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ADAPTATION TO ISCHEMIA WITH SPECIAL EMPHASIS ON NITRIC OXIDE

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CONTENTS

Abstract

Nitric oxide (NO) may play an essential role for maintenance of cardiac function and perfusion. Endothelial dysfunction of atherosclerotic vessels may aggravate cardiac ischemia/reperfusion injury. Adaptation to ischemia may be induced by short episodes of ischemia and reperfusion before sustained ischemia, which may be performed on the heart itself or another organ to protect the heart. NO has been suggested to be a signal molecule initiating this adaptation, while inducible nitric oxide synthase (iNOS) has been suggested as a mediator of delayed preconditioning of the heart. The protection afforded by preconditioning may potentially be associated with changes of vessel tone. Most investigations of the role of cardiac nitric oxide and ischemic preconditioning in ischemia/reperfusion injury has been conducted in animals with normal vessels. With the development of recombinant DNA technology, animal models of atherosclerosis have emerged. One of these models is the apolipoprotein E and LDL receptor double knockout (ApoE/LDLr KO) mouse, which develops atherosclerosis with a distribution pattern of lesions similar to humans.

In the present studies, we demonstrate endothelial dysfunction in ApoE/LDLr KO mice and that the tolerance of their hearts to ischemia was decreased (Langendorff model). A NO donor attenuated the decreased ischemic tolerance of ApoE/LDLr KO mice hearts, while the same concentration of the donor was detrimental in normal hearts, probably due to NO-overproduction and peroxynitrate formation. We also demonstrated several kinds of adaptation to ischemia by preconditioning. Classic ischemic preconditioning could protect not only normal mice hearts but also atherosclerotic mice hearts. Exposure to hyperoxia exerted a similar protection as ischemic preconditioning in atherosclerotic mice. Spontaneous heart infarctions in vivo adapted the heart of atherosclerotic mice to induced ischemia ex vivo analogous to delayed ischemic preconditioning. Spontaneous ischemic events in the brain also protected the hearts of atherosclerotic mice, and this effect could be mimicked by inducing brain ischemia by bilateral internal carotid artery ligation in normal mice. Spontaneous and induced brain ischemia attenuated vessel contractility in vitro, increased vessel dilatation, and upregulated iNOS in the vessel wall as well as in the heart. Pharmacological and genetical inhibition of iNOS abolished the protective effects and changed vessel reactivity. These findings suggest that delayed ischemic preconditioning effects evoked by brain ischemia may mediated by iNOS.

The present study indicates that patients with coronary atherosclerosis may benefit from treatment with NO-enhancing agents or preconditioning techniques. The organ protection is generated by ischemia rather than by ischemia and reperfusion and may mediated by iNOS.

LIST OF ORIGINAL ARTICLES

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals:

I. Wang QD, **Tokuno S**, Valen G, Sjöquist PO, Thorén P
Cyclic fluctuations in cardiac performance of the isolated Langendorff-Perfused mouse heart: The effect of pyruvate
Manuscript

II. **Tokuno S**, Thorén P, Löwbeer C, Valen G
The role of nitric oxide in ischemia/reperfusion injury of isolated hearts from severely atherosclerotic mice
Life Science 2001; in press

III. Jiang J, Valen G, **Tokuno S**, Thorén P, Pernow J
Endothelial dysfunction in atherosclerotic mice: improved relaxation by combined supplementation with L-arginine-tetrahydrobiopterin and enhanced vasoconstriction by endothelin
British Journal of Pharmacology 2000; 131: 1255-1261

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

V. **Tokuno S**, Hinokiyama K, Tokuno K, Löwbeer C, Hansson LO, Valen G
Spontaneous ischemic events in the heart and brain adapts the hearts of severely atherosclerotic mice to ischemia
Manuscript

VI. **Tokuno S**, Chen F, Jiang J, Pernow J, Valen G
Effects of spontaneous or induced brain ischemia on vessel reactivity
Manuscript

ABBREVIATIONS

NO	nitric oxide
EDRF	endothelium-derived relaxing factor
NOS	nitric oxide synthase
eNOS	endothelial nitric oxide synthase
nNOS	neuronal nitric oxide synthase
iNOS	inducible nitric oxide synthase
NFkB	nuclear factor kappa-B
ApoE/LDLr KO	apolipoprotein E and low density lipoprotein receptor double knockout
iNOS KO	inducible nitric oxide synthase knockout
HR	heart rate
CF	coronary flow
LVSP	left ventricular systolic pressure
LVEDP	left ventricular end-diastolic pressure
LVDP	left ventricular developed pressure
max dP/dt	maximum first derivative of pressure
negative dP/dt	negative first derivative of pressure
cTnT	cardiac troponin T
TTC	triphenyl tetrazolium chloride
L-NAME	NG-nitro-L-arginine methyl ester
SNAP	S-nitrose-N-acetylpenicillamine
AG	aminoguanidine
ACh	Acetylcholine
BH_4	tetrahydrobiopterin
SNP	sodium nitroprussid
ET-1	endothelin-1
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$

INTRODUCTION

Atherosclerosis and ischemic heart disease

Arteriosclerosis, a generic term for thickening and hardening of the arterial wall, is responsible for the majority of deaths in Europe and most westernized societies [Braunwald, 1997]. One type, a patchy nodular type, of arteriosclerosis is atherosclerosis, the disorder of the larger arteries that underlies most coronary artery disease, aortic aneurysm, arterial disease of the lower extremities and also plays a major role in cerebrovascular disease. Ischemic heart disease, which includes angina pectoris and myocardial infarction, is one of the commonest diagnosis occurring in hospitalized patients in western countries. Atherosclerosis, especially ischemic heart disease, is by far the leading cause of death in Europe [Braunwald, 1997].

Atherosclerosis itself may cause coronary stenosis, while rupture of an atherosclerotic plaque may trigger the formation of a thrombotic occlusion [Davies and Thomas, 1985]. The stenosis or occlusion of a coronary artery may lead to loss of blood flow in the heart muscle supplied by the vessel, cardiac ischemia. Thus, severe tissue injury with subsequent necrosis of myocytes finally occurr. Treatment of ischemic heart disease primarily focuses on reperfusion of the unperfused area by pharmacological or mechanical means, such as thrombolytic agents or coronary artery bypass grafting. Paradoxically, although reperfusion is essential for cell survival, reperfusion also accelerates ischemic injury, so called reperfusion injury [Braunwald and Kloner, 1985]. Reperfusion injury may be manifest as lethal, irreversible injury, as reversible injury with impairment of contraction and relaxation (“stunning”), or as reperfusion

arrhythmias [Vaage and Valen, 1993, Valen and Vaage, 1993]. Ischemia/ reperfusion injury is a form of inflammatory reaction with free radical generation, production of proinflammatory mediators such as cytokines, activation and transmigration of leukocytes, and release of various vasoactive substances [Vaage and Valen, 1993, Valen and Vaage, 1993]. In analogy to the immune response against infectious disease, components of the inflammatory response may induce adaptation to a subsequent ischemia/reperfusion injury. To understand the mechanisms underlying the adaptation would open a door to a plethora of new therapeutic methods against inflammatory disease.

Animal models of atherosclerosis

Investigations of the development and consequence of atherosclerosis have previously been conducted in cholesterol-fed rabbits, monkeys, and rats. However, limitatons of these models are that the animals do not develop advanced fibrofatty lesions as humans do. With the development of recombinant DNA technology, new animal models of atherosclerosis have emerged. One of these models is the apolipoprotein E and low density lipoprotein receptor double knockout (ApoE/LDLr KO) mouse, which develops atherosclerosis with a content and distribution pattern of lesions similar to humans [Brestow et al., 1996, Tangirala et al., 1996, Hofker et ak., 1998]. An atherogenic diet is often employed to speed up the process. When ApoE/LDLr KO mice were fed an atherogenic diet for 7-9 months to accelerate development of atherosclerosis, advanced fibrofatty lesions as well as spontaneous myocardial infarction was observed [see results]. Thus, the ApoE/LDLr KO mouse is suitable as a model to represent the severe atherosclerosis of patients who may develop ischemic heart disease.

General aspects of nitric oxide

Nitric oxide (NO) was discovered as endothelium-derived relaxing factor (EDRF)

by Furchgott [1988], Ignarro [1988], and Murad [1987]. They demonstrated independently of each other that EDRF itself or its active component is NO.

NO has a variety of biologic actions. In the vascular smooth muscle cell, NO activates soluble guanylate cyclase, which elevates the level of cyclic guanosine monophosphate (cGMP) and results in relaxation of the smooth muscle cell and dilatation of the vessel [Ignarro et al., 1988] (Fig.1). The elevation of cGMP also causes inhibition of platelate adherence and aggregation [Yan et al., 1996], and has positive inotropic effects in cardiomyocytes [Mohan et al., 1996]. When NO production is high, NO may have c-GMP-independent actions and may react as a free radical molecule [see below].

NO is formed by the enzyme NO synthase (NOS) which catalyzes the conversion of the amino acid L-arginine to NO and L-citrulline in the presence of calmodulin (CaM) and several cofactors, including nicotinamide adenine dinucleotide phosphate (NADP), tetrahydrobiopterin (BH_4), and flavin mononucleotide flavin adenine dinucleotide (FMN/FAD) [Palmer et al., 1988] (Fig.1). BH_4 has been proposed to exert allosteric actions to stabilize the active dimeric state of the enzyme and to play a redox-active role in stimulating NO synthase [Werener et al., 1998].

Three isoforms of NOS which are endothelial (eNOS), neuronal (nNOS) and inducible NOS (iNOS) have been discovered and cloned [Bredt et al., 1991, Lamas et al., 1992, Xie et al., 1992]. eNOS is the isoform responsible for regulation of vescular tone under physiological conditions. eNOS was previously thought of as a constitutive enzyme, but has lately been shown to be rapidly upregulated by different stimuli [Li et al., 2000]. eNOS appears beneficial for the heart during ischemia/ reperfusion injury, as mice with targeted deletions of the eNOS gene have increased susceptibility towards induced infarction [Summeray and Yellon, 1998]. nNOS, also constitutively expressed, was recently discovered to be present not only in neuronal cells, but also in cardiac sarcoplasmatic

reticulum [Xu et al., 1999]. nNOS, like eNOS, may play a beneficial role in cardiac ischemia-reperfusion injury, as mice deficient of the gene get larger infarctions and have reduced function after induced ischemia [Song et al. 2001].

