EFFECTS OF A NOVEL INHIBITOR OF LIPID PEROXIDATION ON INJURY CAUSED BY OXIDATIVE STRESS AND ISCHEMIA-REPERFUSION

Ph.D. Thesis

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Szeged, Hungary

2002

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ABBREVIATIONS

CF	Coronary flow
H_2O_2	Hydrogen peroxide
H290/51	cis-5, 5a, 6,10,b-tetrahydro-9-methoxy-7-methylindeno [2,1-b]indole
HR	Heart rate
LDH	Lactate dehydrogenase
LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end-diastolic pressure
LVSP	Left ventricular systolic pressure
MDA	Malondialdehyde
PTCA	Percutaneous transluminal coronary angioplasty
ROS	Reactive oxygen substances
SOD	Superoxide dismutase
TBARS	Thiobarbituric-acid reactive substances

ACKNOWLEDGEMENTS

I am most grateful to my tutor Jarle Vaage, who introduced me to the field of heart research and guided, pushed, supported, encouraged me throughout the process of preparing this thesis.

I acknowledge the help of Istvan Lepran, in reviewing my thesis.

I am indebted to Professor Julius Gy. Papp, for accepting me to his octoral school and giving support in submitting my thesis.

I am grateful to Peter Sellei, co-author in my papers and friend, not only for
I am grateful to my

LIST OF PUBLICATIONS OF ANDRÁS NAGY

PAPERS

1. Vécsei, L., Király, Cs., Bollók, I., Nagy, A., Varga, J., Penke, B., Telegdy, Gy.: Comparative studies with somatostatin and cysteamine in different behavioral tests on rats. PHARMACOL. BIOCHEM. BEHAV. 21:833-837, 1984.
2. **Nagy, A., Sellei, P., Valen, G., Sjöquist, P.O., Vaage, J.:Effects of a novel indenoindole derivative on cardiac injury induced by exogenous toxic oxygen metabolites. FREE RAD. BIOL. MED. 20:567-572, 1996.**
3. **Nagy, A., Valen, G., Bengt E., Sellei, P., Sjöquist, P.O., Vaage, J.: Effects of a novel, low-molecular weight inhibitor of lipid peroxidation on ischemia-reperfusion injury in isolated rat hearts and in cultured cardiomyocytes. FREE RAD. BIOL. MED. 24:1462-1469, 1998.**
4. **Nagy, A., Myklebust, R., Valen, G., Sjöquist, P-O., Vaage, J.: The effect of a low-molecular weight inhibitor of lipid peroxidation on ultrastructural alterations to ischemia-reperfusion in the isolated rat heart. ACTA PHYSIOL. HUNG. 88:101-15, 2001.**

QUOTABLE ABSTRACTS

1. Nagy, A., Vécsei, L., Király, Cs., Bollók, I., Telegdy, Gy.: The role of exogenous and endogenous somatostatin in the organisation of behaviour in the rat. ACTA PHYSIOL. HUNG. 63:360, 1984. (abstract)
2. Vécsei, L., Bollók, I., Nagy, A., Telegdy, Gy., Schally, A.V.: The effect of H-PHE-ILE-TYR-HIS-SER-TYR-LYS-OH on behaviour and hypothalamic neurotransmission in the rat. ACTA PHYSIOL. HUNG. 66:305, 1985. (abstract)
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4. **Nagy, A., Sellei, P., Valen, G., Sjöquist, P.O., Vaage, J.: Effects of a novel, small molecular antioxidant on ischemia-reperfusion injury in isolated rat hearts. J. MOL. CELL. CARDIOL. 26:CII, 1994. (abstract)**
5. Nagy, A., Jánosi, A., Timár, S.: Secondary Prevention in CHD Patients in Primary Care in Hungary. J HK Coll Cardiol 9:1, 2001 (abstract)
6. Nagy, A., Jánosi, A., Timár, S.: Deprived socio-economic status as a determinant of coronary risk factor profile. Acta Clin Croat 40(Suppl): 39, 2001. (abstract)

Publications that form the basis of the thesis are shown in **bold**

CONTENTS

SUMMARY

Introduction. Coronary artery disease with an eventual coronary occlusion and subsequent myocardial injury is one of the leading causes of death in the world. Besides spontaneous reperfusion of an occluded artery thrombolysis and percutaneous transluminal coronary angioplasty (PTCA)are two procedures performed widely, that aim for the restoration of blood flow, i.e. also result in reperfusion. Reperfusion may increase injury over and above that sustained during ischemia. One of the suggested mechanisms of so-called reperfusion injury is the generation and action of reactive oxygen species (ROS). Although in clinical conditions timely reperfusion of the ischemic area is the cornerstone of treatment, there is a possible role of antioxidant therapy to limit ischemia-reperfusion injury.

Aims. I. To investigate the protective efficacy of H290/51, a low molecular weight, hydrophilic compound with effects similar to Vitamin E and inhibiting lipid peroxidation, on hypoxia-reoxygenation injury of isolated cardiomyocytes.

II. To study the effect of H290/51 on oxidative injury induced by exogenous ROS in isolated, perfused rat hearts.

III. To examine its effect on ultrastructural changes in isolated rat hearts subjected to ischemia and reperfusion.

IV. To assess the effect of inhibiting lipid peroxidation on functional and biochemical injury caused by global, normothermic ischemia-reperfusion in isolated rat hearts.

Methods. I. Neonatal myocytes were cultured. On day 6 cells were rendered hypoxic for 1 hour followed by 4 hours of reoxygenation. Lactate dehydrogenase (LDH) leakage was assessed.

II. Oxidative injury was induced in isolated rat hearts by perfusion with H_2O_2 for 10 min, followed by 50 minutes of recovery. Functional (heart rate [HR], coronary flow [CF], left ventricular end-diastolic pressure [LVEDP], left ventricular developed pressure [LVDP]) and biochemical ([LDH leakage], thiobarbituric acid parameters were measured.

III. Isolated rat hearts were subjected to 30 minutes of global, normothermic ischemia, followed by 20 minutes of reperfusion. Hearts were sampled for electron microscopy. Quantitative stereological morphometry was employed.

IV. Isolated rat hearts were subjected to 30 minutes of global, normothermic ischemia, followed by reperfusion for 20 minutes. Functional (HR, CF, LVDP, LVEDP) and biochemical (LDH leakage, TBARS) parameters were measured.

Materials. A novel low molecular weight indenoindole derivative, H290/51 (cis-5, 5a, 6,10,b-tetrahydro-9-methoxy-7-methylindeno [2,1-b]indole) was selected as the antioxidant in the experiments. The compound is an inhibitor of lipid peroxidation, with balanced hydro- and lipophilicity, thus it can rapidly penetrate cell membranes to intracellular sites of ROS generation, lipid peroxidation and oxidant injury.

Results. I. H290/51 inhibited LDH leakage in isolated cardiomyocytes induced by hypoxia-reoxygenation in a dose-dependent manner.

II. H290/51 attenuated diastolic dysfunction as it inhibited the increase in LVEDP caused by H_2O_2. The protective effect was further evidenced by the inhibition of both LDH release and accumulation of TBARS after H_2O_2 exposure.

III. Ischemia-induced increase in interstitial volume and in volume fractions of myocytes and interstitium was inhibited by H290/51. The difference was already present at the end of ischemia, but disappeared during reperfusion.

IV. Ischemia caused an increase in LVEDP and decrease in LVDP and CF, and induced malignant arrhythmias. H290/51 effectively inhibited these changes if it was administered throughout the experiment including the stabilization period before ischemia, but was less potent when given only during reperfusion.

Conclusion. Our study indicates that inhibiting lipid peroxidation by H290/51 counteracts several deleterious processes in an experimental myocardial ischemia/reperfusion injury model. Our data indicate that to some extent its antioxidant properties may be effective not only during reperfusion, but already during ischemia. Thus, a better preserved functional and morphological status of the myocardium during ischemia may yield a better capacity to survive reperfusion injury. The chemical properties of the compound, most importantly its balanced hydro- and lipophilicity and its small molecular weight result in its ability to rapidly reach the intracellular sites where induced free radicals are produced. Clinically, such properties might be of therapeutic value, and H290/51 may therefore be a promising candidate for clinical evaluation and further study.

