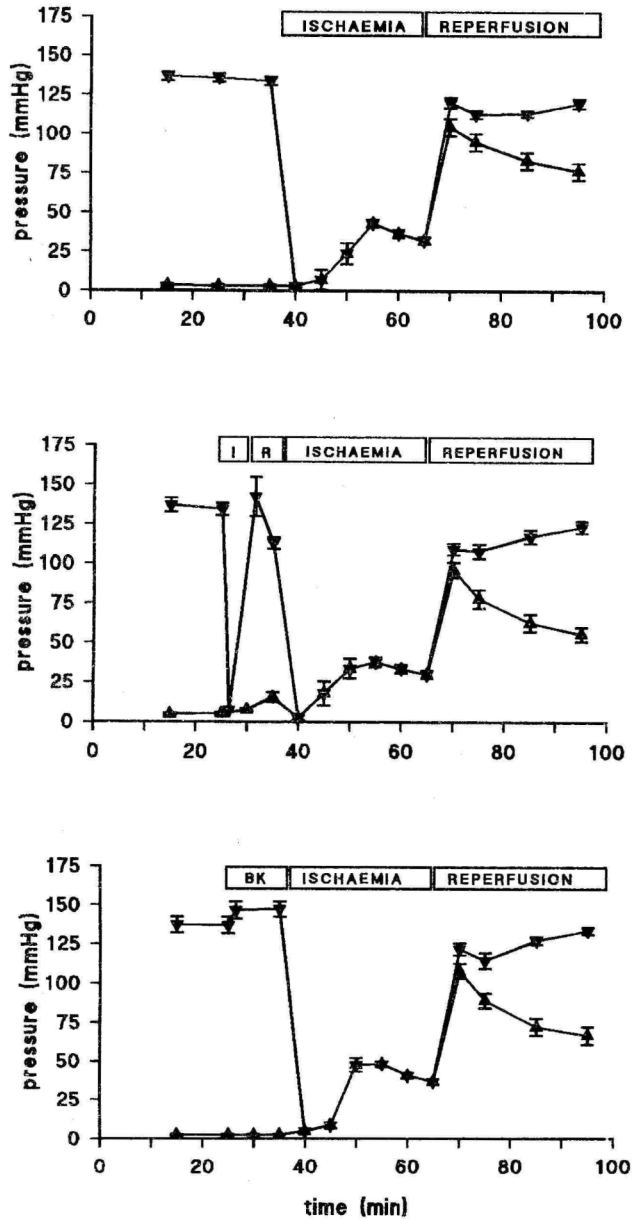


Figure 5. Time course of left ventricular systolic (top tracings) and diastolic pressure (bottom tracing) of hearts subjected to 30 minutes of global ischaemia is shown for 3 groups of hearts. Upper panel control group, middle panel ischemic preconditioning (IP) and lower panel bradykinin treated group (BK). Mean \pm SEM, n=12 in each group.

Table 1. Functional parameters during the experiments in the groups subjected to 30 min of global ischaemia. IP — ischaemic preconditioning, BK — bradykinin.

	Baseline	Stabilisation	Pre-ischaemia	30 min reperfusion
<i>coronary flow (ml/min)</i>				
control	14.1±0.6	14.0±0.6	14.3±0.6	9.6±0.8
IP	14.4±0.5	14.5±0.6	15.8±1.2	12.9±1.6
IP+HOE	14.7±1.1	14.7±1.3	17.8±1.5	14.0±1.0
BK	13.9±0.7	13.8±0.6	16.8±0.7 #	10.6±0.8
BK+HOE	13.2±0.5	13.7±0.7	10.6±0.9 #	8.4±0.5
<i>heart rate (beats/min)</i>				
control	309±11	296±28	313±12	285±16
IP	300±6	305±6	309±11	295±17
IP+HOE	317±18	313±13	328±10	287±11
BK	296±7	298±8	304±7	261±25
BK+HOE	330±14	317±15	301±14	288±15
<i>end diastolic pressure (mmHg)</i>				
control	3.6±0.8	3.3±0.6	3.3±0.6	76.3±5.2
IP	4.9±0.6	5.2±0.5	15.6±3.0 #	55.7±4.6*
IP+HOE	1.7±1.0	1.7±1.0	2.5±1.1	37.5±3.8*
BK	2.6±0.5	2.6±0.5	2.7±0.7	66.8±5.7
BK+HOE	4.4±1.3	4.4±1.3	4.4±1.3	75.6±4.9
<i>developed pressure (mmHg)</i>				
control	133.1±2.7	130.0±2.8	129.2±2.6	43.1±5.9
IP	132.1±4.3	129.3±3.8	97.8±4.7#	67.6±7.1*
IP+HOE	125.9±4.0	134.2±5.4	108.7±4.8#	91.7±3.8*
BK	134.9±5.4	134.6±5.5	145.8±5.0	66.9±6.8*
BK+HOE	120.3±7.3	122.4±7.0	108.3±14.0	40.0±7.8
<i>+dP/dt (mmHg/s)</i>				
control	4792±160	4958±180	4990±186	1298±217
IP	4580±124	4602±148	3114±188#	2284±248*
IP+HOE	4917±201	5333±167	3542±119#	2917±167*
BK	4146±119	4177±128	4458±130	2067±209*
BK+HOE	4361±301	4528±319	3 917±548	1106±258
<i>-dP/dt (mmHg/s)</i>				
control	3365±54	3375±79	3333±50	946±159
IP	3172±93	3261±88	1968±196#	1655±172*
IP+HOE	3083±53	3292±119	2250±65#	2208±140*
BK	3260±120	3292±137	3333±157	1550±140*
BK+HOE	2889±191	3000±186	2417±366	781±175

Baseline — at 15 min of perfusion; Stabilisation — at the end of the 25 min stabilisation period, which is 10 min after drug addition in HOE 140 treated groups; Pre-ischaemia — immediately before the onset of global ischaemia, which is the end of preconditioning cycle or bradykinin infusion, combined with HOE 140 treatment in respective groups. # p≤0.05 vs corresponding baseline value, * p≤0.05 vs control group.

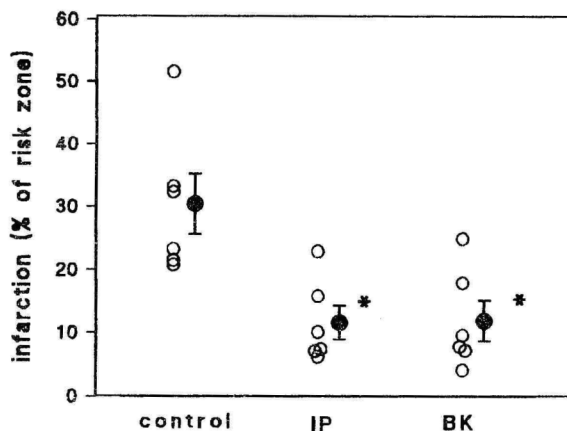


Figure 6. Infarct size expressed as per cent of the risk zone infarcted after 30 minutes of regional ischaemia and 120 minutes of reperfusion. Open symbols represent single hearts while filled symbols with error bars represent means of group \pm SEM. IP — ischaemic preconditioning group, BK — bradykinin-treated group. * $p < 0.05$ compared to control group.

Table 2. Functional parameters during the experiments in the groups subjected to 30 min of regional ischaemia.

	Baseline	Pre-ischaemia	5' ischaemia	120' reperfusion
<i>coronary flow (ml/min)</i>				
control	14.3 \pm 1.6	14.3 \pm 1.5	8.8 \pm 1.6	13.0 \pm 2.1
IP	15.4 \pm 0.7	19.9 \pm 0.1#	11.4 \pm 1.4	12.9 \pm 1.9
BK	13.5 \pm 0.4	16.3 \pm 0.3#	7.2 \pm 0.2	11.8 \pm 1.3
<i>developed pressure (mmHg)</i>				
control	119.3 \pm 12.5	118.5 \pm 12.7	35.2 \pm 5.9	96.0 \pm 4.0
IP	105.8 \pm 6.0	81.3 \pm 6.4#	35.0 \pm 3.4	81.7 \pm 7.4
BK	102.3 \pm 5.0	102.5 \pm 5.5	38.2 \pm 3.4	80.0 \pm 5.1

Baseline — at 15 min of perfusion; Pre-ischaemia — immediately before the onset of regional ischaemia, which is the end of preconditioning cycle or bradykinin infusion in respective groups. IP — ischaemic preconditioning group, BK — bradykinin-treated group. # $p \leq 0.05$ vs corresponding baseline value.

Tissue content of ATP, CP, glycogen, lactate and glucose

Both ischaemic preconditioning and perfusion with bradykinin for 10 minutes resulted in a slight but significant fall in myocardial ATP level, but CP content remained unchanged. At the end of 30 minutes ischaemia or reperfusion, however, no differences in tissue ATP and CP content between the groups were found. Similar, the tissue level of lactate and glycogen were not different between the groups at these time-points.

5.2.2. Lipid peroxidation, arachidonic acid and products of the lipoygenase pathway in ischaemic preconditioning

Fatty acids and peroxidized lipids

Free fatty acid fraction. The changes found in polyunsaturated fatty acids in this fraction of myocardial lipids are illustrated by upper panel on Figure 7. Thus, ischaemic preconditioning with 5 minutes ischaemia and 5 minutes reperfusion increased significantly myocardial level of free arachidonic acid, linoleic acid, and docosahexaenoic acid, as well as the sum of PUFAs.

At the end of 30 minutes of ischaemia unsaturated free fatty acids in both control and IP groups were increased and no difference between these two groups was found. The hearts freeze clamped at the end of 30 minutes ischaemia had significantly higher levels of linolenic acid, arachidonic acid as well as sum of PUFAs when compared to control hearts sampled before ischaemia.

Phospholipid fraction. No significant changes were found in myocardial level of phospholipids, calculated from fatty acids released after treatment of the tissue lipid extract with phospholipase A₂. As expected, there were mostly PUFAs obtained by this enzymatic hydrolysis and no significant differences were found in concentrations of released fatty acids (Figure 7, lower panel).

Lipid peroxidation. The early products of lipid peroxidation, monohydroperoxy and -hydroxy conjugated dienes of polyunsaturated fatty acids, were measured in both free fatty acid and phospholipid fractions and are presented relative to PUFAs in Figure 8.

In the free fatty acid fraction the levels of monohydroxy conjugated dienes were not different between groups before ischaemia. As a result of the increase in total content of PUFAs found at the end of 30 minutes of ischaemia, the relative amount of monohydroxy conjugated dienes at this time-point was reduced. In the control group this tendency reached significance (Figure 8, upper panel). The absolute levels, however, were not different between the groups (data not shown).

With respect to phospholipids, the ischaemic preconditioning cycle of 5 minutes of ischaemia and 5 minutes of reperfusion induced a significant increase of hydroxy conjugated dienes relative to the amount of polyunsaturated fatty acids among fatty acyl residues in the 2-position (Figure 8, lower panel). At the end of the 30 minute ischaemic insult no significant differences between the groups were found (Figure 8, lower panel).

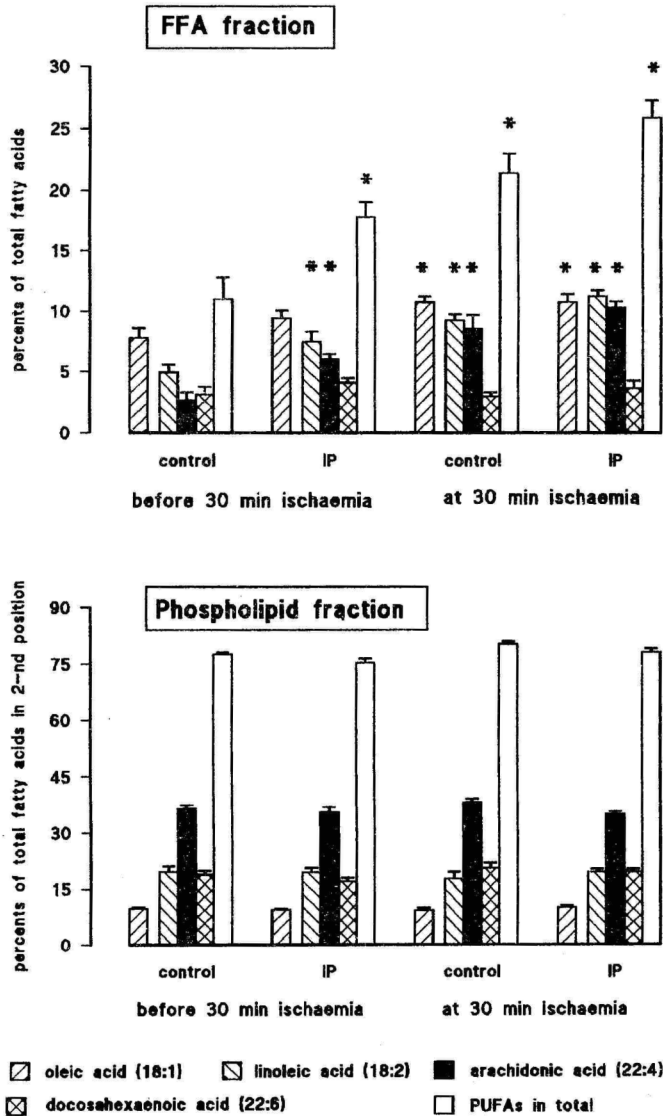


Figure 7. Relative concentrations of fatty acids in free fatty acid and phospholipid fractions extracted from hearts freeze-clamped before and at the end of 30 minutes of global ischaemia. Mean \pm SEM, n=6-10 in each group. IP — ischaemic preconditioning. * p \leq 0.05 compared to control before ischaemia.

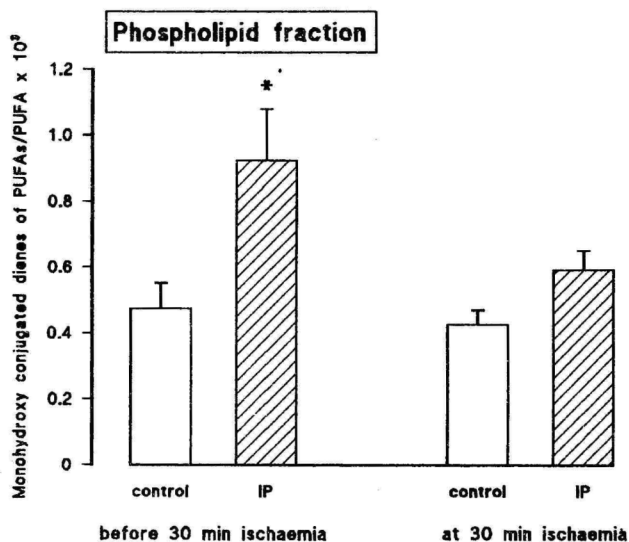
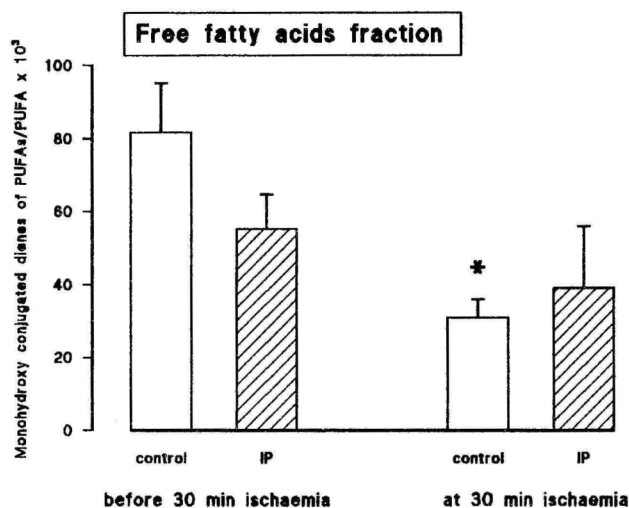


Figure 8. Levels of monohydroxy conjugated dienes of polyunsaturated fatty acids in two fractions of lipid extract from hearts freeze-clamped before and at the end of 30 minutes of global ischaemia. Mean±SEM, n=6–10 in each group. IP — ischaemic preconditioning. * p<0.05 compared to control before ischaemia.

The increase of lipid peroxidation products found in phospholipids of preconditioned hearts was attenuated by the pre-treatment of the hearts with a lipoxygenase inhibitor, NDGA (5 μ M). Thus, the amount of conjugated dienes relative to the amount of polyunsaturated fatty acids in the sample was 0.734 ± 0.130 in IP+NDGA group compared to 0.802 ± 0.107 monohydroxy conjugated dienes of PUFAs/PUFAs $\times 10^3$ in the control+NDGA group, respectively.

NDGA treated hearts subjected to regional ischaemia

Infarct size. Infarct size as percent of risk zone for four groups of hearts is presented in Figure 9. The infarct size limiting effect of ischaemic preconditioning was abolished by the pretreatment of the hearts with NDGA. The drug alone had no effect on development of irreversible myocardial injury. The volume of risk zone was not significantly different among all the groups studied. The overall mean risk zone volume was $298 \pm 12 \text{ mm}^3$ (n=28).

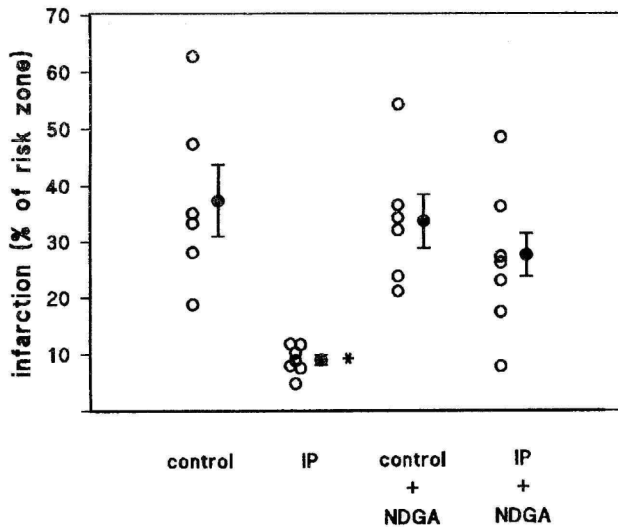


Figure 9. Infarct size expressed as per cent of the risk zone infarcted after 30 minutes of regional ischaemia and 120 minutes of reperfusion. Open symbols represent single hearts while filled symbols with error bars represent means of group \pm SEM. IP — ischaemic preconditioning, NDGA — nordihydroguaiaretic acid. * $p < 0.05$ compared to control group.

Functional recovery. Coronary flow, left ventricular end diastolic pressure and developed pressure of hearts are shown in Table 3. The baseline values for these parameters did not differ between any of the groups. Ischaemic preconditioning led to an increase in coronary flow. This effect was abolished by NDGA: in the IP+NDGA group coronary flow was even significantly decreased when compared to the baseline value. Administration of NDGA for 10 minutes caused a significant decrease in developed pressure which was 13.6 mmHg in average (Table 3). At reperfusion, the NDGA treated hearts (control+NDGA and IP+NDGA, respectively) showed poor recovery of contractile function. In particular, higher levels of end diastolic pressure after reperfusion were observed in these hearts.

5.2.3. Preconditioning with hydrogen peroxide or ischaemia against H₂O₂ induced cardiac dysfunction

Functional recovery

The changes in left ventricular developed pressure and end diastolic pressure are shown in Figure 8. Preconditioning with both 20 μ M H₂O₂ (HPC group) and ischaemia (IPC group) resulted in reduction of developed pressure before perfusion with 140 μ M H₂O₂ (Figure 10, upper panel). During H₂O₂-perfusion, developed pressure was decreased in control hearts, and this effect was attenuated by preconditioning of hearts with low dose H₂O₂. The recovery of developed pressure after high dose H₂O₂ was also improved by H₂O₂ preconditioning, while ischaemic preconditioning had no effect in this respect.

Left ventricular end diastolic pressure was remarkably increased during perfusion with high dose H₂O₂ and had a maximal level after 5 minutes recovery (15 min observation, Figure 10, lower panel). Ischaemic preconditioning attenuated this increase at the end of H₂O₂-perfusion, and after 5 minutes recovery, while preconditioning with low dose H₂O₂ was protective against this effect of H₂O₂ injury at all time points.

Irreversible injury

Release of troponin-T tended to increase during recovery in all groups (not significantly), without significant differences between groups.

Tissue contents of antioxidants

Reduced glutathione (GSH). The baseline contents of GSH in cardiac tissue before preconditioning was 6.5 ± 0.34 mg/g wet weight In control hearts, it decreased to 4 ± 0.25 mg/g wet weight to the end of high dose H₂O₂-perfusion (Figure 11). This decrease was further augmented by H₂O₂ preconditioning.

Table 3. Functional variables of hearts subjected to 30 min of regional ischaemia.

Group	Baseline	Stabilization	Pre-ischaemia	5 min ischaemia	5 min reperfusion	30 min reperfusion	60 min reperfusion	120 min reperfusion
<i>Coronary flow (ml/min)</i>								
Control	12.1±0.6	12.1±0.6	12.1±0.7	7.0±0.6	10.6±1.2	10.0±0.9	9.8±0.7	8.3±0.8
IP	13.0±0.8	12.6±1.0	15.1±1.3#	7.5±0.7	10.6±1.2	10.9±1.4	10.2±1.6	9.9±1.8
Control + NDGA	13.3±0.6	13.3±0.4	13.9±0.7	7.5±0.3	7.1±0.5	6.7±0.6	6.8±1.0	6.7±0.6
IP+ NDGA	13.1±0.5	15.1±0.7	10.0±0.5#	7.4±0.9	7.6±0.7	7.5±0.7	7.7±0.7	7.1±0.8
<i>Left ventricular end diastolic pressure (mmHg)</i>								
Control	3.0±1.0	2.5±1.1	2.5±1.1	8.3±3.3	13.3±5.1	9.2±3.0	10.0±4.1	17.5±7.3
IP	1.0±0.7	1.0±0.7	6.4±4.1	5.4±1.6	10.0±3.9	7.9±3.9	7.9±3.9	9.3±3.9
Control + NDGA	0.5±0.5	0.5±0.5	1.7±1.1	2.5±1.1	24.2±6.9	20.0±5.3*	22.5±6.2	23.0±3.4
IP+ NDGA	1.7±0.8	2.4±0.8	27.8±6.9#	20.6±6.0	43.3±7.7*	33.9±5.3*	33.3±3.0*	35.6±4.9
<i>Left ventricular developed pressure (mmHg)</i>								
Control	161.2±9.7	164.2±7.9	166.6±7.3	67.5±6.4	120.0±12.8	123.3±11.1	114.2±13.2	99.2±12.8
IP	155.4±7.7	151.1±6.0	106.4±3.7#	67.1±7.8	112.1±6.3	115.7±7.8	107.9±8.4	90.7±5.5
Control + NDGA	146.2±8.3	142.8±6.8	129.2±9.2#	52.5±8.5	59.2±7.5*	61.7±7.8*	55.8±7.9*	55.8±6.3*
IP+ NDGA	154.4±5.8	151.1±6.1	71.1±9.2#	51.3±4.4	59.7±5.8*	66.7±4.6*	64.1±5.1*	58.6±5.8*

Baseline — at 15 minutes of perfusion, before drug addition; Stabilization — at 20 minutes of perfusion, which is 5 minutes after drug addition for IP+NDGA group; Pre-ischaemia — immediately before the onset of regional ischaemia, for Control+NDGA group it is 10 minutes after drug addition. IP — ischaemic preconditioning, NDGA — nordihydroguaiaretic acid. # $p \leq 0.05$ vs corresponding baseline value; * $p \leq 0.05$ vs control group at respective time point.

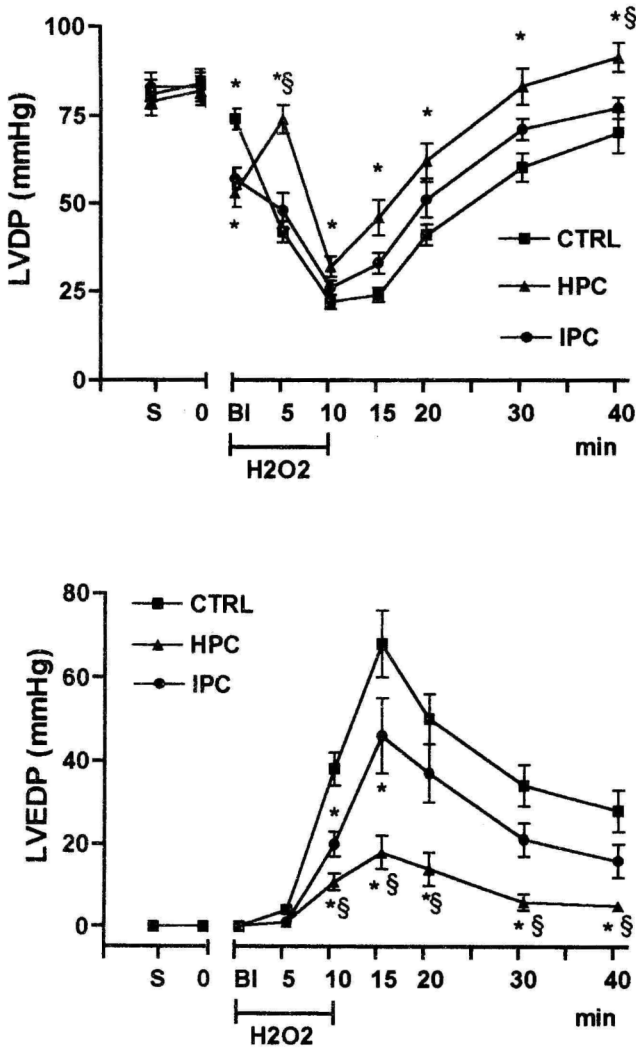


Figure 10. Left ventricular developed (LVDP) and end-diastolic (LVEDP) pressures of hearts subjected to control perfusion (CTRL), pre-treated with 20 μM H_2O_2 for 10 minutes followed by 10 minutes recovery (HPC), or ischaemic preconditioning with 2 episodes of 2 minutes global ischaemia and 5 minutes reperfusion (IPC) before 10 minutes perfusion with 140 μM H_2O_2 and 30 minutes recovery (15–40 min). Mean \pm SEM, $n=10$ in each group. S — stabilization, 0 — before preconditioning, BI — immediately before intervention with 140 μM H_2O_2 . * $p < 0.05$ compared to control group, § $p < 0.05$ between HPC and IPC.