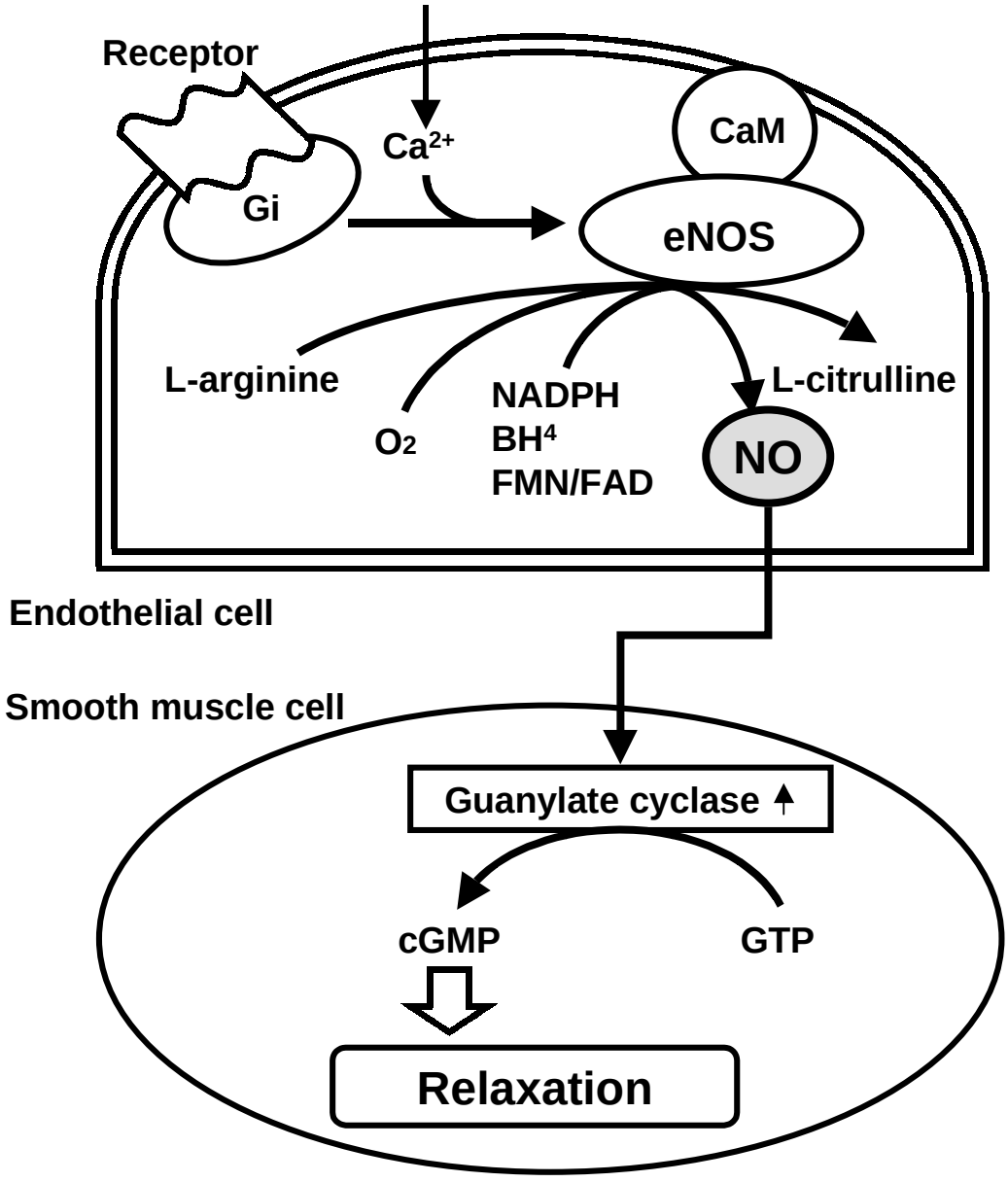

Fig. 1 *Schematic representation of the endothelium dependent vasorelaxation. The constitutive enzyme endotelial NO synthase (eNOS) is activated by Gi protein dependent and independent stimuli. eNOS forms NO and L-citrulline from L-arginine in the presence of calmodulin (CaM) and several cofactors, including nicotinamide adenine dinucleotide phosphate (NADP), tetrahydrobiopterin (BH_4), and flavin mononucleotide/ flavin adenine dinucleotide (FMN/FAD). NO activates soluble guanylate cyclase, which elevates the level of cyclic guanosine monophosphate (cGMP) and results in vasodilatation.*

Inducible nitric oxide synthase

iNOS, as opposed to nNOS and eNOS, is not constitutively expressed. Stimuli such as the proinflammatory cytokines tumor necrosis factor alpha, interferon alpha or gamma, hypoxia, and transactivation of the redox sensitive transcription factors nuclear factor kappa B (NFkB) or activator protein-1 (AP-1) will induce iNOS [Melillo et al 1996]. Induction of iNOS was first found in vascular smooth muscle cells [Geng et al. 1994], and has since then been detected in all cells investigated including macrophages, cardiomyocytes, and endothelial cells [Radomski et al., 1990, Stuehr et al., 1991, Schulz et al., 1992]. iNOS is a much more potent enzyme than eNOS, and it is currently speculated whether induction of iNOS may be detrimental due to overproduction of nitric oxide in some cases such as septic shock [Parrillo 1993]. The role of iNOS in the heart has not been well characterized, although one study investigating its' role for infarct development in knock out mice found that it iNOS was not important [Xi et al., 1999].

Interaction between NO and reactive oxygen metabolites

A key event during reperfusion is the reintroduction of molecular oxygen. Molecular oxygen possesses two electrons in parallel spin-momenta, and therefore easily donates (is oxidized) or accepts (is reduced) an electron to form free radicals [Valen et Vaage 1993]. Free radicals are atoms, ions, or molecules with one or more unpaired electron in their outer orbital, which makes them highly reactive. In situations when the endogenous antioxidant defense is depleted or the free radical production in excess such as during reperfusion, a burst of free radicals may cause chain reactions of lipid peroxidation of inner and outer cell membranes, denaturation of proteins and inactivation of enzymes, breakdown of carbohydrates, and DNA injury [Valen et Vaage 1993]. The main reactive oxygen species generated during reperfusion are the superoxide radical, hydrogen peroxide, and the hydroxyl radical [Zweier et al., 1987, Onodera and

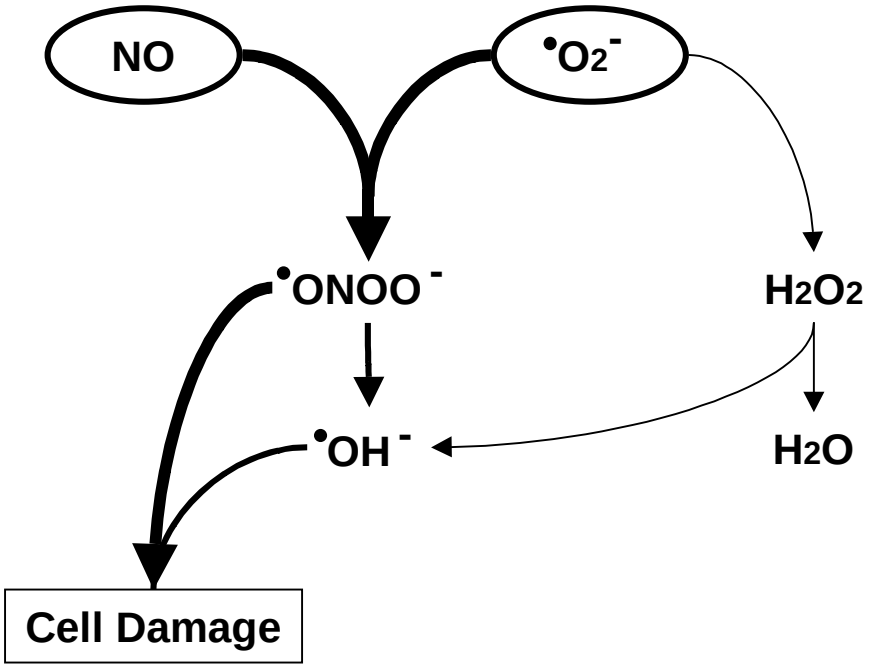

Fig. 2 *Schematic representation of the interaction between NO and reactive oxygen. The superoxide radical ($^{\bullet}O_2^-$) is catalysed to the hydroxyl radical ($^{\bullet}OH^-$). Nitric oxide (NO) reacts with $^{\bullet}O_2^-$ and forms peroxynitrites ($^{\bullet}ONOO^-$). Peroxinitrates will cause chain reactions of nitrosylation leading to tissue injury.*

Ashraf, 1991]. The NO molecule, itself a free radical, can react with the superoxide anion and form a chain reaction of nitrite peroxidation as shown in Figure 2 [Beckman et al., 1990, Maulik et al., 1995, Liu et al., 1997, Xie et al., 1998]. Peroxynitrite formation is associated with depressed cardiac muscle contractility [Matheis et al., 1992, Naseem et al., 1995, Yasmin et al., 1997, Xie et al., 1998], reperfusion arrhythmias [Naseem et al., 1995, Liu et al., 1997], and inhibited mitochondrial respiration [Xie et al., 1998].

Endothelial dysfunction in ischemia/reperfusion injury

The role of NO during reperfusion is controversial. Evidence suggests that endothelium-dependent relaxation is impaired during experimental ischemia, which causes reduction of both basal and stimulated NO release [Tsao et al., 1990ab, Dignan et al.,1992, Giraldez et al., 1997]. This impairment is more

severe during reperfusion than during ischemia [Vinten-Johansen et al., 1999]. While many reports suggest that eNOS activity and NO release is decreased during and after ischemia/reperfusion [Tsao et al., 1990ab, Dignan et al.,1992, Giraldez et al., 1997], Depre et al. [1997] reported that cytosolic NOS activity in the heart is rapidly stimulated by ischemia. It was also reported that eNOS activity decreased during ischemia, but was rapidly reactivated upon start of reperfusion [Giraldez et al., 1997]. NO, measured directly in vitro after ischemia/reperfusion, is releaseed but reduced compared with hormonal conditions [Engelman et al., 1995, Guo et al., 1996].

It has been reported that inhibition of NOS by pharmacological agents improves myocardial function following ischemia/reperfusion [Naseem et al., 1995, Yasmin et al., 1997]. It has also been shown that NO donors or a precurser of NO, L-arginine, had protective effects against ischemia/ reperfusion injury [Siegfried et al., 1992, Johnson et al., 1991, Yasmin et al., 1997, Izhar et al., 1998] and that NOS inhibitors impaired cardiac function after ischemia/ reperfusion injury [du Toit et al., 1998]. Myocardial ischemia/reperfusion injury is exacerbated in endothelial NOS knockout mice in vivo [Jones et al., 1999a] and ex vivo [Flogel et al., 1999]. Possibly the controversial findings may depend on model, and amount of NO inhibited or produced.