INTRODUCTION

Coronary artery disease, in particular in the form of acute myocardial ischemic syndromes, remains the principle cause of mortality, morbidity and early disability in the industrialised world. These events are usually triggered by a coronary artery occlusion at the site of a ruptured plaque that leads to myocardial ischemia and subsequent severe tissue injury and cell death in a time-dependent manner. The major goal of treatment is to restore blood flow to the jeopardised myocardial area. New techniques, such as percutaneous transluminal coronary angioplasty (PTCA) and thrombolysis in the acute phase of an impending myocardial infarction rapidly gained wide acceptance as the appropriate therapy for re-establishing perfusion to ischemic areas of the myocardium. A common example of global reperfusion in clinical practice is open-heart surgery with cardioplegia (Vaage and Valen, 1993).

Reperfusion injury and its clinical relevance

Although an absolute pre-requisite for tissue survival, it is widely accepted that reperfusion may increase injury over and above that sustained during ischemia (Hearse, 1998; Ambrosio and Tritto, 1999). Thus, there exists a paradox in that tissue viability can be maintained only if reperfusion is installed as soon as possible, but only at the risk of extending the injury beyond that already having occurred during ischemia. As injurious components may partly counteract the otherwise beneficial effects of reperfusion, this phenomenon has thus been termed “reperfusion injury” (Braunwald and Kloner, 1985; Becker and Ambrosio, 1987). This phenomenon may have major clinical implications in such frequent and important clinical conditions as angina or in procedures performed to recover vascular patency like thrombolysis, angioplasty and cardiac surgery, where ischemia is followed by reperfusion.

The consequences of ischemia-reperfusion injury include a series of events: (a) reperfusion arrhythmias, (b) myocardial stunning, a reversible impairment of contraction, (c) microvascular damage and no-reflow, and probably (d) lethal reperfusion injury where irreversible injury with cell death occurs (Dhalla et al., 2000; Ferrari and Hearse, 1997). These phenomena have been described in humans and may partly be reproduced in various animal models (Hearse, 1990).

Pathophysiology of reperfusion injury, oxygen free radicals

Ischemia-reperfusion injury can be explained by two hypotheses: the oxygen paradox and Ca^{2+}-overload. During reperfusion, sequential reduction of molecular oxygen occurs ending in formation of reactive oxygen substances (ROS). This forms the basis of the oxygen paradox, on the premise that while oxygen is essential for tissue survival, it can be injurious during reperfusion of ischemic myocardium (Park and Lucchesi, 1999). Oxidative stress damages the sarcolemmal Ca^{2+} pump regulatory mechanisms which results in intracellular Ca^{2+} overload (Kaplan et al., 1997). These mechanisms are most likely related to each other. Substantial evidence indicates that ROS are important mediators of myocardial ischemia-reperfusion injury (Bolli, 1990). ROS are chemical species with one or more unpaired electrons in their outer orbit, which highly increases their reactivity and makes them capable of inducing oxidative modification of other molecules (Blake et al., 1987) The addition of a single electron to molecular oxygen results in the production of superoxide anion radical (O_2^-). Formation of superoxide anion is the first of several steps in forming other oxygen-derived reactive products, which include hydrogen peroxide (H_2O_2) and hydroxyl radical. H_2O_2 is not a radical by itself, but is capable of causing cell damage by interacting with transition metals such as iron. A single electron reduction of H_2O_2 results in the formation of the hydroxyl radical, which is highly reactive and has a very short half-life. Peroxynitrite ($ONOO^-$) is another possible contributor to reperfusion injury. The first excited state of O_2 is a singlet oxygen ($^1O_2$) that is non-radical in itself, but can also initiate radical chain reactions.

In the heart ROS can be produced in the sarcoplasmic reticulum, the mitochondria, the vascular endothelium and by aggregating neutrophils (Nayler, 1992; Thompson and Hess, 1986). It should be noted that a number of sites, at which ROS may be produced, are intracellularily located, thus not easily available to exogenous antioxidant compounds. There are different sources of ROS during ischemia-reperfusion. Oxygen is essential for ATP production in the mitochondria. Under normal conditions some ROS are formed during mitochondrial electron transport, and these are inactivated by intracellular antioxidant systems. During hypoxia and ischemia, however, the mitochondrial electron transport chain in the myocyte is disrupted and a massive accumulation of ROS occurs. Other sources of ROS are endothelial cells and activated leukocytes. Most likely H_2O_2 is the ROS species that is produced by activated leukocytes during ischemia-reperfusion (Kraemer et al., 1990). ROS,

through the formation of lipid peroxides and hydrogen peroxide, inhibit membrane bound enzymes and initiate chain-propagation reactions, which cause diminishing membrane fluidity and increasing membrane permeability. Intracellularily, the sarcolemma and mitochondrial membrane structures are most prone to ROS-induced damages (Hess et al., 1983). Membrane abnormalities may cause a serious defect in calcium regulation and cellular volume, inducing mitochondrial dysfunction, calcium overload and cellular swelling. These injuries can eventually lead to cell death (Steenbergen and Jennings, 1991, Ambrosio et al., 1991). ROS can also damage nucleic acids causing mutations and cell death (Singal et al., 1999). ROS also increase leukocyte activation, chemotaxis, and leukocyte-endothelial adherence after ischemia-reperfusion (Collard and Gelman, 2001).

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Role of ROS

The role of ROS in the pathogenesis of myocardial reperfusion injury is substantiated experimentally by a) the detection of ROS in the reperfused myocardium; b) exposure of myocardium to exogenous ROS yields biochemical and functional dysfunction similar to that caused by ischemia-reperfusion; and c) treatment with antioxidants affords protection against reperfusion injury.

Production of ROS can be measured directly and indirectly (Saran and Bors, 1991). Production of ROS was detected in small amounts in vivo in the intact beating heart (Grill et al., 1992). ROS production increases during ischemia at least three-fold, and subsequently there is an additional burst in production during reperfusion which has been measured in vivo by electron paramagnetic resonance spectroscopy (Zweier et al., 1987). Severe ischemia induces a reduction in the tissue concentration of various scavenger compounds that protect against oxygen toxicity (Ferrari et al., 1985). Lipid peroxidation is detected through the estimation of malondialdehyde (MDA) either by chemiluminescence, HPLC or the thiobarbituric acid (TBARS) method. MDA is an end-product of oxidative lipid degradation, itself biologically active. TBARS are usually regarded as an indicator of MDA production, despite their limitations due to lack of specificity (Esterbauer et al., 1991). Biological end-points of ROS can be determined as functional, metabolic and morphologic parameters. The generation of ROS was found in humans also by coronary sinus sampling during primary PTCA for myocardial infarction (Grech et al., 1995). Experimentally, estimation of the role of

ROS is possible by pharmacological addition of scavengers or antioxidants, or manipulation with endogenous protection. Usually a combination of indirect measurements is employed to study the effects of ROS.

Endogenous defence against ROS

Under physiological conditions the tissue concentration of free radicals is maintained by a system of enzymatic and non-enzymatic antioxidants. It is estimated that approximately 5% of the oxygen consumed by normal tissues is transformed into ROS (Lefer and Granger, 2000). Superoxide dismutase (SOD) enzymatically protects against $\cdot O_2^-$ (Thompson and Hess, 1986). H_2O_2 is enzymatically scavenged by gluthathione peroxidase or catalase (Thompson, 1986). Ischemia causes a decrease in mitochondrial glutathione peroxidase activity, thus detoxification of H_2O_2 is impaired (Shlafer et al., 1987). SOD and catalase have recently been found to be protective in myocardial ischemia-reperfusion injury in a genetically engineered animal model (Chen et al., 1998). There is no physiological scavenger against ·OH, and the defence is directed against preventing its formation. The enzymatic defence predominates within the cell. Extracellular defence is mainly exerted through metal binding proteins such as transferrin and ceruloplasmin (Thompson, 1986).

Among the non-enzymatic antioxidants, α-tocopherol (Vitamin E) is the most efficient in the lipid phase. It is a fat-soluble antioxidant, which is mainly present in the plasma, low-density lipoprotein particles and the cell membrane (Burton et al. 1982). In comparison with striated muscular tissue the myocardium has a high concentration of Vitamin E (Kornbrust and Mavis, 1979). It has recently been shown that plasma Vitamin E levels correlate well with coronary endothelial function (Kinlay et al., 1999). Other important endogenous antioxidants are Vitamin C (Packer et al., 1979) and ubiquinone (Forsmark et al., 1991).