Hearts preconditioned with ischaemia had GSH level at the end of H₂O₂-perfusion significantly lower than the baseline value, however, it remained higher than in control hearts at corresponding time-points.

Glutathione peroxidase, total antioxidant status, antioxidant capacity and catalase did not differ significantly within or between groups.

Superoxide dismutase (SOD). Baseline SOD was 2.5±0.2 U/mg protein, and it was increased 3.1±0.2 U/mg protein by 140 µM H₂O₂. The increase was not altered by H₂O₂-preconditioning, but ischaemic preconditioned hearts had lower contents of SOD than controls at the end of H₂O₂-perfusion.

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS). Baseline TBARS was 34±2 nmol/g wet weight, and was not changed by H₂O₂-perfusion or preconditioning.

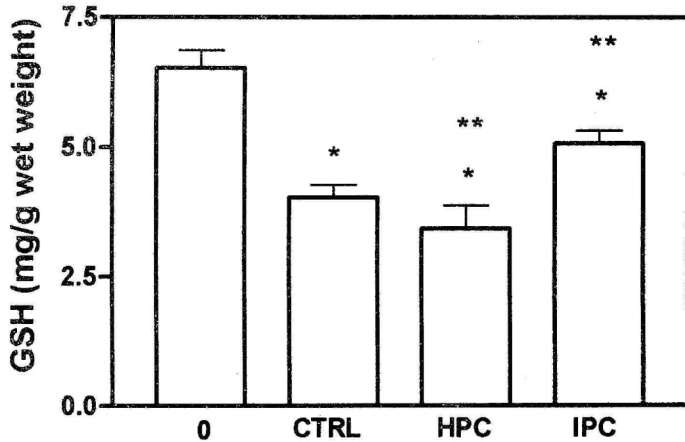


Figure 11. Myocardial content of reduced glutathione (GSH) in hearts before intervention (0), after 20 minutes control perfusion and 10 minutes perfusion with 140 µM H₂O₂ (CTRL), after pretreatment with 20 µM H₂O₂ for 10 minutes followed by 10 minutes recovery and 10 minutes perfusion with 140 µM H₂O₂ (HPC), or ischemic preconditioning with 2 episodes of 2 minutes global ischaemia and 5 minutes reperfusion and 10 minutes perfusion with 140 µM H₂O₂ (IPC). Mean±SEM, n=10 in each group. * p<0.05 compared to baseline level (0), ** p<0.05 compared to control group (CTRL).

5.3. Methodological considerations

The isolated perfused heart model. This model was first described more than hundred years ago (Langendorff, 1895), is well characterized, and is still widely used in basic cardiac research. The main advantage of the model is that it is easy to control and standardize, and it is technically easy to perform. Functional parameters like coronary flow, heart rate and ventricular pressures can be easily monitored. However, perfusion with buffer eliminates the activation of blood components and blood-endothelial interaction as well as influences by circulating hormones (e.g. catecholamines, insulin), sympathetic and parasympathic innervation, and peripheral resistance. Thus, the model greatly simplifies “real life” and findings in the isolated heart model are necessarily not directly applicable to the intact organism. Also, the conclusions obtained by research in one species are not directly applicable to another species. Thus, research based on the rat heart has to be used with great care when the human heart is considered.

Infarct size measurement. The histochemical method used in present work for infarct size measurements is based on separating viable from nonviable myocardium by triphenyl-tetrazoliumchloride (TTC) staining. In viable myocardium, the TTC is reduced to red pigment by intracellular dehydrogenases and NADH. In nonviable myocardium these reducing equivalents are lost, and, consequently, TTC negative tissue can safely be considered dead. This method, however, lacks the ability to detect scattered cell damage. Light microscopic examination of tissue therefore remains as a reliable technique for infarct size assessment, but it requires at least two or three days of reperfusion before necrotic area is clearly demarcated (Ytrehus *et al.* 1993). With respect to control and preconditioned hearts, however, 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 *et al.* 1993). Pharmacological agents (NDGA and bradykinin in present thesis) may interfere with TTC staining, and therefore we cannot completely exclude the possibility that infarct size measured by histology after prolonged reperfusion could lead to other results than those reported in the present work.

Lipid peroxidation assays. In the present thesis, two assays for lipid peroxidation assessment were used. In paper IV, monohydroperoxy and -hydroxy conjugated dienes of fatty acids were determined with HPLC technique supplemented with gas chromatography. With this method the early step in the lipid peroxidation reactions is quantified. This step is probably subject of rapid changes. This method is suggested to have superior specificity (Hegstad *et al.* 1994b; Draper *et al.* 1993; Romaschin *et al.* 1990) and sensitivity as peroxidized lipids

in picomolar range can be detected. However, the proceeding of the sample has many steps and is relatively long-lasting, and this increases theoretical possibility for artifactual changes during the procedure, in particular, when dealing with highly reactive compounds as peroxidation products. In paper V myocardial lipid peroxides were detected by TBARS spectrophometric assay. This method is aimed to detect (accumulating) end-products of lipid peroxidation, specifically malondialdehydes (MDA). The method is relatively simple and easy to proceed. Artifactual changes during the procedure could thus be minimized, some even avoided. However, the method has received argued criticism by some authors due to low specificity (Pincemail *et al.* 1996; Ceconi *et al.* 1991). It is known that measurement of MDA by HPLC detects lower levels of lipid peroxidation end-products than TBARS, suggesting that TBARS takes more than just MDA in the sample. On the other hand, it has been suggested that remarkable and physiologically relevant changes in lipid peroxidation could be detectable by this assay (Sies, 1985). In addition, data from simultaneous measurements of antioxidant parameters would provide supplemental information for objective and complete assessment of oxidative stress status.

Myocardial content of high energy phosphates, glycogen and lactate. In order to evaluate whether the achieved cardioprotective effect coexists with alterations in biochemical energy status, we measured myocardial content of high energy phosphates, glycogen and lactate in bradykinin pre-treated and preconditioned hearts. These data, although not proposed to give detailed information about metabolic activity, provide a picture of energy status in the heart at the sampling point. Interpretation of the data should be carried out with some reservation. With respect to ATP, for instance, we have measured total tissue content, but production/utilization, concentrations in different cell compartments, etc. were not measured.

Drugs. In all papers included in this part of the thesis various drugs have been used. Although these drugs are all claimed to exert specific effects, there will always be a possibility that effects other than that/those expected might to some degree contribute to the results obtained.

5.4. Discussion

In the second part of the present thesis we found that various interventions, including preischaemic bradykinin, ischaemic preconditioning, and low dose of hydrogen peroxide, can induce cardioprotection in experimental model of isolated rat heart. Our results also indicate that oxidative stress appears to have dual role in ischaemia-reperfusion of the heart. In the following, underlying mechanisms of protection are discussed.

Myocardial protection by preischaemic bradykinin

In the paper III of the present thesis we found that a short preischaemic bradykinin infusion improved the postischaemic functional recovery in isolated rat hearts. Based on the corresponding data on infarct size this indicates that less cell death during the ischaemia reperfusion insult explains this finding. Our results are consistent with previous studies demonstrating that bradykinin can reduce postischaemic contractile dysfunction in rats (Brew *et al.* 1995), dogs (Ehring *et al.* 1994) and pigs (Tio *et al.* 1991). HOE 140, reported to be a potent and highly specific bradykinin B₂-receptor antagonist (Hock *et al.* 1991), completely reversed the protective effect of bradykinin, revealing that the afforded protection was mediated by B₂-bradykinin receptors. Bradykinin B₂-receptors therefore seem to be coupled to a potent endogenous system for protection against ischaemic cell death.

Stimulation of B₂-receptors on endothelial cells results in release of nitric oxide and prostacyclin from these cells. Although there is no consensus with respect to cardioprotective properties of nitric oxide so far, accumulating evidence suggests that inhibition of NO synthesis does not influence the cardioprotective effect of bradykinin (Patel *et al.* 1993; Ehring *et al.* 1994). Prostacyclin seems to possess anti-ischaemic properties, partly reversed by the cyclo-oxygenase inhibitor indomethacin (Martorana *et al.* 1991). This could suggest involvement of cyclo-oxygenase plus an additional mechanism in the cardioprotective effect of bradykinin. In rat hearts an independent kallikrein-kinin system has been demonstrated, suggesting that locally generated kinins may regulate cardiac functions (Nolly *et al.* 1994). The presence of bradykinin B₂-receptors on cardiomyocytes, which are functionally coupled to the production of inositol trisphosphate IP₃, has recently been demonstrated in several species (Minshall *et al.* 1995). The IP₃ production is believed to be coupled to the same signal transduction pathway as protein kinase C (PKC) activation. It has been demonstrated that inhibition of PKC completely blocked the effect of bradykinin on infarct size in rabbits (Goto *et al.* 1995) as well as on contractile dysfunction in isolated rat hearts (Brew *et al.* 1995). Bugge and Ytrehus have recently reported similar findings in the infarct model in isolated perfused rat hearts (Bugge *et al.* 1996b). Therefore our results support the view that re-

ceptors coupled to intracellular signal transduction through the PKC system are able to protect the heart against ischaemic cell death.

Ischaemic preconditioning — cellular mechanisms beyond myocardial protection

In present work, two aspects of cellular mechanisms proposed to be involved in ischaemic preconditioning were considered. At first, the role of bradykinin in ischaemic preconditioning's effect on postischaemic functional recovery was examined, and, second, the involvement of oxidative stress, arachidonic acid and its lipoxygenase derived metabolites was studied. We found that pretreatment of preconditioned hearts with the bradykinin B₂-receptor antagonist HOE 140 did not abolish the effect of preconditioning on postischaemic functional recovery. In the following experiments we found that preconditioning with one cycle of 5 minutes of global ischaemia and 5 minutes of reperfusion resulted in a significant increase in fatty acid hydroxy conjugated dienes in myocardial phospholipids, and there was concomitant increase in arachidonic acid as well as in other PUFAs in the free fatty acid fraction of myocardial tissue. We also found that the infarct size limiting effect of ischaemic preconditioning in the isolated rat heart could be blocked by nordihydroguaiaretic acid (NDGA), an inhibitor of the lipoxygenase pathway of arachidonic acid metabolism.

These our results altogether support the view that metabolites of arachidonic acid derived by lipoxygenase are important intracellular messengers responsible for the cellular adaption leading to protection against ischaemic cell death in ischaemic preconditioning of the rat heart. Bradykinin, although it can induce protection of the rat heart through a mechanism similar to ischaemic preconditioning, is evidently not the only or sole trigger of this phenomenon. Our results support, however, that oxidative stress could be a trigger of preconditioning in rat heart.

Our finding that B₂-receptor antagonist HOE 140 did not abolish the cardioprotective effect of ischaemic preconditioning corresponds with other studies, in which infarct size as endpoint of ischaemic injury was measured either in isolated rat hearts (Bugge *et al.* 1996b) or rabbit hearts (Goto *et al.* 1995). However, in contrast to these findings, Brew *et al.*, using an experimental model of global ischaemia comparable to that one used in the present study, have reported that the effect of preconditioning in rat hearts was significantly attenuated, although not completely abolished, by blockage of B₂-receptors (Jeroudi *et al.* 1994). The use of a different bradykinin B₂-antagonists (NPC-349 by Brew *et al.* and HOE 140 in the present study) could influence the results, but also the difference in experimental protocols might be causal for this controversy. Brew *et al.* have used 2 minutes ischaemia and 8 minutes reperfusion to precondition the hearts, while we used 5 minutes ischaemia plus 5 minutes reperfusion. Furthermore, Brew *et al.* have used 20 minutes sustained

ischaemia instead of 30 minutes in the present study, and therefore the achieved cardioprotection in their model is probably more dependent on absence or presence of stunning. Pharmacological interventions might influence contractile function partly independent of the effect upon ischaemic preconditioning and confound the results in models using contractile function as end point.

Although we believe that bradykinin is not the only or sole mediator of ischaemic preconditioning in the isolated perfused rat heart the possibility exists that bradykinin can operate in concert with other endogenous substances released during short preconditioning ischaemia and that blockage of a single trigger is insufficient to restrain the cardioprotective effect of preconditioning. The common feature for these substances (i.e. adenosine, noradrenaline, acetylcholine, angiotensin II, endothelin, opioid peptides) is that their sarcolemmal receptors are all coupled to the intracellular signalling pathway for protein kinase C activation, which, as demonstrated in many recent studies, is crucial for ischaemic preconditioning (Ytrehus *et al.* 1994; Speechly Dick *et al.* 1995; Cohen *et al.* 1996). However, simultaneous blocking of bradykinin, adenosine and α -adrenoreceptors did not blocked ischaemic preconditioning of rat heart (Bugge *et al.* 1996b). This suggests the presence of alternative or additional stimulators linked to intracellular signalling pathways responsible for PKC activation.

From this point of view, our finding of enhanced phospholipid peroxidation and an increase in free arachidonic acid as well as other PUFAs by the preconditioning procedure seems important. It suggests that oxidative stress and phospholipase A₂ activation may be involved as additional pathway in cellular signal transduction in ischaemic preconditioning. Possible mechanisms explaining how oxidative stress could be involved in the physiology of ischaemic preconditioning have been debated in a number of previous studies (Richard *et al.* 1993; Tanaka *et al.* 1994; Zhou *et al.* 1996). Firstly, it has been suggested that the beneficial effect of preconditioning is the consequence of an ischaemia induced increase in myocardial antioxidant activity. However, many authors have failed to detect any changes, at least in studies on the early protection, in heart antioxidant status after preconditioning (Turrens *et al.* 1992; Richard *et al.* 1993; Fu *et al.* 1993b). Another possibility, supported also by our results, is that free radicals participate in cellular signal transduction and are in this way involved in the adaptive responses against ischaemic injury. Thus, on the cellular level, the following events is suggested to occur in ischaemic preconditioning (see Figure 12 for illustration). Short ischaemia-reperfusion episode(s) required to precondition the heart induce oxidative stress and subsequent lipid peroxidation. Oxidation of lipids by free radicals can activate phospholipase A₂ (Potts *et al.* 1992), which releases polyunsaturated fatty acids from phospholipids. Arachidonic acid, released by PLA₂, is shown to activate protein kinase C, in some circumstances even synergistically with diacylglycerol (Khan *et al.* 1995; Nishizuka, 1995). Protein kinase C, once activated, can

further phosphorylate PLA₂ among other proteins and this generates a positive feedback system for cellular signalling (Nishizuka, 1995) through protein kinase C. A likely candidate for an effector protein, activated by protein kinase C dependent phosphorylation during ischaemic preconditioning, is the K_{ATP}-channel (Tsuchida *et al.* 1994; Speechly Dick *et al.* 1995; Hu *et al.* 1996; Bugge *et al.* 1996a). The K_{ATP}-channels, however, may be activated also by nonesterified polyunsaturated fatty acids in a PKC dependent manner (Müller *et al.* 1992) or directly by arachidonic acid, as well as HETEs and HPETEs (Buttner *et al.* 1989; Xu *et al.* 1996). Involvement of the latter hypothesis is supported by our finding that the infarct size limiting effect of preconditioning was blocked by NDGA, a lipoxygenase inhibitor.

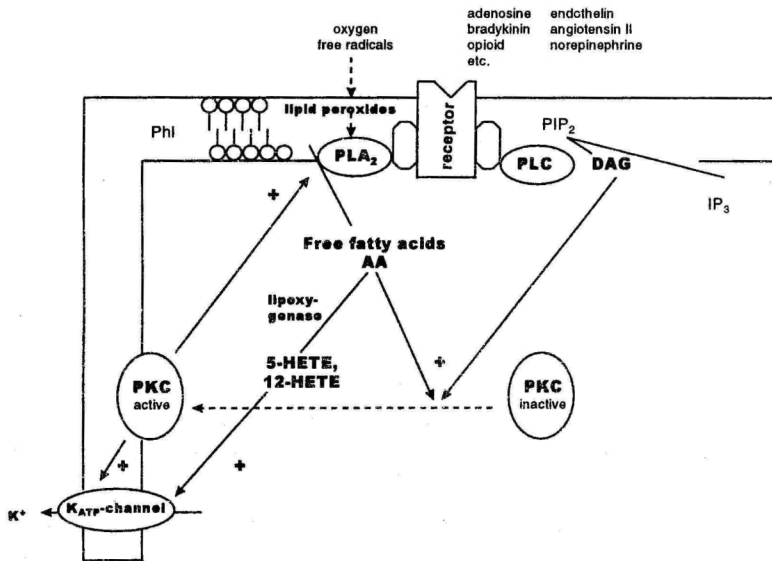


Figure 12. The proposed intracellular signalling pathway for ischaemic preconditioning. Phl — phospholipids, PLA₂ — phospholipase A₂, PIP₂ — phosphoinositol-diphosphate, PLC — phospholipase C, DAG — diacylglycerol, IP₃ — inositol-trisphosphate, AA — arachidonic acid, 5-HETE — 5-hydroxyeicosatetraenoic acid, 12-HETE — 12-hydroxyeicosatetraenoic acid, PKC — protein kinase C. See text for further discussion.

Postischaemic functional recovery, cell death, and myocardial energy status

A critical question for any study, investigating potentially cardioprotective interventions and using functional parameters as an endpoint, depends on clarifying whether the improved functional recovery is due to limited extent of cell death or achieved by reduced stunning (or inotropic stimulation at reperfusion) or is a mixture of both. In the study investigating the effects of bradykinin on functional recovery after 30 minutes of global ischaemia we used mea-

surements of creatine kinase leakage during the first 30 minutes of reperfusion as a standard for assessment of myocardial necrosis. We did not find any significant differences in this parameter, suggesting that neither bradykinin nor preconditioning influenced the extent of cell necrosis. To confirm this finding, we performed an additional series of experiments with exactly the same protocols of ischaemic preconditioning or bradykinin treatment but followed by 30 minutes of regional ischaemia. We found that both interventions reduced infarct size substantially in rat hearts. Based on these results, we think that measurement of creatine kinase release can sometimes lead to inappropriate interpretations, probably due to delay in creatine kinase washout. We therefore suppose that the improved functional recovery in our model of global ischaemia is related to reduction in ischaemic cell death.

In order to evaluate whether the achieved cardioprotective effect coexists with alterations in biochemical energy status in the heart tissue, we measured tissue content of high energy phosphates, glycogen and lactate. One cycle of 5 minutes of ischaemia and 5 minutes of reperfusion caused decrease in myocardial glycogen with concomitant increase in tissue free glucose, which is in accordance with previous studies (Schaefer *et al.* 1995). However, despite preischaemic difference in the measured tissue metabolites (decreased glycogen and increased glucose content, reduced ATP level) the preconditioned and bradykinin treated hearts did not exhibit any difference in myocardial ATP and CP contents when compared to control neither at the end of ischaemia nor at 30 minutes of reperfusion. Furthermore, we did not find any correlation between recovery of function during reperfusion and level of high energy phosphates and glycogen. Especially it was surprising to see that hearts with less cell death and better recovery of postischaemic contractile function (preconditioned and bradykinin pre-treated hearts) did not have an higher tissue level of ATP and CP compared to controls. In summary, we believe that alterations found in high energy phosphates and glycogen stores are not prominent factors for explaining the protection observed after ischaemic preconditioning.

In the experiments where NDGA was used in the setting of regional ischaemia, we found that NDGA had a some detrimental effect on contractile function during reperfusion. In particular, the diastolic pressure at reperfusion was higher in these hearts, and it appeared to be independent of the duration of the preceding ischaemia (30 minutes in control group or 5 minutes during preconditioning). One could speculate that the blocking effect of NDGA in preconditioned hearts is due to the effect on contractile function. However, under control conditions infarct size was not influenced by NDGA, while contractile performance of these hearts was similarly deteriorated. On the other hand, the untreated preconditioned hearts, although having significantly less tissue infarcted, did not show better recovery of contractile function during 120 minutes of reperfusion compared to untreated non-preconditioned hearts. These findings stress the fact that there appears to be poor correlation between the

extent of ischaemic cell death and global contractile performance during reperfusion in isolated rat heart subjected to regional ischaemia.

Myocardial protection by low dose of hydrogen peroxide

The main findings from paper V of the present thesis were that preconditioning with a low dose of H_2O_2 improved functional recovery after cardiac injury induced by higher dose H_2O_2 , while ischaemic preconditioning with a 2 minutes ischaemia cycle was only minimally protective in this experimental setting. There was no difference between groups in the cardiac release of troponin-T during reperfusion, indicating that reduction of myocardial necrosis was not an important mechanism for the protection afforded. Thus, it appears that ischaemic preconditioning with 2 minutes ischaemia cycle does not efficiently protects the heart against cardiac injury induced by exogenous ROS. Some studies indicate that ischaemic preconditioning could protect against myocardial stunning (Cave *et al.* 1993; Lawson *et al.* 1993; Cave *et al.* 1994; Vuorinen *et al.* 1995), and this phenomenon is believed to be mediated by ROS generated at reperfusion (Bolli, 1992; Mitchell *et al.* 1993; Jeroudi *et al.* 1994).

Moderate oxidative stress as possible cardioprotective intervention, in particular the effect of low dose hydrogen peroxide, has been increasingly studied during the last few years. Very low dose of hydrogen peroxide while administered during reperfusion has been shown to limit infarct size in isolated perfused rabbit heart (Ytrehus *et al.* 1995). In isolated rat heart, however, controversial results have been reported. In some studies the low dose of hydrogen peroxide have been found to provide myocardial protection (Miyawaki *et al.* 1996; Hegstad *et al.* 1994a), while some studies report opposite results (Takeshima *et al.* 1997). Thus, a very narrow threshold seems to exist with respect to this type of protection.

The exact mechanisms of cardiac dysfunction induced by high doses of H_2O_2 are not fully clarified. Hydrogen peroxide itself may be the main injurious agent, since catalase inhibits its functional as well as biochemical changes (Takemura *et al.* 1993; Skjelbakken *et al.* 1996). H_2O_2 penetrates biological membranes and high concentrations can be reached in the interior of the cell. H_2O_2 accumulating in mitochondria is known to influence mitochondrial redox state and impair oxidative phosphorylation. The hydroxyl radical may also play a role, as hydroxyl radical scavengers partially attenuate the cardiac dysfunction induced by H_2O_2 (Takemura *et al.* 1993; Skjelbakken *et al.* 1996). In the present study 140 μM H_2O_2 did not induced detectable lipid peroxidation, and thus lipid peroxidation does not appear to be an important mechanism of injury which may explain the functional impairment in the present model of ROS-induced injury.

In contrast to lipid peroxidation, tissue glutathione was reduced by H_2O_2 in present model. The reduction was exacerbated by pretreatment with H_2O_2 , but was attenuated by ischaemic preconditioning. These our findings suggests that

the thiol-groups of peptides and proteins are more easily exposed to oxidative injury than myocardial lipids. In accordance with our findings, Ambrosio *et al.* have found that perfusing rat hearts with H_2O_2 increased the release of glutathione into the coronary effluent (Ambrosio *et al.* 1992). Using another model of ROS injury, perfusion with hypoxanthine/xanthine oxidase, Vaage *et al.* have found that oxidized glutathione remained unchanged at the end of intervention, but was increased after 30 minutes recovery, while reduced glutathione was decreased after 30 minutes recovery (Vaage *et al.* 1997). However, it is not clear whether myocardial level of glutathione has any important role in functional recovery after cardiac injury. Increased myocardial glutathione level, achieved by induction of endogenous catalase activity, did not protect against H_2O_2 -induced injury in isolated rat hearts (Stearns *et al.* 1994). In another study exogenous glutathione was found to attenuate hypoxia-induced stunning in isolated rat hearts (Seiler *et al.* 1996). In the present study, depletion of endogenous glutathione seemed not to be critical for cardiac function, as the most depleted hearts had the best function. It could be speculated that the changes in GSH level are important for induction of adaptive changes. Availability of GSH in cellular compartments might also be of importance to explain the observations. Depletion of GSH might also reflect better protection of proteins SH-groups in expense of GSH oxidation, and this, consequently, could explain the improved contractile function in the H_2O_2 pre-treated group.

With respect to mechanisms whereby low dose of hydrogen peroxide protected the heart against high dose H_2O_2 injury we can propose only speculative explanations. We found that the beneficial functional effects were not due to reduction of necrosis, inhibition of lipid peroxidation, or increases of antioxidant levels. In previous study, Valen *et al.* have found that perfusing isolated rat hearts with low concentrations of H_2O_2 induces a vasodilatation which is partly dependent on nitric oxide, and inhibition of nitric oxide aggravates functional effects of H_2O_2 (Valen *et al.* 1996). Therefore, release of nitric oxide in hearts pre-treated with H_2O_2 may have contributed to the protective effects in the present study. In present study, however, coronary flow before inducing injury with high H_2O_2 was lowest in hearts preconditioned with H_2O_2 , and this means that NO production was very unlikely increased in this group. Another possibility is that low dose hydrogen peroxide can modify cellular signal transduction and this generates myocardial adaption against subsequent injury. The activation of phospholipases and thereby an increase in the level of lipid second messengers by hydrogen peroxide have been proposed by several authors (Larsson *et al.* 1989; Meij *et al.* 1994), whereas others have found a decrease in phospholipase activity related to thiol group modification. Dose dependent relations could explain this diversity. Activation of phospholipases C or D and/or A_2 could result in activation of protein kinase C, which, as discussed above, is proposed to occur also in ischaemic preconditioning. In support to

this hypothesis Miyawaki *et al.* have reported that pre-treatment of isolated rat hearts with 20 μM H_2O_2 attenuates calcium paradox injury through protein kinase C activation (Miyawaki *et al.* 1996). Furthermore, the role for K_{ATP} -channels in this protection has been suggested by these authors. With regards to this, however, it seems controversial why ischaemic preconditioning afforded only marginal protection against H_2O_2 -injury in the present study. The most likely explanation is that although preconditioning of hearts with 2 episodes of 2 minutes ischaemia + 5 minutes reperfusion is clearly protective against ischaemia-reperfusion injury under some circumstances it is not sufficient to generate signal activation threshold necessary to protect the heart against H_2O_2 -injury. Most laboratories agree that a 2 minutes ischaemia cycle is at the threshold and therefore often not producing enough stimulation for adaption.