Endothelial dysfunction and atherosclerosis

During atherogenesis, morphological, physiological or biochemical changes in blood vessels, such as increase in thickness and stiffness of the vessel wall [Imai et al., 1966, Parker and Odland, 1966, Cox and Detweiler, 1979], decrease of selective permeability of the endothelial layer to lipoproteins [Shimamoto, 1972] or increase of arterial permeability to albumin [Chobanian et al., 1983] have been observed.

Endothelial dysfunction of atherosclerotic vessels coincides with decreased release of nitric oxide in human clinical research [Zeiher et al., 1991, Oemar et al.,

1998] and in cholesterol-fed rabbits [Kolodgie et al., 1990, Galle et al., 1991, Boger et al., 1998]. Kolodgie et al. [1990] suggested that endothelium-dependent relaxation of vascular smooth muscle cells is reduced with the progression of athrosclerosis primarily due to a loss of endothelial cells. However, Galle et al. [1991] and Boger et al. [1998] suggested that hypercholesterolemia and atherosclerosis alter vascular reactivity by different mechanisms: via oxidative stress due to oxidized low-density lipoproteins and via reduction of NO release due to loss of endothelial cells.

This atherosclerosis-associated endothelial dysfunction, especially reduction of NO release, may itself potentially influence ischemia/reperfusion injury of the heart with atherosclerotic vessels. In humans ischemia/reperfusion injury normally occurs in hearts of patients with severe coronary atherosclerosis, most of them with a long-standing hypercholesterolemia. However, most experimental studies of the role of cardiac nitric oxide in ischemia/reperfusion injury have been conducted in animals with normal vessels and normal cholesterol levels. Thus, in light of the controversial results discussed above, it is really crucial to understand the differences of the role of NO in ischemia/reperfusion injury of hearts with normal vessels and those with severely atherosclerotic vessels.

Ischemic preconditioning

In 1986, Murry and colleagues discovered that the canine heart could be adapted to ischemia by preceeding periods of brief ischemia [Murry et al., 1986]. This phenomenon has been termed ischemic preconditioning and shown to occur in several animal models such as pigs [Schott et al., 1990], rabbits [Cohen et al., 1991], rats [Li et al., 1992], humans [Yellon et al., 1993], and mice [Guo et al., 1998, Xi et al., 1998, Sumeray et al., 1998]. Ischemic preconditioning offers a profound protection against ischemia/reperfusion injury improving contractile function, increasing coronary flow, and reducing infarct size, and is potentially of great clinical interest for treatment of patients with unstable angina or imminent

myocardial infarction, or in open heart surgery with cardioplegic arrest. Adaptation can be achieved when preconditioning of the heart takes place less than two hours (classic preconditioning) [Murry et al., 1986] or 24-72 hours (delayed preconditioning) before sustained ischemia [Kuzuya et al., 1993, Marber et al., 1993, Guo et al., 1996].

The results of clinical investigations of ischemic preconditioning attempted during open heart surgery are controversial [Zeeuw et al., 1999]. Both beneficial effects on myocardial contractility and hemodynamics [Lu et al., 1997, Illes et al., 1998] and no improvement of hemodynamics or cardiac isoenzyme release has been found [Kaukoranta et al., 1997, Malkowski et al., 1998]. In theory the chronic inflammation of atherogenesis [Hansson, 1999] could itself modify the cardiac response to ischemia, so that the benefit of preconditioning is vastly reduced. No investigation has evaluated preconditioning in the severely atherosclerotic experimental animal, although one factor contributing to atherosclerosis, hypercholesterolemia, has been studied [Kremastinos et al., 2000]. The importance of coronary atherosclerosis per se rather than hypercholesterolemia for the preconditioning response has not been investigated.

Unstable angina may represent a clinical correlate to preconditioning. Several studies indicate that having unstable angina prior to acute myocardial infarction improves morbidity and mortality compared to patients with infarctions of sudden onset [Kloner et al., 1995, Noda et al., 1999], where unstable angina may cause short episodes of spontaneous ischemia due to intermittent hypoperfusion. Indeed, cardiac tissue of patients with unstable angina have increased amounts of cardioprotective proteins associated with the preconditioning response such as heat shock protein 72 and endothelial nitric oxide synthase, while NFkB, believed to be important for preconditioning signalling (see below), is activated [Valen et al. 2000]. One can speculate that in analogy to the immune system protecting against infectious agents, it would make sense for the atherosclerotic

body to protect itself against ischemia. However, no animal model has so far been forwarded to investigate if spontaneously occurring ischemic events can adapt the animal towards ischemia.

Remote ischemic preconditioning

Ischemic preconditioning has not only received wide attention in heart reserch, but has also been a topic of extensive studies involving protection of other organs such as brain [Kitagawa et al., 1990, Perez-Pinzon et al., 1997], kidney [Zager et al., 1984], skeletal muscle [Mounsey et al., 1992], and liver [Lloris-Carsi et al., 1993]. Przyklenk and colleagues showed that a brief coronary artery occlusion preconditioned the myocardium outside its own perfusion territory (“protection of virgin myocardium”) [1993]. From their findings, the hypothesis of remote preconditioning (that short episodes of ischemia and reperfusion in other organs protects the heart against ischemia/reperfusion injury) was forwarded. The possibility of interorgan protection was first addressed by McClanahan et al., who showed that in rabbits a brief renal artery occlusion followed by reperfusion reduced myocardial infarction size [1993]. After their preliminary data, several kinds of remote preconditioning models have been reported. Thus, short episodes of ischemia and reperfusion in limb [Loke et al., 1996, Oxman et al., 1997], skeletal muscle [Birnbaum et al., 1997], colon [Gho et al., 1996], and kidney [McClanahan et al., 1993, Takaoka et al., 1999] protects the heart, which can be either classic or delayed. The mechanisms underlying this endogenous protection are not fully understood and may differ between model, but must be determined to exploit the cardioprotective actions pharmacologically in patients. It is possible that also spontaneous ischemia of shorter or longer duration in any human organ may influence heart function and necrosis analogous to remote preconditioning, but this is not feasible to investigate.

It has been demonstrated that the brain itself can be preconditioned by cerebral ischemia both as immediate [Kitagawa et al., 1990] and delayed protection

[Perez-Pinzon et al., 1997]. In this respect the influence of cerebral ischemia on the heart may be of special interest, because cerebral infarction and myocardial infarction are main causes of death in atherosclerosis, and are frequent complications to each other. Additionally, donor hearts for transplantation are often obtained from patients with brain death. Evidence from clinical studies indicate that some of the transplanted hearts exhibit poor function, despite the fact that no immunological, surgical, or technical reason is apparent [Fragomeni & Kaye, 1988, Hauptman et al., 1994]. Some experimental studies indicate that brain death leads to severe impairment of myocardial contractility, irreversible myocardial damage and reduction of ischemic tolerance [Biswas et al., 1996. Herijgers et al,. 1998, Szabo et al., 1998]. In contrast to the influence of prolonged cerebral ischemia on myocardial function and structure, the effect of focal, moderate, or brief cerebral ischemia on the former has not been investigated.

Nitric oxide in delayed ischemic preconditioning

The mechanisms underlying ischemic preconditioning are complex and not completely understood, and so far only investigated in models preconditioning the organ to protect itself. Only the possible pathways relevant to the present work are shown in Figure 3 and discussed in this chapter. During ischemia and reperfusion substances such as NO and reactive oxygen metabolites are released. These and other trigger substances cause activation of protein kinase C, and downstream MAPkinases and/or tyrosine kinase [Ping et al., 1999]. One of the cellular effects of these kinases is activation of NFkB. During ischemic preconditioning of the heart NFkB is activated, and pharmacological inhibition of NFkB activation abolishes the preconditioning effect [Maulik et al., 1998, Xuan et al., 1999, Morgan et al., 1999]. One of the genes NFkB regulates is iNOS. Our laboratory has additionally developed a model of preconditioning by hyperoxia, where both immediate and delayed myocardial protection can be evoked in rats

by hyperoxic exposure. A systemic oxidative stress evident as increased serum lipid peroxidation products after 60 minutes of hyperoxia occurs [Tähepôld et al., 2001]. NFkB is activated in cardiac tissue, and pharmacological inhibition of its activation abolishes the functional effects [Tähepôld et al., 2001b]. It has

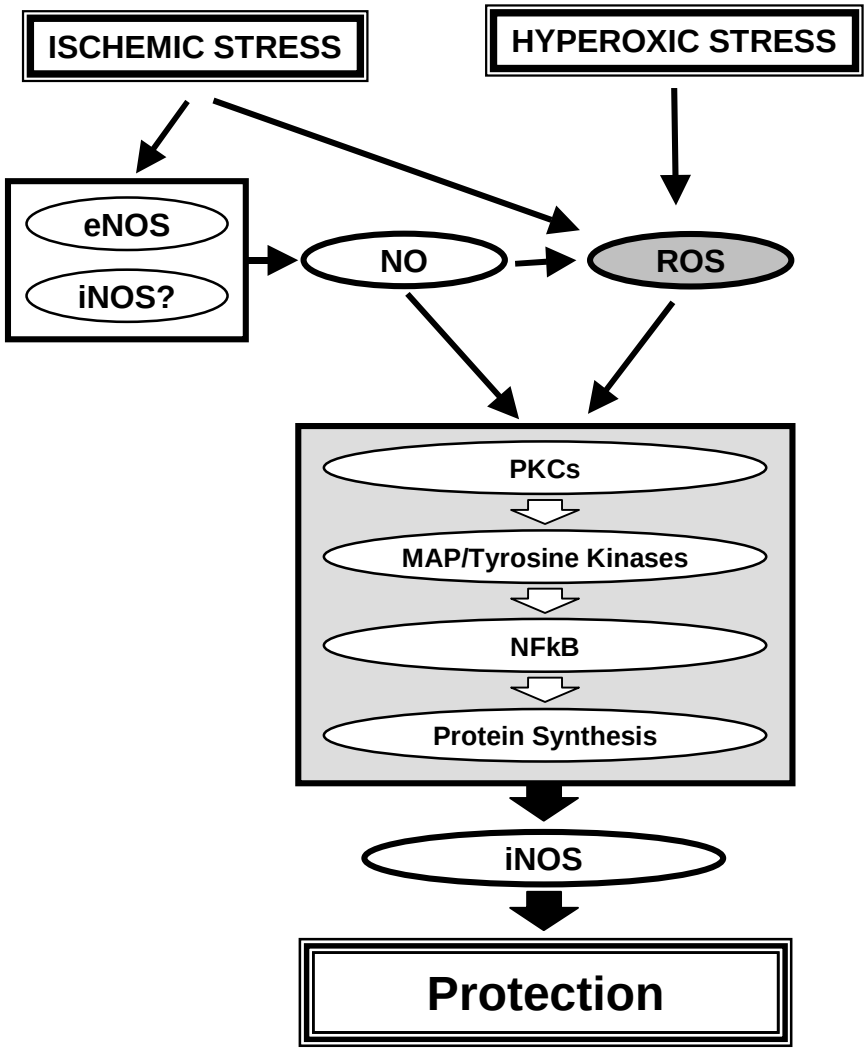

Fig. 3 *Schematic representation of the nitric oxide hypothesis in late preconditioning. A brief episode of ischemia/reperfusion causes increased production of nitric oxide (NO) and reactive oxygen spicies (ROS) which activate the signal transduction cascade involving protein kinase C (PKC), MAP and tyrosine kinases which transactivate nuclear factor kappa-B (NFkB). NFkB upregulates the iNOS which may mediate protective effects in late preconditioning.*