Lipid peroxidation

Lipid peroxidation has been suggested to be a main mechanism of oxidative injury (Noronha-Dutra and Steen, 1982; Ceconi et al., 1992; Parinandi et al., 1991). The removal of a hydrogen atom from the unsaturated site in a fatty acid results in the production of a lipid radical. The addition of ROS to lipid radicals results in the formation of lipid peroxides (Singal 1999). Lipid peroxidation is accompanied by an altered distribution of membrane-located receptors

(Davies, 1987), denaturation of proteins and inactivation of enzymes (Kramer et al., 1986), alterations of cellular membrane lipoproteins causing functional disturbances (Manciet and Copeland, 1992) and ultimately the destruction of membranes and cell necrosis (Bolli, 1991; Southorn and Powis, 1988). Inhibition of lipid peroxidation has been shown to be protective against reperfusion-induced myocardial damage (Janero and Burghardt, 1989; Ferrari et al., 1991). Lipid peroxidation is characterised by three phases: initiation, propagation and chain termination (Esterbauer, 1991). Chain-breaking antioxidants can suppress free radical chain oxidation during the oxidation of lipids. *In vivo*, ascorbic acid is an outstanding antioxidant in the plasma. However, it may not be an ideal antioxidant against lipid peroxidation of lipoproteins and membranes due to its hydrophilicity. The most important lipid-soluble chain-breaking antioxidant in biological membranes *in vivo* is Vitamin E, and it has been tested and proven effective in protecting from oxidative stress (Halliwell, 1990). Vitamin E-treated rats showed a decrease in mortality and infarct size due to coronary occlusion by approximately 50% compared to the control group (Sethi et al., 2000). In another study, however, Vitamin E was not effective in reducing infarct size after ligation of a coronary artery (Bellows et al., 1995). The disadvantage of Vitamin E is that its extreme lipophilicity causes a slow availability to cardiomyocytes. This can explain why treatment with Vitamin E just prior to the ischemia-reperfusion episode is of limited value only, and why effective pretreatment needs large doses employed (Axford-Gately et al., 1993). These pharmacokinetic characteristics of Vitamin E render it less feasible in acute ischemic situations; hence other drugs should be investigated with similarity in action to Vitamin E but with a more favourable pharmacokinetic profile.

Therapeutical possibilities

It is important to establish optimal conditions for reperfusion so as to preserve as much of the myocardium as possible, particularly in view of the fact that the prognosis following myocardial infarction is highly influenced by the remaining left ventricular function. As a growing body of evidence exists that oxidative processes play a role in cardiac diseases, it seems reasonable to include antioxidants among therapeutic strategies. Some of the most prominent pharmaceuticals currently employed in cardiovascular medicine, like captopril or carvedilol are already known to have antioxidant properties as well (Wattanapitayakul and Bauer, 2001).

A number of antioxidants have been tested for their possible protective effect in ischemia-reperfusion injury of the heart (Opie, 1989, Manson et al., 1993). The results are far from consistent, ranging from a marked protection to no protective effects at all (Uraizee, 1987, Klein et al., 1991). Experimentally, SOD and catalase have gained the most attention as of possible value in the treatment of reperfusion injury. These antioxidant enzymes have been found to be cardioprotective in a number of experimentally induced ischemia-reperfusion models (Jolly et al., 1984; Mehta et al., 1989). Unfortunately, SOD treatment was reported to be ineffective in many other publications. Moreover, recombinant SOD (h-SOD) provided no additional protection in a large, multicenter clinical trial in patients undergoing angioplasty (Flaherty, 1994; Uraizee et al., 1987). Consequently, it has been suggested that antioxidant strategies should involve low molecular weight compounds that are capable of both scavenging ROS and gaining access to the intracellular compartments where ROS are produced and where a major part of the deleterious action of ROS is effected (Lesnefsky, 1992; Maxwell and Lip, 1997).

H290/51

A novel indenoindole derivative, H290/51 (cis-5, 5a, 6,10,b-tetrahydro-9-methoxy-7-methylindeno [2,1-b] indole), a low molecular weight (288 MW) inhibitor of lipid peroxidation, is an effective quencher of radical chain propagation rather than an inhibitor of free radical production. Similarly to Vitamin E, it has the ability to recycle with ascorbate (Björquist et al., 1996). The lipophilicity of H290/51 is considerably lower than that of vitamin E. The log P value (-log[compound soluble in octanol]/[compound soluble in water] is 3.5 for H290/51. Consequently, H290/51 is much more hydrophilic than vitamin E (log P > 12), but more lipophilic than Vitamin C (log P < 2.9). The inhibitory potency, IC_{50}: -log[compound] to reduce TBARS formation by 50% in Fe/ascorbate-induced production in phospholipid vesicles, is 8.2 for H290/51 compared with 5.6 for Vitamin E (Westerlund et al., 1996). This drug is of particular interest, since its small molecular weight and balanced hydro- and lipophilicity make it suitable for rapid cellular uptake. It can rapidly penetrate cell membranes to intracellular sites of ROS generation, lipid peroxidation, and oxidant injury. Theoretically H290/51 may effectively give protection to jeopardised subcellular compartments and protect the myocardium fromischemia- reperfusion injury.

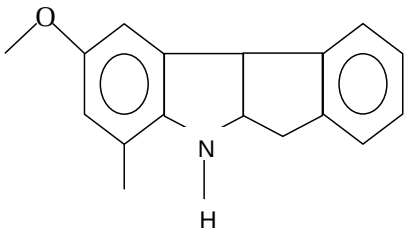

Fig. 1
Chemical structure of indenoindole derivative H29/51

AIMS OF THE STUDY

The main objective of the present investigation was to study the effects of inhibiting lipid peroxidation by a novel, low molecular weight antioxidant in different models of ischemia-reperfusion.

The individual studies were conducted with particular emphasis on the following objectives:

I. To investigate the protective efficacy of H290/51, a hydrophilic compound with similar effects to Vitamin E on hypoxia-reoxygenation injury of isolated cardiomyocytes.

II. To study its effect on oxidative injury induced by exogenous ROS in isolated, perfused rat hearts.

III. To examine its effect on ultrastructural changes in isolated rat hearts subjected to ischemia and reperfusion.

IV. To assess the effect of inhibiting lipid peroxidation on functional and biochemical injuries caused by global, normothermic ischemia-reperfusion in isolated rat hearts

METHODS

Animals

The experiments were approved by the Regional Ethics Committee at the Karolinska Institute, Stockholm, Sweden. All animals received care in compliance with *the Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. .

Experimental set-up

Cultured cardiomyocytes

Neonatal hearts were collected from rats between 2 and 6 days of age. The ventricles were dissected free, placed in Hanks balanced salt solution without Ca^{2+} and Mg^{2+}, but with 0.35 g/l sodium carbonate, and cut in small pieces. The cells were dispersed in the same solution, but supplemented with collagenase solution (type 1, 0.8 mg/ml) at 37 C for 10 min, and centrifuged at 160 g for 5 min. Subsequently, five serial 20 minutes digestions were performed, and the cells from the initial digestion were discarded. After each digestion period the cells were collected by centrifugation and suspended in Ham's F10 culture medium, supplemented with 10% fetal bovine serum, glutamine 2 mmol/l, penicillin 50 IU/ml, and streptomycin 50 μg/ml. To enrich the isolation of myocytes, the cells were replated twice for 30 and 90 min. The myocytes were plated on 35 mm plastic culture dishes at a density of approximately 9×10^5 cells/ml (1.8×10^6 cells/dish). The cells were kept in culture for 6 days at 37 ^oC and 5% CO_2 under aerobic conditions in a carbon dioxide incubator (Forma Scientific). During this period the cells were beating spontaneously at a frequency of 20-40 beats/min. Each culture dish contained 0.9 ± 0.2 mg protein ($n = 9$). The supplemented F10 medium was changed each day. Before starting the experiments, the medium was removed and changed to 4 ml F10 medium without antioxidant and serum, but supplemented with glutamine. To obtain hypoxic conditions, the medium was saturated by bubbling argon. The culture plates were transferred to specially designed, air tight, thermostat-controlled chambers with the ability to withdraw samples from each culture dish (Ek, 1994). The experiment lasted for 5 h, including 1 h hypoxia, when a mixture of 95% N_2 and 5% CO_2 was slowly gassed above the cells, followed by 4 h reoxygenation (95% O_2 and 5 % CO_2). The hypoxic

procedure reduced oxygen from 20 to 5% after 3 min, and to 1.2% after 10 min. Reoxygenation increased oxygen to 24% after 5 min, and was 80% within 30 min. Cell damage was assessed by leakage of lactate dehydrogenase (LDH) to the medium, and was analysed in aliquots of 100 µl after 1, 2, 3, 4, and 5 h.