In summary, we found that pretreatment of hearts with a low dose of H_2O_2 attenuated cardiac dysfunction induced by high dose of H_2O_2 in isolated rat hearts. The mechanism of protection was not through reduction of necrosis, limitation of lipid peroxidation, or influence on cardiac antioxidant activity. These findings support the hypothesis that moderate oxidative stress can induce beneficial adaptive responses against subsequent cardiac injury.

6. CONCLUSIONS

1. Open-heart surgery is associated with significant and time dependent increase in blood lipid peroxidation products and simultaneous decrease in blood antioxidant capacity. These findings are indicative of the presence of oxidative stress in this clinical setting.
2. The severity of oxidative stress found in cardiac surgery patients is characterized by the finding that changes of comparable extent occurred in critically ill septic patients. The data provide evidence for systemic inflammatory response syndrome caused by cardiopulmonary bypass as an important contributing factor for oxidative stress in open heart surgery. The clinical implication of these findings would be that adjuvant use of antioxidant drugs might attenuate the some of harmful effects of cardiopulmonary bypass and thus improve the outcome of patients after heart surgery.
3. Preconditioning of isolated rat hearts with one cycle of 5 minutes of global ischaemia and 5 minutes of reperfusion provides potent protection against myocardial infarction and postischaemic contractile dysfunction. Preischaemic bradykinin can improve postischaemic contractile dysfunction similarly, and this effect is mediated through bradykinin B₂-receptors. At the same time, the pre-treatment of hearts with B₂-receptor antagonist did not alter the protection afforded by ischaemic preconditioning. A clinical implication for these findings is support for direct cardioprotective properties of ACE-inhibitors, since these drugs are known to increase the tissue levels of bradykinin.
4. Preconditioning of rat hearts with a short ischaemia-reperfusion episode resulted in significant increase in phospholipid peroxidation and in myocardial level of free arachidonic acid. The effect of ischaemic preconditioning against infarction can be blocked by nordihydroguaiaretic acid, a lipoxygenase inhibitor. These findings support evidence for a role of oxidative stress, PLA₂ and lipoxygenase-derived lipid second messengers in ischaemic preconditioning. This phenomenon might modulate the proposed harmful effects of oxidative stress in clinical settings.
5. Correlation between the extent of ischaemic cell death and global contractile performance seems not to be present in the preconditioned or pharmacologically pretreated isolated rat heart. The attenuation of ischaemic injury by both bradykinin or preconditioning is not associated with improved tissue ATP or CP levels at the end of ischaemia and reperfusion indicating that this form of energy conservation has not a prominent role in preserving tissue survival with these preischaemic treatments.
6. Pre-treatment of hearts with a low dose of H₂O₂ significantly improved functional recovery after myocardial injury induced by high dose of H₂O₂.

The mechanism of protection was not through reduction of necrosis, limitation of lipid peroxidation, or significant influence on cardiac antioxidant activity. These findings support the hypothesis that moderate and controlled oxidative stress can induce beneficial adaptive responses as exposure to low dose of H₂O₂ protected the heart against subsequent oxidative injury.

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OKSÜDATIIVNE STRESS JA SÜDAME ISHEEMIA-REPERFUSIOON

Kliinilis-eksperimentaalne uurimus

Kokkuvõte

Oksüdatiivse stressi (OS) all mõistetakse organismi pro- ja antioksidantide tasakaalu häiret, mis viib potentsiaalsele koekahjustusele. Oksüdatiivsel stressil arvatakse olevat osa mitmete koekahjustuste/haiguste, muu hulgas isheemia-reperfusiooni kahjustuse patogeneesis. Südamelihase isheemia järgse reperfusiooni (reoksügenisatsiooni) käigus produtseeritud hapniku reaktiivsete osakeste tähtsus südamelihase isheemilise kahjustuse eri vormide puhul on erinev. Nii ei saa väita, et pikaajalise isheemia tagajärjel tekkiva letaalse kahjustuse e. infarkticolde suuruse määraaks reperfusioonil esinev OS. Kuni 20 minutit kestva isheemiaepisoodi põhjustatud täielikult pöörduva kontraktiilse funktsiooni languse ("oimetu" müokard) keskseks põhjuseks peetakse aga just reperfusiooni käigus tekkinud OS-i. Üheks kliiniliseks situatsiooniks, kus südamelihase allutatakse plaanilisele isheemia-reperfusioonile, on kehavälise vereringe tingimustes toimuv südameoperatsioon. On näidatud, et kehavälise vereringe põhjustab paljude põletikumediaatorite aktiveerumise, kutsudes esile (sarnaselt septilise seisundiga) üldise, kogu organismi haarava põletikureaktsiooni. Muu hulgas aktiveeruvad neutrofiilsed granulotsüüdid, mille produtseerivad hapniku reaktiivsed osakesed (hapniku vabad radikaalid) süvendavad isheemia-reperfusiooniepisoodidega kaasnevat oksüdatiivset stressi.

Lühiajaline isheemia võib aga olla ka organismile soodsate tagajärgedega, mobiliseerides südame endogeenseid kaitsemehhanisme. Isheemiline eelkohastumus (*ischaemic preconditioning*, lühendatult IE) on tuntud kui fenomen, mille puhul lühiajaline (-sed) isheemiaepisood(id) vähendavad oluliselt järgneva, pikaajalisema isheemia põhjustatud müokardikahjustuse ulatust. IE on efektiivne kõigi isheemilise kahjustuse vormide puhul — väheneb nii isheemiajärgsete rütmihäirete esinemine kui ka infarkticolde suurus, paraneb oluliselt südame kontraktiilse funktsiooni taastumine. Arvatakse, et selle võimsa adaptatiivse endogeense mehhanismi käivitumisel on tähtis osa adenosiin-A₁-, α₁-adrenoretseptorite jt. membraaniretseptorite stimulatsioonil. Samas puudub ühtne arusaam, milline roll võiks IE füsioloogias olla OS-ga seoses olevatel protsessidel.

Uurimuse eesmärgid

Käesoleva töö põhieesmärkideks oli uurida järgmisi asjaolusid:

1. Oksüdatiivse stressi esinemise perioperatiivset dünaamikat haigetel kehavälise vereringe tingimustes tehtud südameoperatsioonide korral. Oksüdatiivse stressi markerid määrati vereplasmas kuuel ajamomendil. Oksüdatiivse stressi ulatuse iseloomustamiseks määrati samad parameetrid võrdlevalt septilistel üldkirurgilistel haigetel.
2. Kas preisheemiline bradükiniinireseptorite stimulatsioon kaitseb südant isheemia-reperfusiooni kahjustuse eest ja kas bradükiniin osaleb isheemilise eelkohastumuse tekkimisel Langendorffi järgi perfundeeritud, isoleeritud rotisüdamel?
3. Oksüdatiivse stressi rolli südamelihase isheemilise eelkohastumuse tekkemehhanismides. Selleks on määratud müokardi lipiidse peroksüdatsiooni taset ning antioksidantsete süsteemide aktiivsust roti südamelihases nii isheemilise eelkohastumuse eel kui ka järel. Lisaks on uuritud, kas lühiajaline ja mõõdukas oksüdatiivne stress ise, antud juhul madalas doosis H_2O_2 , on võimeline aktiveerima endogeenseid kaitsemehhanisme ning seeläbi kaitsma südamelihast järgneva, potentsiaalselt letaalse kahjustuse eest.

Uurimuse peamised tulemused

Uurimuse tulemusena selgus, et patsientidel, kes teevad läbi südameoperatsiooni kehavälise vereringega, esineb oluline oksüdatiivne stress. Vereseerumi lipiidide peroksüdatsiooniproduktide hulk suureneb juba 15 minuti jooksul pärast kehavälise vereringe algust ning saavutab maksimumi pärast aordiklemmi eemaldamist. Esimese operatsioonijärgse päeva hommikuks on muutused taandarenenud operatsioonieelsele tasemele. Vereseerumi antioksidantse mahtuvuse langus on samuti suurim pärast aordiklemmi eemaldamist, jääb püsima aga ka esimese operatsioonijärgse päeva hommikul. Nimetatud muutuste ulatus on võrreldav kriitilises seisundis olevate septiliste haigete omaga. Tulemuste põhjal saab väita, et operatsioonidega avatud südamel kaasneb raske oksüdatiivne stress, mis oma ulatuselt on sarnane raskes septilises seisundis olevatel haigetel esinevaga. Seega, mõlema kliinilise seisundi korral oleks argumenteeritud adekvaatse (preventiivse) antioksidantse ravi kasutamine.

Uurimuse eksperimentaalses osas leidsime, et südamelihase bradükiniini B_2 -retseptorite isheemiaeelne stimulatsioon vähendab nii regionaalse kui ka üldise isheemia põhjustatud müokardikahjustust. Nimetatud efekt ei olnud seotud müokardi ATP ja kreatiinfosfaadi varude soodustatud säilimisega. Isheemiline eelkohastumus osutus efektiivseks nii regionaalse kui ka üldise isheemia puhul, kuid roti südamel ei ole bradükiniini B_2 -retseptoritel olulist rolli saavutatud kardioproteksioonis. Samas selgus, et isheemilise eelkohastumuse

puhul tekib lühiajalise isheemia järgse reperfusiooni käigus sarkolemma fosfolipiidide peroksüdatsioon, mis võivad olla tähtsad protektsiooni tagavate rakusiseste signaalsüsteemide käivitumisel. Mõõduka, kontrollitud oksüdatiivse stressi olulisust adaptatiivsete muutuste esilekutsumisel toetavad ka käesolevas uuringus saadud teised tulemused. Nimelt leiti, et vesinikperoksiid, manustatuna väikses doosis, parandab oluliselt südame oksüdatiivse kahjustuse järgset funktsionaalset taastumisvõimet. Seega võib isheemia-reperfusiooni ning oksüdatiivse stressi patofüsioloogias leida ühe sarnase ja olulise põhimõtte: südamelihase lühiajaline, subletaalne ekspositsioon kahjustavale faktorile (vastavalt isheemia või väikses doosis H_2O_2) kutsub esile adaptatiivsed muutused, mis kaitsevad oluliselt müokardi järgneva potentsiaalselt letaalse kahjustuse vastu. Nimetatud adaptatiivsete muutuste rakusiseste mehhanismide täpsem tundmaõppimine võib osutada perspektiivseks uute müokardi protektsiooniks kasutatavate strateegiade väljatöötamisel.

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TIME COURSE OF OXIDATIVE STRESS DURING OPEN-HEART SURGERY

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Abstract. Oxidative stress and subsequent lipid peroxidation have been suggested as pathogenetically important for postischaemic reperfusion injury. We studied the time-course of oxidative stress in 14 adults undergoing cardiac surgery, evaluating serum levels of lipid peroxidation products—diene conjugates (DC) and basal and Fe-stimulated thiobarbituric acid reactive substances (TBARS, Fe-TBARS)—as well as markers of blood antioxidant status—serum antioxidative capacity (AOC) and red blood cell glutathione (RBC-GSH) at 6 perioperative time-points. Arterial TBARS were significantly increased 15 minutes after start of cardiopulmonary bypass, 5 minutes after release of aortic cross-clamp and 15 minutes after cessation of bypass, compared with the preoperative levels (respective means 20.8, 38.5, 34.8 vs 7.5 nmol/g protein, $p < 0.05$). AOC had decreased at these times (means 21.3, 18.1, 23.2 vs 34.9%, $p < 0.05$). The TBARS changes correlated with AOC decrease ($r = 0.30$, $p < 0.001$). Changes in serum DC and RBC-GSH were not statistically significant. All lipid peroxidation parameters had returned to preoperative levels on the following morning, while antioxidative capacity remained suppressed (28.1%, $p < 0.05$). These data demonstrate a definite time-course of oxidative stress markers in arterial blood during open-heart surgery.

Key words: Oxidative stress, cardiac surgery, cardiopulmonary bypass, reactive oxygen species.

Many tissues may be damaged after cardiopulmonary bypass (CPB) during open-heart surgery. In particular, depression of cardiac function (myocardial “stunning”) and impaired pulmonary function in the early postoperative period have great clinical importance (2, 9, 12, 16). The myocardial damage, which probably occurs during reperfusion of the cardioplegic heart and also after cessation of CPB with reperfusion of lungs and other tissues, may play a substantial role in development of organ damage in these patients (8, 17, 27). It has also been suggested that “whole body inflammatory response” to CPB, which includes the changes

in circulating neutrophils, platelets, components of the complement system, etc., is in part responsible for organ dysfunction following open-heart surgery (15, 16, 27).

Oxidative stress, mediated by reactive oxygen species (ROS), was shown to play a crucial role in the pathogenesis of reperfusion injury in numerous experimental studies with various animal models (1, 10, 23). From observations in these animal studies, it is generally accepted that in certain circumstances, especially with respect to postischaemic myocardial stunning, antioxidant agents may promote recovery of the heart's contractile function (2, 14, 22). Moreover, the activation of neutrophils (in response to the CPB and to any other activating factor) results in generation of ROS (15, 20, 28). At cellular level, the ROS-induced damage to membrane lipids (e.g. lipid peroxidation), proteins and other cellular structures, leads to tissue injury (2, 10, 30).

Some studies have aimed to evaluate the evidence for ROS generation and subsequent lipid peroxidation during ischaemia/reperfusion injury of the human heart as well as in CPB (4, 5, 18, 25, 26). Measurements of lipid peroxidation products as malondialdehydes (MDA) or diene conjugates (DC) were mostly used for oxidative stress monitoring in these studies. To our knowledge, however, no reports have described measurement of lipid peroxidation markers with simultaneous assessment of blood antioxidant status during cardiac operations. To obtain more detailed information on the role of oxidative stress in patients undergoing cardiac surgery, we therefore attempted to evaluate the time course of three separate markers of lipid peroxidation together with changes in blood antioxidant status.

Table I. General and clinical data

Female/male patients	5/9
Preoperative NYHA class:	
II	1
III	9
IV	4
Surgery:	
Mitral valvuloplasty	1
Mitral valve replacement	3
Aortic valve replacement	4
Aortic valve replacement & mitral valvuloplasty	1
Aortic & mitral valve replacement	5
Aortic cross-clamp time (min)	84.9 ± 8.9
Cardiopulmonary bypass time (min)	119.0 ± 10.6
Operating time (min)	251.9 ± 15.1

MATERIAL AND METHODS

The study was performed on 14 adults undergoing elective heart valve surgery. General data are summarized in Table I. The study was approved by the Ethics Committee and all patients gave informed consent to blood sampling during the operative procedure.

Surgical procedure. No cardiac medication was stopped prior to surgery. Premedication (c. 1 hour preoperatively) was given with morphine and dehydrobenzperidol (Droperidol). Anaesthesia was induced with benzodiazepine and barbiturate or ketamine, and muscle relaxation was achieved with pancuronium bromide (Pavulon). Fentanyl 50 µg/kg was given for maintenance of anaesthesia. The lungs were ventilated with O₂:N₂O 1:1 to maintain normocarbic ventilation, with halothane up to 0.5 vol% as required for additional anaesthesia.

Perfusion for cardiopulmonary bypass was performed with pulsatile flow at 2.4 l/min/m², using standard roller pumps (Gambro) and maintaining mean arterial pressure at 60 mm Hg. A bubble oxygenator (Jostra) was used for 11 patients and a membrane oxygenator (Compactflo, Dideco) for three, with total prime 2 liters, consisting of 5% glucose, dextran, mannitol and sodium bicarbonate. Systemic hypothermia up to 24.4 ± 0.5°C (mean, SEM) was employed for myocardial preservation, with cardioplegic arrest (cold St Thomas Hospital solution II), repeated antegrade every 20 minutes. The lungs were not ventilated during CPB and the endotracheal

tube was open to air. Systemic heparinization was instituted shortly before aortic cannulation, to achieve activated clotting time not less than 400 seconds and was reversed with protamine (2 mg/kg) immediately before aortic decannulation. All operations were performed by the same surgical and anaesthetic team.

Blood sampling. Blood was sampled from the arterial line 1) 1 hour before operation, 2) 15 minutes after induction of anaesthesia, 3) 15 minutes after the start of CPB, 4) 5 minutes after release of aortic cross-clamp, 5) 15 minutes after cessation of CPB and 6) in the morning of the first postoperative day. The blood was drawn into plastic syringes and the serum was separated by centrifugation and kept in plastic tubes at -4°C until analysis.

Analyses

Markers of lipid peroxidation. The reagents used for the analyses were purchased from Sigma (St Louis, USA), except isopropanol and butanol, obtained from Aldrich (Milwaukee, USA). The samples were immediately treated with antioxidant (butylated hydroxytoluene, BHT), before adding the test reagents, to suppress artifactual changes during handling and assay procedures (13).

Thiobarbituric acid reactive substances (TBARS) were measured according to Okhawa et al. (21), with modifications. Briefly, samples were incubated at 37°C for 30 min, after which BHT (0.25%) was added, then treated with acetate buffer to achieve pH 3.5 at 80°C and heated with thiobarbituric acid solution (1%) at 80°C for 70 min. The reaction mixture was then cooled and acidified (5 N HCl). The red pigment was extracted with n-butanol and measured spectrophotometrically by absorbance at 534 nm.

Fe-stimulated TBARS (FeTBARS) were assessed

in samples treated with $475 \mu\text{M Fe}^{2+}$ (FeSO_4) as prooxidant at 37°C for 30 min. Thereafter BHT (0.25%) was added and TBARS were measured as described above.

For study of diene conjugates (DC), serum samples were incubated at 37°C for 25 min, BHT (0.25%) was added and lipids were extracted by heptane/isopropanol, acidified with 5 N HCl and extracted by heptane. After centrifugation samples were spectrophotometrically analyzed at 233 nm (24).

To correct the results for haemodilution, the protein content in samples was determined according to Lowry et al. (19) and values are presented as per gram of protein.

Markers of antioxidant status. To assess serum antioxidative capacity (AOC), standard linolenic acid in 96% ethanol (1:100) was diluted in isotonic saline. This test solution in sodium dodecyl sulphate and serum (in isotonic saline 1:1) was incubated in the presence of $200 \mu\text{M Fe}^{2+}$ (37°C , 60 min). Then BHT (0.25%) was added and the reaction mixture was treated with acetate buffer ($\text{pH} = 3.5$) and heated with thiobarbituric acid solution (1%) at 80°C for 35 min. This was followed by the above-described assessment of TBARS. The results were expressed as % inhibition of linolenic acid peroxidation induced by serum samples. Linolenic acid peroxidation in the presence of isotonic saline served as a control value.

For calculation of glutathione content in red blood cells (RBC-GSH), blood was drawn into heparinized tubes, centrifuged, erythrocytes separated and RBC-GSH was measured according to Ellman (7).

All measurements of lipid peroxidation products and antioxidant markers were performed in triplicate within 4 hours after blood sampling. Means were calculated and used for statistical analysis.

Statistical analysis. Values are presented as mean \pm standard error of the mean. One-way analysis of variance was used to test the time-dependent changes in oxidative stress markers. Statistically significant changes ($p \leq 0.05$) were determined with Student's *t*-test, using Bonferroni correction for multiple comparisons.

RESULTS

There were no complications associated with surgery and no deaths during the study period, although one patient died on postoperative day 5, due to thrombosis of mitral valve prosthesis and acute heart failure. Dopamine infusion ($2\text{--}4 \mu\text{g}/\text{kg}/\text{min}$) and intravenous digoxin were given as postoperative routine, and in three cases dobutamine and/or adrenaline was used for additional inotropic support. The mean duration of postoperative pulmonary mechanical ventilation was 18 hours. Raised serum levels of bilirubin ($>25 \mu\text{mol}/\text{l}$) were found post-

operatively in five patients and heightened serum urea and creatinine (>10 and $>100 \text{ mmol}/\text{l}$, respectively) in four, with no other indications of hepatic or renal failure.

Time-course of oxidative stress markers. Table II surveys the perioperative dynamics of measured markers. Significant increase in serum TBARS level, compared with preoperative values, was found 15 minutes after the start of CPB, 5 minutes after release of aortic cross-clamp and 15 minutes after cessation of CPB. At these times no statistically significant changes in Fe-TBARS or DC levels were detected.

The serum antioxidant capacity, i.e. the ability of the blood serum to inhibit linolenic acid peroxidation in vitro (Table II), was significantly attenuated at 15 minutes after start of CPB compared with preoperative values and remained suppressed at the later measuring points. The observed rise in RBC-GSH content (Table II) was not statistically significant. The pattern of AOC decrease correlated with the changes in TBARS and DC, $r = 0.30$ ($p < 0.001$) and $r = 0.46$ ($p < 0.02$), respectively. On the morning of postoperative day 1, all the parameters except AOC had returned to almost preoperative levels.

DISCUSSION

Cardiopulmonary bypass in cardiac surgery can cause diffuse tissue damage in lungs, myocardium, kidneys and brain by mechanisms not well understood. It is assumed that oxidative stress mediated by ROS may be substantially involved in this pathologic phenomenon (16, 27). One of the main mechanisms in ROS toxicity is lipid peroxidation. The intermediates of this process—conjugated dienes, or biochemical end-products—malondialdehydes (measured by TBARS assay) can be used as indirect markers of ROS generation. In this respect the chief findings in the present study, viz. significantly increased serum TBARS with highest peak at release of aortic cross-clamp together with sustained decrease of plasma AOC, supports the concept of oxidative stress in patients undergoing open-heart surgery.

Although the observed changes in serum

Table II. Perioperative dynamics of serum basal (TBARS) and Fe stimulated (Fe-TBARS) levels of thiobarbituric acid reactive substances, diene conjugates (DC), antioxidative capacity (AOC) and red blood cells glutathione content (RBC-GSH). Values are presented as mean \pm SEM

	1 h before operation	15 min. after induction of anaesthesia	15 min. at CPB	5 min. after release of aortic cross-clamp	15 min. after CPB	1st post operative day (morning)
TBARS (nmol/g protein)	7.46 \pm 1.46	11.42 \pm 3.25	20.82 \pm 1.89*	38.55 \pm 3.42*	34.83 \pm 3.20*	10.11 \pm 2.73
Fe-TBARS (nmol/g protein)	11.01 \pm 1.05	11.42 \pm 3.25	13.83 \pm 1.83	7.91 \pm 1.96	6.74 \pm 1.68	16.34 \pm 1.65
DC (μ mol/g protein)	358.8 \pm 31.1	372.3 \pm 47.8	455.8 \pm 45.7	488.9 \pm 61.1	493 \pm 59.3	353.1 \pm 26.1
AOC (%)	34.93 \pm 0.80	30.82 \pm 1.23	21.29 \pm 1.45*	18.14 \pm 1.54*	23.21 \pm 1.54*	28.14 \pm 1.60*
RBC-GSH (mg/dl cells)	62.88 \pm 4.40	61.68 \pm 4.12	68.20 \pm 5.40	71.79 \pm 7.00	76.38 \pm 6.73	65.45 \pm 4.84

* Different from preoperative value ($p \leq 0.05$).

diene conjugates were not statistically significant, the finding of correlation between changes in DC and AOC could also support the oxidative stress hypothesis. This discrepancy between TBARS and DC may be partly explained as an accumulation of end-products in response to high-rate peroxidation processes. The wide variability between single values in DC measurements, however, should also be noted.

Measurement of Fe-TBARS was performed for complex characterization of serum lipid status. It reflects the peroxidative capacity of lipids not yet peroxidized at the time of blood sampling. No significant changes were found in this parameter. The tendency of Fe-TBARS to decrease during CPB (Table II) may reflect the consumption of substrates (unsaturated fatty acids) in response to high-rate peroxidation processes.

Our findings in lipid peroxidation markers correspond principally with those in earlier studies (5, 18), both of which demonstrated marked increase of lipid peroxidation products during coronary bypass surgery. Despite use of mannitol (known to have antioxidant properties), however, we found a three-fold increase in serum TBARS as early as 15 minutes after the start of CPB, whereas in one of the earlier studies (5) increase of lipid peroxidation products was shown only after release of aortic cross-clamp. Differences in oxygenator types could possibly be one explanation for this discrepancy. Moreover, it is not known with certainty

how the degree of systemic hypothermia influences free radical reactions. The temperature during CPB was kept substantially lower in the present trial than previously (5), 24.4 \pm 0.5 vs 32–34°C, and a study demonstrating increase in arterial blood malonaldehydes already after 20 minutes of CPB (26), also used deeper systemic hypothermia (28–30°C).

Very few data are available on the antioxidant status in patients undergoing cardiac surgery. Recent authors stressed that on the microvascular level the endothelium plays a crucial role in organ functioning (3, 29). The endothelial dysfunction was stated to be an important factor in further tissue damage in heart, lungs and other organs (6, 29). To avoid endothelial damage by free radicals from the vascular side, human plasma possesses an array of antioxidant defence mechanisms (10, 11), which are based on integrated actions of molecules such as ascorbate, α -tocopherol, carotenoids, proteins (transferrin, ceruloplasmin, proteins with free sulphhydryl groups), etc. Because of this complexity, it is difficult to identify the most important antioxidant to be measured in human plasma for characterizing the course of oxidative stress (11). Consequently, we evaluated the changes in whole serum antioxidative capacity during surgery, on the hypothesis that, as in blood acid-base status, there may be a critical point also for this capacity. In our preliminary (unpublished) studies, two patients with antioxidative capacity less than 12%, compared with 38 \pm 4% in 25

healthy volunteers, died in the intensive care unit. Although in the present study the remarkable reduction in serum antioxidative capacity could be partly attributed to the haemodilution accompanying CPB, it reflects actual capacity of serum antioxidative defence mechanisms and is therefore indicative of increased susceptibility to oxidative stress at corresponding time-points. Furthermore, the correlation between changes in serum antioxidative capacity and two of the three assessed lipid peroxidation markers (TBARS and DC) can be interpreted as consumption of antioxidant reserves in response to enhanced free radical production.