previously been shown by others that iNOS is upregulated after preconditioning of the heart [Guo et al., 1999, Jones et al., 1999b, Wildhirt et al,. 1999]. NO has been suggested to be a signal molecule initiating adaptation to ischemia [Qiu et al., 1997], while delayed preconditioning may be mediated by iNOS induced by the preconditioning episode [Takano et al., 1998] (Fig.3). Evidence for the latter is provided by studies pharmacologically blocking preconditioning with iNOS inhibitors [Takano et al., 1998, Wildhirt et al,. 1999, Imagawa et al., 1999], or by preconditioning mice with targeted disruption of the iNOS gene [Guo et al., 1999].

Pharmacological preconditioning by lipopolysaccharide or monophosphoryl lipid A not only protects the heart against ischemic injury [Xi et al., 1999, Zacharowski et al., 1999], but also has systemic vasodilatory effects [Weigert et al., 1995, Yen et al., 1995, Gunnett et al., 1998]. The beneficial effects of these agents appear to be mediated by iNOS, as the effects are abolished by pharmacological [Weigert et al., 1995, Yen et al., 1995, Xi et al., 1999] or genetical iNOS inhibition [Gunnett et al., 1998, Xi et al., 1999]. Cardiac iNOS may protect the heart through coronary vasodilatation [Loke et al., 1996], reduction of myocardial oxygen consumption, or by opening K_{ATP} channels [Gho et al., 1996, Takaoka et al., 1999]. How signalling from other organs to the heart takes place is currently unknown. Preconditioning of other organs to evoke myocardial protection indicates that a general as well as an organ specific protection is induced. The role of NO and iNOS in mediating this protection indicates that preconditioning may affect vascular tone; however, there are no publications on this.

AIMS

These studies were initiated to investigate the mechanism of adaptation to ischemia with special emphasis on nitric oxide with the following specific aims:

- To establish Langendorff-perfusion in mice.
- To investigate the role of the endothelium for cardiac performance and infarct development during ischemia and reperfusion in isolated hearts from mice with severe atherosclerosis.
- To study the role of the endothelium for vessel reactivity in aortic rings from mice with severe atherosclerosis.
- To investigate whether hearts of animals with severe atherosclerosis can be adapted to ischemia by preconditioning.
- To evaluate if spontaneous ischemic events occurred in vivo in severely atherosclerotic animals, and the possible effects of such events on cardiac performance and vessel reactivity.

METHODOLOGICAL CONSIDERATIONS

Animal models

In the present studies mice have been employed as experimental animals. The advantage with mice as a species is that its genes are so well characterized, and a large range of genetically engineered animals are easily available. The disadvantage of the mouse is its size, which challenges the technology and dexity of physiology research. The model of Langendorff-perfusion, induced brain ischemia, and vessel reactivity employed in the present work were, with some effort, successfully scaled down to employ in the mouse.

The advantage of genetically engineered animals is that they can be designed to overexpress or be deficient for a specific gene of interest, which at least in theory is superior to pharmacological approaches where unspecific drug effects is an enimy to clear-cut results. However, one must keep in mind that animals deficient for one substance may compensate by overexpression something else. In the present study mice with targeted deficiency for iNOS were one of the animals models employed. As iNOS is an inducible gene, the question of compensation might not be such a problem in this case.

The other mouse model employed were ApoE/LDLr knockout mice fed an atherogenic diet (21% fat, 0.15 % cholesterol) for 7-9 months to speed up the process of atherosclerosis, which were compared with wild-types 3-4 months old and fed on chow. An age- and diet matching of the animals would have been preferable, but this was not performed due to limitations in animal housing space

as well as high housing cost.

Perfusion of the isolated mouse heart

To evaluate cardiac performance, we employed the retrograde isolated perfusion model (modified Langendorff model). Hearts were isolated from anesthetized mice and perfused retrogradely with Krebs-Henseleit (K-H) buffer at a constant pressure of 55 mmHg. This is lower than the physiological blood pressure in mice which is approximately 100 mmHg [Doevendans et al., 1998]. However this pressure was chosen to have reproducible results as discussed in the results chapter. The K-H buffer is designed to mimic blood in contents of nutrients and

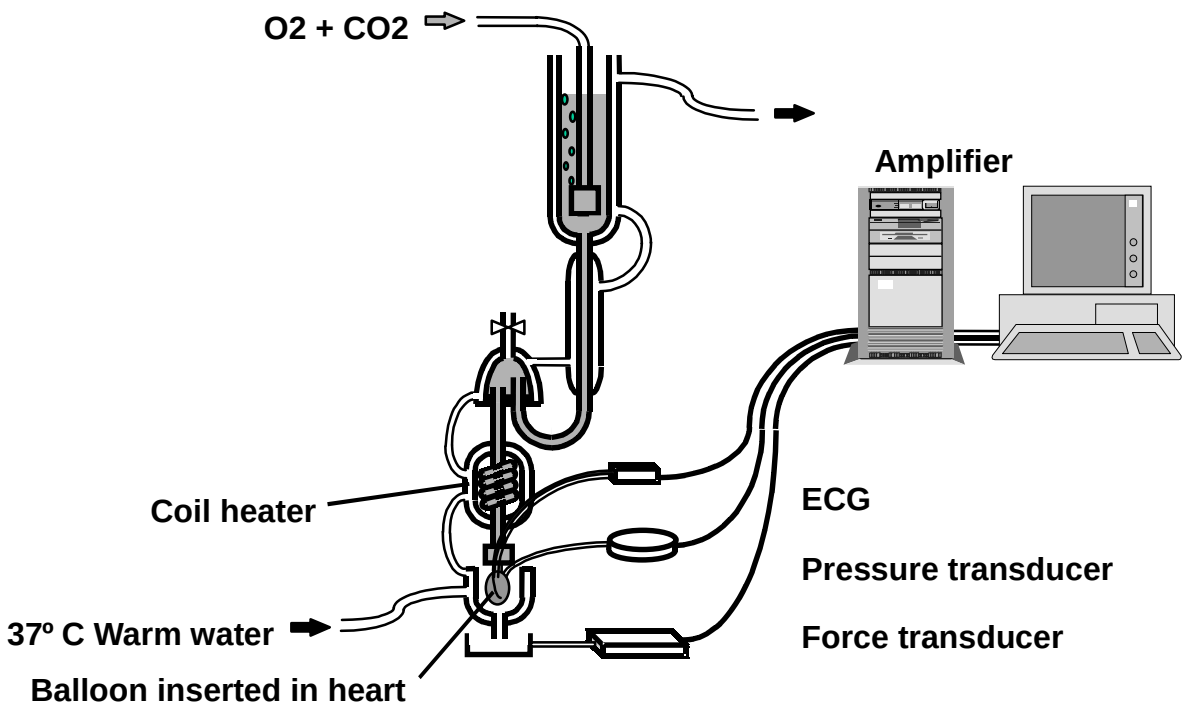

Fig. 4 *Computerized Langendorff system for mice. The cannulated hearts were retrogradely perfused with gassed (5% CO_2, 95% O_2) Krebs-Henseleit buffer at a constant pressure of 55 mmHg, with a core temperature of the heart maintained at 37°C. Left ventricular pressures and coronary flow was measured and ECG was registered. The data were continuously collected and calculated by a data collection system.*

electrolytes, but does not contain cells. It is employed at body temperature, and is oxygenated sufficiently to maintain tissue oxygen delivery as in vivo while maintaining a physiological pH. Left ventricular isovolemic pressures were measured via an intraventricular balloon, coronary flow (CF) by timed collections of the coronary effluent, and ECG was recorded by a computerized continuous data collection system (Fig.4). The great advantage of the Langendorff-system is that it gives a good assessment of heart function. Furthermore, it is simple to use, and possible to conduct a large number of experiments without any assistance and in short time. The disadvantage is that one is working with a denervated, buffer-perfused heart lacking the influence of circulating blood cells and components. Furthermore the Langendorff model is non-working. The latter is partially compensated by the intraventricular balloon, which fills up an otherwise empty left ventricle. Fig. 5 shows the protocols for heart perfusion [see paper I, II, VI and V for details]. To investigate the role of nitric oxide in the hearts from severely atherosclerotic mice, some of the hearts were given a NO donor or NOS inhibitor only during reperfusion. Thus, a possible NO formation during ischemia which has reported [Giraldez et al., 1997, Engelman et al., 1995, Guo et al., 1996] was not influenced. We employed NG-nitro-L-arginine methyl ester (L-NAME) as NOS inhibitor. It is reported that L-NAME has a NO-independent vasoconstrictive effect which may lead to depression of cardiac function [Nakaike et al. 1995]. We found vasoconstrictive effects of L-NAME in the in vitro vessel reactivity studies (data not shown). However, when employed in the heart, no influence of cardiac performance was detected in the concentrations employed (data not shown). Due to the intervention of ischemic preconditioning, the duration of stabilization in the classical ischemic preconditioning study was different from the others (Fig 5).