Isolated rat heart

Male Sprague-Dawley rats, weighing 250-300 g, anesthetised with diethyl ether were used. Heparin (200 IU) was injected into the femoral vein. The hearts were rapidly excised through a median sternotomy and immediately immersed into ice-cold Krebs-Henseleit buffer during preparation. The ascending aorta was cannulated for retrograde perfusion as a modified Langendorff preparation (Langendorff 1895). Perfusion pressure (100 cm H_2O) and temperature (37 ^oC) were kept constant.

In experiments II and IV left ventricular systolic (LVSP) and end-diastolic pressures (LVEDP) were measured isovolumetrically via a fluid filled latex balloon introduced into the left ventricle through the left atrium. Left ventricular developed pressure (LVDP) was calculated (LVSP-LVEDP). Heart rate (HR) was counted from the pressure curves. Coronary flow (CF) was measured by timed collections of the coronary effluent.

The hearts were perfused with Krebs-Henseleit buffer containing glucose (NaCl 118.5mM, $NaHCO_3$ 25.0 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgSO_4$ x7H_2O 1.2 mM, $CaCl_2$ 2.4 mM, Glucose x H_2O 11.1 mM) and bubbled with a gas mixture containing 95% O_2 and 5% CO_2. Coronary flow (CF) was measured by collecting the coronary effluent every 2.5 min to adjust drug infusion to changes in flow (see page 19, “Chemicals”). The perfusion protocol started with a 25 minutes stabilisation period. Only hearts that met the following criteria at the end of stabilisation were included: CF: 7-15 ml/min, HR: 260-360 beats/min, LVSP: 50-150 mmHg and LVEDP: 0 mm Hg. In Study III the insertion of the intraventricular balloon was omitted and only CF was used to evaluate stability. In experiments III and IV global, normothermic ischemia was induced by clamping the inflow tubing for 30 minutes, followed by 20 minutes of reperfusion. In study II hydrogen peroxide (H_2O_2) was added to the perfusate to groups as listed below.

In Study IV LVEDP, CF, and HR were measured at 0, 2, 5, 10, 15, 20, 30, 40 and 60 min, and in Study II at the same time points except 2 and 40 min. At time 0, and after 2, 5, 10, 20 and 60 minutes of reperfusion aliquots of coronary effluent were collected in precooled tubes, rapidly frozen, and stored at –80ºC until analysed for LDH activity. (Sampling was not done at 2 and 5 min in Study IV).

In Studies II and IV hearts were freeze-clamped with liquid nitrogen at the end of the experiments (60 min), and stored at –80 ºC before processing and analysis of tissue contents of thiobarbituric acid-reactive substances (TBARS). Additional hearts were freeze-clamped after 15 and 20 min (n = 8 in each group at each time point for freeze clamping). Results from hearts in group 1 obtained at time 0 served as baseline values for all freeze-clamped groups, because all hearts were treated equally during stabilisation. Hemodynamic data from the freeze-clamped hearts are not included.

Preparation for Electron Microscopy

At the end of experiments hearts were perfusion-fixed with McDowel's fixative for 10 min at room temperature and at a perfusion pressure of 60 cm H_2O (McDowel and Trump, 1976) . Only the ventricular myocardium was used for morphometry. The volume of the ventricular myocardium was measured by liquid displacement and expressed in µl for further calculations. The hearts were then kept in fixative. At the final processing the ventricles were cut into 1 and 2 mm thick parallel slices with a razor blade apparatus (Baddeley et al., 1986) . All the 1 mm thick slices were then placed at random under a regularly perforated Plexiglas plate. The perforations were placed in a square (32mm × 32 mm) and separated from each other by a distance of 2 mm. Each perforation had a diameter of 1 mm. One biopsy was taken at every second perforation using a biopsy needle. Postfixation was performed in 1% aqueous OsO_4 for 2 hours. After a subsequent wash in buffer the pieces were stained in 2% uranyl sulphate for 1.5 h. The pieces were dehydrated in a series of graded alcohol before embedding in a mixture of Epon and Araldite. Eight pieces of ventricular biopsies were taken from each animal, and out of this, five samples were selected randomly for further stereologic processing. Thin sections were stained with Reynolds lead citrate for 8 min, and subsequently

with 5% uranyl sulphate for 5 min. A randomised procedure was used to select fields to be photographed. A section from each randomly selected biopsy was covered by a grid consisting of 10 fields and every second field was photographed totalling 5 fields per sample. This procedure ensured randomness at all steps in the process. All micrographs were taken at a magnification of ×4000. Thus, a total of 25 micrographs per heart were the basis of quantitative evaluation.

Stereology

Volume fractions were estimated using the point counting method (Weibel, 1973). A counting grid with sampling points was laid on the micrographs and points overlying on different tissue and cell components were counted and related to points falling on the whole myocardium. Volumes of mitochondria, altered mitochondria and cellular edema were estimated as fractions of myocyte volume and denoted: V_v (mito/myocyte), V_v (alt mito/myocyte), V_v (cell edema/myocyte). Volume fractions of myocytes, interstitium and capillaries in total myocardium were estimated: V_v (myocyte /myocard), V_v (interstitium /myocard), V_v (capillary /myocard). The absolute volume of different tissue components was calculated by multiplying the volume fraction of the tissue component with the respective measured ventricular volume and given as µl. In addition, the planar sections of cells appearing on micrographs from different groups were inspected. A semiquantitative evaluation was undertaken. Rounded mitochondria with reduced contrast (loss of matrix density) and more pronounced changes in mitochondria were counted as altered mitochondria. Cellular edema was defined as clear spaces in the cytosol of the myocytes.

Experimental groups

Studies with cultured myocytes (Study I)

Compound H290/51 was added to the culture medium for the 6 days of culturing at a final concentration of 10^{-8}, 10^{-7}, 10^{-6}, and 5×10^{-6} mol/l in 0.1% ethanol. The medium was subjected to 1 hour of hypoxia. LDH leakage was assessed from samples taken after 1, 2, 3 and 4 hours after reoxygenation.

Isolated rat hearts subjected to oxidant stress by hydrogen peroxide (Study II)

Group I: H_2O_2 was given for 10 minutes, followed by 50 minutes of recovery (n = 9)
Group II: Vehicle of H290/51 was given to the buffer for 10 minutes (n = 9)
Group III: H290/51 was added for 10 minutes (n = 8)
Group IV: Like group I, and H290/51 was added from time 0 onwards (n = 12)
Group V: Like group I, and H290/51 was added from 10 minutes onwards only (n = 12)

Isolated rat hearts subjected to ischemia-reperfusion – quantitative electron microscopical morphometry (Study III)

Group A: control hearts perfused with buffer and vehicle subjected to ischemia and reperfusion. Hearts were sampled at the end of stabilisation (time 0), at the end of ischemia (30 minutes) and at the end of reperfusion (50 minutes; n=6 at each time point in each group).
Group B: like Group A, but with H290/51 added throughout both stabilisation and reperfusion. Hearts were sampled as in Group A.
Group C: hearts were not subjected to ischemia, but H290/51 was added throughout the experiment. Hearts in this group were only sampled for morphometric evaluation at 50 min.