In regard to red blood cells, glutathione is essential to avoid haemoglobin denaturation. It also reconverts methaemoglobin to haemoglobin and preserves the integrity of the red blood cell membranes during peroxidative injury. The enzyme glutathione peroxidase utilizes hydrogen peroxide to convert reduced glutathione to oxidized glutathione, while glutathione reductase reverses the process at the expense of oxidizing nicotinamide adenine dinucleotide phosphate. Since the method we used for measuring the glutathione content of red blood cells is based on detection of thiol groups after precipitation of proteins, our results principally reflect the changes in reduced glutathione. A slight, though not statistically significant increase of that parameter was found during surgery. One may speculate that an increase in glutathione reductase activity occurred as an adaptive response to blood injury during CPB, a consequence of which is increased erythrocyte-glutathione pool. The detailed mechanism of these changes remains to be elucidated, however, as does the relevance of the erythrocyte redox status to the redox status of other tissues.

Since it is difficult to measure the free radicals directly, their origin is not defined in the present study. The activation of neutrophils in response to CPB, and also ischaemia/reperfusion of the heart and lungs, are assumed to be the main events leading to the generation of ROS. Further, the tissue hypoperfusion during hypothermic extracorporeal circulation can lead to reperfusion injury and subsequent stimulation

of lipid peroxidation also in liver, kidneys and pancreas.

Methodologic considerations. Although it has been suggested that ROS contribute to many pathologic states, their exact role is difficult to quantify. Free radical activity was indirectly assessed in most of the studies, by measurement of damaged biologic molecules, especially lipids. The TBARS spectrophotometric assay used for evaluation of lipid peroxides is known to have nonspecific properties, including formation of adducts with sugars, nucleic acids and proteins. To avoid such possible errors, we introduced several methodologic modifications, such as heating samples up to 80°C instead of boiling them and addition of antioxidant immediately after drawing the sample.

In conclusion, we demonstrated time-dependent increase in lipid peroxidation products with simultaneously decreasing serum antioxidant capacity in patients undergoing open-heart surgery. These findings are suggestive of oxidative stress, and it should prove interesting to assess the usefulness of preventive antioxidant therapy in this clinical setting. Further studies are also required to resolve the role of oxidative stress in the postoperative course of cardiac surgery.

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The evidence of oxidative stress in cardiac surgery and septic patients: a comparative study

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Abstract

In present study, lipid peroxidation products (thiobarbituric acid reactive substances (TBARS) and diene conjugates (DC)) and markers of blood antioxidant status (serum antioxidative capacity (AOC) and red blood cells glutathione (RBC-GSH)) were measured to compare the extent of oxidative stress in 12 cardiac surgery and 10 septic general surgery patients. In heart surgery, arterial TBARS were significantly increased 15 min after the start and 15 min after cessation of cardiopulmonary bypass, while AOC at these times was decreased. Eighteen hours after surgery all parameters, except antioxidative capacity, had returned to preoperative levels. In septic patients, the preoperative level of lipid peroxidation was significantly higher and antioxidative capacity lower than in heart surgery patients. Surgery had no influence on oxidative stress markers in this group of patients. Increase in lipid peroxidation and reduction in blood antioxidant capacity, induced either by sepsis or cardiopulmonary bypass, were of comparable extent. © 1997 Elsevier Science B.V.

Keywords: Cardiopulmonary bypass; Cardiac surgery; Systemic inflammatory response syndrome; Sepsis; Oxygen free radicals; Malondialdehydes; Antioxidant capacity; Glutathione

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1. Introduction

Cardiopulmonary bypass (CPB), a necessary and integral part of cardiac surgery, itself has deleterious effects, which can result in diffuse damage of many tissues. In addition to the unphysiological hemodynamic conditions, a systemic inflammatory response induced by activation of circulating neutrophils, platelets, complement system and other blood components in the extracorporeal circuit is known to be one of the main factors determining the harmful effects of CPB [1–4]. A similar systemic inflammatory response syndrome (SIRS), known to have complicated and multifactorial physiology, occurs in patients during sepsis, multiple trauma and other critical illnesses and can lead to multiple organ failure [5,6].

Another major pathological phenomenon contributing to organ damage in open heart surgery patients is reperfusion injury. Cold cardioplegic arrest, commonly employed for myocardial protection, could itself be considered as a form of reperfusion injury, causing complement and neutrophil activation, as well as release of lipid peroxidation products [6–8]. Clinically it is expressed as depressed contractile function of the heart (myocardial ‘stunning’), which may have critical importance in the early postoperative period [1,9,10]. In addition, the reperfusion injury, which probably occurs after cessation of CPB due to reperfusion of lungs as well as other organs that have been hypoperfused during by-pass, is also suggested to be important in development of post-operative organ failure in these patients [11–13].

Evidence suggests that reactive oxygen species (ROS) may play important roles in the pathogenesis of both of these above-mentioned phenomena [14,15]. It has been shown that activation of neutrophils (in response to any inflammatory reaction) results in generation of oxygen free radicals [16,17]. Systemic increase of H_2O_2 and lipid peroxidation products has been demonstrated to occur during CPB, so giving supportive evidence for oxidative stress in open heart surgery [18,19]. In turn, from experimental studies it is known that ROS generation takes place during ischaemia as well as at reperfusion in various species [20,21]. On the basis of these animal studies it is generally accepted that in certain circumstances the antioxidant agents could have a beneficial effect for the postischaemic recovery of the contractile function of the heart [22,23]. At the cellular level, the excessive production of oxygen free radicals causes damage to membrane lipids (lipid peroxidation), denaturation of proteins and inactivation of enzymes, breakdown of carbohydrates and, consequently, tissue injury [9,10,24].

Some previous studies have demonstrated oxidative stress and subsequent lipid peroxidation during CPB as well as at ischaemia/reperfusion of the human heart [7,19,25,26]. However, there are very few data available comparing the extent of oxidative stress in cardiac surgery patients with that in septic patients.

Therefore, the aim of the present study was two-fold. First, to evaluate the time-course of oxidative stress markers in patients undergoing open heart surgery and, second, to compare this profile to that of septic surgical patients with systemic inflammatory response syndrome.

Measurements of lipid peroxidation products, such as malondialdehydes (e.g., thiobarbituric acid reactive substances, TBARS) or diene conjugates (DC), also applied in present study, are mostly used in previous clinical trials to evaluate occurrence of oxidative stress. To more fully evaluate oxidative stress we have also assessed the blood antioxidant status by measuring serum total antioxidant capacity as well as red blood cell glutathione content.

2. Materials and methods

2.1. Patients

Twelve non-septic patients scheduled for elective heart valve surgery with CPB (seven male, five female, mean age of 47.9, range 20–64 years, preoperative NYHA III–IV) and 10 septic patients, admitted to ICU and who required general surgery (six male, four female, mean age of 48.7, range 30–75 years) were studied. Among the septic patients, eight had soft tissue infection of different body regions (four of the lower extremity, one of the upper extremity, one of the neck, one of the abdominal wall and one of the back) and two had diffuse peritonitis. Diagnosis of sepsis was established when the patient met all the following criteria [27]: (1) clinically evident focus of infection, (2) fever $> 38^{\circ}\text{C}$ (rectal) or hypothermia $< 35.5^{\circ}\text{C}$ (rectal), (3) tachycardia > 90 beats/min, (4) tachypnea > 20 breaths/min in spontaneously breathing patients, (5) at least one organ dysfunction. The onset of septic illness was assessed to be 70.5 h (mean, range 24–135 h) before arrival to the hospital. Mean APACHE II score of septic patients was 17.4 (range 8–28). The study was approved by the institutional Ethics Committee and all patients, or, if necessary, next of kin or legally authorised guardian, gave informed consent to blood sampling during the operative procedure.

2.2. Cardiac surgery

One hour before operation morphine and dehydrobenzperidol (Droperidol) were given for premedication. Induction of anaesthesia was performed with benzodiazepine and barbiturate or ketamine, and muscle relaxation was achieved with pancuronium bromide (Pavulon). Fentanyl $50\ \mu\text{g}/\text{kg}$ was used as a main anaesthetic. The lungs were ventilated with $\text{O}_2:\text{N}_2\text{O}$ 1:1 to maintain normocarbic ventilation, with halothane up to 0.5 vol.% as required for additional

anaesthesia. Pulsatile flow at 2.4 l/min/m^2 , by standard roller pumps (Gambro), was used for perfusion during cardiopulmonary bypass. Mean arterial pressure was kept at 60 mmHg. A bubble oxygenator (Jostra, for ten patients) or a membrane oxygenator (Compactflo, Dideco, for two patients) was used. Total prime of 2 l consisted 5% glucose, dextran, mannitol and sodium bicarbonate. Systemic hypothermia up to $24.6 \pm 0.5^\circ\text{C}$ (mean \pm S.E.M.) was employed for myocardial preservation, with cardioplegic arrest (cold St Thomas Hospital solution II), repeated antegrade every 20 min. Systemic heparinization was instituted shortly before aortic cannulation, to achieve activated clotting time not less than 400 s, and was reversed with protamine (2 mg/kg) immediately before aortic decannulation. All operations were performed by the same surgical and anaesthetic team. The mean aortic cross-clamp time was 76.5 ± 8.0 min, CPB time was 107.4 ± 8.5 min and operating time was 222.3 ± 9.8 min.

2.3. General surgery

Surgical treatment in septic patients was aimed to remove the source of infection and included: fasciotomies, amputation of the infected limb and resection of the small intestine with drainage of the peritoneal cavity. All were emergency operations performed by different surgeons of the hospital. Endotracheal anaesthesia was performed according to the routine of the hospital, using fentanyl, halothane and N_2O as main anaesthetics. The mean operating time was 122.6 ± 15.3 min.

2.4. Blood sampling

Samples were obtained from arterial line (cardiac surgery patients, group C) or from v. cubitalis (septic patients, group S), according to the protocol described in Table 1. The blood was drawn into plastic syringes and the serum was separated by centrifugation and kept in plastic tubes at -4°C until analysis.

Table 1

Blood sampling protocol for comparative measurements of oxidative stress markers in cardiac surgery (group C) and septic patients (group S)

Time	Group C	Group S
T0	1 h before surgery	1 h before surgery
T1	15 min after start of CPB	—
T2	15 min after cessation of CPB	15 min after surgery
T3	18 h after surgery (morning of the first postoperative day)	18 h after surgery

2.5. Analyses

The methods for measurements of oxidative stress markers have been previously described in detail [28]. Briefly, the thiobarbituric acid reactive substances (TBARS) were measured spectrophotometrically according to Okhawa et al. [29] with modifications. For study of diene conjugates (DC), serum samples were incubated at 37°C for 25 min, the reaction was stopped by addition of antioxidant (butylated hydroxytoluene, BHT) and lipids were extracted. Thereafter the samples were spectrophotometrically analysed at 233 nm [30].

To correct the results for hemodilution, the protein content in samples was determined according to Lowry et al. [31] and values for both TBARS and DC are presented per gram of protein.

To assess serum antioxidative capacity (AOC), the ability of serum to inhibit *in vitro* linolenic acid peroxidation in the presence of 200 $\mu\text{mol/l}$ Fe^{2+} was evaluated. The level of linolenic acid peroxidation end-products was measured by TBARS assay and results were expressed as percent of inhibition of linolenic acid peroxidation induced by serum samples. Glutathione content in red blood cells (RBC-GSH) was measured according to Ellman [32].

The reagents used for the analyses were purchased from Sigma Chemical Co. (St Louis, USA), except isopropanol and butanol, obtained from Aldrich (Milwaukee, USA).

All measurements of lipid peroxidation products and antioxidant markers were performed in triplicate within 4 h after blood sampling. Means were calculated and used for statistical analysis.

2.6. Statistical analysis

Values are presented as mean \pm standard error of the mean. One-way analysis of variance was used to test the time-dependent changes in oxidative stress markers. Statistically significant changes ($P < 0.05$) were determined with Student's *t*-test and Bonferroni correction was used in case of multiple comparisons.

3. Results

3.1. Cardiac surgery

The anaesthetic and surgical procedures were uncomplicated in all cases. During the study period there was no mortality and no patients had infectious complications. Dopamine infusion (2–4 $\mu\text{g/kg/min}$) and intravenous digoxin

were used as a routine postoperatively; in two cases dobutamine and/or adrenaline was required for additional inotropic support. The mean duration of postoperative mechanical ventilation of lungs was 18 h. In four patients increased serum bilirubin ($> 25 \mu\text{mol/l}$) and in two patients raised serum urea and creatinine levels (> 10 and $> 100 \text{ mmol/l}$, respectively) developed postoperatively without any other symptoms of hepatic or renal failure.

3.2. General surgery

The anaesthetic procedure was uncomplicated in all cases. In four out of 10 patients studied, re-operation was required 24 h after the initial surgery or later, suggesting incomplete removal of septic focus in these cases. One patient died during the study period due to circulatory shock. Three more patients died later (on the 3rd, 6th and 14th postoperative days, respectively) due to multiple organ failure. The postoperative ventilation of the lungs was required for at least 24 h (range 1–58 days), while adrenomimetic support was employed in 3–31 postoperative days. All the patients had organ dysfunction (one to six organs) for 2–43 days.

3.3. Changes in oxidative stress markers

Comparison of perioperative dynamics of oxidative stress markers is presented in Table 2.

In cardiac surgery patients, a significant increase in serum TBARS level compared to preoperative values was found 15 min after the start of CPB and 15 min after cessation of CPB. At these times no statistically significant changes in DC levels were detected. The serum antioxidant capacity in these patients, i.e., the ability of blood serum to inhibit linolenic acid peroxidation *in vitro*, was significantly decreased at 15 min after introduction of CPB in comparison with the preoperative level, and remained suppressed in all points assessed. The increase in RBC-GSH content was statistically non-significant. Eighteen hours after cardiac surgery all the measured parameters, except AOC, had returned almost to preoperative levels. The changes in OS markers were independent of the type of oxygenator used.

Throughout the study period there were no statistically significant changes in measured parameters in septic patients (Table 2). When compared to cardiac surgery patients, before surgery the septic patients had significantly higher levels of serum TBARS, diene conjugate and red blood cell glutathione, with concomitant reduction in antioxidant capacity. At all other times the changes in OS markers were of comparable extent.

Table 2
Comparative dynamics of oxidative stress markers in blood in cardiac surgery (group C) and septic patients (group S)

Parameter	Group	Time			
		1 h before surgery	15 min after start of CPB	15 min after CPB/general surgery	18 h after surgery
TBARS (nmol/g protein)	C	7.71 ± 1.69	20.54 ± 2.03*	34.83 ± 3.20*	10.47 ± 3.22
	S	16.75 ± 1.20**	—	25.25 ± 2.92	17.44 ± 3.69
DC (μmol/g protein)	C	360.5 ± 36.4	463.5 ± 32.4	74.7 ± 65.6	355.3 ± 28.5
	S	530.0 ± 63.6**	—	514.0 ± 61.7	451.0 ± 60.7
AOC (%)	C	34.5 ± 0.9	21.1 ± 1.6*	23.9 ± 1.7*	27.5 ± 1.8*
	S	27.0 ± 2.6**	—	28.0 ± 1.9	27.6 ± 1.5
RBC-GSH (mg/dl cells)	C	62.85 ± 4.78	66.68 ± 5.63	76.31 ± 7.32	65.82 ± 5.28
	S	83.29 ± 4.86**	—	81.66 ± 6.82	72.46 ± 4.12

TBARS, thiobarbituric acid reactive substances; DC, diene conjugates; AOC, antioxidative capacity; and RBC-GSH, red blood cell glutathione content; CPB, cardiopulmonary bypass. Values are presented as mean ± S.E.M.
*P ≤ 0.05 compared with preoperative value; **P ≤ 0.05 between groups.

4. Discussion

Systemic inflammatory response during cardiopulmonary bypass is assumed to be one of the major factors responsible for the diffuse tissue damage in lungs, myocardium, kidneys and brain, described in patients undergoing open heart surgery [1,2]. Similarly to septic patients, several inflammatory mediators became activated during CPB with subsequent generalisation of inflammatory response [2,3]. Evidence suggests that reactive oxygen species produced by activated neutrophils or by tissue reperfusion injury may be involved in pathogenesis of diffuse tissue damage in these patients [6,13,18]. The major mechanism for ROS toxicity is peroxidation of membrane lipids, which may lead to injury of cellular organelles and membrane-bound enzymes. In turn, lipid peroxidation products, such as conjugated dienes or malondialdehydes (measured by TBARS assay), may be used as markers of oxygen free radical generation.

In the present study, we demonstrate a significant increase in serum TBARS levels in response to CPB, together with simultaneous decrease in serum antioxidant capacity (AOC). These results support the hypothesis of oxidative stress in patients undergoing open heart surgery and are in accordance with previous studies [25,26]. In the present study, the increase in diene conjugates during CPB remained non-significant. However, Davies and co-authors [26] have shown an increase in diene conjugates between 2 and 8 h after CPB, so our failure to do so might be caused by an insufficient blood sampling protocol.

To estimate the extent of oxidative stress occurring during CPB, we performed comparable analysis of OS markers in septic patients. Sepsis has been described as a classical and undoubted example of systemic inflammatory response syndrome, known to be associated also with oxidative stress [5]. Such comparative measurement of OS markers, performed by the same laboratory, could therefore reliably assess the extent of oxidative stress in cardiac surgery patients: thus, our finding of an identical increase in lipid peroxidation products in CPB and septic patients seems important. It shows that the load of free radicals during CPB can temporarily reach the same level as in severely ill septic patients, but in the absence of infection it spontaneously abates.

There are several potential sources of free radical production during CPB, including activated neutrophils, xanthine oxidase of endothelial cells, and damaged heart mitochondria. Protection against free radical attack is offered by a wide spectrum of antioxidants and scavengers, distributed in all tissues. In human plasma, integrated actions of molecules such as ascorbate, α -tocopherol, carotenoids, proteins (transferrin, ceruloplasmin, proteins with free sulphhydryl groups), etc., are designed to defend the endothelium from free radical load [16,33]. In some earlier studies, decrease in plasma levels of α -tocopherol during CPB has been demonstrated [19,25]. In our opinion, however, it is hard

to pick out a single agent from the complex of antioxidants which could fully reflect the changes in whole blood antioxidant status. Therefore we have assessed the serum total antioxidative capacity during surgery. In the present study, the hemodilution accompanying CPB, could be in part responsible for remarkable reduction in serum antioxidative capacity in cardiac surgery patients. On the other hand, our results reflect an actual capacity of serum antioxidative defence mechanisms and, thus, increased susceptibility to oxidative stress during CPB is demonstrated. Furthermore, evidently due to consumption of antioxidant reserves, AOC was still depressed on the morning of postoperative day 1. This finding of insufficient capacity of physiological defence systems in the early postoperative period may influence the clinical outcome of patients after cardiac surgery.

Glutathione is the main factor preventing haemoglobin denaturation in red blood cells. Hydrogen peroxide, a highly reactive intermediate of free radical metabolism, is neutralised when glutathione peroxidase converts reduced glutathione to the oxidised form. Glutathione reductase in turn reverses the reaction at the expense of oxidising NADPH. The method used by us for measurements of glutathione content in red blood cells is based on determination of protein free thiol groups. Consequently, our results principally reflect the changes in reduced glutathione. The slight increase in this parameter in cardiac surgery patients was not significant.

In septic patients, there were no significant differences in pre- and postoperative levels of lipid peroxidation products. However, preoperative values of both TBARS and DC were considerably higher in septic than in cardiac surgery patients. Furthermore, the preoperative antioxidant capacity in the group of septic patients was significantly attenuated when compared to levels in the heart surgery group. The onset of septic illness was assessed to be at least 24 h before admission to the ICU. Thus, our findings could be interpreted as evidence of sustained oxidative stress during septic illness, which results in enhanced lipid peroxidation and severe consumption of antioxidant reserves. The time-course of oxidative stress might explain the tendency to a higher level of erythrocyte-reduced glutathione among septic patients. It could be speculated that, as an adaptive response to oxidative stress, the activity of glutathione reductase in cells increases, subsequently leading to a rise in the erythrocyte-reduced glutathione pool. Since adaptive changes imply a definite time, the higher content of reduced glutathione in red blood cells in septic patients may be contributed to by a longer duration of oxidative stress in this group of patients.

Removal of the infectious focus has critical importance for septic patients. In the present study, four out of 10 septic patients underwent early re-operation, indicating incomplete treatment of infected tissues at first operation. These two subgroups — patients with complete versus incomplete removal of infectious focus — also had different trends in dynamics of OS markers during the later

postoperative period. When compared to the preoperative level, patients with successful first operation showed a gradual decrease in serum TBARS after surgery. In contrast, after operations without removal of infectious focus, the serum TBARS remained at or above the preoperative level until sufficient surgery was performed. This finding emphasises once more the importance of early and proper surgery in management of septic patients.

In conclusion, we demonstrate a significant temporary increase in lipid peroxidation products with a simultaneous decrease in serum antioxidant capacity in patients undergoing open heart surgery. The extent of these changes was similar to that in septic general surgery patients. These results suggest oxidative stress in both clinical conditions. The clinical implication of these findings is that adjuvant use of antioxidant drugs might attenuate the harmful effects of cardiopulmonary bypass, as well as improve the outcome of patients with systemic inflammatory response syndrome associated with sepsis.

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Preischaemic bradykinin and ischaemic preconditioning in functional recovery of the globally ischaemic rat heart

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Abstract

Objectives: Substantial release of bradykinin has been demonstrated to occur during short periods of myocardial ischaemia in various species. The aim of the present study was to investigate the protective effect of bradykinin in ischaemia and whether bradykinin could be involved in ischaemic preconditioning in the rat heart. **Methods:** Isolated, buffer-perfused hearts were subjected to 30 min of global ischaemia, followed by 30 min of reperfusion. Postischaemic functional recovery was recorded in the following groups: (1) control; (2) treatment with 0.1 μ M bradykinin for 10 min before ischaemia (BK); (3) bradykinin treatment combined with pretreatment with the specific bradykinin B₂-receptor antagonist, HOE 140; (4) ischaemic preconditioning by 5 min ischaemia + 5 min reperfusion prior to sustained ischaemia (IP); and (5) ischaemic preconditioning combined with HOE 140 administration. **Results:** Postischaemic myocardial function was significantly improved in both BK and IP groups (developed pressure 66.9 ± 6.8 and 67.6 ± 7.1 mmHg, respectively, vs. 43.1 ± 5.9 mmHg in controls, $P < 0.05$). Pretreatment with 1 μ M HOE 140 completely abolished the effect of bradykinin, while protection achieved by IP was unaltered by this drug. None of the protective interventions was associated with any significant improvement in myocardial adenosine triphosphate, creatine phosphate, glycogen, lactate or glucose tissue levels, detected either at the end of ischaemia or after 30 min of reperfusion. **Conclusions:** Bradykinin, acting via B₂-receptors, can protect against postischaemic contractile dysfunction to a similar extent as IP. An involvement of B₂-receptors in the ischaemic preconditioning phenomenon could, however, not be demonstrated.

Keywords: Bradykinin; Contractile function; Glycogen; HOE 140; Ischemic preconditioning; Rat, heart; Myocardial ischemia; Adenosine triphosphate

1. Introduction

Previous studies have shown that both endogenously released and exogenously administered bradykinin can be protective in ischaemic myocardium [1–4]. Bradykinin, acting via B₂-receptors on the endothelial cell, promotes the release of nitric oxide and prostacyclin from these cells; both agents are postulated to have cardioprotective properties. These data, and also demonstration of substantial release of bradykinin during short cardiac ischaemia in man [5], in dogs [6] as well as in isolated rat hearts [7], have led us and other investigators to the hypothesis that endogenous bradykinin might act as a modulating factor for ischaemic preconditioning. Recently it has been re-

ported that HOE 140, a specific bradykinin B₂-receptor antagonist, abolished the infarct size limiting effect of preconditioning in anaesthetised, open-chest rabbits [8] as well as attenuated the antiarrhythmic effect of preconditioning in dogs [9]. However, when isolated, buffer-perfused rabbit hearts were used as experimental model, the preconditioning was not blocked by this drug [10]. This finding has been supported also by Bugge and Ytrehus, who demonstrated that ischaemic preconditioning's effect on infarct size in isolated rat hearts was not influenced by HOE 140 pretreatment [11]. In contrast, using postischaemic functional recovery for assessment of ischaemic injury, Brew et al. have reported that bradykinin mediates preconditioning in isolated rat hearts through a protein kinase C dependent mechanism [12].

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Ischaemic preconditioning has been defined as the phenomenon in which sublethal episode(s) of ischaemia result in increased tolerance to a later, potentially lethal episode of ischaemia. This beneficial effect can be expressed as significant reduction in the extent of myocardial cell necrosis [13], attenuated incidence of reperfusion arrhythmias [14] as well as improved postischaemic functional recovery of the heart [15]. Results from several studies suggest that stimulation of membrane-bound receptors by agents released during the short, preconditioning ischaemia, is one of the key events leading to the subsequent cardioprotective effect. Adenosine A₁ or A₃, muscarinic M₁ or M₂, angiotensin AT₁-receptors or α_1 -adrenoreceptors are reported to be involved in the mechanisms of ischaemic preconditioning (see [16] for review). Many authors have demonstrated that adenosine plays a key role in this process in rabbits [17,18], dogs [16] and pigs, while overwhelming data indicate that it does not play a role in rats [15,19,20].

In addition to the theory of receptor activation, a metabolic basis for the ischaemic preconditioning phenomenon has been proposed. Ischaemic preconditioning has been shown to reduce myocardial ATP consumption, limit intracellular acidosis and reduce glycogenolysis during ischaemia [21–23]. However, a causal role for these metabolic changes in the mechanisms of preconditioning is still debated.