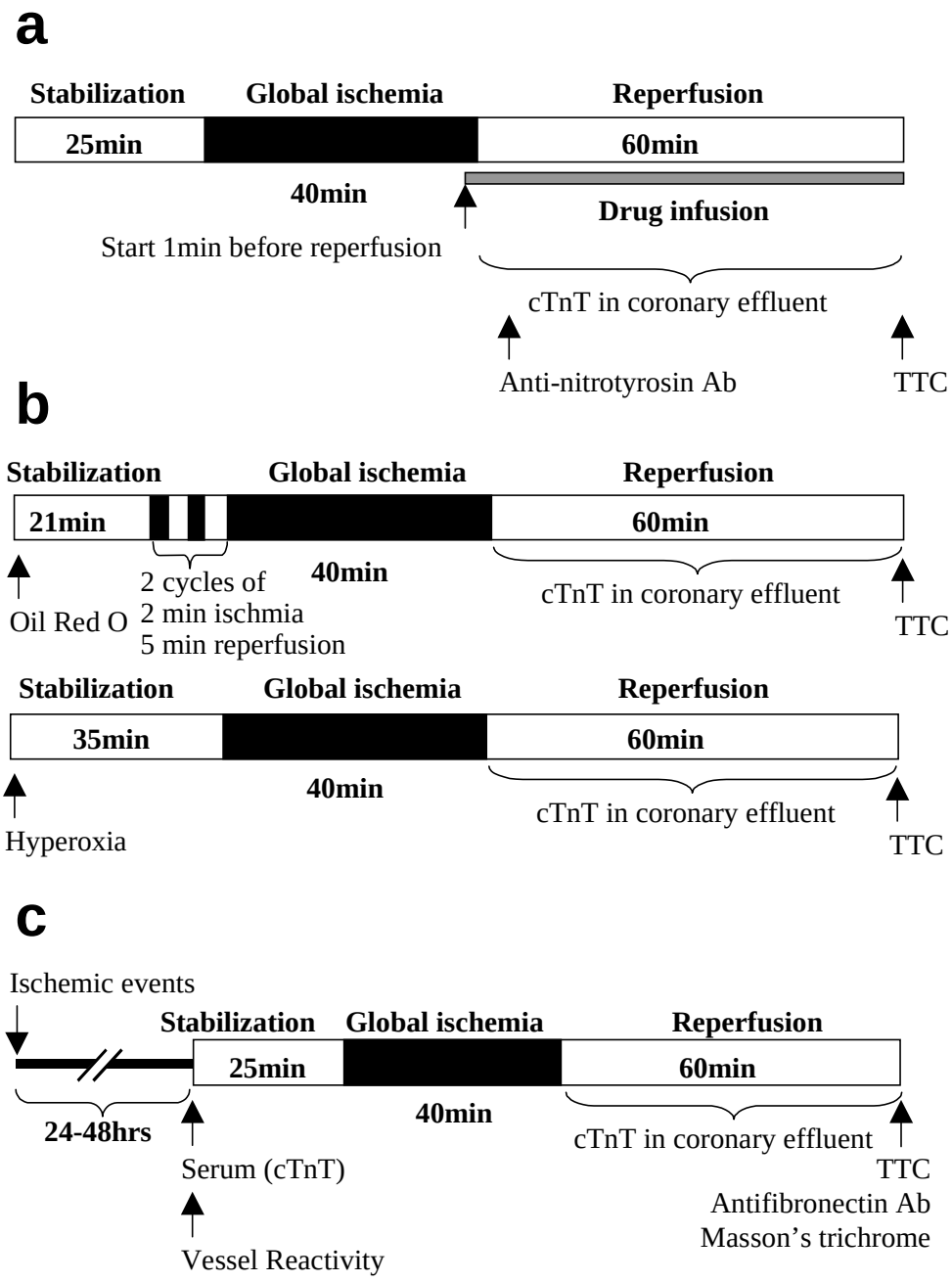

Fig. 5 *After stabilization, all hearts were subjected to 40 minutes of global ischaemia and 60 minutes reperfusion.* ***a*** *To investigate the role of nitric oxide in the hearts from severely atherosclerotic mice (apolipoprotein E and low density lipoprotein receptor double knockout (ApoE/LDL KO) mice fed an atherogenic diet 7-9 months) and compared with hearts of C57BL6 mice, some of the hearts were given a NO donor or a NOS inhibitor.* ***b*** *To study classic ischemic preconditioning in mice with severe atherosclerosis, hearts were subjected to two episodes of 2 minutes global ischemia followed by 5 minutes reperfusion before sustained ischemia, or mice were kept in a hyperoxic enviroment for 60 minutes immediately before heart perfusion and compared with C57BL6 mice hearts.* ***c*** *To investigate the effect of spontaneous or induced ischemic events, hearts of ApoE/LDL KO animals were isolated and perfused 24 – 48 hours after the ischemic events. The same protocol was used to study the role of iNOS in iNOS KO animals, while thoracic aortas were collected for vessel reactivity studies as shown in Fig. 4.*

In vitro vessel reactivity

To investigate the influence of atherosclerosis and brain ischemia on vessel reactivity, the isometric tension of thoracic aortic rings was recorded in the classic organ bath model. After anesthetizing, the descending thoracic aorta was isolated and mounted onto two thin stainless steel holders connected to a force transducer which measures the isometric tension (Fig.6). The thoracic aorta was selected due to the fact that in the mice, this vessel has a caliber of 1-2 mm, and the technical challenge of mounting smaller vessels was too high. However, the reactivity of the thoracic aorta may be different from peripheral arteries including coronary arteries, as the aorta has much less reserve for dilatation and contraction than regulatory vessels.

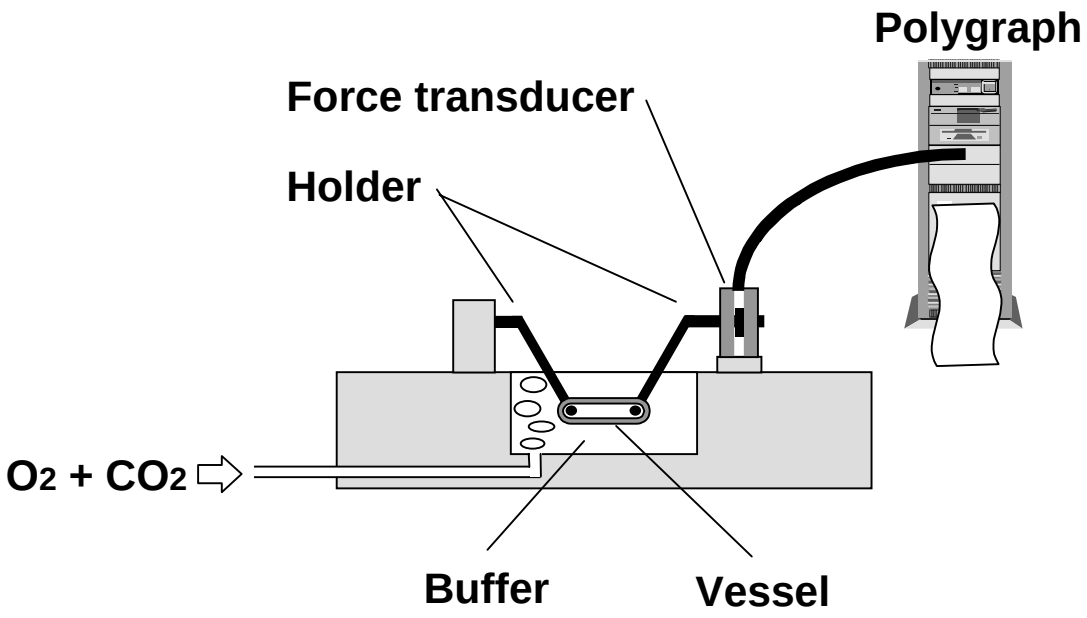

Fig. 6 *Thoracic aortic rings were mounted onto two thin stainless steel holders, one of which was connected to a force displacement transducer. The mounted rings were kept in 2 ml organ baths containing Krebs-Henseleit solution at 37 °C and continuously bubbled with a gas mixture of 95 % O_2 and 5 % CO_2 to maintain a pH of 7.4. After the application of a passive tension of 1.5-2.0 g, the isometric tension after relaxing and contractile agents was recorded on a polygraph.*

Fig. 7 shows the protocols for vessel reactivity investigations [see paper III and VI in detail]. Relaxations were investigated after precontraction with phenylephrine. We employed preincubation with aminoguanidine (AG) for inhibition of iNOS. However, AG is a relatively unselective inhibitor of iNOS, which can also inhibit eNOS [Wolff et al., 1995]. Therefore we chose to employ also iNOS KO mice to investigate clearly the role of iNOS for vessel reactivity.

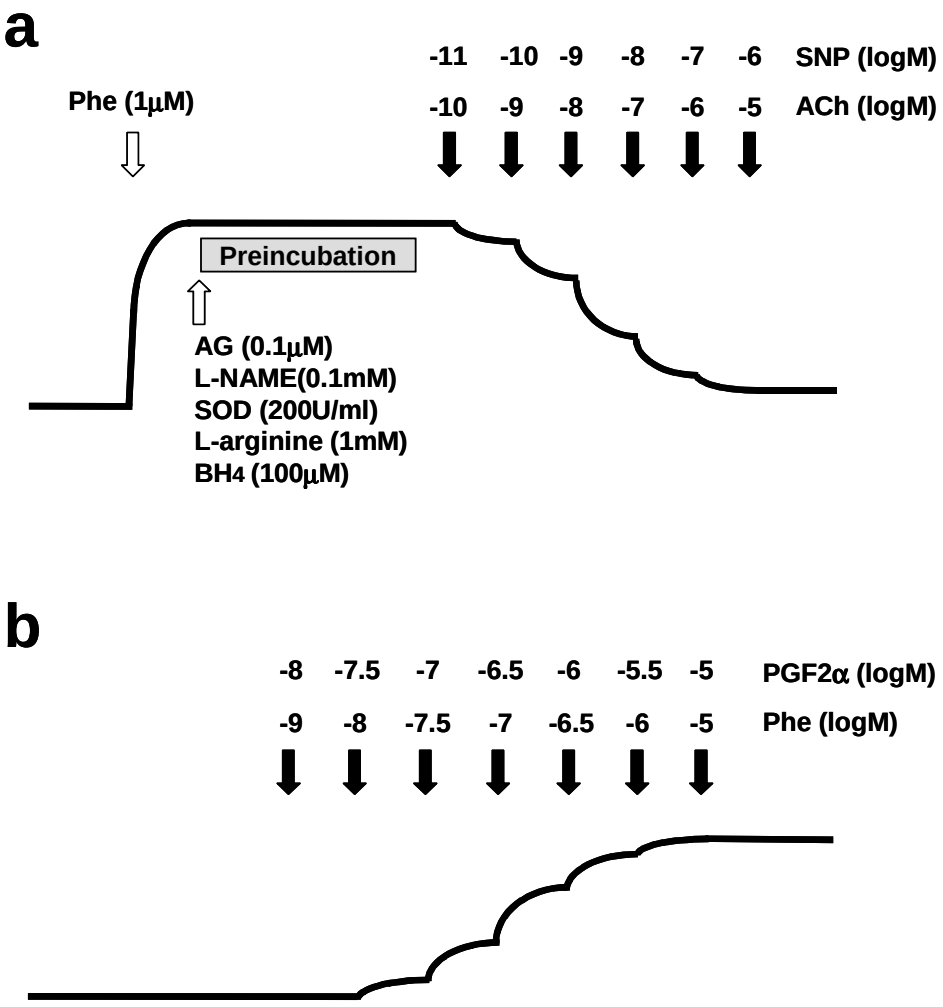

Fig. 7 *After an equilibration period of 30 min, the contractile function of the vessel was tested by KCl solution as the reference contraction.* ***a*** *Following washout, the vessels were contracted once with 1 µmol/L phenylephrine (Phe) and preincubated for 10 min with 0.1 mmol/L aminoguanidine (AG) or NG-nitro-L-arginine methyl ester (L-NAME) or for 30 min with one or a combination of substances which were L-arginin (1 mM), BH_4 (100 µM), and superoxide dismutase (SOD: 200U/ml). Relaxations were determined by application of 10^{-10}-10^{-5} mol/L acetylcholine (ACh) or 10^{-11}-10^{-6} mol/L sodium nitroprussid (SNP) to precontracted vessels.* ***b*** *Contractile responses were determined by application of 10^{-8}-10^{-5} mol/L prostaglandin F2α (PGF2α) or 10^{-9}-10^{-5} mol/L phenylephrine (Phe).*