Isolated rat hearts subjected to ischemia-reperfusion – biochemical and functional analysis (Study IV)

Group 1: ischemia – reperfusion with buffer only (n = 25)
Group 2:Like 1, but vehicle added throughout 25 min stabilisation and reperfusion (n = 18)
Group 3: Like group 2, but H290/51 added throughout reperfusion (n = 21)
Group 4:Like 1, but H290/51 added throughout both stabilization and reperfusion (n = 13)

Biochemistry

LDH activity in the coronary effluent and cell medium was measured in a Cobas Bio centrifugal analyser (Hoffmann-La Roche, Switzerland) by using a commercial reagent kit (Boehringer-Mannheim, Germany, Cat. No. 191353). LDH in the coronary effluent is presented as activity in units (U) released per minute.

In the study on isolated myocytes the LDH values were expressed as reoxygenation/hypoxia ratios, calculated from the LDH leakage (U/l medium) after different times of reoxygenation divided by the LDH leakage after 1 hour of hypoxia.

TBARS. Myocardial samples were homogenised in a glass/glass homogeniser using a 0.1 M potassium phosphate buffer at pH 7.4. The tissue weight/volume ratio was ½, and the buffer contained 2.5 mg/ml desferoxamine. The formation of TBARS was quantified using an automated continuous-flow technique (Svensson et al., 1993).

Chemicals

H290/51 (AstraZeneca R & D, Mölndal, Sweden), was dissolved in a vehicle containing polyethylene glycol, ethanol and water in a 40:10:50 w/w percentage ratio. H290/51 or vehicle alone was administered with an infusion pump at a rate of $1/20^{th}$ of coronary flow into a mixing chamber immediately upstream to the aortic cannula, to obtain a concentration of 10^{-6} mol/l in the coronary circulation. In experiment II H_2O_2 was added directly to the perfusate at a concentration of 180 μM.

Statistical methods

Data in Studies I, II and IV were presented as mean ± standard error of the mean (SEM). In Study III values were given as mean ± standard deviation (SD). The differences between groups were calculated by the non-parametric Mann-Whitney test. In Study I *p*-values were corrected according to the Bonferroni method. In Study I for analysis of arrhythmias, data were pooled according to groups to raise the number of observations in each category, and the chi-square test with Yates correction was used. The pairwise Wilcoxon Signed Rank Test was used to analyse differences within groups. In all statistical tests $p < 0.05$ was considered significant.

RESULTS

Hypoxia-reoxygenation injury of isolated cardiomyocytes

The effect of H290/51 on LDH leakage induced by reoxygenation is presented in Fig. 2. The time related increase in LDH leakage was reduced by increasing the concentration of H290/51 in the medium. At a concentration of 10-6 mol/l, LDH leakage was almost totally inhibited. The pIC50 to reduce LDH leakage by 50% was calculated as 7.2 ± 0.4.

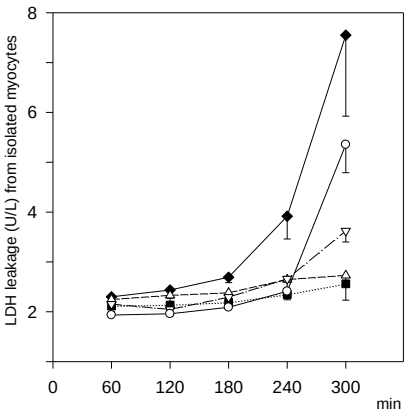

Fig. 2. Effect of H290/51 on reoxygenation induced LDH leakage in isolated myocytes. LDH-values are expressed as reoxygenation/hypoxia ratios, calculated from the LDH-leakage expressed in U/L after l, 2, 3 and 4 hours reoxygenation divided by the LDH-leakage after 1 hour hypoxia. Control (◆-◆), 0.01(○ - ○), 0.1(▽- ▽), 1.0 (■ - ■) and 5.0 (△-△) μM H290/5l. Mean values ± SEM, n=3.

Isolated rat hearts subjected to oxidant stress by hydrogen peroxide

Functional measurements

Left ventricular end-diastolic pressure

LVEDP remained at baseline level (0 mmHg) throughout perfusion in hearts given either H290/51 (group III) or vehicle alone (group II) (Fig. 3). LVEDP increased significantly already after 5 minutes of H_2O_2 administration (group I) to 6±1 mmHg and remained elevated throughout the experiment with a maximum of 32±7 mmHg at 20 min. Administration of H290/51 (group IV) attenuated the increase in LVEDP. At 15 min LVEDP was 16±3 mmHg in group V and then gradually decreased, being less than in group I at 20 min (9±3 mmHg, $p<$ 0.006), 30 min (3±1 mmHg, $p<0.001$) and 60 min (2±1 mmHg, $p<0.002$)(Fig. 3). At 30 and

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60 min LVEDP in group IV was not different from controls (group III). Addition of H290/51 to perfusate after the end of H_2O_2 perfusion (group V) resulted in an intermediate course as compared to group I and group IV (Fig. 3), without being significantly different, except for group 4 at 30 min (p<0.02).

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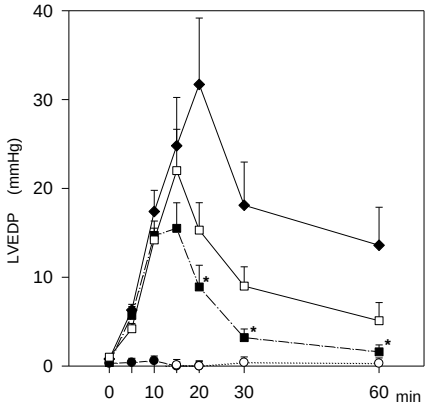

Fig. 3. Left ventricular end-diastolic pressure (LVEDP) in isolated, perfused rat hearts. Group I (◆—◆): H_2O_2 (180 μM) for 10 min followed by 50 min recovery. Group II (○—○) and III (●—●) were given vehicle and H290/51, respectively, for 10 min. Group IV (■—■) was perfused with H_2O_2 like group I, as well as H290/51. In group V perfusion with H290/51 started when H_2O_2 perfusion ended (□—□). *denotes p<0.025 compared to H_2O_2-perfused hearts. Values are mean ± SEM.

Left ventricular developed pressure

Administration of H290/51 and/or vehicle (groups II and III) alone had no significant effects on LVDP. H_2O_2 (group I) reduced LVDP to a minimum of 25±2 mmHg after 10 min. Between 5 and 20 min LVDP was significantly reduced as compared to the baseline value (p< 0.001). LVDP gradually recovered to 84±7 mmHg after 60 minutes. Neither addition of H290/51 during H_2O_2 perfusion and recovery (group IV), nor during recovery only (group V) significantly influenced the H_2O_2-induced reduction in LVDP (Table 1).

Coronary flow

CF was 11±0, 10±1, 11±1, 11±1 and 12±1 ml/min at time 0 in groups I-V, respectively (Table 1). H_2O_2 (group I) increased CF to 15±1 ml/min after 5 min (p<0.001), and it remained higher than the basal value (time 0) throughout the observation period. Addition of H290/51 or the vehicle to the perfusate (groups II and III) did not significantly influence CF. In group IV CF was similar to group I or even higher (20 min, p<0.02)(Table 1). In group V CF was not different from group I).

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Heart rate

There was a tendency of HR to increase during H_2O_2 perfusion in groups I, IV and V, but no significant intra- or intergroup differences were recorded during the observation period.