Thus, the aim of the present study was twofold. Firstly, to determine whether bradykinin could be involved in ischaemic preconditioning in the rat heart by pretreating preconditioned hearts with the specific bradykinin B₂-receptor antagonist, HOE 140 [24]. A model of 30 min global ischaemia in which postischaemic contractile function could be measured was chosen and supplemented with a model of regional ischaemia. Additional groups of hearts were subjected to short preischaemic infusion of bradykinin alone or in combination with HOE 140, to evaluate protective properties of bradykinin independent of preconditioning on postischaemic contractile dysfunction. Secondly, we wanted to examine how myocardial energy status correlated with contractile function after either ischaemic preconditioning or bradykinin treatment. For this purpose, tissue myocardial content of high-energy phosphates, glycogen, lactate and glucose were measured at the end of ischaemia, at the end reperfusion and at the end of preischaemic exposure to either bradykinin or ischaemic preconditioning.

2. Methods

2.1. Perfusion procedure

The investigation conforms with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-233,

revised 1985). Male Wistar rats weighting 270–340 g, fed a standard diet, were heparinized with 200 IU and anaesthetized with Na-pentobarbital 50 mg/kg intraperitoneally. The hearts were rapidly excised, placed in ice-cold buffer and perfused within 60 s in a non-recirculating Langendorff perfusion system maintained at 37°C. The perfusion pressure was kept at 100 cm H₂O. The Krebs-Henseleit buffer (pH = 7.4, oxygenated with 95% O₂/5% CO₂) contained 2.4 mM calcium and 11.1 mM glucose. A water-filled latex balloon, connected to a pressure transducer and coupled to a Gould recorder, was inserted into the left ventricle through an incision in the left atrium. The volume of the balloon was adjusted to assure that the balloon was unstretched and that an end-diastolic pressure below 10 mmHg was obtained. Heart rate, left ventricular systolic pressure (LVSP) and end-diastolic pressure (LVEDP), positive (+dP/dt) and negative (–dP/dt) first derivatives of pressure were recorded. Left ventricular developed pressure (LVDP) was calculated as the difference between LVSP and LVEDP, and coronary flow was measured by timed collections of effluent. At the end of each experiment the heart was freeze-clamped and stored at the temperature of liquid nitrogen for subsequent biochemical analysis. In separate series of experiments with regional ischaemia, a 3-0 silk thread was passed around the main branch of the left coronary artery, and the ends were threaded through a small vinyl tube to form a snare. Regional ischaemia was achieved by pulling the snare. Ischaemia was confirmed by a substantial fall in both left ventricular developed pressure and coronary flow.

2.2. Experimental protocol

2.2.1. Global ischaemia

All hearts experienced an initial 25 min stabilisation period. Baseline values for functional parameters were obtained after 15 min of perfusion. In the control group ($n = 12$) the stabilisation period was extended by 10 min ordinary perfusion. Thereafter the hearts were subjected to a standard ischaemic insult of 30 min global ischaemia and 30 min reperfusion. In the second group (IP, $n = 12$) ischaemic preconditioning was achieved by 5 min ischaemia and 5 min reperfusion prior to the standard ischaemic insult. In the bradykinin-treated group (BK, $n = 12$) the hearts were perfused for 10 min with 0.1 μ M bradykinin before the sustained 30 min ischaemic period. In two additional groups (IP + HOE and BK + HOE, respectively, $n = 8$ in both) the hearts were pretreated with the bradykinin B₂-receptor antagonist, HOE 140 (1 μ M), before interventions. In the IP + HOE group the infusion of HOE 140 was started 10 min prior to the IP cycle and was continued until onset of the 30 min prolonged ischaemic period. Similarly, the hearts in the BK + HOE group were subjected to HOE 140, starting 10 min before the bradykinin administration and continued until the prolonged ischaemic period was established.

2.2.2. Regional ischaemia

In this series of experiments hearts were subjected to 30 min regional ischaemia and 120 min reperfusion. In the control group ($n = 6$) the initial stabilisation period of 25 min was followed by 10 min ordinary perfusion prior to regional ischaemia. In the preconditioning group 5 min global ischaemia and 5 min reperfusion was applied before occlusion of the left coronary artery, and in the third group hearts were perfused with $0.1 \mu\text{M}$ bradykinin for 10 min immediately prior to regional ischaemia ($n = 6$ in both groups).

Both bradykinin and HOE 140 were dissolved in saline and kept as stock solutions, which were added to the perfusion buffer immediately before use. Bradykinin was administered by switching to a separate perfusion reservoir. HOE 140 was delivered into an infusion port directly above the aortic cannula by an infusion pump (B. Braun Melsungen AG, Germany).

The concentrations of drugs used in the study were chosen on the basis of previous reports [24,25]. 10 min of perfusion with $0.1 \mu\text{M}$ bradykinin resulted as expected in an increase of coronary flow ($13.9 \pm 0.7 \text{ ml/min}$ at baseline versus $16.8 \pm 0.7 \text{ ml/min}$ after drug administration; $P < 0.05$). This effect was completely reversed by $1 \mu\text{M}$ HOE 140.

2.3. Assessment of irreversible ischaemic injury

2.3.1. Global ischaemia

In order to assess the extent of cell necrosis in hearts exposed to global ischaemia, release of creatine kinase (CK) was measured in the control, IP and BK group. Coronary effluent from these hearts was collected continuously during the reperfusion period. CK in effluent was measured spectrophotometrically [25] at room temperature and expressed as IU released during 30 min of reperfusion (IU/30 min/heart).

2.3.2. Regional ischaemia

In hearts subjected to regional ischaemia, infarct size was measured by a technique described in detail previously [20]. The risk zone was determined by fluorescent particles and infarct size by tetrazolium staining. Infarct size was expressed as a percentage of the risk zone infarcted.

2.4. Tissue content of adenosine triphosphate (ATP), creatine phosphate (CP), glycogen, lactate and glucose

For metabolic assays, the hearts were freeze-clamped and stored in liquid nitrogen. The hearts from which the functional variables were obtained were freeze-clamped at the end of 30 min reperfusion. In addition, three subgroups, perfused identically to the control, IP and BK protocols, were freeze-clamped at the end of 30 min of

global ischaemia ($n = 11, 10$ and 8 , respectively). Six hearts, frozen immediately after 25 min of normal perfusion, formed a baseline group in this setting. Seven hearts were frozen at 5 min of reperfusion in the IP cycle to investigate the alterations associated with preconditioning per se. Seven hearts were frozen at 10 min of $0.1 \mu\text{M}$ bradykinin administration. Adenosine triphosphate (ATP) and creatine phosphate (CP) were measured by HPLC technique as described by Sellevold et al. [26]. Briefly, the ventricular part of the freeze-clamped heart was pulverised in liquid nitrogen, freeze-dried, homogenised and extracted in perchloric acid. After centrifugation the samples were neutralised before being applied to the HPLC system which made use of a reverse phase C-18 column and a spectrophotometer set at 206 nm. The mobile phase consisted of KH_2PO_4 (215 mM), tetrabutylammonium hydrogen sulphate (2.3 mM) and acetonitrile (3.5%) at pH 6.25. Standard solutions of the assay substances were dissolved in the extraction agent, perchloric acid, and then neutralised. Five standard concentrations were used to establish the standard curves.

For tissue glycogen and glucose detection an enzymatic method based on hexokinase and glucose-6-phosphate dehydrogenase was used [27]. Prior to measurements the tissue was extracted and hydrolyzed by perchloric acid and HCl. Separation of unbound glycogen from protein-bound glycogen by tissue extraction with perchloric acid did not change the differences between groups and therefore only total glycogen is presented. Myocardial content of lactate was measured spectrofluorometrically according to Passonneau [28].

2.5. Materials

All chemicals used in the present study were obtained from the Sigma Chemical Company (St. Louis, MO, USA), except for HOE 140 which was a generous gift from Hoechst AG (Frankfurt, Germany).

2.6. Statistics

Results are expressed as mean \pm standard error of the mean (s.e.m.). One-way analysis of variance was performed and Tukey's test was applied to identify significant differences ($P < 0.05$) between groups. The paired *t*-test was used for within-group analyses to test for drug effects on functional parameters prior to ischaemia.

3. Results

3.1. Functional parameters

3.1.1. Global ischaemia

Functional parameters measured at baseline, after 10 min of HOE 140 administration, immediately before the

Table 1
Functional parameters during the experiments in the groups subjected to 30 min of global ischaemia

	Baseline	Stabilization	Pre-ischaemia	30' reperfusion
Coronary flow (ml/min)				
Control	14.1±0.6	14.0±0.6	14.3±0.6	9.6±0.8
IP	14.4±0.5	14.5±0.6	15.8±1.2	12.9±1.6
IP+HOE	14.7±1.1	14.7±1.3	17.8±1.5	14.0±1.0
BK	13.9±0.7	13.8±0.6	16.8±0.7*	10.6±0.8
BK+HOE	13.2±0.5	13.7±0.7	10.6±0.9*	8.4±0.5
Heart rate (beats/min)				
Control	309±11	296±28	313±12	285±16
IP	300±6	305±6	309±11	295±17
IP+HOE	317±18	313±13	328±10	287±11
BK	296±7	298±8	304±7	261±25
BK+HOE	330±14	317±15	301±14	288±15
End-diastolic pressure (mmHg)				
Control	3.6±0.8	3.3±0.6	3.3±0.6	76.3±5.2
IP	4.9±0.6	5.2±0.5	15.6±3.0*	55.7±4.6*
IP+HOE	1.7±1.0	1.7±1.0	2.5±1.1	37.5±3.8*
BK	2.6±0.5	2.6±0.5	2.7±0.7	66.8±5.7
BK+HOE	4.4±1.3	4.4±1.3	4.4±1.3	75.6±4.9
Developed pressure (mmHg)				
Control	133.1±2.7	130.0±2.8	129.2±2.6	43.1±5.9
IP	132.1±4.3	129.3±3.8	97.8±4.7*	67.6±7.1*
IP+HOE	125.9±4.0	134.2±5.4	108.7±4.8*	91.7±3.8*
BK	134.9±5.4	134.6±5.5	145.8±5.0	66.9±6.8*
BK+HOE	120.3±7.3	122.4±7.0	108.3±14.0	40.0±7.8
+dP/dt (mmHg/s)				
Control	4792±160	4958±180	4990±186	1298±217
IP	4580±124	4602±148	3114±188*	2284±248*
IP+HOE	4917±201	5333±167	3542±119*	2917±167*
BK	4146±119	4177±128	4458±130	2067±209*
BK+HOE	4361±301	4528±319	3917±548	1106±258
-dP/dt (mmHg/s)				
Control	3365±54	3375±79	3333±50	946±159
IP	3172±93	3261±88	1968±196*	1655±172*
IP+HOE	3083±53	3292±119	2250±65*	2208±140*
BK	3260±120	3292±137	3333±157	1550±140*
BK+HOE	2889±191	3000±186	2417±366	781±175

Baseline = at 15 min of perfusion; Stabilisation = at the end of the 25 min stabilisation period, which is 10 min after drug addition in HOE-140-treated groups; Pre-ischaemia = immediately before the onset of global ischaemia, which is the end of the preconditioning cycle or bradykinin infusion, combined with HOE 140 treatment in the respective groups.

* $P < 0.05$ vs corresponding baseline value; * $P < 0.05$ vs control group.

onset of 30 min ischaemia and at 30 min reperfusion are shown in Table 1. There were no differences between the groups concerning baseline values.

The preconditioning cycle (5 min ischaemia followed by 5 min reperfusion) resulted in a significant reduction in contractile function (decrease in developed pressure, +dP/dt and -dP/dt, and increase in end-diastolic pressure, respectively) when compared to control hearts. Similar changes, except for end-diastolic pressure, were found also in the IP+HOE group. Treatment with 0.1 μ M

bradykinin increased coronary flow significantly. HOE 140 alone did not cause any changes in functional variables, but prevented the bradykinin-induced increase in coronary flow (BK+HOE group) (Table 1).

Fig. 1 demonstrates the time course of left ventricular pressures in the control, IP and BK groups, respectively. Compared to the control group, an earlier onset of ischaemic contracture occurred in both the preconditioned and the bradykinin-treated groups. During the reperfusion period, however, the increase in end-diastolic pressure was substantially lower in preconditioned hearts than in controls, which resulted in a significantly higher developed pressure at 30 min of reperfusion in this group (Table 1). At the end of reperfusion +dP/dt had reached $50.2 \pm 5.9\%$ and -dP/dt $51.3 \pm 5.6\%$ of the baseline levels, compared to $26.2 \pm 4.3\%$ and $28.0 \pm 4.8\%$ in controls, respectively ($P < 0.05$), indicating improved recovery of both systolic and diastolic function after global ischaemia with ischaemic preconditioning. Hearts receiving

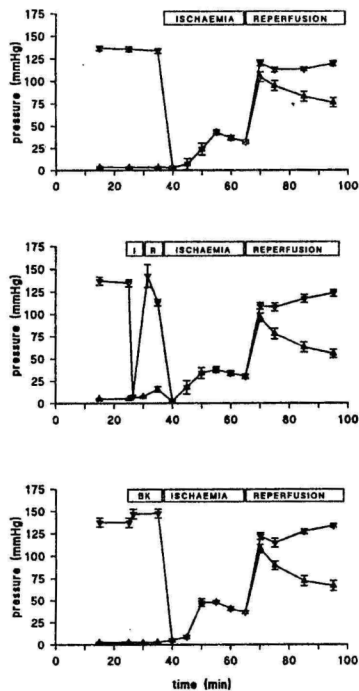


Fig. 1. Time course of left ventricular systolic (top tracings) and diastolic pressure (bottom tracing) of hearts subjected to 30 min of global ischaemia is shown for 3 groups of hearts. Upper panel control group, middle panel ischemic preconditioning (IP) and lower panel bradykinin-treated group (BK). Mean \pm s.e.m.; $n = 12$ in each group.

Table 2
Functional parameters during the experiments in the groups subjected to 30 min of regional ischaemia

	Baseline	Pre-ischaemia	5' ischaemia	120' reperfusion
<i>Coronary flow (ml/min)</i>				
Control	14.3 ± 1.6	14.3 ± 1.5	8.8 ± 1.6	13.0 ± 2.1
IP	15.4 ± 0.7	19.9 ± 0.1*	11.4 ± 1.4	12.9 ± 1.9
BK	13.5 ± 0.4	16.3 ± 0.3*	7.2 ± 0.2	11.8 ± 1.3
<i>Developed pressure (mmHg)</i>				
Control	119.3 ± 12.5	118.5 ± 12.7	35.2 ± 5.9	96.0 ± 4.0
IP	105.8 ± 6.0	81.3 ± 6.4*	35.0 ± 3.4	81.7 ± 7.4
BK	102.3 ± 5.0	102.5 ± 5.5	38.2 ± 3.4	80.0 ± 5.1

Baseline = at 15 min of perfusion; Pre-ischaemia = immediately before the onset of regional ischaemia, which is the end of the preconditioning cycle or bradykinin infusion in the respective groups.

* $P < 0.05$ vs corresponding baseline value.

bradykinin for 10 min before ischaemia expressed a similar improvement in postischaemic functional recovery (Table 1).

The effect of ischaemic preconditioning on postischaemic contractile dysfunction was not significantly influenced by HOE 140 (Table 1). In contrast, HOE 140 completely abolished the protective effect of bradykinin on functional recovery of ischaemic myocardium (Table 1).

3.1.2. Regional ischaemia

Coronary flow and left ventricular developed pressure during regional ischaemia are shown in Table 2. The baseline values did not differ between the three groups. The ischaemic preconditioning protocol with one cycle of 5 min of ischaemia + 5 min of reperfusion led to a significant decrease in developed pressure with a concomitant increase in coronary flow. Perfusion with 0.1 μ M bradykinin increased coronary flow significantly. Occlusion of the coronary artery caused a substantial and similar fall in coronary flow and developed pressure in all three groups. There was no significant difference in recovery of function at 120 min of reperfusion (Table 2). The baseline values for heart rate and end-diastolic pressure were also not different between groups (heart rate: 294 ± 22 in control, 320 ± 8 in IP and 309 ± 10 beats/min in BK group; end-diastolic pressure: 3.5 ± 0.5 in control, 1.3 ± 0.9 in IP and 2.8 ± 0.7 mmHg in BK group, respectively), and did not differ throughout the experiments. At the end of reperfusion the end-diastolic pressure in the control group was 9.5 ± 1.4 compared to 5.8 ± 0.8 in preconditioned and 3.0 ± 0.9 mmHg in bradykinin treated hearts.

3.2. Irreversible cell injury

3.2.1. Global ischaemia

There were no significant differences in release of creatine kinase between the groups subjected to global ischaemia. In the control group the release was 16.8 ± 2.5 IU/30 min/heart, whereas a release of 19.0 ± 2.0 IU was

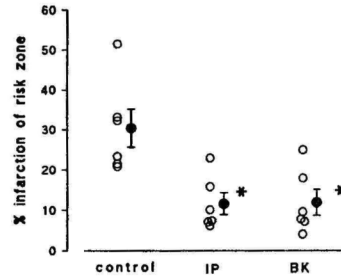


Fig. 2. Infarct size expressed as percent of the risk zone infarcted after 30 min of regional ischaemia and 120 min of reperfusion. Open symbols represent single hearts while filled symbols with error bars represent means of group \pm s.e.m. IP = ischaemic preconditioning group; BK = bradykinin-treated group. * $P < 0.05$ versus control group.

found in preconditioned and 16.5 ± 2.0 in bradykinin-treated hearts, respectively.

3.2.2. Regional ischaemia

Both ischaemic preconditioning and bradykinin treatment, when applied before 30 min of regional ischaemia, significantly reduced infarct size. In the IP group $11.6 \pm$

Table 3
Myocardial content of adenosine triphosphate (ATP), creatine phosphate (CP), glycogen, lactate, and free glucose in groups before and after 30 min of global ischaemia and at 30 min of reperfusion

	Pre-ischaemia	30' ischaemia	30' reperfusion
<i>ATP (nmol/mg dw)</i>			
Control	25.18 ± 0.86	2.66 ± 0.16	5.83 ± 0.80
IP	18.69 ± 2.97*	2.48 ± 0.33	6.68 ± 0.75
BK	19.61 ± 1.12*	2.67 ± 0.20	5.89 ± 0.59
<i>CP (nmol/mg dw)</i>			
Control	40.73 ± 3.03	7.29 ± 0.49	23.84 ± 4.08
IP	51.00 ± 9.25	8.96 ± 1.75	27.25 ± 3.74
BK	34.85 ± 2.72	6.95 ± 0.51	19.78 ± 1.88
<i>Glycogen (nmol/mg dw)</i>			
Control	112.00 ± 5.96	20.73 ± 1.51	27.91 ± 1.96
IP	55.86 ± 4.72*	18.70 ± 1.41	30.22 ± 2.49
BK	87.54 ± 3.79*	17.29 ± 1.41	26.83 ± 2.27
<i>Lactate (nmol/mg dw)</i>			
Control	22.0 ± 13.8	221.1 ± 55.6	152.3 ± 14.1
IP	78.0 ± 32.4	126.8 ± 26.5	155.6 ± 19.2
BK	42.4 ± 9.9	260.1 ± 53.6	189.3 ± 4.9
<i>Glucose (nmol/mg dw)</i>			
Control	25.0 ± 1.75	4.23 ± 1.08	52.09 ± 2.88
IP	40.43 ± 1.62*	1.79 ± 0.44	56.00 ± 2.33
BK	38.41 ± 1.63*	3.53 ± 0.46	42.92 ± 1.18*

Pre-ischaemia = at 25 min of normal perfusion in control group, at the end of the preconditioning cycle in the IP group and at 10 min of bradykinin infusion in the BK group; IP = ischemic preconditioning group; BK = bradykinin-treated group.

* $P < 0.05$ vs control group.

2.7% and in BK group $11.9 \pm 3.2\%$ of the area at risk was infarcted, compared to $30.4 \pm 4.8\%$ in controls (Fig. 2). Risk zone volume in the three groups was not statistically different, the combined mean value being $356.9 \pm 30.6 \text{ mm}^3$.

3.3. Tissue content of ATP, CP, glycogen, lactate and glucose

Both ischaemic preconditioning and perfusion with bradykinin for 10 min resulted in a slight but significant fall in myocardial ATP level, but CP content remained unchanged. There were no differences in tissue ATP and CP content between the groups at the end of ischaemia or end of reperfusion (Table 3).

Both procedures—the IP cycle and bradykinin treatment—significantly increased the free glucose level and simultaneously reduced the glycogen content in heart tissue (Table 3). After 30 min of global ischaemia, there were no differences in myocardial glycogen content between control, preconditioned and bradykinin-treated hearts. The tissue level of lactate did not differ significantly between the groups (Table 3) at any timepoints.

4. Discussion

A main finding in the present study was that a short preischaemic bradykinin infusion improved the postischaemic functional recovery in isolated rat hearts. Based on the corresponding data on infarct size this indicates that less cell death during the ischaemia reperfusion insult explain this finding. The biochemical measurements support a mechanism of cell salvage partly independent of energy conservation. Similar results were obtained in the groups where ischaemic preconditioning was used instead of bradykinin pretreatment. Our results are consistent with previous studies demonstrating that bradykinin can reduce postischaemic contractile dysfunction in rats [12], dogs [29] and pigs [3]. HOE 140, reported to be a potent and highly specific bradykinin B_2 -receptor antagonist [24], completely reversed the protective effect of bradykinin, revealing that the afforded protection was mediated by bradykinin B_2 -receptors. Bradykinin B_2 -receptors therefore seem to be coupled to a potent endogenous system for protection against ischaemic cell death.

Stimulation of B_2 -receptors on endothelial cells results in release of nitric oxide and prostacyclin from these cells. Although there is no consensus so far with respect to cardioprotective properties of nitric oxide, accumulating evidence suggests that inhibition of NO synthesis does not influence the cardioprotective effect of bradykinin [29–31]. Prostacyclin (PGI_2) seems to possess anti-ischaemic properties, partly reversed by the cyclo-oxygenase inhibitor, indomethacin [32]. This could suggest involvement of cyclo-oxygenase plus an additional mechanism in the car-

dioprotective effect of bradykinin. In rat hearts an independent kallikrein-kinin system has been demonstrated, suggesting that locally generated kinins may regulate cardiac functions [33]. The presence of bradykinin B_2 -receptors on cardiomyocytes, which are functionally coupled to the production of inositol trisphosphate IP_3 , has recently been demonstrated in several species [34]. The IP_3 production is believed to be coupled to the same signal transduction pathway as protein kinase C (PKC) activation. It has been demonstrated that inhibition of PKC completely blocked the effect of bradykinin on infarct size in rabbits [10] as well as on contractile dysfunction in isolated rat hearts [12]. We have recently reported similar findings in the infarct model in isolated perfused rat hearts [11]. Therefore our results support the view that receptors coupled to intracellular signal transduction through the PKC system are able to protect the heart against ischaemic cell death. The two characteristics—i.e., coupling to PKC and ability to protect the heart—are shared by a variety of receptors in addition to the bradykinin receptor—i.e., adenosine [35], acetylcholine [36], opioid [37], endothelin [38], angiotensin II [39] and norepinephrine receptors [40].

The other main purpose of the present study was to investigate a possible role for bradykinin in ischaemic preconditioning's effect on postischaemic functional recovery. Pretreatment of preconditioned hearts with the bradykinin B_2 -receptor antagonist, HOE 140, did not abolish the effect of preconditioning. This corresponds with other studies in which infarct size as endpoint of ischaemic injury was measured either in isolated rat hearts [11] or in rabbit hearts [10]. In contrast to these findings, Brew et al., using an experimental model comparable to the one used in the present study, have reported that the effect of preconditioning in rat hearts was significantly attenuated, although not completely abolished, by blockage of B_2 -receptors [12]. The different bradykinin B_2 antagonists (NPC-349 by Brew et al. and HOE 140 in the present study) could influence the results, but also the difference in the experimental protocol might be causal for this controversy. Brew et al. have used 2 min ischaemia and 8 min reperfusion to precondition the heart, while we used 5 min ischaemia plus 5 min reperfusion. Furthermore, Brew et al. have used 20 min sustained ischaemia instead of 30 min in the present study, and therefore the achieved cardioprotection in their model is probably more dependent on the absence or presence of stunning. Pharmacological interventions might influence contractile function partly independent of the effect upon ischaemic preconditioning and confound the results in models using contractile function as endpoint. On the other hand, one could assume that a higher dose of blocking agent is required to block the effects of bradykinin in the case of 5 min compared to 2 min of preconditioning ischaemia. However, the dose of HOE 140 used in the present study was well above the reported IC_{50} [24]. Although there are no exact data about the amount of bradykinin released during the first minutes

of ischaemia, it seems very unlikely that the concentration of HOE 140 was not sufficient enough to block endogenous bradykinin since it was sufficient to block exogenous bradykinin at the level of 0.1 μM (123 ng/ml). Reported release of bradykinin in rat hearts subjected to regional ischaemia was 7 ng/ml/g wet weight [7]. Thus we believe that although bradykinin can induce protection in the rat heart through a mechanism similar to ischaemic preconditioning, it is not the only or sole mediator of ischaemic preconditioning in the isolated perfused rat heart. The possibility exists that bradykinin can operate in concert with other endogenous substances such as adenosine, noradrenaline, acetylcholine, angiotensin II, endothelin and opioid peptides, and blockage of a single mediator is insufficient to restrain the cardioprotective effect of preconditioning. Also, it could be tempting to propose that yet unknown factors related to the activation of the PKC system participate in ischaemic preconditioning in the rat heart.

A critical question for any study investigating potentially cardioprotective interventions and using functional parameters as an endpoint depends on clarifying whether the improved functional recovery is due to a limited extent of cell death or achieved by reduced stunning (or inotropic stimulation at reperfusion) or is a mixture of both. In this study, we first used measurements of creatine kinase leakage as a standard for assessment of myocardial necrosis. We did not find any significant differences in this parameter, suggesting that neither bradykinin nor preconditioning influenced the extent of cell necrosis. To confirm this finding, we performed an additional series of experiments with exactly the same protocols of ischaemic preconditioning or bradykinin treatment but followed by 30 min of regional ischaemia. We found that both interventions reduced infarct size substantially in rat hearts. Based on these results, we suppose that the creatine kinase release measurement led to inappropriate interpretations, probably due to delay in creatine kinase washout. We therefore think that the improved functional recovery in our model of global ischaemia is related to reduction in ischaemic cell death. In our previous study we used three cycles of IP or preischaemic bradykinin to protect isolated perfused rat hearts against infarction. In the present study we have been able to show protection based on only one cycle of IP or 10 min preischaemic bradykinin infusion. The decrease in infarct size was, however, somewhat less.