Measurement of infarct size

When evaluating infarct size after isolated heart perfusion with induced global ischemia and reperfusion, we employed triphenyl tretrazolium chloride (TTC) staining by intracoronary perfusion of TTC solution after one hour reperfusion. TTC is based on lactate dehydrogenase activity, and stains viable cells red while necrotic cells become unstained. Hearts were thereafter sectioned, a digital image obtained, and the unstained areas quantified semiautomatically with the aid of Adobe photoshop as percentage of total ventricular area. Although TTC staining is a “golden standard” of experimental infarction, it may retrospectively have been a better choice to perform this staining not by perfusion but on sectioned tissue as coronary stenosis in the ApoE/LDLr KO mice may have influenced the distribution of solution and thus the evaluation of infarct size.

Evaluation of in vivo infarctions

During housing for 7-9 months, some ApoE/LDLr KO mice developed signs of disease. To evaluate if cardiac necrosis observed after reperfusion in these mice was due to the Langendorff-intervention, recent spontaneous infarctions in vivo, or infarction of older age, different staining techniques were employed. The areas unstained by TTC included all these types of myocardial necrosis. Antifibronectin staining was employed for evaluate recent infarction in vivo and Masson’s trichrome staining was employed for evaluating collagen formation, which comes in later stage of scar formation. We employed sections from the aortic root, the middle region of the heart, and the apex region in hearts with suspected in vivo infarctions and their controls. As the whole hearts were not sectioned and stained, there is a possibility of underestimation of in vivo infarctions. As a support serum cardiac troponin T (cTnT) level was measured. To do this blood was collected from the thoracic cavity after heart isolation. The serum cTnT levels in control mice was higher than to be expected based on levels in humans or other experimental animals [O'Brien et al., 1997, Aartsen et al.,

2000]. This may have been due to an acute release due to the intervention of heart isolation or cross reaction with skeletal muscle troponin. Indeed, a pilot study performed demonstrated that serum cTnT levels in blood from the thoracic cavity was higher than in blood from the jugular vein (Fig. 8). However, cTnT was not detectable in homogenisates of mouse skeletal muscle (not shown). Thus, the increased level we are detecting due to the sampling procedure should be evenly distributed between groups, as we used the same procedure in all animals. Additionally, according to the results of pilot data, C57BL6 mice had higher serum levels of cTnT compared with ApoE/LDLr KO mice. The reason for this difference is still unknown. Thus, we could not compare serum cTnT levels between groups.

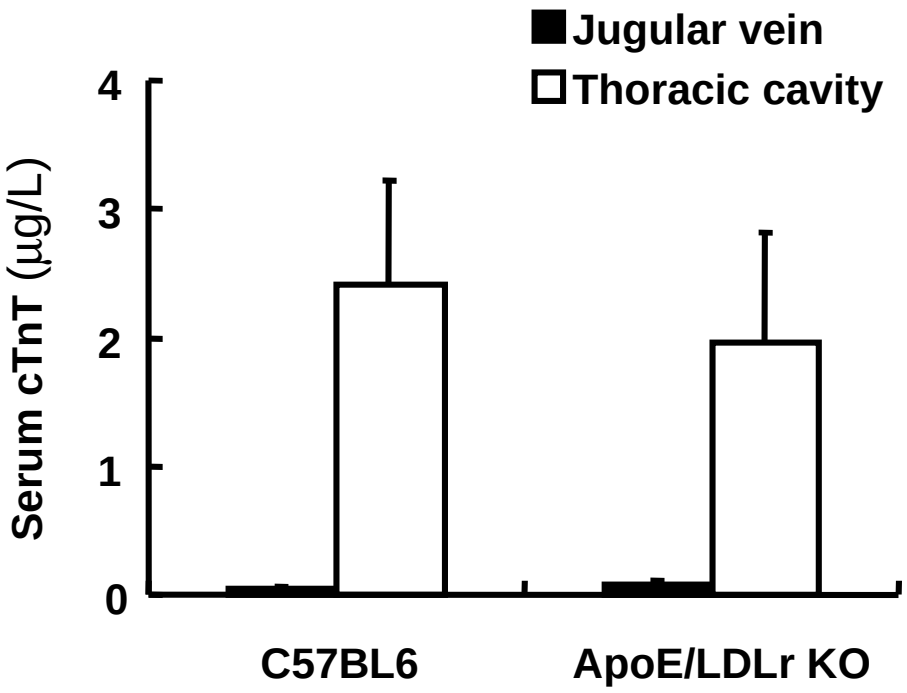

Fig. 8 *Serum cardiac troponin T (cTnT) in blood from the thoracic cavity compared with from jugular vein in ApoE/LDLr KO or C57BL6 mice. Jugular vein blood samples were collected by puncture before the chest was opened. After that, blood samples were collected from the thoracic cavity at the time of heart isolation with left atrial incision.*

Induced brain ischemia

In some of the ApoE/LDLr KO mice with advanced atherosclerosis and signs of disease, ischemic events in the brains was suspected. To address this, we first attempted a similar approach as with the hearts, that is to section in three different areas (cerebellar, middle region, and forebrain) and stain with hematoxilin/eosin to evaluate morphology. We could not find any morphological evidence of brain infarction with histological analysis (results not shown). We collected all brains 24-32 hours after onset of signs of disease or induced brain ischemia. Morphological changes of the brain after infarction, however, becomes clear 48-72 hours after onset. Thus our time point of investigating might have been too early to find morphological changes in the brains. As another approach, we measured serum S100B as a marker of brain damage. S100B belongs to a family of intracellular calcium-binding proteins originating from astroglial and Schwann cells, and leaks to serum if the permeability of the blood-brain barrier is increased [Persson et al., 1987]. S100B has been found not only in brain but also in mediastinal tissue [Anderson et al., 2000], and we collected blood from the thoracic cavity. Thus, possibly the base line of serum S100B became higher than the physiological level. However, since we employed the same procedure of blood collection in all groups, the possible source of error should be reproducible between individuals and not influence the end result. To investigate the influence of brain ischemia on cardiac function and vessel reactivity, bilateral ligation of the internal carotid arteries 24-32 hours before heart isolation was performed.

Immunoblotting and immunohistochemical analysis

To evaluate whether changes in heart function and vessel reactivity between atherosclerotic mice and mice with normal vessels could be due to an influence on NO production by eNOS, we employed immunoblotting of eNOS protein on the thoracic aortas sampled from ApoE/LDLr KO mice and C57BL6 mice. The density of the bands were scanned and measured to quantify differences between

groups. Likewise, to investigate the involvement of iNOS in remote preconditioning evoked by brain ischemia, immunoblots with antibody against iNOS were performed. As the amount of detectable bands was low, probably due to small amounts of tissue iNOS, the gels were also stained with Ponceau solution which unspesifically stains proteins. The band for iNOS was measured and corrected for the amount of protein per lane before quantification. We also evaluated iNOS in the thoracic aortas after brain ischemia.

Nitric oxide may react with superoxide generated upon reperfusion and form peroxynitrites, which depresses cardiac muscle contractility [Matheis et al., 1991, Naseem et al., 1995, Yasmin et al., 1997, Xie et al., 1998]. Peroxynitrite reacts with an aromatic amino acid, and leaves key markers in biological systems. Nitrate tyrosine is one of the important markers, formed by the reaction of peroxynitrite with tyrosine [Pryor et al., 1995]. To evaluate whether the deteriorated cardiac function in control hearts perfused with the high concentration of NO donor, S-nitrose-N-acetylpenicillamine (SNAP), could be due to NO-overproduction with peroxynitrite formation, immunohistochemical analysis of hearts with a nitrate tyrosine antibody was employed [Paper II].

RESULTS

Cyclic fluctuations in performance of the isolated Langendorff-Perfused mouse heart (Paper I)

After establishing a model of Langendorff-perfusion in the mouse heart, a phenomenon of cyclic fluctuations of coronary flow and left ventricular systolic pressure became apparent after approximately 20 minutes perfusion. Parallel deviations of these parameters with 20-50% changes appeared every eight to ten minutes throughout a two-hour perfusion period. The fluctuations were aggravated with high perfusion pressure (100 mmHg), and abolished at a low pressure (55 mmHg). Pyruvate in the perfusate abolished the fluctuations, but had an anti-ischemic effect. Thus, for the future studies (papers II, VI, and V) we chose to employ 55 mmHg as a perfusion pressure, and omit adding pyruvate in the perfusate.

Evaluation of atherosclerosis (Paper III, IV)

The degree of atherosclerosis in the ApoE/LDLr KO mouse was evaluated by sectioning hearts and thoracic aortas, and staining with oil Red-O. The atherosclerotic lesions in the hearts of ApoE/LDLr KO mice after 6-8 months on an atherogenic diet were widespread. In the thoracic aorta and aortic root advanced fibrofatty lesions were observed, with lipid deposits in the proximal coronary arteries. Atherosclerotic lesions in distal coronary arteries could be observed in the middle and apex regions of the heart.