	Group	0 min	5 min	10 min	15 min	20 min	30 min	60 min
CF (ml/min)	I	11 ± 0	15 ± 1**	14 ± 0**	14 ± 1*	14 ± 1*	15 ± 1*	13 ± 1*
	II	10 ± 1	13 ± 1	13 ± 1	11 ± 1	11 ± 1	12 ± 1	12 ± 1
	III	11 ± 1	13 ± 1	12 ± 1	13 ± 1	13 ± 1	13 ± 1	13 ± 1
	IV	11 ± 1	15 ± 0**	14 ± 0*	14 ± 0*	16 ± 0**♥	17 ± 1**	13 ± 1
	V	12 ± 1	15 ± 0**	13 ± 1	12 ± 1	13 ± 1	14 ± 1*	11 ± 0
LVSP (mmHg)	I	90 ± 6	55 ± 4**	42 ± 2**	60 ± 3**	82 ± 5*	95 ± 3	97 ± 4
	II	88 ± 6	104 ± 6	109 ± 5	102 ± 11	103 ± 6	107 ± 5	102 ± 4
	III	95 ± 8	107 ± 11	114 ± 12	110 ± 10	111 ± 10	111 ± 8	104 ± 8
	IV	90 ± 4	62 ± 5**	45 ± 2**	62 ± 2**	73 ± 2**	93 ± 5	86 ± 4
	V	91 ± 5	58 ± 3**	49 ± 2**	63 ± 3**	71 ± 2**	83 ± 4*	85 ± 6
LVDP (mmHg)	I	90 ± 6	48 ± 4**	25 ± 2**	35 ± 3**	50 ± 4**	77 ± 6	83 ± 6
	II	88 ± 6	103 ± 6	108 ± 6	102 ± 11	103 ± 6	107 ± 5	103 ± 4
	III	95 ± 8	107 ± 11	114 ± 11	110 ± 10	111 ± 9	111 ± 8	104 ± 8
	IV	90 ± 4	56 ± 6**	30 ± 3**	46 ± 4**	64 ± 4*	90 ± 5	85 ± 4
	V	90 ± 5	53 ± 3**	35 ± 2**	41 ± 5**	55 ± 3**	74 ± 5	80 ± 6

Table 1. Hemodynamic measurements in Langendorff-perfused rat hearts perfused with H_2O_2 alone or with addition of the antioxidant H290/51. Values are mean ± SEM of left ventricular systolic pressure (LVSP), coronary flow (CF), left ventricular developed pressure (LVDP). *, ** and *** denote $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with initial value, ♥ denotes $p<0.05$ compared to H_2O_2 alone. For details about groups, see methods.

Biochemical data

Release of lactate dehydrogenase

At time 0 there was no significant difference between groups in LDH activity release. LDH release did not change during perfusion in groups II and III (Fig. 4). After 10 min perfusion with H_2O_2 (group I), LDH activity was similar to controls. However, LDH increased during recovery (maximum 64 ± 29 U/min × 10^{-3} at 30 min, $p < 0.02$ compared to baseline value). H290/51 (group IV) significantly inhibited the H_2O_2 –induced increase in LDH, but not when it was given after H_2O_2 perfusion (group V).

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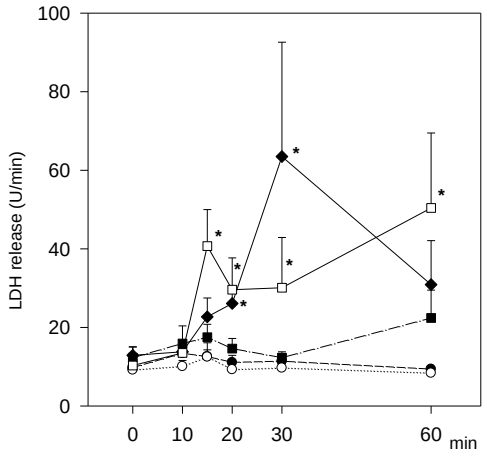

Fig. 4 Lactate dehydrogenase (LDH) activity released into the coronary effluent from V groups of isolated, perfused rat hearts. Group I (◆—◆) was given H_2O_2 (180 μM) for 10 min followed by 50 min recovery. Group II (○—○) and III (●—●) were given vehicle and H290/51, respectively, for 10 min. Group IV (■—■) was perfused with H_2O_2 like group I with the addition of H290/51 from time 0. In group V perfusion with H290/51 started when H_2O_2 perfusion ended (□—□). * denotes $p<0.05$ compared to baseline value. Values are mean ± SEM.

Tissue content of TBARS

The basal level of TBARS (time 0) was 0.6 ± 0.04 nmol/g tissue. The level of TBARS in groups II and III did not change throughout the experiments. After perfusion with H_2O_2 for 10 min TBARS increased to 3.1 ± 0.4 nmol/g ($p<0.001$). Five minutes later TBARS had returned to baseline level. Addition of H290/51 (group IV) significantly inhibited the H_2O_2-induced increase in TBARS at 10 min (0.9 ± 0.03 nmol/g, $p < 0.001$)(Fig. 5).

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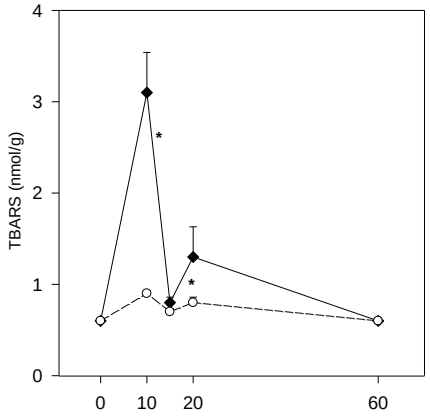

Fig. 5 Tissue levels of thiobarbituric acid-reactive substances (TBARS) in 2 groups of isolated rat hearts. One group is perfused with H_2O_2 (180 μM) from time 0 to 10 min, followed by recovery (◆—◆). The other group (○—○) was given H290/51 from time 0, in addition to H_2O_2 as described. Compared to baseline value TBARS were significantly elevated after 10 min in the group given H_2O_2 alone. * denotes that H290/51 inhibited the H_2O_2-induced increase in TBARS ($p<0.001$). Values are mean ± SEM.

Isolated rat hearts subjected to ischemia-reperfusion – quantitative electron microscopical morphometry.

Morphometry

Absolute volume of ventricular myocardium and different tissue components

There was no significant change in the volume of ventricular myocardium, myocytes, myofilaments, mitochondria or capillaries after 30 minutes of global ischemia in either group Table 2). However, the cytosolic volume was significantly increased in both groups after ischemia and returned to baseline after reperfusion. The extracellular interstitium increased in the control group compared to baseline at the end of ischemia. Treatment with H290/51 inhibited this increase. At the end of reperfusion the interstitium of both groups had increased compared to baseline, but there was no difference between the two groups. In addition to the increases of the volume of the interstitium, both the ventricular volume and the volume of mitochondria had increased after reperfusion in control hearts, but not in the H290/51 treated group. This difference was not significant between groups (Table 2).

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		0 min	30 min end of ischemia	50 min end of reperfusion
Ventricular volume	Group A	663 ± 92	768 ± 104	955 ± 84*
	Group B	775 ± 87	782 ± 94	922 ± 146
Myocyte	Group A	430 ± 81	473 ± 43	532 ± 69
	Group B	460 ± 80	554 ± 90	472 ± 139
Myofil	Group A	266 ± 65	219 ± 40	295 ± 63
	Group B	271 ± 36	286 ± 57	253 ± 94
Mitochondria (total)	Group A	142 ± 22	179 ± 27	204 ± 30*
	Group B	161 ± 36	206 ± 48	181 ± 55
Cytosol	Group A	19 ± 10	68 ± 33*	23 ± 15
	Group B	14 ± 4	47 ± 21*	24 ± 15
Interstitium	Group A	177 ± 47	247 ± 80*#	342 ± 109*
	Group B	232 ± 31	159 ± 50	389 ± 64*
Capillaries	Group A	62 ± 20	47 ± 20	80 ± 38
	Group B	82 ± 35	68 ± 17	61 ± 24

Table 2. Effects of 30 minutes of ischemia followed by 20 minutes of reperfusion on absolute volumes (µl) of ventricular myocardium and different tissue components in isolated, perfused rat hearts (controls = group A and hearts treated with H290/51 = group B) (n=6 in each group at each time point). * Denotes $p < 0.05$ compared to baseline value. # Denotes $p < 0.05$ between groups.

	0 min	30 min end of ischemia		50 min end of reperfusion	
Vv (myocyte/myocard)					
Group A	0.645 ± 0.060	0.622 ± 0.071		0.562 ± 0.090	
Group B	0.591 ± 0.036	0.707 ± 0.052	* #	0.505 ± 0.079	*
Vv (interstitium/myocard)					
Group A	0.355 ± 0.060	0.318 ± 0.062	#	0.356 ± 0.105	
Group B	0.305 ± 0.064	0.206 ± 0.067		0.431 ± 0.093	*
Vv (capillary/myocard)					
Group A	0.091 ± 0.022	0.059 ± 0.019	*#	0.083 ± 0.038	
Group B	0.104 ± 0.034	0.087 ± 0.021		0.065 ± 0.017	

Table 3. Effects of 30 minutes of ischemia followed by 20 minutes of reperfusion on the calculated volume fractions of different tissue components in isolated, perfused rat hearts (controls = group A and hearts treated with H290/51 = group B) (n=6 in each group at each time point). * denotes $p < 0.05$ compared to baseline value. # denotes $p < 0.05$ between groups.