In order to evaluate whether the achieved cardioprotective effect coexists with alterations in biochemical energy status in the heart tissue, we measured the tissue content of high-energy phosphates, glycogen and lactate. One cycle of 5 min of ischaemia and 5 min of reperfusion caused a decrease in myocardial glycogen with concomitant increase in tissue free glucose, which is in accordance with previous studies [41]. In contrast, there is very little known about the metabolic effects of bradykinin. In bradykinin-treated hearts we observed alterations in glycogen and

ATP partly similar to those in preconditioned hearts. Significant changes in glucose metabolism after bradykinin treatment have been reported by Rösen et al. [42], the underlying mechanism, however, remains poorly understood. Further investigation is needed to understand the effects of bradykinin on energy metabolism. Despite a preischaemic difference in the measured tissue metabolites (decreased glycogen and increased glucose content, reduced ATP level) the IP- and BK-treated hearts did not exhibit any difference in myocardial ATP and CP contents when compared to control either at the end of ischaemia or at 30 min of reperfusion. Also, there was no correlation between recovery of function during reperfusion and level of high-energy phosphates and glycogen in the present study. Therefore, we believe that alterations found in high-energy phosphates and glycogen are not causal factors for the protection observed after ischaemic preconditioning. It was especially surprising that hearts with less cell death and better recovery of postischaemic contractile function (IP and BK groups in the present study) did not have a higher tissue level of ATP and CP than controls.

In conclusion, we found that preischaemic bradykinin can improve postischaemic contractile dysfunction significantly, and that this effect is mediated through bradykinin B_2 -receptors. Ischaemic preconditioning was found clearly protective, but the pretreatment of hearts with B_2 -receptor antagonist did not alter the protection. None of the interventions was associated with improved ATP or CP levels at the end of ischaemia and reperfusion, indicating that energy conservation plays a minor role in the protective effects of either preconditioning or bradykinin. A clinical implication of the present study is that the findings support the assumption of cardioprotective properties of ACE-inhibitors, drugs which are known to increase the tissue levels of bradykinin.

Acknowledgements

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IV

Lipid peroxidation, arachidonic acid and products of the lipoxygenase pathway in ischaemic preconditioning of rat heart

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Abstract

Objective: Preconditioning with brief intermittent periods of ischaemia is known to provide protection against ischaemic injury. It has been suggested that myocardial ischaemia also activates phospholipase A₂, which releases arachidonic acid from phospholipids. In the present study the possible role of phospholipid peroxidation, arachidonic acid and products of the lipoxygenase pathway in cellular mechanisms of ischaemic preconditioning was examined. **Methods:** Isolated, buffer-perfused rat hearts were freeze-clamped at the end of preconditioning (a cycle of 5 min global ischaemia + 5 min reperfusion) and at the end of 30 min global ischaemia were analysed for non-esterified fatty acids and fatty acids in the 2-position of phospholipid. In a separate set of experiments, hearts pretreated with a lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), were subjected to 30 min regional ischaemia and 120 min reperfusion. Infarct size was determined by tetrazolium staining and the ischaemic risk zone with fluorescent particles. **Results:** Myocardial levels of arachidonic as well as of linoleic and docosahexaenoic acid were significantly elevated by preconditioning. Also, the level of peroxidized polyunsaturated fatty acids (measured as hydroxy conjugated dienes) in myocardial phospholipid was significantly increased: 101.4 ± 16.8 nmol/g versus 51.2 ± 7.3 nmol/g tissue dw in the control group, $p < 0.05$. Pre-treatment of hearts with 5 μM NDGA blocked the infarct limiting effect of preconditioning: infarct size was 37.4 ± 6.4% of risk zone in control, 9.0 ± 0.9% in the preconditioning group and 27.7 ± 3.8% in the preconditioning + NDGA group ($p < 0.05$ vs. IP, n.s. vs. control). **Conclusion:** Our findings provide evidence for the involvement of phospholipase A₂ and lipoxygenase derived lipid second messengers in ischaemic preconditioning of the isolated rat heart. © 1997 Elsevier Science B.V.

Keywords: Ischaemic preconditioning; Arachidonic acid; Lipoxygenase; Phospholipid peroxidation; Nordihydroguaiaretic acid; Rat heart

1. Introduction

Preconditioning with brief intermittent periods of ischaemia has been demonstrated to provide protection against ischaemic injury [1–3]. Another consequence of brief periods of ischaemia is generation of free radicals [4]. The relationship between these two phenomena has been debated but not fully explored.

The mechanism through which preconditioning protects the myocardium has been examined in a wide variety of

experimental models [1,2,4,5]. Regardless of the model used, increasing evidence suggests that stimulation of intracellular signalling events by endogenous agents released during the short, preconditioning ischaemia, is important for subsequent cardioprotection [6–8]. This is partly based on identification of membrane bound receptors linked to protein kinase C (PKC) activation and subsequent cardioprotection [9,10]. PKC activation seems to play a central role in myocardial protection afforded by ischaemic preconditioning in many species including the rat [11–13]. However, the receptors responsible for initiating signal transduction for PKC activation in the preconditioned rat

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heart have not been clarified and seem to be highly dependent on the experimental model [14,15].

Recently Murphy et al. have hypothesised that metabolites of arachidonic acid derived from the lipoxygenase pathway could be involved in preconditioning of rat heart [6]. They proposed that during short ischaemia, phospholipase A₂ becomes activated, arachidonic acid is released, and metabolites of arachidonic acid modulate cellular signalling pathways that are responsible for the cardioprotection by preconditioning. Moreover, arachidonic acid itself is also known to be a potent intracellular second messenger [16,17]. It has been reported that PKC is activated by *cis*-unsaturated fatty acids such as arachidonic acid and oleic acid [16,18]. Thus arachidonic acid could be one possible candidate explaining the physiology of ischaemic preconditioning of rat heart. It is also possible that free radicals and lipid peroxidation products, produced after short-lasting ischaemia, generate reversible changes in cellular unsaturated fatty acids which are responsible for activation of phospholipases and subsequent release of substrates for the lipoxygenase pathway.

The aim of the present study was to examine whether alteration in myocardial content (relative or absolute) of arachidonic acid or other unsaturated fatty acids occurs in preconditioning. For this purpose arachidonic acid, oleic acid, linoleic acid, and docosahexaenoic acid were measured in the free fatty acid fraction and the polar lipid fraction of myocardial lipid extracts. As polyunsaturated fatty acids are easily peroxidized, another objective of our study was to evaluate the occurrence of oxidative stress in ischemic preconditioning. For this purpose we performed measurements of hydroxy conjugated dienes of polyunsaturated fatty acids in free fatty acid and phospholipid fractions using the HPLC technique to separate unchanged and peroxidized polyunsaturated fatty acids. In a separate set of experiments in order to further clarify the role of lipoxygenase metabolites of arachidonic acid in ischaemic preconditioning, we used nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, to block preconditioning against infarction in an isolated rat heart model of regional ischaemia.

2. Materials and methods

2.1. Perfusion procedure

The investigation conforms with the guidelines on accommodation and care of animals formulated by the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. Male Wistar rats weighing 270–340 g, fed a standard diet, were heparinized with 200 IU and anaesthetized with Na-pentobarbital 50 mg/kg intraperitoneally. The hearts were rapidly excised, placed in an ice-cold buffer and perfused within 60 s in a non-recirculating Langendorff perfusion

system maintained at 37°C. The perfusion pressure was kept at 100 cm H₂O. The Krebs–Henseleit buffer (pH = 7.4, oxygenated with 95% O₂/5% CO₂) contained 2.4 mM calcium and 11.1 mM glucose. A water filled latex balloon, connected to a pressure transducer and coupled to a Gould recorder, was inserted into the left ventricle through an incision in the left atrium. The volume of the balloon was adjusted to assure that the balloon was unstretched and that an end-diastolic pressure below 10 mmHg was obtained. Heart rate, left ventricular systolic pressure (LVSP) and end diastolic pressure (LVEDP) were recorded. Left ventricular developed pressure (LVDP) was calculated as the difference between LVSP and LVEDP, and coronary flow was measured by timed collections of effluent. In a separate series of experiments with regional ischaemia, a 3-0 silk thread was passed around the main branch of the left coronary artery and the ends threaded through a small vinyl tube to form a snare. Regional ischaemia was achieved by pulling the snare. Ischaemia was confirmed by a substantial fall in both left ventricular developed pressure and coronary flow.

2.2. Experimental protocol

In the first part of the study we investigated alterations in myocardial lipid status associated with ischaemic preconditioning. For these purposes, a model of global ischaemia was chosen. The hearts were freeze-clamped at different time points of the experimental protocol and stored in liquid nitrogen for subsequent biochemical analysis.

The sampling protocol is illustrated in Fig. 1. All hearts underwent an initial 20 min stabilizing perfusion. In the first group (controls, before ischaemia), the stabilization period was extended by 10 min of ordinary perfusion, after which the hearts were frozen to obtain baseline values for normal hearts. In the second group (ischaemic preconditioning (IP), before ischaemia) the hearts were subjected to 5 min global ischaemia followed by 5 min of reperfusion and then freeze-clamped to investigate the alterations caused by the preconditioning procedure. Two groups were included for analysis of myocardial lipids after sustained ischaemia in hearts with and without IP. Thus, in the third group (controls at 30 min of ischaemia) the stabilization period was followed by 10 min of ordinary perfusion after which the hearts ($n = 10$) were subjected to a standard ischaemic insult of 30 min of global ischaemia, at the end of which the hearts were freeze-clamped. In the fourth group (IP at 30 min of ischaemia), the standard ischaemic insult was preceded by ischaemic preconditioning. At the end of sustained ischaemia the hearts ($n = 10$) were freeze-clamped. In order to clarify whether nordihydroguaiaretic acid (NDGA, 5 μ M) influences myocardial lipid alterations caused by ischaemic preconditioning two additional groups were included. In the fifth group (control + NDGA) hearts were freeze-clamped after 10 min of NDGA

infusion. In the sixth group (IP + NGDA) hearts preconditioned in the presence of NDGA, were freeze-clamped at 5 min of reperfusion.

An additional set of experiments was included to demonstrate that the preconditioning protocol induced myocardial protection under the condition of global ischaemia, which was used for the tissue lipid analysis. These hearts were reperfused for 30 min and LVDP at the end of reperfusion served as an endpoint.

In the second part of the study we investigated the influence of nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, on protection by preconditioning. For these purposes an isolated rat heart model of regional ischaemia with assessment of infarct size was chosen. The protocol for these experiments is illustrated in Fig. 2. In the control group ($n = 6$), the initial stabilization period of 20 min was followed by 10 min of ordinary perfusion prior to 30 min of regional ischaemia. In the preconditioning group, 5 min of global ischaemia and 5 min of reperfusion was applied before occlusion of the left coronary artery ($n = 7$). In the third group, 5 μ M NDGA was administered for 10 min prior to regional ischaemia ($n = 6$). In the fourth group, the preconditioning protocol was combined with 5 μ M NDGA ($n = 9$). After 30 min of regional ischaemia, all these hearts were reperfused for 120 min.

NDGA (Sigma, St. Louis, MO, USA) was dissolved in DMSO, further diluted in buffer (final concentration of DMSO 0.001%), and delivered into an infusion port directly above the aortic cannula by an infusion pump (B. Braun Melsungen, Germany). The infusion speed was

adjusted to the coronary flow to achieve the required final dilution of the drug. The concentration used was chosen based on the previous report from Murphy et al. [6].

2.3. Assays for fatty acids and peroxidized lipids

Myocardial tissue was homogenised under liquid nitrogen and approximately 250 mg of the tissue extracted according to Folch et al. [19].

Following extraction, the lipids were dissolved in chloroform and applied to hexan-conditioned solid phase extraction silica columns (Bond Elut, Varian) using the method of Kaluzny et al. [20] with modifications to separate phospholipids and free fatty acids.

The phospholipid fraction was evaporated to dryness under nitrogen and redissolved in a small amount of diethyl ether. 125 μ l Methanol, 250 μ l CaCl_2 (0.01 M), 500 μ l sodium borate buffer (0.1 M, pH 8.5) and 0.18 mg phospholipase A_2 from *Naja mozambique* (Sigma, St. Louis, MO, USA, 1540 u/mg protein) in 75 μ l borate buffer were added. The tubes were flushed with argon, capped and put on a shaking bath (25°C, 30 min). The hydrolysis was stopped with acetic acid (0.2M). Released free fatty acids were extracted with diethyl ether, then evaporated to dryness and redissolved in chloroform/methanol 1:2.

Lipid peroxidation was detected by assessment of monohydroxy-fatty acid conjugated dienes in both the nonesterified lipid fraction as well as in fatty acid released after enzymatic hydrolysis. Fatty acid samples were chro-

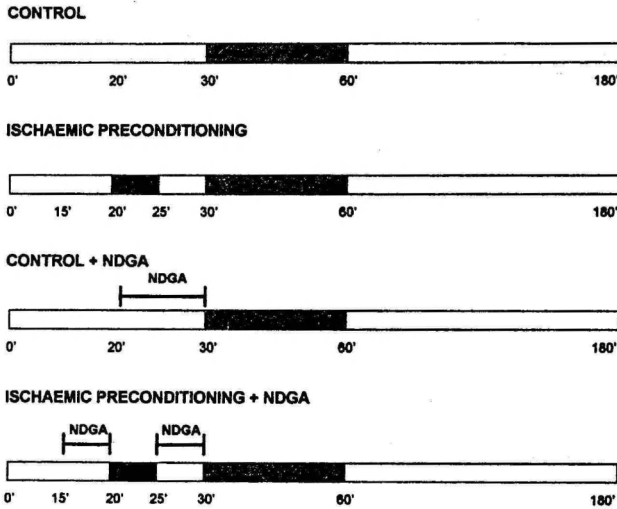


Fig. 2. Experimental protocol for infarct size measurements. Black bars — global ischaemia; hatched bars — regional ischaemia; open bars — perfusion. Perfusion with nordihydroguaiaretic acid (NDGA) is indicated by a line above the bars.

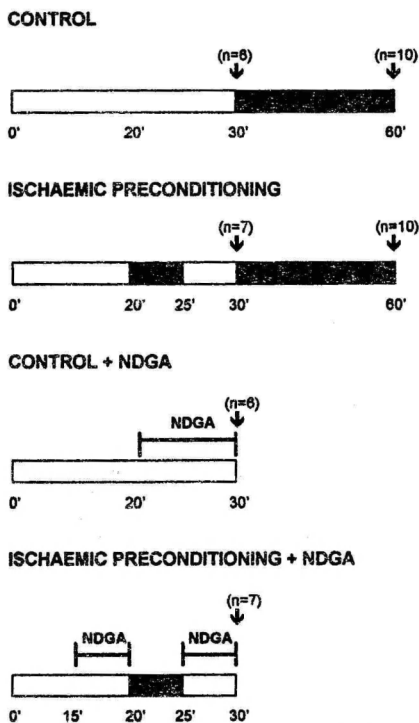


Fig. 1. Experimental protocol for assessment of myocardial lipid status. Black bars — global ischaemia; open bars — perfusion. Perfusion with nordihydroguaiaretic acid (NDGA) is indicated by a line above the bars. Hearts were freeze-clamped as indicated by the arrows.

matographed by reverse phase HPLC in order to separate the different components of the sample; i.e. monohydroxy fatty acids were eluted after 15–30 min. Conjugated dienes were detected at 235 nm using a variable UV-detector (Model 481, Waters). The elution pattern was confirmed by applying purified commercially available unsaturated fatty acids and monohydroxy fatty acids (5-HETE, 12-HETE) to the HPLC system. Identification of the retention-time interval was based on the use of monohydroperoxy conjugated diene isomers of linoleic acid, docosahexaenoic acid and arachidonic acid as reported by Van Rollins and Murphy [21]. Quantification was based on integration of all peaks corresponding to the retention-time interval. 5-HETE was used as a standard, and the levels were expressed in units/mg dry wt. based on the absorbance produced by 1 pmol of 5-HETE in the HPLC system.

Samples from the non-esterified fatty acid fraction and the fatty acids released from the 2-position of phospho-

lipids were analysed by gas chromatography. Total amount of polyunsaturated fatty acids (PUFA) was calculated as the sum of measured concentrations of linoleic acid, arachidonic acid, and docosahexaenoic acid.

2.4. Infarct size

In hearts subjected to regional ischaemia, infarct size was measured by the technique described in detail previously [22]. The risk zone was determined by fluorescent particles and infarct size by tetrazolium staining. Slices (2 mm) of heart tissue were subjected to computer-based planimetry. Infarct size was expressed as the percentage of risk zone infarcted.

2.5. Statistics

Results are expressed as the mean \pm standard error of the mean (SEM). Paired *t*-tests were used to identify significant differences ($p < 0.05$) within groups before prolonged ischaemia. Between group differences were analyzed by one way analysis of variance combined with Tukey's test.

3. Results

3.1. Free fatty acid fraction

The myocardial level of free fatty acids was not significantly altered by an ischaemic preconditioning cycle of 5 min of ischaemia and 5 min of reperfusion, and no differences were found between preconditioned and control hearts at the end of 30 min of prolonged ischaemia (Table 1). The preconditioned hearts, however, had a significantly higher content of free fatty acids at the end of the sustained ischaemia period when compared to controls before ischaemia (Table 1).

Significant changes were found in both absolute and

Table 1
Myocardial concentrations ($\mu\text{mol/g}$ dry wt.) of phospholipids (calculated from fatty acids released from polar lipid fraction by treatment with phospholipase A_2 from *Naja mombasica*) and free fatty acids

Group	Phospholipids	Free fatty acids
<i>Before 30 min of ischaemia</i>		
Control	143.8 \pm 7.0	2.02 \pm 0.25
IP	147.4 \pm 2.4	2.57 \pm 0.10
Control + NDGA	133.7 \pm 1.9	2.33 \pm 0.18
IP + NDGA	141.5 \pm 4.3	2.82 \pm 0.21*
<i>At 30 min of ischaemia</i>		
Control	149.9 \pm 3.9	2.66 \pm 0.15
IP	158.5 \pm 3.9	2.89 \pm 0.20*

Hearts were freeze-clamped as described in experimental protocol. IP — ischaemic preconditioning, NDGA — nordihydroguaiaretic acid.

* $p < 0.05$ compared to control before ischaemia.

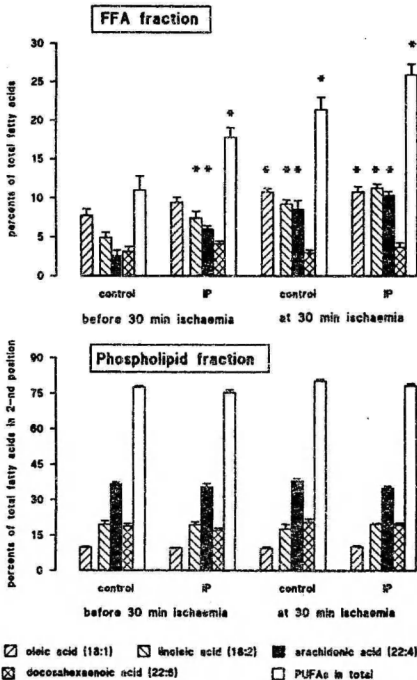


Fig. 3. Relative concentrations of fatty acids in phospholipid and free fatty acid fractions extracted from hearts freeze-clamped before and at the end of 30 min of ischaemia. IP — ischaemic preconditioning. * $p < 0.05$ compared to control before ischaemia.

relative (Fig. 3, upper panel) concentrations of polyunsaturated fatty acids in this fraction of myocardial lipids. Ischemic preconditioning with 5 min ischaemia and 5 min reperfusion resulted in significant increase in free arachidonic acid (from 58 ± 18 to 157 ± 14 nmol/g dry wt.), linoleic acid (from 105 ± 24 to 195 ± 30 nmol/g dry wt.) and docosahexaenoic acid (from 65 ± 14 to 105 ± 8 nmol/g dry wt.). As a result of the preconditioning procedure the sum of PUFAs expressed as percent of all free fatty acids was significantly increased (Fig. 3, upper panel).

At the end of 30 min of ischaemia the unsaturated free fatty acids in both control and IP groups were increased and no difference between these two groups was found. The hearts freeze clamped at the end of 30 min ischaemia had significantly higher relative (Fig. 3, upper panel) and absolute (data not shown) levels of linolenic acid, arachidonic acid as well as sum of PUFAs when compared to control hearts sampled before ischaemia.

Treatment with $5 \mu\text{M}$ NDGA for 10 min increased the level of free arachidonic acid from 58 ± 148 nmol/g to

168 ± 27 nmol/g in control and from 157 ± 10 nmol/g to 187 ± 27 nmol/g dry wt. in preconditioned hearts ($p < 0.05$ for both group). The level of other fatty acids was not significantly influenced (data not shown). As a result of increased arachidonic acid content, the content of total free fatty acids was increased by NDGA treatment (Table 1).

3.2. Phospholipid fraction

No significant changes were found in myocardial level of phospholipids, calculated from fatty acids released after treatment of tissue lipid extract with phospholipase A_2 . Treatment with phospholipase A_2 is assumed to release fatty acids from the 2nd position of myocardial phospholipids. As expected, there were mostly PUFAs obtained by this enzymatic hydrolysis and no significant differences were found in absolute (data not shown) or in relative concentrations (Fig. 3, lower panel) of released fatty acids. Treatment of both control and preconditioned hearts with $5 \mu\text{M}$ NDGA did not cause any changes in the level (Table 1) or in the composition (data not shown) of myocardial phospholipids when compared with non-treated hearts.

3.3. Monohydroperoxy and -hydroxy conjugated dienes of polyunsaturated fatty acids

The products of lipid peroxidation, monohydroperoxy and -hydroxy conjugated dienes of fatty acids, were measured in both free fatty acid and phospholipid fractions and are presented relative to PUFAs in Fig. 4.

In the free fatty acid fraction the levels of monohydroxy conjugated dienes were not different between groups before ischaemia. As a result of increase in total content of PUFAs found at the end of 30 min of ischaemia, the relative amount of monohydroxy conjugated dienes at this time point was reduced. In the control group this tendency reached significance (Fig. 4, upper panel). The absolute levels, however, were not different between the groups (data not shown).

With respect to phospholipids, ischaemic preconditioning cycle of 5 min of ischaemia and 5 min of reperfusion induced a significant increase of hydroxy conjugated dienes relative to the amount of polyunsaturated fatty acids among fatty acyl residues in the 2-position (Fig. 4, lower panel). The absolute level of hydroxy conjugated dienes was also significantly increased by ischaemic preconditioning: from 51.2 ± 7.3 nmol/g dry wt. in control to 101.4 ± 16.8 nmol/g dry wt. in IP group, respectively. At the end of the 30 min ischemic insult no significant differences between the groups were found (Fig. 4, lower panel).

The increase of lipid peroxidation products found in phospholipids of preconditioned hearts was attenuated by NDGA treatment. Thus, the level of monohydroperoxy and -hydroxy conjugated dienes of fatty acids among fatty acyl residues at the 2-position was 82.9 ± 16.1 nmol/g dry wt. in the IP + NDGA group versus 84.1 ± 11.9 nmol/g dry

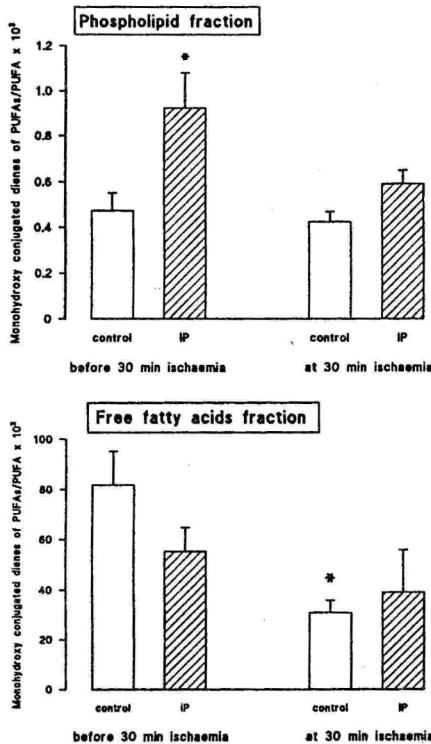


Fig. 4. Levels of monohydroxy conjugated dienes of polyunsaturated fatty acids in two fractions of lipid extract from hearts freeze-clamped before and at the end of 30 min of ischaemia. IP — ischaemic preconditioning. * $p < 0.05$ compared to control before ischaemia.

wt. in the control + NDGA group, respectively. Also, the amount of conjugated dienes relative to the amount of polyunsaturated fatty acids in the sample was not altered by preconditioning in the presence of NDGA: 0.734 ± 0.130 in IP + NDGA compared to 0.802 ± 0.107 monohydroxy conjugated dienes of PUFAs/PUFAs $\times 10^3$ in the control + NDGA group, respectively.

3.4. Functional recovery after global ischaemia

Functional parameters measured at the end of stabilization, immediately before the onset of 30 min of global ischaemia and at 30 min of reperfusion are shown in Table 2. Control hearts recovered to 29.0% of their preischaemic LVDP at the end of the 30 min of reperfusion period. Hearts preconditioned with one cycle of 5 min of ischaemia and 5 min of reperfusion expressed significantly better functional recovery as, in this group, the LVDP at

Table 2

Functional parameters of hearts subjected to 30 min of global ischaemia

	Stabilization	Pre-ischaemia	30 min reperfusion
Coronary flow (ml/min)			
Control	14.1 ± 0.7	14.3 ± 0.7	8.8 ± 0.9
IP	14.3 ± 0.6	14.3 ± 1.1	10.8 ± 1.2
Heart rate (beats/min)			
Control	316 ± 19	304 ± 14	268 ± 18
IP	304 ± 5	298 ± 12	293 ± 17
End diastolic pressure (mmHg)			
Control	3.4 ± 0.8	3.5 ± 0.8	81.4 ± 5.1
IP	4.9 ± 0.6	$18.8 \pm 3.5^{\#}$	$63.1 \pm 3.2^*$
Developed pressure (mmHg)			
Control	129.9 ± 4.2	130.3 ± 3.7	37.9 ± 3.1
IP	120.3 ± 5.0	98.1 ± 6.4	$56.0 \pm 3.4^*$

At the end of stabilization; pre-ischaemia, which is the end of the preconditioning cycle for preconditioned hearts; and at 30 min of reperfusion. IP — ischaemic preconditioning.