The role of nitric oxide in ischemia/reperfusion injury of isolated hearts from severely atherosclerotic mice (Paper II)

Hearts of animals with atherosclerosis were more susceptible to ischemia/reperfusion injury than hearts of animals with healthy vessels. Expression of eNOS in the wall of the thoracic aorta was reduced in ApoE/LDLr KO animals. When the NO-donor SNAP was given immediately before start of reperfusion, left ventricular function was protected and infarct size and cTnT release into the coronary effluent reduced in hearts of atherosclerotic mice. The same concentration of SNAP depressed left ventricular pressure and increased infarct size and cTnT release during reperfusion compared with animals subjected to ischemia/reperfusion only. When hearts were harvested after 5 min reperfusion and immunostained with an anti-nitrotyrosine antibody, more positive staining was found in hearts of animals perfused with the high dose of SNAP. A low concentration of SNAP protected against ischemia/reperfusion induced dysfunction and infarction in normal hearts. The NOS-inhibitor L-NAME depressed left ventricular performance in atherosclerotic hearts without significantly influencing infarct size.

These findings suggest that increase of endogenous NO through SNAP was beneficial against ischemia/reperfusion injury in hearts of severely atherosclerotic animals with loss of eNOS, indicating a potential therapeutic gain of NO-donors. Additionally, a concilatory solution to the controversial role of NO in ischemia/reperfusion injury may be offered, indicating a dose-dependent beneficial or detrimental effect of NO.

Endothelial dysfunction in atherosclerotic mice (Paper III)

Relaxations induced by acetylcholine (ACh), but not those to sodium nitroprusside (SNP), were impaired in ApoE/LDLr mice compared to control mice, indicating impaired endothelial dependent relaxation. Preincubation with the NO substrate L-arginine did not affect, whereas the cofactor for NO synthase,

tetrahydrobiopterin (BH_4) slightly improved the relaxations induced by ACh in ApoE/LDLr KO mice. However, the impairment of endothelium dependent relaxation in ApoE/LDLr KO mice was abolished by combined administration of L-arginine and BH_4. In addition, the endothelin A (ETA) receptor mediated vasoconstriction by ET-1 was enhanced in ApoE/LDLr KO mice.

Preconditioning hearts of severely atherosclerotic mice (Paper IV)

Hearts of mice with coronary atherosclerosis (ApoE/LDLr KO mice) had worse postischemic function and increased infarct size and cTnT release compared to hearts of mice with normal vessels. Ischemic preconditioning, which was two episodes of 2 minutes global ischemia followed by 5 minutes reperfusion before sustained ischemia, improved post-ischemic ventricular function, and reduced myocardial infarct size and cTnT release in both normal and ApoE/LDLr KO mice. The effects were most pronounced in ApoE/LDLr KO hearts. Sixty minutes exposure to hyperoxia immediately before heart perfusion exerted a similar protection of function and cell viability of ApoE/LDLr KO mice hearts. These findings suggest that the severely atherosclerotic heart may be protected by preconditioning induced by ischemia or hyperoxia.

Spontaneous ischemic events in the heart and brain adapts the hearts of severly atherosclerotic mice to ischemia (Paper V)

Some of the ApoE/LDLr KO mice fed an atherogenic diet for 7-9 months developed signs of disease which was suspected to be spontaneous ischemic events. Spontaneous heart infarctions were evaluated with anti-fibronectin staining and elevation of serum cardiac troponin T levels, while brain infarctions were evaluated with serum S100B level and neurological signs such as paraplegia. Of 12 hearts from animals with signs of disease, 6 hearts had positive antifibronectin staining and/or high serum cTnT levels while 6 animals had neurological signs and/or increased serum S100B. The mice with spontaneous

ischemic events in heart or brain had improved cardiac function, and reduced infarct size and cTnT release during reperfusion after induced global ischemia. To investigate the remote preconditioning effect of brain ischemia itself and the mechanism of this effect, brain ischemia was induced by ligation of both internal carotid arteries in C57BL6 mice and iNOS KO mice. C57BL6 mice hearts with induced brain ischemia were protected against ischemia reperfusion injury analogous to hearts of animals with spontaneous infarctions. In C57BL6 mice, cardiac iNOS was upregulated 24 hours after inducing brain ischemia. In iNOS KO mice, the induced brain ischemia could not protect their hearts. These findings suggest that spontaneous ischemic events in the brain and heart produce a preconditioning-like effect in hearts of mice with coronary atherosclerosis, and that iNOS is a key factor of the remote preconditioning effect of brain ischemia. These findings place the triggers of preconditioning in the ischemia rather than the reperfusion phase.

Effects of spontaneous or induced brain ischemia on vessel reactivity (Paper VI)

In isolated thoracic aortic rings from ApoE/LDLr KO mice with spontaneous ischemic events, contraction to prostaglandin F2α (PGF2α) was reduced, but relaxation to ACh unchanged. When brain ischemia was induced in C57BL6 mice 24 hours before vessel isolation, both impaired contractility to PGF2α and enhanced relaxation to ACh were found. In the vessel wall of C57BL6 mice with induced brain ischemia, eNOS protein was downregulated and iNOS protein was upregulated compared with mice without brain ischemia. The changes of vessel reactivity in animals with induced brain ischemia were abolished by pre-incubation with aminoguanidine, a relatively selective iNOS inhibitor. When employing iNOS KO mice, impaired contractility to PGF2α and enhanced relaxation to ACh by induced brain ischemia could not be observed. In the vessel wall of iNOS KO mice with induced brain ischemia, eNOS protein was

upregulated. These findings suggest that induced brain ischemia as a model of delayed remote preconditioning protects vessel reactivity, and this protection is mediated by iNOS.

DISCUSSION

ApoE/LDLr KO mice as an animal model of atherosclerosis

The ApoE/LDLr KO mouse is a novel tool as an animal model of atherosclerosis, and adaptation of heart physiology techniques to the mouse may open new perspectives in basic research and provide new therapeutic targets [paper I]. The ApoE/LDLr KO mice fed an atherogenic diet for 7 months had severe atherosclerosis from the thoracic aorta throughout the distal coronary arteries [paper III, IV], and an accompanying impaired endothelial function in vitro [paper III, VI]. The animals had reduced eNOS in the thoracic aortas, and reduced coronary flow [paper II]. These findings confirm that the ApoE/LDLr KO mouse is a suitable model of human atherosclerosis, where reduced eNOS expression and NO-production as well as impaired regulation of coronary blood flow have been reported [Zeiher et al., 1991, Oemar et al., 1998].

Isolated mouse heart perfusion

Establishing the isolated perfusion model of mice hearts was a methodological challenge due to size (the mouse heart is 0.09 g in weight and has a left ventricular volume of 20 μl). We used a computerized data collection system to standardize data collection because the heart rate is rapid (300-500 beats/min) and coronary flow is low (0.5-3 ml/min). After acquiring manual skills and surgical routine, a phenomenon of cyclic fluctuations in left ventricular pressure and coronary flow appeared [Paper I]. The phenomenon of cyclic fluctuations in heart performance has not been published in any species, including the mouse. However the phenomenon has been observed in other laboratories in Europe as

well as USA (information collected at international research conferences). We did not attempt to elucidate the mechanisms of the fluctuations in paper I. Pilot studies indicate that they may be endothelium dependent, because the fluctuations increase when employing the NO inhibitor, L-NAME, and decrease when employing an ET-1 agonist (not published). Furthermore, the fluctuations never appear after global ischemia exept in preconditioned animals with preserved endothelial function (not shown). We abolished this phenomenon by employing low perfusion pressure (55 mmHg) [Paper I, II, VI and V] or with pyruvate in the perfusate [Paper I]. However, for future studies we chose not to employpyruvate because pyruvate had an anti-ischemic effect [Paper I].

The effect of atherosclerosis on heart function and vessel reactivity

Evidence suggest that NO may have both protective and detrimental effects in ischemia/reperfusion injury in hearts of mice with normal vessels. We investigated the role of NO in ischemia/reperfusion injury of hearts from animals with normal or with severely atherosclerotic vessels.

Hearts of mice with severe atherosclerosis had depressed left ventricular performance during reperfusion compared with mice with normal vessels [paper II, IV]. We demonstrated that eNOS expression was reduced in thoracic aortas from ApoE/LDLr KO mice compared with C57BL6 mice [paper II]. Endothelial relaxation induced by acetylcholine (ACh) were impaired in ApoE/LDLr mice compared to control mice [paper III]. In previous studies investigating the role of cholesterol on left ventricular performance, no significant differences between long-term (more than 5 weeks) cholesterol fed and normal rabbits during reperfusion were found [Tilton et al., 1987, Le Grand et al., 1995]. The discrepancy between the present study and previous findings may be due to the severity of atherosclerosis in the present animals. When ApoE/LDLr KO mice hearts were perfused with the NO-donor SNAP, cardiac dysfunction during reperfusion was attenuated, while infarct size was reduced [paper II]. This is in

accordance with several previous studies in animals with normal vessels, where protective effects of NO-donors in ischemia/reperfusion injury have been reported [Siegfried et al., 1991, Johnson et al., 1991, Hoshida et al., 1996, Yasmin et al., 1997, Izhar et al., 1998]. Furthermore, we demonstrated that the impairment of endothelial relaxation in atherosclerotic vessels was abolished by combined administration of the NO substrate L-arginine and the cofactor for NO synthase, tetrahydrobiopterin (BH_4) [paper III]. These findings suggest that a possible mechanism for reduction of heart function during reperfusion or impaired vessel reactivity may be decreased NO production due atherosclerosis-induced loss of endothelium and eNOS.

In the C57BL6 hearts, the dose of SNAP which protected the ApoE/LDLr KO heart aggravated reperfusion injury, while a ten-fold lower dose of SNAP protected it [paper II]. Peroxynitrite formation in hearts given the high dose of SNAP was demonstrated by increased nitrosylated tyrosine with immunohistochemical staining [paper II]. L-NAME protected the heart of C57BL6 mice especially during the early period (until 15 minutes) of reperfusion but did not decrease infarct size. It appears that NO modulates cardiac contractility in a biphasic manner: at a low concentration a positive inotropic effect may be found [Kojda et al., 1995], while a negative inotropic effect may be found by a high concentration [Kojda et al,. 1996]. Whether these are direct NO-effects or secondary to i.e. vasodilation or peroxynitrite formation remain to be determined.

These findings suggest that endogenous NO was beneficial against ischemia/reperfusion injury in hearts of severely atherosclerotic animals with loss of eNOS, indicating a potential therapeutic gain of NO-donors. Likewise, impaired reactivity of atherosclerotic vessels could be altered by pharmacological enhancing substrates for NO-production. Additionally, a conciliatory solution to the controversial role of NO in ischemia/reperfusion injury may be offered,

indicating a dose-dependent beneficial or detrimental effect of NO.