	0 min	30 min end of ischemia		50 min end of reperfusion	
Vv (mito/myocyte)					
Group A	0.333 ± 0.023	0.380 ± 0.052	*	0.386 ± 0.049	*
Group B	0.347 ± 0.021	0.367 ± 0.040		0.387 ± 0.064	
Vv (alt mito/myocyte)					
Group A	0 ± 0	0.307 ± 0.094	*	0.158 ± 0.187	*
Group B	0 ± 0	0.233 ± 0.145	*	0.200 ± 0.139	*
Vv (cell edema/myocyte)					
Group A	0.047 ± 0.025	0.144 ± 0.070	*#	0.044 ± 0.030	
Group B	0.031 ± 0.012	0.083 ± 0.033	*	0.054 ± 0.039	

Table 4.

Effects of 30 minutes of ischemia followed by 20 minutes of reperfusion on the calculated volume fractions of different subcellular organelles in myocytes in isolated, perfused rat hearts (controls = group A and hearts treated with H290/51 = group B) (n=6 in each group at each time point). * Denotes $p < 0.05$ compared to baseline value. # Denotes $p < 0.05$ between groups.

Volume fractions of different tissue components in the myocardium

Relative volumes of tissue components calculated as volume fractions (V_v) of the myocardium are presented in (Table 3). The volume fraction of myocytes increased after ischemia in the treated group, and at this time point it was also higher than in the control

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group. During reperfusion it decreased in both groups without any difference between groups. Ischemia increased the volume fraction of the interstitium in control hearts compared to baseline. This increase was inhibited in the group given H290/51, but during reperfusion the volume fraction of the interstitium increased in the treated group. The decreased volume fraction of capillaries in the myocardium after ischemia was inhibited by H290/51(Table 3).

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Volume fractions of subcellular components versus myocytes

Volume fractions of mitochondria increased in control hearts at the end of ischemia and remained high during reperfusion (Table 5). This increase was prevented by H290/51, but there was no difference between groups. Alterations of mitochondria were observed at the end of ischemia and during reperfusion in both groups without any effect of H290/51. Ischemia increased the relative volume of cytosol within the cardiomyocytes (V_v cell edema/myocyte) in both groups, and this increase was significantly higher in hearts not pretreated with H290/51 (Table 5). During reperfusion the volume fraction of cytosol normalized in both groups.

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	0 min	30 min end of ischemia	50 min end of reperfusion
Vv (mito/myocyte)			
Group A	0.333 ± 0.023	0.380 ± 0.052 *	0.386 ± 0.049 *
Group B	0.347 ± 0.021	0.367 ± 0.040	0.387 ± 0.064
Vv (alt mito/myocyte)			
Group A	0 ± 0	0.307 ± 0.094 *	0.158 ± 0.187 *
Group B	0 ± 0	0.233 ± 0.145 *	0.200 ± 0.139 *
Vv (cell edema/myocyte)			
Group A	0.047 ± 0.025	0.144 ± 0.070 *#	0.044 ± 0.030
Group B	0.031 ± 0.012	0.083 ± 0.033 *	0.054 ± 0.039

Table 5. Effects of 30 minutes of ischemia followed by 20 minutes of reperfusion on the calculated volume fractions of different subcellular organelles in myocytes in isolated, perfused rat hearts (controls = group A and hearts treated with H290/51 = group B) (n=6 in each group at each time point). * denotes $p < 0.05$ compared to baseline value. # denotes $p < 0.05$ between groups.

Ultrastructural changes in the myocardium

Before ischemia myocytes with mitochondria and other cell organelles appeared normal as did the endothelial cells. There was no difference in control hearts perfused with H290/51

(Group C) at the end of perfusion compared to preischemic micrographs. The results of Group C are not further commented upon.

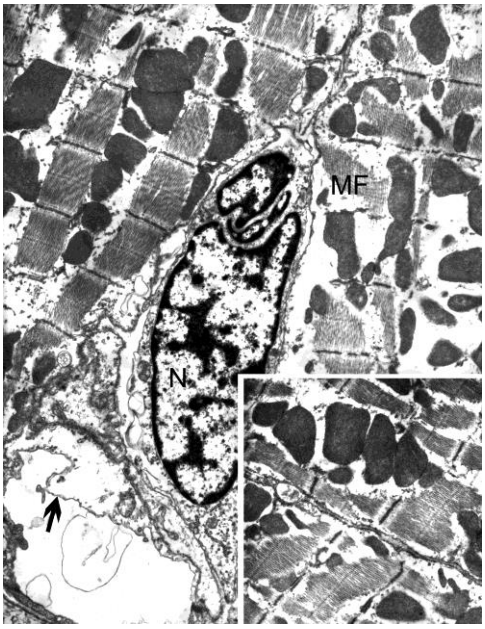

Fig. 6. Two myocytes are shown on the micrograph sampled from control group (Group A) after 30 min ischemia. One of them reveals a moderate intracellular edema with moderate mitochondrial swelling and intact sarcolemma. The other is damaged, edematous, the myofilamental pattern (MF) is disrupted. The cytoplasm of the endothelial cell is edematously swollen with luminal protrusions (arrow). Chromatin margination of the nucleus (N) is observed. The inserted micrograph shows a fairly well maintained structure, apart from fragmentation of the sarcolemma. ×10000.

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After ischemia areas with damaged cells were observed on most micrographs. In control hearts (Group A) myocytes were swollen with perimitochondrial and perimyofibrillar edema (Fig. 6). In severely damaged myocytes the sarcolemma was fragmented. Subsarcolemmal blebs occurred in more severely injured myocytes with massive cell swelling. The myofilamental pattern became irregular and Z lines were distorted. In more severely injured myocytes myofilaments were disrupted with a loss of Z lines. Mitochondria generally appeared swollen and rounded. The mitochondrial matrix showed a loss of electron density. Occasionally, mitochondrial cristae were broken or amorphous matrix densities appeared showing severe damage. Cristal adhesions were found in severely altered mitochondria. There was also evidence of tissue edema and endothelial cells showed swelling with luminal protrusions. Cellular debris accumulated where severe damage was evident. Thirty minutes of ischemia also caused margination of nuclear chromatin in both endothelial and myocardial cells. On micrographs sampled from hearts treated with H290/51 (Group B) the ischemic damage was less apparent, the structure remained fairly normal (Fig. 7.). In most myocytes

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myofilamental pattern was better preserved and edematous changes were less marked both intracellularily and interstitially. However, foci of damaged myocytes were observed.

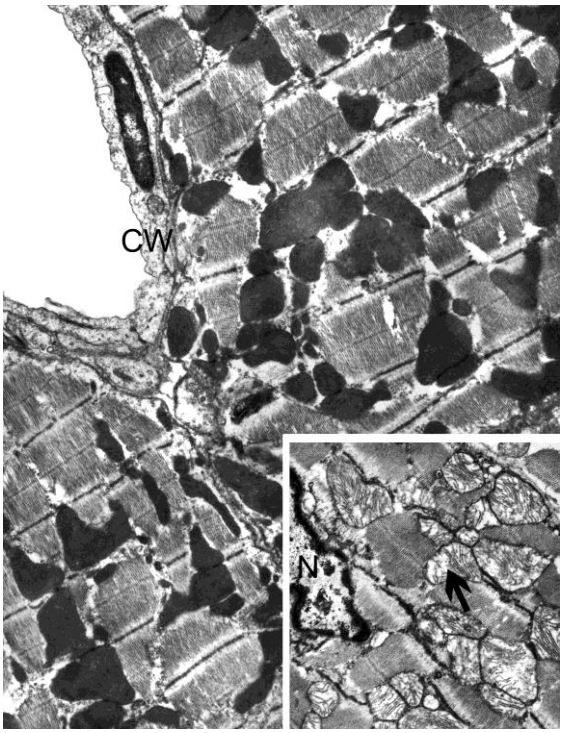

Fig. 7. Electron micrograph taken of rat heart treated with H290/51 exposed to 30 min ischemia. The structure is fairly well maintained, apart from a mild intracellular edema. The capillary wall (CW) reveals a normal structure, the chromatin is finely dispersed in the nucleus of the endothelial cell. On the insert the myocardium is damaged. The mitochondrial cristae are disrupted (arrow). The remnants of myofilaments are only partly preserved. The nuclear chromatin margination is conspicuous (N). ×10000.