$^{\#} p \leq 0.05$ compared to corresponding baseline value.

$^* p \leq 0.05$ compared to control group.

the end of reperfusion was 43.7% of baseline value.

3.5. NDGA treated hearts subjected to regional ischaemia

3.5.1. Infarct size

Infarct size as a percent of risk zone for four groups subjected to 30 min of regional ischaemia is presented in Fig. 5. Ischaemic preconditioning significantly reduced infarct size. This effect of preconditioning was abolished by the treatment of the hearts with lipoxygenase inhibitor NDGA ($5 \mu\text{M}$). The drug alone had no effect on development of irreversible myocardial injury. The volume of risk zone was not significantly different among all the groups studied. The overall mean risk zone volume was $298 \pm 12 \text{ mm}_3$ ($n = 28$).

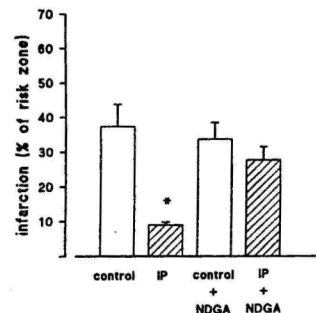


Fig. 5. Infarct size expressed as per cent of the risk zone infarcted after 30 min of regional ischaemia and 120 min of reperfusion. Mean \pm SEM, $n = 6-9$ in each group. IP — ischaemic preconditioning, NDGA — nordihydroguaiaretic acid. * $p < 0.05$ compared to control group.

3.5.2. Functional variables

Coronary flow, left ventricular end diastolic pressure and developed pressure of hearts subjected to 30 min of regional ischaemia are shown in Table 3. The baseline values for these parameters, obtained after 15 min of perfusion, did not differ between any of the groups. Ischaemic preconditioning led to an increase in coronary flow. This effect was abolished by NDGA: in the IP + NDGA group coronary flow was even significantly decreased when compared to the baseline value. Administration of NDGA for 10 min caused a significant decrease in developed pressure in average of 13.6 mmHg (Table 3). At reperfusion, the NDGA treated hearts (control + NDGA and IP + NDGA, respectively) showed poor recovery of contractile function. In particular, higher levels of end diastolic pressure after reperfusion were observed in these hearts.

The baseline values for heart rate were not different between any of the groups (data not shown) and did not differ between the groups throughout the experiments. Mean heart rate for all groups at baseline was 307 ± 6 beats/min, while at 5 min of regional ischaemia it was 277 ± 9 beats/min, and at the end of experiments 286 ± 8 beats/min, respectively.

4. Discussion

One of the main findings of this study was that preconditioning with one cycle of 5 min of global ischaemia and

5 min of reperfusion resulted in a significant increase of hydroxy conjugated dienes of fatty acids in the phospholipids, and there was concomitant increase in arachidonic acid as well as in other PUFAs in the free fatty acid fraction of myocardial tissue. As hydroxy conjugated dienes of fatty acids are markers of lipid peroxidation, it is evident from our results that the short ischaemia-reperfusion episode required to precondition the heart is enough to induce oxidative stress. This finding is in accordance with the study of Zhou et al. who very recently demonstrated that anoxic preconditioning of isolated rat myocytes was accompanied by a burst of oxygen-free radicals [23]. We also found that the infarct size limiting effect of ischaemic preconditioning in the isolated rat heart can be blocked by nordihydroguaiaretic acid (NDGA), an inhibitor of the lipoxygenase pathway of arachidonic acid metabolism.

Possible mechanisms explaining how oxidative stress could be involved in the physiology of ischaemic preconditioning have been debated in a number of studies [4,23,24]. Firstly, it has been suggested that the beneficial effect of preconditioning is the consequence of an ischaemia induced increase in myocardial antioxidant activity. However, many authors have failed to detect any changes, at least in studies on the early protection, in heart antioxidant status after preconditioning [24–26]. Another possibility is that free radicals participate in cellular signal transduction and are in this way involved in adaptive responses on the cellular level. Oxidation of lipids by free radicals can activate phospholipase A₂ [27], which releases polyunsatu-

Table 3
Functional variables of hearts subjected to 30 min of regional ischaemia

Group	Baseline	Stabilization	Preischaemia	5 min ischaemia	5 min reperfusion	30 min reperfusion	60 min reperfusion	120 min reperfusion
<i>Coronary flow (ml/min)</i>								
Control	12.1 ± 0.6	12.1 ± 0.6	12.1 ± 0.7	7.0 ± 0.6	10.6 ± 1.2	10.0 ± 0.9	9.8 ± 0.7	8.3 ± 0.8
IP	13.0 ± 0.8	12.6 ± 1.0	15.1 ± 1.3 [#]	7.5 ± 0.7	10.6 ± 1.2	10.9 ± 1.4	10.2 ± 1.6	9.9 ± 1.8
Control + NDGA	13.3 ± 0.6	13.3 ± 0.4	13.9 ± 0.7	7.5 ± 0.3	7.1 ± 0.5	6.7 ± 0.6	6.8 ± 1.0	6.7 ± 0.6
IP + NDGA	13.1 ± 0.5	15.1 ± 0.7	10.0 ± 0.5 [#]	7.4 ± 0.9	7.6 ± 0.7	7.5 ± 0.7	7.7 ± 0.7	7.1 ± 0.8
<i>Left ventricular end diastolic pressure (mmHg)</i>								
Control	3.0 ± 1.0	2.5 ± 1.1	2.5 ± 1.1	8.3 ± 3.3	13.3 ± 5.1	9.2 ± 3.0	10.0 ± 4.1	17.5 ± 7.3
IP	1.0 ± 0.7	1.0 ± 0.7	6.4 ± 4.1	5.4 ± 1.6	10.0 ± 3.9	7.9 ± 3.9	7.9 ± 3.9	9.3 ± 3.9
Control + NDGA	0.5 ± 0.5	0.5 ± 0.5	1.7 ± 1.1	2.5 ± 1.1	24.2 ± 6.9	20.0 ± 5.3*	22.5 ± 6.2	23.0 ± 3.4
IP + NDGA	1.7 ± 0.8	2.4 ± 0.8	27.8 ± 6.9 [#]	20.6 ± 6.0	43.3 ± 7.7*	33.9 ± 5.3*	33.3 ± 3.0*	35.6 ± 4.9
<i>Left ventricular developed pressure (mmHg)</i>								
Control	161.2 ± 9.7	164.2 ± 7.9	166.6 ± 7.3	67.5 ± 6.4	120.0 ± 12.8	123.3 ± 11.1	114.2 ± 13.2	99.2 ± 12.8
IP	155.4 ± 7.7	151.1 ± 6.0	106.4 ± 3.7 [#]	67.1 ± 7.8	112.1 ± 6.3	115.7 ± 7.8	107.9 ± 8.4	90.7 ± 5.5
Control + NDGA	146.2 ± 8.3	142.8 ± 6.8	129.2 ± 9.2 [#]	52.5 ± 8.5	59.2 ± 7.5*	61.7 ± 7.8*	55.8 ± 7.9*	55.8 ± 6.3*
IP + NDGA	154.4 ± 5.8	151.1 ± 6.1	71.1 ± 9.2 [#]	51.3 ± 4.4	59.7 ± 5.8*	66.7 ± 4.6*	64.1 ± 5.1*	58.6 ± 5.8*

Baseline — at 15 min of perfusion, before drug addition; Stabilization — at 20 min of perfusion, which is 5 min after drug addition for IP + NDGA group; Pre-ischaemia — immediately before the onset of regional ischaemia, for Control + NDGA group it is 10 min after drug addition. IP — ischaemic preconditioning, NDGA — nordihydroguaiaretic acid.

[#] $p \leq 0.05$ vs. corresponding baseline value.

* $p \leq 0.05$ vs. control group at respective time point.

rated fatty acids from phospholipids. Nonesterified polyunsaturated fatty acids, in particular arachidonic acid and its cyclo- and lipoxygenase metabolites, are implicated as important cellular second messengers [18,28], which could also be involved in mechanisms of ischaemic preconditioning. Our present finding of enhanced phospholipid peroxidation and an increase in free arachidonic acid, as well as other PUFAs by the preconditioning procedure, is supportive for this hypothesis. The theory is further emphasised by the fact that the infarct size limiting effect of preconditioning was blocked by NDGA, a lipoxygenase inhibitor. NDGA, however, is also known as an antioxidant [29], attenuating free radical production in different experimental conditions [30,31]. Thus, it could theoretically restrain the effect of preconditioning by interfering on the signal transduction level above the lipoxygenase reaction (i.e. by indirect PLA_2 inhibition). Therefore, the influences of NDGA on lipids in the myocardium in preconditioned hearts was also examined. We found that, under control conditions, perfusion of hearts with NDGA resulted in an increase in myocardial free fatty acids, in particular of arachidonic acid, and this tended to add to the changes induced by the preconditioning procedure. The increase in phospholipid peroxidation products induced by preconditioning, however, was attenuated by NDGA pretreatment.

In general our findings are in accordance with a study by Murphy et al. [6]. They showed that NDGA blocked the accumulation of 12-hydroxyeicosatetraenoic acid (12-HETE) during the preconditioning protocol as well as restrained the effect of preconditioning on functional recovery after global ischaemia. However, in this study it remained unclear whether the alterations in functional recovery were due to limited extent of cell necrosis or achieved by reduced stunning, or was a mixture of both. Therefore, we have chosen the experimental model of regional ischaemia to demonstrate that NDGA specifically blocks the effect of preconditioning on ischaemic cell death.

Our method for lipid peroxidation assessment will detect monohydroxy conjugated dienes of fatty acids including 5-HETE and 12-HETE. It has been shown that production of both 5-HETE and 12-HETE in isolated cardiac myocytes is enhanced during reoxygenation after hypoxia [32]. Therefore, the measurements of hydroxy conjugated dienes of fatty acids in the free fatty acid fraction partly correspond with detection of HETEs in the study of Murphy et al. [6]. However, in contrast to their results, we did not find any changes in hydroxy conjugated dienes in the free fatty acid fraction in relation with ischaemic preconditioning. This controversy can be explained by the difference in methodology as our assay is not aimed at detecting specific HETEs and/or by the difference in experimental protocols. The detection of 12-HETE by Murphy and coworkers was made in hearts with arachidonic acid added to the buffer solution, which was probably responsible for

amplifying the activity in the lipoxygenase pathway. In their study, Murphy et al. have used four cycles of 5 min of ischaemia and 5 min of reperfusion to precondition the heart while we have used only one such cycle. An increase in IP cycles could amplify the signal beyond the detection limit for such short-lived and reactive compounds as HETEs.

Myocardial ischaemia-reperfusion injury is associated with subsequent accumulation of leucocytes in injured tissue [33]. HETEs, precursors of mediators of inflammation, are generated by activated leucocytes, and leukotrienes are suggested to contribute to the myocardial cell damage by ischaemia-reperfusion [34]. Attenuation of neutrophil function by the lipoxygenase inhibitors is in some studies shown to reduce myocardial infarction *in vivo* [35,36]. The cells of blood origin are not present in the isolated heart model, and the lipoxygenase inhibitor NDGA exhibited no protection in the present study. Thus, it is reasonable to propose that controlled production of HETEs by myocardial cells during short preconditioning ischaemia is able to induce cellular adaptation without any harmful effect.

With respect to functional variables, we found that NDGA had a somewhat detrimental effect on the contractile function during reperfusion. In particular, the diastolic pressure at reperfusion was higher in these hearts and it appeared to be independent of the duration of preceding ischaemia (30 min in control group or 5 min during preconditioning). From these findings one could speculate that the effect of NDGA in preconditioned hearts is due to its effect on contractile function. However, under control conditions the infarct size was not influenced by NDGA, while the contractile performance of these hearts was similarly deteriorated. On the other hand, the untreated preconditioned hearts, although having significantly less tissue infarcted, did not show better recovery of contractile function during 120 min of reperfusion compared to untreated non-preconditioned hearts. In previous studies we have demonstrated poor correlation between the extent of ischaemic cell death and global contractile performance during reperfusion in isolated rat heart subjected to regional ischaemia [37]. Therefore, we suggest that the effect of NDGA on preconditioning against infarction was not affected by the effect on contractile function in reperfused hearts.

Altogether, our results support the view that metabolites of arachidonic acid are important intracellular messengers in ischaemic preconditioning. Many recent studies have demonstrated that protein kinase C activation, occurring in ischaemic preconditioning, is crucial for protection against ischaemic cell death [11,12,38]. The most common view has been that activation of phospholipase C in response to stimulation of a variety of membrane-bound receptors, with subsequent release of inositoltrisphosphate (IP_3) and diacylglycerol (DAG), are the steps responsible for signal transduction leading to protein kinase C activation. How-

ever, in a previous study we failed to block ischemic preconditioning by simultaneous blocking of several different G_i/G_o coupled receptors [22], which suggests the presence of some additional intracellular signalling pathways for PKC activation. Hence, *in vitro* studies have revealed that also arachidonic acid, released by PLA_2 , can activate protein kinase C, in some circumstances even synergistically with diacylglycerol [16,18]. Protein kinase C, once activated, can further phosphorylate PLA_2 among other proteins and this generates a positive feedback system for cellular signalling [18]. The most likely candidate for effector protein, activated by protein kinase C dependent phosphorylation during ischaemic preconditioning, is the K_{ATP} -channel [39,40]. The K_{ATP} -channels, however, may be activated also by nonesterified polyunsaturated fatty acids in PKC dependent manner [41] or directly by arachidonic acid, as well as HETEs and HPETEs [42,43]. Thus, the activation of a signalling pathway involving phospholipase A_2 activation and arachidonic acid release could be one important factor responsible for the cellular adaption leading to protection against ischaemic cell death in the rat heart.

In conclusion, the present study demonstrated that an ischaemic preconditioning cycle of 5 min of ischaemia plus 5 min of reperfusion was associated with an increase in phospholipid peroxidation and increase in free arachidonic acid in myocardial tissue. We also found that ischaemic preconditioning could be blocked by nordihydroguaiaretic acid, a lipoxygenase inhibitor. These findings support evidence for a role of PLA_2 and lipoxygenase-derived lipid second messengers in ischaemic preconditioning in rat hearts.

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PRECONDITIONING WITH HYDROGEN PEROXIDE (H₂O₂) OR ISCHEMIA IN H₂O₂-INDUCED CARDIAC DYSFUNCTION

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ABSTRACT

The possible cardioprotective effects of preconditioning by ischemia (IPC) or a low dose of H₂O₂ (HPC) prior to a high dose of H₂O₂ was investigated in Langendorff-perfused rat hearts. Groups (n=10 in each) were subjected to 10 min of 140 µmol/L H₂O₂ and 30 min recovery after either 1) Control perfusion, 2) 20 µmol/L H₂O₂ for 10 min, recovery 10 min, or 3) 2 × 2 min global ischemia and 5 min reperfusion. 140 µmol/L H₂O₂ increased left ventricular end-diastolic pressure from 0 to 68±8 mmHg in controls (mean±SEM), which was attenuated by IPC (46±9 mmHg, p<0.001) and HPC (18±4 mmHg, p<0.001 compared to controls, p<0.01 compared to IPC). HPC, but not IPC, improved coronary flow (p<0.02) and left ventricular developed pressure (p<0.001) during recovery. Troponin T release was similar in all groups. Tissue thiobarbituric acid reactive substances, antioxidant capacity, catalase, and glutathione peroxidase were not influenced by 140 µmol/L H₂O₂. H₂O₂ decreased the level of tissue glutathione. This reduction was augmented by HPC (p<0.02) and attenuated by IPC (p<0.02). H₂O₂ increased superoxide dismutase (p<0.04). The increase was attenuated by IPC (p<0.05), but not influenced by HPC. In conclusion, HPC efficiently protected cardiac function in H₂O₂-induced cardiac injury, while IPC had only a small protective effect. The functional protection cannot be explained by reduction of cell necrosis, attenuation of lipid peroxidation, or modification of tissue antioxidant parameters. It appears that it is not ischemic preconditioning *per se* which protects the heart against functional injury, but repetition of a stimuli participating in both preconditioning and injury.

Key words: Catalase, glutathione, glutathione peroxidase, superoxide dismutase, thiobarbituric acid reactive substances,

INTRODUCTION

Ischemic preconditioning reduces infarct size and improves cardiac function in experimental models of global and regional ischemia.^{1, 2} Despite intensive investigations into the underlying mechanisms of the preconditioning response, this is far from clarified and appears to differ between species. Reactive oxygen species (ROS), including hydrogen peroxide, have been suggested as possible mediators.³ ROS are produced during short episodes of ischemia and reperfusion.⁴ Some investigators have found antioxidants to reduce the acute, beneficial effects of preconditioning,⁵⁻⁸ while others did not.⁹⁻¹¹ Ischemic preconditioning has been reported to reduce the lipid peroxidation product malondialdehyde¹² and influence myocyte antioxidant status,^{5, 13, 14} but these findings were not confirmed in other models.^{15, 16} The effect of preischemic exposure to low doses of exogenous ROS on the preconditioning response is controversial.¹⁷⁻¹⁹

Preconditioning has been suggested to be an universal mode of myocardial protection.²⁰ If so is the case, ischemic preconditioning should protect against cardiac injury induced by other agents than ischemia. Ischemic preconditioning has been shown to protect the heart against cardiac dysfunction induced by anthracyclin toxicity,²¹ but not against dysfunction induced by hypoxia²² or exogenous ROS.²³ Studies using the model of cardiac injury induced by exogenous ROS are mostly aimed to investigate reversible postischemic contractile dysfunction of the heart (myocardial stunning), as oxidative stress has been suggested to have a crucial role in this phenomenon.²⁴ Although a variety of stimuli have been employed to mimic preconditioning, success in this matter might depend on finding a stimuli which triggers mediators or intracellular signalling systems that are activated and repeated during the sustained ischemia.

The present study tests the hypothesis that it is not ischemic preconditioning *per se*, but repeated exposure to the same injurious agent which primarily triggers the preconditioning response. Cardiac function and release of troponin T were investigated in Langendorff-perfused rat hearts subjected to either a low dose of hydrogen peroxide or ischemic preconditioning prior to exposure to a higher dose of hydrogen peroxide. Tissue contents of lipid peroxidation products and antioxidative parameters were measured to study the role of oxidative stress in functional protection.

MATERIALS AND METHODS

Heart perfusion

Male Sprague Dawley rats (200–300 g) were anesthetized with diethyl ether, and 200 IU heparin was injected into the femoral vein. The hearts were then rapidly excised through a median sternotomy and placed in ice-cold buffer during preparation for aortic cannulation. The hearts were retrogradely perfused with gassed (5% CO₂, 95% O₂) Krebs Henseleit buffer (NaCl 118.5 mmol/L,

NaHCO₃ 25.0 mmol/L, KCl 4.7 mmol/L, KH₂PO₄ 1.2 mmol/L, MgSO₄ • 7H₂O 1.2 mmol/L, Glucose•H₂O 11.1 mmol/L, CaCl₂•2H₂O 1.8 mmol/L) as a modified Langendorff - preparation. The perfusion pressure (100 cm H₂O) was kept constant. Water jackets round the perfusate reservoirs and heart chamber kept the temperature at 37°C throughout the experiments. A balloon was inserted into the left ventricle via the left atrium for isovolumetric recordings of left ventricular systolic (LVSP) and end-diastolic (LVEDP) pressures. Coronary flow (CF) was measured by timed collections of the coronary effluent. Heart rate (HR) was counted from the pressure curves. Left ventricular developed pressure (LVDP) was calculated (LVDP = LVSP – LVEDP). Two perfusate reservoirs were employed in order to rapidly change between buffer and H₂O₂-perfusion. H₂O₂ (3% solution) was purchased at the Karolinska Hospital's Pharmacy, and mixed directly into the buffer to achieve final concentrations of either 20 or 140 µmol/L. Global ischemia was induced by clamping the inflow tubing.

Experimental protocol

The hearts were stabilized for 20 minutes before start of the experiments (defined as time 0). Only hearts with LVSP between 60–160 mmHg, LVEDP 0 mmHg, CF 8–16 ml/min, and HR 240–360 beats/min at the end of stabilization were included in one of the experimental groups below:

GROUP 1: (n=10) Control perfusion for 20 min before 10 min perfusion with 140 µmol/L H₂O₂ and recovery with buffer only for 30 min.

GROUP 2: (n=10) Ischemic preconditioning with 2 episodes of 2 min global ischemia and 5 min reperfusion before H₂O₂ and recovery as group 1.

GROUP 3: (n=10) Pretreatment with 20 µmol/L H₂O₂ for 10 min, followed by 10 min buffer perfusion only before H₂O₂ and recovery as in groups 1 and 2.

LVSP, LVEDP, LVDP, HR, and CF were measured after 15 min stabilization (S), at time 0, immediately before infusion of 140 µmol/L H₂O₂, after 5 and 10 min of H₂O₂-infusion, and after 5, 10, 20, and 30 min recovery. Samples of the coronary effluent were collected for measurement of troponin T (TnT) at time 0, at the end of H₂O₂-infusion, and at the end of recovery. Additional hearts were perfused and freeze-clamped in liquid nitrogen cooled thongs at time 0 (n=10) and at the end of infusion of 140 µmol/L H₂O₂ (n=10 in all 3 groups) for analysis of cardiac antioxidative parameters and lipid peroxidation products. The freeze-clamped hearts were not included in the hemodynamic comparisons, or in measurements of TnT. Tissue reduced glutathione content (GSH), glutathione peroxidase (GSH-Px), total antioxidant status (TAS), catalase (CAT), superoxide dismutase (SOD), and antioxidant capacity were measured. Lipid

peroxidation was evaluated by tissue contents of thiobarbituric acid reactive substances (TBARS).

Laboratory methods

1 ml samples of the coronary effluent were collected in precooled tubes, stored on ice, and rapidly frozen at -70°C . The freeze-clamped hearts were immediately stored at -70°C , transported on dry ice to Estonia, and kept frozen (-70°C) until analysis. Heart tissue was homogenized in 10 volumes of ice-cold 1.15 % KCl, and filtered to get a homogenous mixture. To one aliquot of homogenate butylated hydroxytoluene (v/v ratio 11 μl /1000 μL KCl) was added to suppress artefacts during handling of the samples in the TBARS measurements. Protein was measured according to Lowry *et al.*²⁵

TBARS were measured according to Okhawa *et al.*,²⁶ with modifications previously described by us.²⁷ Assays were performed in triplicates with mean values used for comparisons, and malonaldehyde was used as a standard. TBARS are expressed in nmol/g wet weight.

Antioxidant capacity was determined by the ability of the samples to inhibit lipid peroxidation induced by linolenic acid.²⁷ Lipid peroxidation was assessed by contents of TBARS. Antioxidant capacity is expressed as percent inhibition of linolenic acid standard peroxidation in the sample.

Total antioxidant status (TAS) was measured by the commercially available kit (Randox Laboratories Ltd, Ardmore, United Kingdom) for assay of total antioxidant status. The values are expressed in mmol/l.

Tissue content of reduced glutathione (GSH) was measured with Ellman reagent as described by Beutler *et al.*²⁸ Briefly, tridistilled water (1800 μL) and 100 μl of 500 mmol/L perchloric acid were added to 500 μL sample. After 5 min protein sediment was eliminated by centrifugation (3000 rpm, 10 min). 300 μL of the supernatant was mixed with 600 μL of 300 m Na_2HPO_4 and 150 μL Ellman reagent, and incubated for 10 min at 37°C . The absorbance of yellow dye was measured at 412 nm. GSH content was calculated by means of a standard plot (1–20 mg GSH/dl).

Superoxide dismutase (SOD) activity of the sample was measured by a commercial kit (RANSOD, Randox Laboratories Ltd, Ardmore, United Kingdom) and expressed as SOD units/mg protein.

Glutathione peroxidase (GSH-Px) activity was measured by a commercial kit (RANSEL, Randox Laboratories Ltd, Ardmore, United Kingdom) and expressed as GSH-Px units/mg protein.

Catalase (CAT) activity was measured with the method described by Goth,²⁹ and expressed as CAT units/mg protein.

Troponin T was measured with the second generation of the cardiac troponin T ELISA.³⁰ The assays were performed using the Enzymum-Test Troponin-T (Boehringer Mannheim GmbH, Mannheim, Germany) on ES300

Enzymum Immunoassay system (Boehringer Mannheim). Troponin T in the coronary effluent was calculated as amount released per minute [troponin T ($\mu\text{g/L}$) \times CF (ml/min) $\times 10^{-3} = \text{ng/min}$].

Statistics

A Mann Whitney U-test was employed to compare differences between groups, and a Wilcoxon Signed rank sum test for differences within groups. $P < 0.05$ was considered significant. Data are presented as mean \pm SEM.

RESULTS

Left ventricular developed pressure

LVDP was reduced by preconditioning with both 20 $\mu\text{mol/L}$ H_2O_2 and ischemia compared to controls before perfusion with 140 $\mu\text{mol/L}$ H_2O_2 ($p < 0.02$ and $p < 0.03$, respectively) (Fig. 1). LVDP decreased during H_2O_2 -perfusion in controls. Hearts preconditioned with H_2O_2 had higher LVDP than controls during perfusion with 140 $\mu\text{mol/L}$ H_2O_2 and the subsequent recovery ($p < 0.01$ at all time points), and higher than ischemic preconditioned hearts after 5 min of H_2O_2 -perfusion ($p < 0.005$) and 30 min recovery ($p < 0.03$). Ischemic preconditioned hearts were not significantly different from controls after start of intervention (Fig. 1).