Preconditioning the heart with atherosclerotic vessels

Although preconditioning is a powerful mode of protection in experimental models, results of clinical studies in patients undergoing open heart surgery are controversial [Zeeuw et al., 1999]. This may be due to the fact that the patients we want to protect have atherosclerosis. Possible adaptation of the heart with ischemic heart disease to ischemia has already happened in vivo which may be partially due to loss of endothelium and NO release, as NO may be a signal molecule initiating adaptation to ischemia [Qiu et al., 1997]. A further possible reason of the inconsistent protection found in clinical studies may be targeting of patients groups with short surgical procedures where loss of function and necrosis is negligible.

Ischemic preconditioning has previously been shown to reduce infarct size and protect the function of murine hearts with normal vessels [Guo et al., 1998, Xi et al., 1998, Sumeray et al., 1998]. In the present study the protection of function and cell survival was more evident in hearts of ApoE/LDLr KO mice than mice with normal vessels [paper IV]. The most likely explanation is that the ApoE/LDLr KO had larger infarcts and deteriorated cardiac performance, and therefore benefited more from endogenous protection. In our laboratory adaptation to ischemia through exposing animals to hyperoxia in vivo prior to heart excision has been developed, where both an immediate and a delayed protection has been evoked in the rat [Tähepôld et al 2000]. Although the mediators of hyperoxic cardioprotection are not determined, the mechanism of protection is dependent on activation of nuclear factor kappa-B (NFkB) [Tähepôld et al., 2001]. In the present study hyperoxia protected postischemic function, reduced infarct size, and reduced release of cTnT analogous to classic preconditioning in the ApoE/LDLr KO mouse heart. Hyperoxia is a mode of myocardial protection which potentially may be of direct clinical applicability, as

opposed to ischemic preconditioning where the mechanisms must be determined for pharmacological exploitation. These findings have implications to the therapeutic use of ischemic preconditioning on atherosclerotic patients.

Heart protection by spontaneous ischemic events

During housing for 7-9 months, some ApoE/LDLr KO mice developed paraplegia or general malaise with reduced mobility, shallow breathing, some with chest scratching as if having chest pain. When the animals developed signs of disease, their hearts were isolated and perfused with induced global ischemia, reperfusion, and TTC staining, and their tissues and serum was investigated for evidence of spontaneous ischemic events. We decided to target ischemic events in hearts or brain as these are the most commonly occurring in patients; however, we do not know if other organs may also have been of importance.

We found evidence of spontaneous heart infarction by immunohistochemical analysis of hearts as well as increased serum cTnT levels in 6 of all 12 animals with signs of disease. These hearts were protected against injury induced by global ischemia analogous to ischemic preconditioning [paper V]. According to the authors' knowledge, this is the first evidence that spontaneously occurring ischemic events may protects the heart. The present findings pinpoint that organ protection is generated by ischemia rather than by ischemia and reperfusion, although the possibility of reperfusion via collateral flow remains. One possible clinical correlate to both ischemic preconditioning and the present findings is evidence of pre-infarction or unstable angina protecting the heart [Kloner et al., 1995, Noda et al., 1999]. We also found the evidence of spontaneous brain ischemia, evident as either paraplegia and/or increased serum S100B in 6 of 12 animals with sign of disease. Hearts of these animals were protected against injury induced by global ischemia in a manner analogous to delayed ischemic preconditioning [paper V]. It has been demonstrated that the brain itself can be preconditioned by cerebral ischemia both as immediate [Kitagawa et al., 1990]

and delayed protection [Perez-Pinzon et al., 1997]. However, there are no publications which demonstrate a remote preconditioning effect of brain ischemia. Additionally, aortic rings from animals with spontaneous ischemic events had reduced contractility to PGF2α, while relaxation to ACh was not influenced [paper VI]. We speculated that the adaptation to ischemia initiated by ischemic preconditioning may manifest itself not only in the heart, but in other organs such as vessels due a possible whole body effect of endogenous protection.

Remote preconditioning by induced brain ischemia and its influence on vessel reactivity

In order to investigate whether brain ischemia directly could protect the heart according to the findings described above, a group of C57BL6 mice subjected to induced brain ischemia by bilateral ligation of the internal carotid arteries were included in the study. Bilateral ligation of the internal carotid arteries caused transitory neurologic disturbances 24 hours later. Heart function, infarct size, cTnT release, as well as serum levels of S100B were very similar in animals with induced and spontaneous ischemic events [paper V]. Thoracic aortic rings from these animals had reduced contractility to PGF2α and improved relaxation to ACh in vitro [paper VI]. These findings suggest that brain ischemia protects heart against ischemia. These findings may possibly be secondary to changes of vascular tone if the thoracic aorta is representative of the coronary circulation. For instance, a reduced vessel contractility could explain some of the beneficial effects observed in the isolated hearts during reperfusion, as this might lead to a reduction of the no-reflow phenomenon and to increased oxygen supply to the heart muscle. However, this is at present only a speculation which remains to be conclusively shown.

The role of iNOS in remote ischemic preconditioning

The mechanisms of remote preconditioning are not clarified and probably differ

between preconditioning models. Remote preconditioning evoked by brain ischemia has not previously been demonstrated. It has been reported that focal cerebral ischemia upregulates iNOS [Iadecola et al., 1996] in the brain, and that ischemia/reperfusion in the heart upregulates myocardial iNOS [Guo et al., 1999, Jones et al., 1999b, Wildhirt et al,. 1999]. Evidence indicates that delayed ischemic preconditioning initiated in the heart is mediated by iNOS [Weigert et al., 1995, Yen et al., 1995, Xi et al., 1999, Gunnett et al., 1998, Xi et al., 1999]. We investigated if iNOS could mediate remote preconditioning evoked by brain ischemia.

When cytoplasmatic myocardial proteins were extracted, electrophoresed, and incubated with an iNOS antibody, increased cardiac iNOS was found in hearts of animals preconditioned with brain ischemia 24 hours earlier [Paper V]. Likewise, when immunoblotting was performed on protein extracts from thoracic aortas, preconditioned animals had increased iNOS [paper VI]. When brain ischemia was induced in mice with targeted disruption of the iNOS gene, no protection of heart function or reduction of myocardial necrosis was apparent [paper V]. Moreover, the in vitro impaired contractility to PGF2α and enhanced relaxation to ACh observed in thoracic aortic rings of remote preconditioned wild types were abolished by pre-incubation with aminoguanidine, a relatively selective of iNOS, or by employing iNOS KO mice. These findings indicate that iNOS is a key mediator of remote preconditioning in this model.

In vitro relaxation to ACh was enhanced in C57BL6 animals with induced brain ischemia, but not in ApoE/LDLr KO with spontaneous ischemia. The difference in response to ACh between ApoE/LDLr KO mice and C57BL6 mice with ischemia could be due to atherosclerosis, which is associated with loss of endothelium, reduced release of NO, and/or impaired NOS activity [Dusting et al., 1998]. Although the amount of eNOS evaluated by immonoblotting were not altered, the enzyme activity might have been influenced, or the cooperation with

cofactors altered. For instance, BH_4, which is an essential cofactor for NO synthesis from both eNOS and iNOS [Shimizu et al., 1999], reversed dysfunction to the response to ACh in ApoE/LDLr KO mice when given in combination with L-arginine [paper III]. Possibly the atherosclerotic mice lacked BH_4 as observed in patients with atherosclerosis [Stroes et al., 1997].

Mechanism of preconditioning and implications of the present findings

Although the mechanisms of preconditioning in the mouse heart are largely unknown, we may speculate on a recently discovered factor. Maulik et al. [1998] found that classic ischemic preconditioning in the rat was dependent on NFkB, which was activated during preconditioning, and inhibition of this activation abolished the functional effects of classic preconditioning in rabbits [Xuan et al., 1999] and rats [Maulik et al., 1998, Morgan et al., 1999], or of hyperoxia in rats [Tähepôld et al., 2001]. It has also reported that focal cerebral ischemia activates NFkB [Schneider et al., 1999]. Two NFkB-regulated genes, inducible nitric oxide synthase and inducible cyclooxygenase, have recently been suggested to mediate delayed preconditioning in studies of knock out mice [Guo et al., 1999, Shinmura et al., 2000]. Cardiac iNOS may protect the heart through coronary vasodilatation [Loke et al., 1996], reduction of myocardial oxygen consumption, or by opening K_{ATP} channels [Gho et al., 1996, Takaoka et al., 1999]. How signalling from the preconditioned brain (or other organs) to the heart takes place is currently unknown, and will be the subject of future investigations.

Induction of brain ischemia for myocardial protection does not have an obvious appeal as a therapeutic agent. However, systemic as well as local effects of ischemic adaptation are implicated with the present findings, indicating that when fully elucidated, the principles underlying endogenous ischemic adaptation may be exploited pharmacologically to treat a wide spectra of patients with inflammatory disease, and thus has implications may beyond treatment of

ischemic heart disease.

CONCLUSIONS

Hearts of animals with atherosclerosis were more susceptible to ischemia/reperfusion injury than hearts of animals with healthy vessels. This may be due to endothelial dysfunction of atherosclerotic coronary arteries. Evidence supporting this is that the aortic vessel wall contained less endothelial nitric oxide synthase expression than normal vessels, and that the relaxations to acetylcholine was reduced in the thoracic aortas of ApoE/LDLr KO mice. The impaired relaxation could be attenuated by the substrates for NO-synthesis L-arginin and BH_4.

The NO-donor SNAP protected function and reduced infarct size of atherosclerotic hearts subjected to induced ischemia. The same concentration of SNAP was detrimental in normal hearts, probably due to NO-overproduction and peroxynitrate formation demonstrated immunohistochemically as nitrate tyrosine.

Classic ischemic preconditioning improved heart function and reduced infarct size in ApoE/LDLr KO mice hearts. In addition, exposure to hyperoxia exerted a similar protection of function and cell viability of ApoE/LDLr KO mice hearts.

Spontaneous ischemic events in hearts and brains of severely atherosclerotic ApoE/LDLr KO animals protected function and reduced necrosis when hearts were isolated and exposed to global ischemia 24-48 hours later, and reduced the contractility of thoracic aortic rings in vitro analogous to delayed or remote preconditioning. These findings could be mimicked by inducing brain ischemia

in C57BL6 mice, where also a protection of endothelium-dependent relaxation was evident.

iNOS increased in the heart and the vessel wall after induced brain ischemia. The hearts and vessels of animals with targeted disruption of the iNOS gene could not be preconditioned by induced brain ischemia, while inhibition of iNOS by pharmacological means also abolished the protective effects on vessels. These findings suggest that iNOS is an important mediator of cardiac and vascular protection induced by brain ischemia .

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