Left ventricular end-diastolic pressure

Neither preconditioning with ischemia nor H_2O_2 influenced LVEDP before perfusion with 140 $\mu\text{mol/L}$ H_2O_2 (Fig. 1). 140 $\mu\text{mol/L}$ H_2O_2 increased LVEDP in controls, with a maximal level after 5 min recovery (15 min observation). Ischemic preconditioning attenuated this increase at the end of H_2O_2 -perfusion ($p < 0.0004$), and after 5 min recovery ($p < 0.03$). Preconditioning with 20 $\mu\text{mol/L}$ H_2O_2 attenuated the H_2O_2 -induced increase of LVEDP at all time points compared to H_2O_2 controls ($p < 0.0006$ at all time points) and compared to ischemic preconditioning ($p < 0.01$ at all time points) (Fig. 1).

Coronary flow

Preconditioning with 20 $\mu\text{mol/L}$ H_2O_2 reduced CF before intervention compared to controls ($p < 0.02$), and compared to ischemic preconditioning ($p < 0.002$) (Fig. 1). 140 $\mu\text{mol/L}$ H_2O_2 increased CF both during infusion and recovery. Hearts pretreated with H_2O_2 had higher CF than controls after 10 min infusion of 140 $\mu\text{mol/L}$ H_2O_2 , and after 5 and 10 min recovery ($p < 0.02$ at all times). Ischemic preconditioning did not significantly influence CF at any time point (Fig. 1).

Heart rate

HR was 290 ± 7 , 296 ± 11 , and 288 ± 9 beats/min in controls, IPC and HPC at time 0, respectively. HR was 321 ± 12 , 313 ± 19 , and 303 ± 9 beats/min at the end of recovery, with no significant difference within or between groups during preconditioning or perfusion with $140 \mu\text{mol/L H}_2\text{O}_2$.

Troponin T

Release of troponin T tended to decrease at the end of H_2O_2 -perfusion, and to increase during recovery (Fig. 1). In controls the increase was not significant compared to either time 0 or to the end of H_2O_2 -perfusion. In ischemic preconditioned hearts TnT at the end of recovery was not different from time 0, but increased compared to release at the end of H_2O_2 -perfusion ($p < 0.03$). Likewise, TnT release after pretreatment with H_2O_2 increased compared to the end of H_2O_2 -perfusion ($p < 0.004$), but not compared to time 0 (Fig. 1). There was no difference between groups at any time point.

Tissue contents of antioxidants

Reduced glutathione (GSH). The baseline contents of GSH in cardiac tissue before preconditioning was 6.5 ± 0.3 mg/g wet weight, and decreased to 4.0 ± 0.3 mg/g wet weight in controls at the end of H_2O_2 -perfusion ($p < 0.004$) (Fig. 2). Preconditioning with H_2O_2 decreased GSH compared to baseline value at the end of H_2O_2 -infusion, (3.4 ± 0.5 mg/g wet weight, $p < 0.002$), and compared to H_2O_2 controls ($p < 0.02$). In hearts preconditioned with ischemia GSH decreased compared to baseline value (5.1 ± 0.2 mg/g wet weight, $p < 0.008$), but GSH was increased at the end of H_2O_2 -perfusion compared to H_2O_2 controls ($p < 0.02$) (Fig. 2).

Glutathione peroxidase (GSH-Px). Baseline GSH-Px was 112 ± 8 U/g protein, and did not change significantly in any group (Fig. 2).

Total antioxidant status (TAS). Baseline TAS in cardiac tissue was 3.5 ± 0.3 $\mu\text{mol/g}$ wet weight. TAS was not significantly reduced by H_2O_2 -perfusion, and was not influenced by preconditioning with H_2O_2 or ischemia (Fig. 2).

Catalase (CAT). Baseline CAT was 55.9 ± 6.3 U/g protein, and did not change significantly within or between groups (Fig. 2).

Superoxide dismutase (SOD). Baseline SOD was 2.5 ± 0.2 U/mg protein. $140 \mu\text{mol/L H}_2\text{O}_2$ increased SOD to 3.1 ± 0.2 U/mg protein ($p < 0.04$). The increase was not modified by H_2O_2 -preconditioning, but ischemic preconditioning abolished the H_2O_2 -induced increase of SOD ($p < 0.04$) (Fig. 3).

Antioxidant capacity. Antioxidant capacity was not influenced by H_2O_2 -perfusion or preconditioning (Fig. 3).

Thiobarbituric acid reactive substances (TBARS)

Baseline TBARS were 34 ± 2 nmol/g wet weight, and did not change after H_2O_2 -perfusion or preconditioning (Fig. 3).

DISCUSSION

The main findings of the present study were that cardiac dysfunction induced by H_2O_2 was only minimally influenced by ischemic preconditioning, while preconditioning with a low dose of H_2O_2 prior to the higher dose of H_2O_2 attenuated the increase of LVEDP, the decrease of LVDP, and increased CF during recovery. We have previously shown that the ischemic preconditioning model employed in the present study protects isolated rat hearts against global ischemia.¹⁹ There was no difference between groups in the cardiac release of troponin T during recovery, indicating that reduction of myocardial necrosis was not an important mechanism for the functional protection afforded. One previous study has investigated the effect of ischemic preconditioning prior to hydrogen peroxide. In accordance with the present findings, Gan *et al.* found that ischemic preconditioning prior to 30 min 200 $\mu\text{mol/L}$ H_2O_2 had only marginally beneficial effects on the function of isolated rat hearts.²³

Ischemic preconditioning protects against myocardial stunning.² Many studies have shown that preconditioning can be achieved by simulating other stimuli than oxidative stress prior to ischemia-reperfusion. Thus pretreatment with bradykinin,³¹ acidosis,³² acetylcholine,³³ hypoxia,³⁴ interleukin- 1α ,³⁵ or α_1 adrenoagonists³⁶ have all been protective against ischemia-reperfusion injury. All these stimuli are known to be mediators or phenomena of the subsequent ischemia-reperfusion injury, while oxidative stress is believed to be related to the reperfusion component of this injury.²⁴ Consequently, it could be speculated whether the adaptive events responsible for protection against myocardial stunning occur during the ischemic episode, rather than are related to oxidative stress induced by reperfusion. Furthermore, it may be speculated that ischemic preconditioning per se is not an universal mode of myocardial protection, but that the protective effect of preconditioning is dependent on a low dose of a participant of the subsequent cardiac injury.

Moderate oxidative stress as a possible cardioprotective intervention is not well studied. We have previously found in the isolated rat heart that 20 $\mu\text{mol/L}$ H_2O_2 , as employed in the present study, only protected against severe reperfusion arrhythmias, but not against dysfunction after global ischemia-reperfusion injury.^{18, 19} Ischemic preconditioning was protective in the same model.¹⁹ Another ROS-generating system (xanthine oxidase/purine) reduced infarct size and improved cardiac function after regional ischemia in rabbits.¹⁷ The infarct-limiting effect was dependent on protein kinase C.¹⁷ It is possible that the superoxide anion might be crucial for the cardioprotective effects of ischemic preconditioning, and that this anion was not produced in our systems.^{18, 19}

The exact mechanisms of cardiac dysfunction induced by high doses of H_2O_2 are not fully clarified. H_2O_2 itself may be the main injurious agent, since catalase inhibits its functional as well as biochemical changes.³⁷⁻³⁹ The hydroxyl radical may have been generated, as hydroxyl radical scavengers partially attenuate the cardiac dysfunction induced by H_2O_2 .^{37, 38} In the present study

140 $\mu\text{mol/L}$ H_2O_2 did not induce lipid peroxidation. We have previously found that perfusing rat hearts with 180 $\mu\text{mol/L}$ H_2O_2 for 10 min increased TBARS at the end of intervention.⁴⁰ The lipid peroxidation product malondialdehyde increased in isolated rat hearts perfused with 600 $\mu\text{mol/L}$ H_2O_2 ,⁴¹ and released into the coronary effluent of hearts perfused with either 200 or 300 $\mu\text{mol/L}$, but not 100 $\mu\text{mol/L}$ H_2O_2 .⁴² In rat hearts perfused with xanthine oxidase/hypoxanthine, tissue contents of malondialdehyde was increased at the end of a 10 min intervention, as were contents of H_2O_2 .⁴³ The concentration of H_2O_2 employed in the present study was evidently not high enough to induce a significant lipid peroxidation as evaluated by TBARS and indirectly by antioxidative markers. Consequently, lipid peroxidation may not explain the functional impairment after ROS-induced injury in the present model.

Total antioxidant status and capacity were unchanged, as were myocardial contents of catalase and glutathione peroxidase. H_2O_2 increased tissue SOD, and this increase was abolished by ischemic preconditioning. In a previous study, perfusing rat hearts with hypoxanthine/xanthine oxidase reduced tissue contents of SOD at the end of intervention.⁴³ This finding may be due to augmented production of the superoxide anion by hypoxanthine/xanthine oxidase, and subsequent depletion of cardiac SOD to protect against superoxide-induced injury.

Tissue glutathione was decreased by H_2O_2 , suggesting that in the present model, thiol-groups were more easily exposed to H_2O_2 -induced injury than myocardial lipids. The reduction was augmented by pretreatment with H_2O_2 , and was attenuated by ischemic preconditioning. Perfusing rat hearts with H_2O_2 has previously been found to increase release of glutathione into the coronary effluent,⁴⁴ which may explain the reduced tissue contents found in the present study. Depletion of endogenous glutathione seemed not to be critical for cardiac function in our study, as the most depleted hearts had the best function. Accordingly, Steare and Yellon found that increased endogenous glutathione levels prior to perfusion with H_2O_2 in isolated rat hearts did not protect against H_2O_2 -induced functional injury or release of lactate dehydrogenase.⁴⁵ However, exogenous glutathione attenuated hypoxia-induced stunning in another isolated rat heart model.⁴⁶ Loss of glutathione has been found in conjunction with ischemic preconditioning.⁸ N-acetylcysteine blocked both the loss of glutathione and the beneficial effects of preconditioning on cardiac function.⁸ It may be speculated that changes in glutathione levels are adaptive in a certain range, but impaired myocardial function may be evident when glutathione decreases below a critical concentration. On the other hand, the availability of glutathione in cellular compartments might be of importance. The depletion of glutathione could reflect improved protection of protein SH-groups at the expense of glutathione oxidation, and, consequently, could explain the improved contractile function in the HPC group.

We can only speculate on the possible mechanisms of attenuating H₂O₂-induced injury by preconditioning, as the beneficial functional effects were not due to reduction of necrosis, inhibition of lipid peroxidation, or increase of antioxidant levels. We have previously found that perfusing isolated rat hearts with low concentrations of H₂O₂ induces a vasodilation which is partly dependent on nitric oxide, and inhibition of nitric oxide aggravated functional effects of H₂O₂.⁴⁷ Thus, release of nitric oxide in hearts pretreated with H₂O₂ may have contributed to the protective effects in the present study. However, before inducing injury with 140 µmol/L H₂O₂, CF was lowest in hearts preconditioned with H₂O₂. A possible role of endogenous adenosine in ischemic preconditioning prior to injury induced by exogenous H₂O₂ has been suggested by Gan *et al.*,²³ but exogenous adenosine did not significantly reduce dysfunction caused by H₂O₂.⁴⁸ We have previously found that vasoactive eicosanoids play a role in H₂O₂-induced cardiac injury.⁴⁹ As prostacyclin has been suggested as a mediator of ischemic preconditioning,²⁰ it may possibly have contributed to protection against H₂O₂-induced dysfunction.

In summary, ischemic preconditioning offered only marginal protection against dysfunction induced by H₂O₂ in isolated rat hearts, while pretreatment with a low dose of H₂O₂ attenuated cardiac dysfunction. The mechanism of protection was not through reduction of necrosis, limitation of lipid peroxidation, or influence on cardiac antioxidant activity. It appears that it is not ischemic preconditioning *per se*, but rather repetition of a stimuli participating in both preconditioning and injury, which is crucial for the preconditioning response and protection of the heart.

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ABBREVIATIONS

CAT = catalase
CF = coronary flow
GSH = glutathione
GSH-Px = glutathione peroxidase
H₂O₂ = hydrogen peroxide
HPC =preconditioning with 20 µmol/L H₂O₂

HR = heart rate
IPC = ischemic preconditioning
LVEDP = left ventricular end-diastolic pressure
LVDP = left ventricular developed pressure
LVSP = left ventricular systolic pressure
ROS = reactive oxygen species
SOD = superoxide dismutase
TAS = total antioxidant status
TBARS = thiobarbituric acid reactive substances
TnT = troponin T

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FIGURE LEGENDS

FIG. 1. Left ventricular developed (LVDP) and end-diastolic (LVEDP) pressures, coronary flow (CF), and release of troponin T (TnT) into the coronary effluent of Langendorff-perfused rat hearts subjected to control perfusion (CTRL, n=10), pretreated with 20 $\mu\text{mol/L}$ H_2O_2 for 10 min followed by 10 min recovery (HPC, n=10), or ischemic preconditioning with 2 episodes of 2 min global ischemia and 5 min reperfusion (IPC, n=10) before 10 min perfusion with 140 $\mu\text{mol/L}$ H_2O_2 and 30 min recovery (10–40 min). Values are mean \pm SEM. * denotes $p<0.05$ compared to CTRL, ** denotes $p<0.05$ compared to value at end of H_2O_2 -perfusion (TnT), § denotes $p<0.05$ between HPC and IPC. S=stabilization (see methods), 0=start of the experiments, BI=immediately before intervention with 140 $\mu\text{mol/L}$ H_2O_2 .

FIG. 2. Tissue contents of glutathione (GSH), glutathione peroxidase (GSH-Px), total antioxidant status (TAS) and catalase (CAT) in Langendorff-perfused rat hearts before intervention (0, n=10), after 20 min control perfusion and 10 min perfusion with 140 $\mu\text{mol/L}$ H_2O_2 (CTRL, n=10), after pretreatment with 20 $\mu\text{mol/L}$ H_2O_2 for 10 min followed by 10 min recovery and 10 min perfusion with 140 $\mu\text{mol/L}$ H_2O_2 (HPC, n=10), or ischemic preconditioning with 2 episodes of 2 min global ischemia and 5 min reperfusion and 10 min perfusion with 140 $\mu\text{mol/L}$ H_2O_2 (IPC, n=10). Values are mean \pm SEM. * denotes $p<0.05$ compared to 0, ** denotes $p<0.05$ compared to CTRL.

FIG. 3. Tissue contents of superoxide dismutase (SOD), antioxidant capacity as measured by in vitro inhibition of linoleic acid peroxidation, and thiobarbituric acid reactive substances (TBARS) in Langendorff-perfused rat hearts before intervention (0, n=10), after 20 min control perfusion and 10 min perfusion with 140 $\mu\text{mol/L}$ H_2O_2 (CTRL, n=10), after pretreatment with 20 $\mu\text{mol/L}$ H_2O_2 for 10 min followed by 10 min recovery and 10 min perfusion with 140 $\mu\text{mol/L}$ H_2O_2 (HPC, n=10), or ischemic preconditioning with 2 episodes of 2 min global ischemia and 5 min reperfusion and 10 min perfusion with 140 $\mu\text{mol/L}$ H_2O_2 (IPC, n=10). Values are mean \pm SEM. * denotes $p<0.05$ compared to 0, ** denotes $p<0.05$ compared to CTRL.

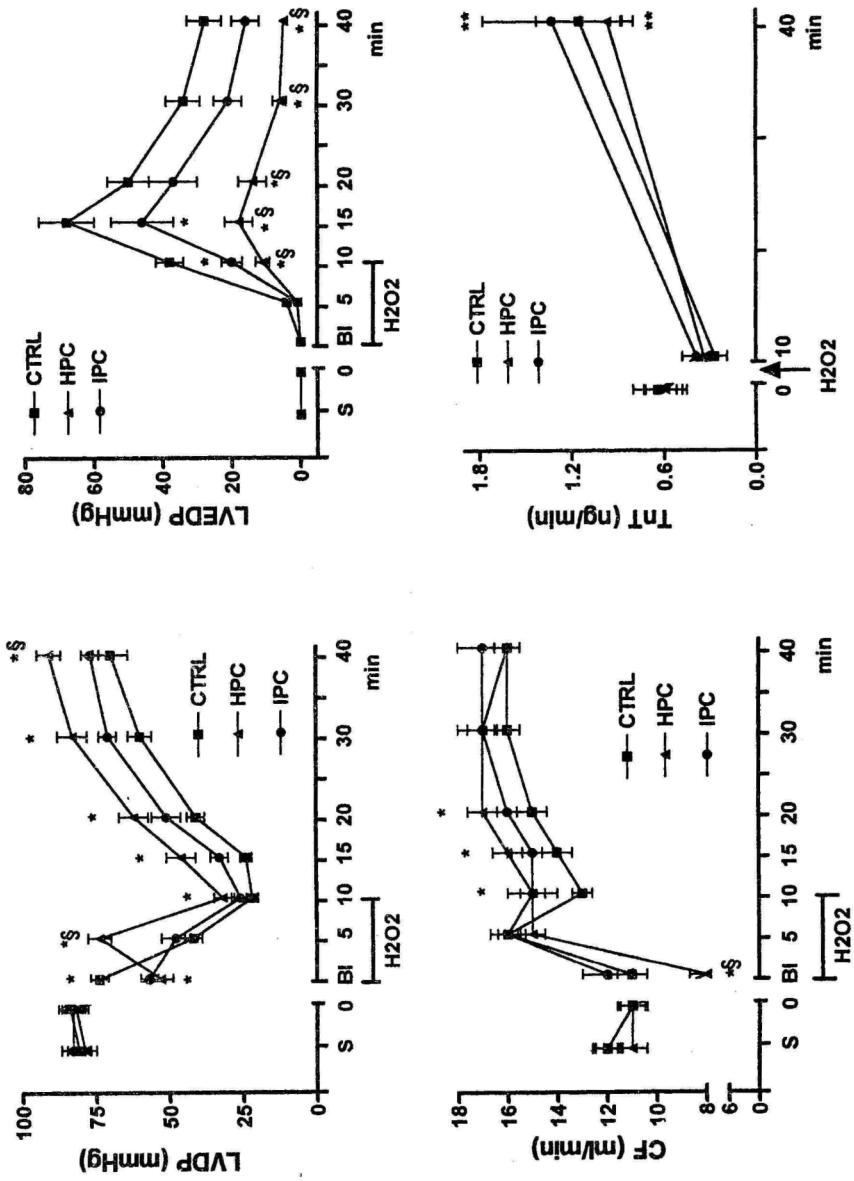


FIG. 1.

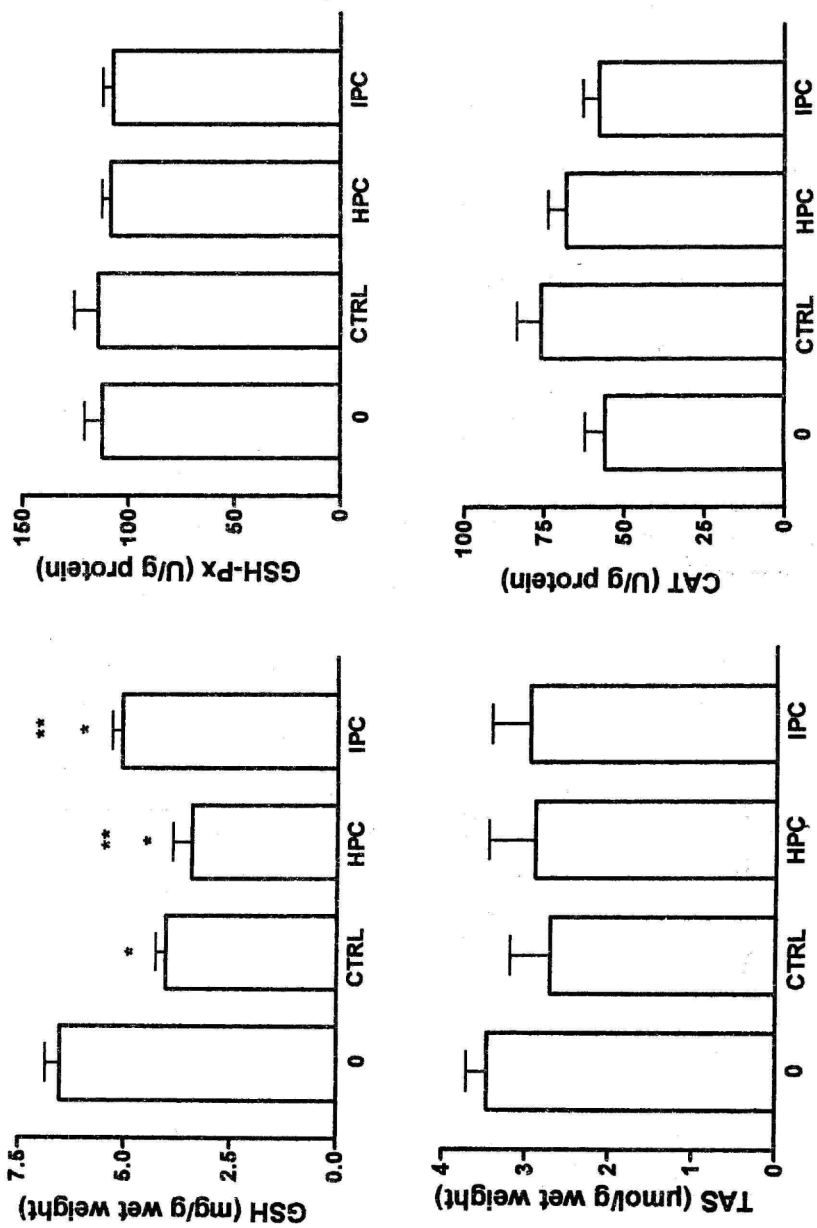


FIG. 2.

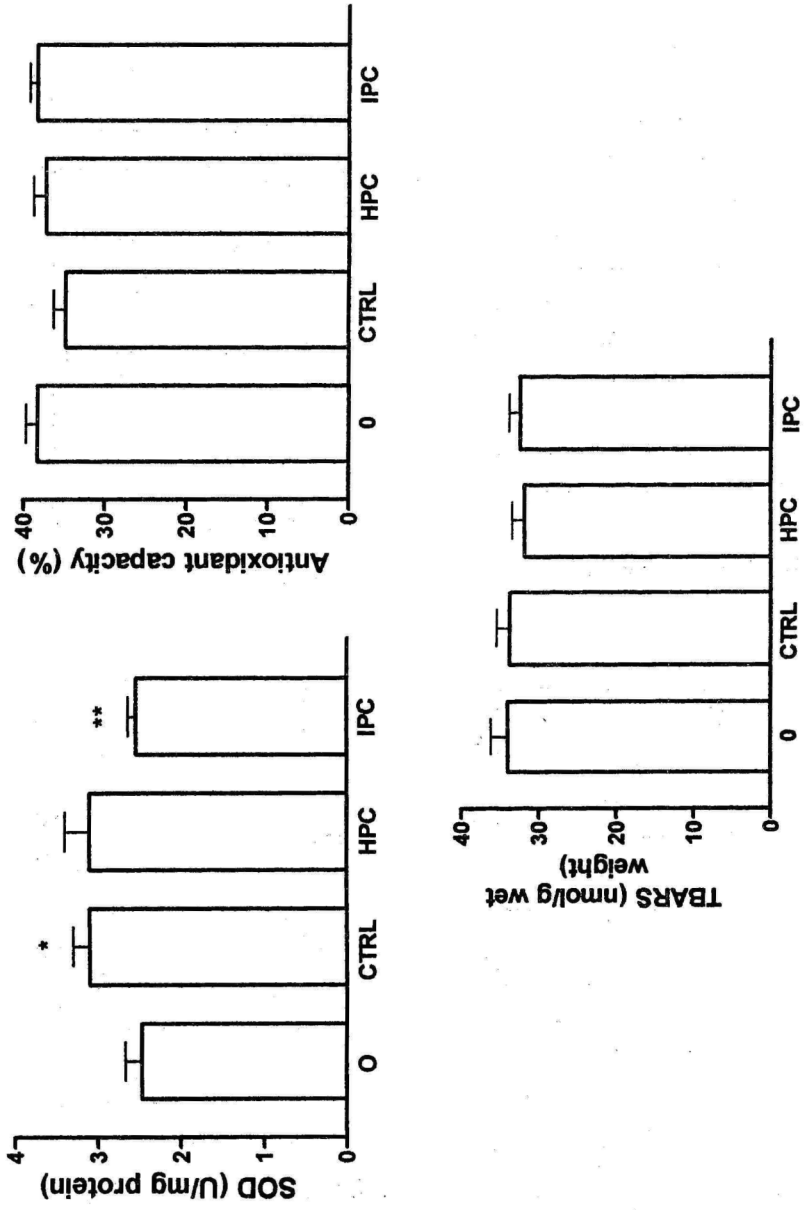


FIG. 3.

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- 1972–1983 Tallinn, Secondary School No. 43
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1991–1992 internship in anaesthesiology
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Special courses

- 1994 6 months in the Department of Medical Physiology, Institute of Medical Biology, University of Tromsø, Norway (scholarship from the Research Council of Norway)

Professional employment

- 1993– anesthesiologist, Department of Cardiac Surgery, Tartu University Hospital
1996– assistant, Department of Biochemistry, University of Tartu, and Department of Anaesthesiology and Intensive Care, Tartu University Hospital

Scientific work

Research field: ischaemia-reperfusion injury of the heart, with focus on the role of oxidative stress and strategies for myocardial protection.

Total: 21 scientific publications, 6 presentations in international scientific conferences.

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| 1993– | Tartu Ülikooli Maarjamõisa Haigla kardiokirurgiaosakond, anestesioloog |
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Teadustegevus

Põhiliseks uurimisvaldkonnaks südamelihase isheemia-reperfusiooni kahjustus, oksüdatiivne stress, kardioproteksiooni võimalused. Ilmunud 21 publikatsiooni, koos ettekannet rahvusvahelistel konverentsidel.

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