Most ischemic changes persisted after reperfusion. There were no signs of progressive damage during reperfusion. Interstitial edema tended to increase in the control group. Within the myocytes the fluid accumulation seen after ischemia decreased during reperfusion. Myofilamental pattern was restored on many micrographs.

Isolated rat hearts subjected to ischemia-reperfusion – biochemical and functional analysis

Functional measurements

Heart rate and severe arrhythmias

No significant intra- or intergroup differences were recorded during the observation period. Severe arrhythmias occurred early in reperfusion in all groups. Significantly less severe arrhythmia occurred in hearts treated with H290/51 throughout perfusion (group 4) compared to controls (p<0.0001). (Fig. 8).

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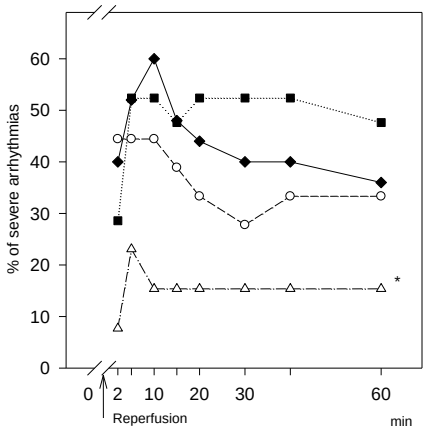

Fig. 8. Proportion of hearts with severe arrhythmias in isolated, Langendorff perfused rat hearts subjected to 30 min global ischemia followed by 60 min reperfusion. Group 1: Ischemic controls (◆-◆). Group 2: Vehicle added throughout perfusion (○ - ○). Group 3: Vehicle added before ischemia, and H290/51 added during reperfusion (■ - ■). Group 4: H290/51 added throughout the experiments (△-△). * denotes p<0.0001 compared to controls.

Left ventricular developed pressure

LVDP was 99±5, 94±5, 90±4 and 91 ±4 mmHg at time 0 in groups 1-4, respectively. LVDP decreased significantly during reperfusion to 62±3 mmHg at 90 minutes in ischemic controls (p<0.004, at 70 min and 90 min, group 1). LVDP was significantly higher at 70 and 90 minutes (p<0.008 and p<0.003, respectively) in hearts given H290/51 throughout perfusion (group 4) (Fig. 9). H290/51 given only during reperfusion had no significant effect on LVDP.

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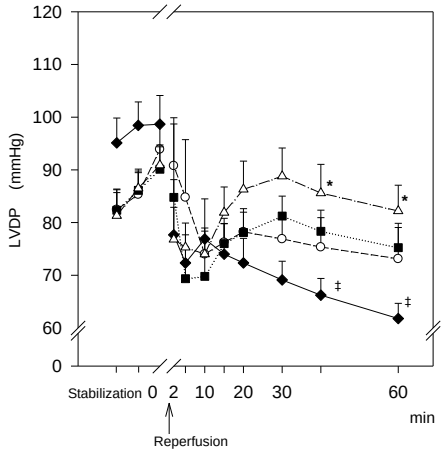

Fig. 9. Left ventricular developed pressure in isolated, Langendorff perfused rat hearts subjected to 30 min global ischemia followed by 60 min reperfusion. Group 1: Ischemic controls (◆-◆). Group 2: Vehicle added throughout perfusion (○ - ○). Group 3: Vehicle added before ischemia, and H290/51 added during reperfusion (■ - ■). Group 4: H290/51 added throughout the experiments (△-△). n=10 in all groups, values are mean ± SEM. ‡ denotes $p<0.05$ compared to initial value, * denotes $p<0.05$ compared to controls.

Left ventricular end-diastolic pressure

LVEDP was 0 mmHg at time 0 in all groups. LVEDP increased during reperfusion in group 1 to a maximum of 31 ± 12 mmHg after 5 min reperfusion (35 minutes of observation) ($p<0.001$), and remained high throughout the experiment. H290/51 (group 4) attenuated the increase of LVEDP after 32 and 35 minutes of observation ($p<0.001$ and $p<0.008$ respectively) (Fig. 10).

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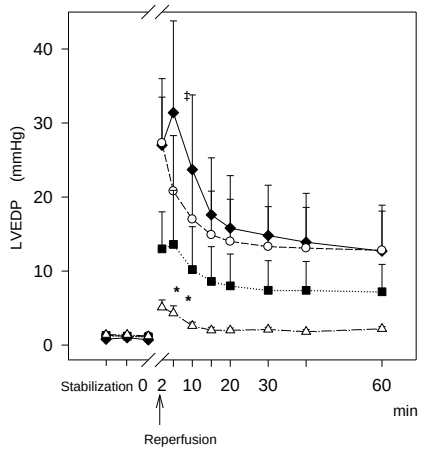

Fig. 10. Coronary flow in isolated, Langendorff perfused rat hearts subjected to 30 min global ischemia followed by 60 min reperfusion. Group 1: Ischemic controls (◆-◆). Group 2: Vehicle added throughout perfusion (○ - ○). Group 3: Vehicle added before ischemia, and H290/51 added during reperfusion (■ - ■). Group 4: H290/51 added throughout the experiments (△-△). n=10 in all groups, values are mean ± SEM. * denotes $p<0.05$ compared to controls.

Coronary flow

CF was 11±1, 13±0, 12±1 and 13±1 ml/min at time 0 in groups 1-4, respectively. CF gradually decreased to 6.1 ±0 ml/min after 90 min observation in ischemic controls (p<0.0001 from 45 minutes onwards). Addition of H290/51 (group 4) significantly attenuated the ischemia-induced decrease in CF from 45 minutes onwards (p<0.006 at all time points)(Fig. 11). Delayed administration of H290/51 (group 3) or adding vehicle only (group 2) also attenuated, although less pronounced, the ischemia-induced decrease in CF (p<0.01 at 50, 60 and 70 minutes) (Fig. 5).

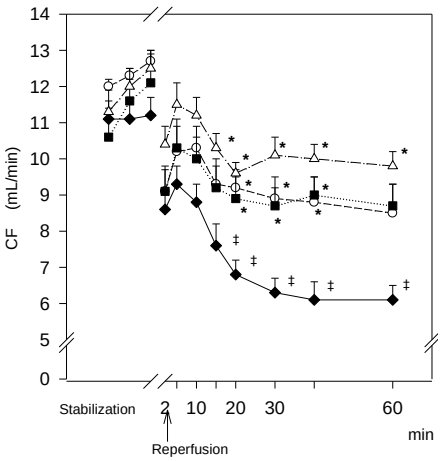

Fig. 11. Coronary flow in isolated, Langendorff perfused rat hearts subjected to 30 min global ischemia followed by 60 min reperfusion. Group 1: Ischemic controls (◆-◆). Group 2: Vehicle added throughout perfusion (○ - ○). Group 3: Vehicle added before ischemia, and H290/51 added during reperfusion (■ - ■). Group 4: H290/51 added throughout the experiments (△-△). n=10 in all groups, values are mean ± SEM. ‡ denotes p<0.05 compared to initial value, * denotes p<0.05 compared to controls.

Biochemical data

Release of LDH

LDH activity in the effluent was 20.2±2.2, 19.3±2.2, 18.4±2.1 and 20.5±3.2 U/min* 10^{-3} at time 0 in groups 1-4, respectively. LDH increased during reperfusion in ischemic controls (group 1) (maximum 94.5±36.0 U/min* 10^{-3} at after 5 minutes of reperfusion, p<0.002)(Fig. 12). Vehicle only did not influence LDH release (maximum 79.1 ±13.0 U/min* 10^{-3} at 5 min reperfusion, p<0.001)(Fig. 12). H290/51 throughout perfusion (group 4) inhibited the ischemia-induced LDH release, and was significantly different from controls after reperfusion

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for 5 minutes (p<0.002). Delayed administration of H290/51 (group 3) resulted in higher LDH release than group 4, but lower than groups 1 and 2.

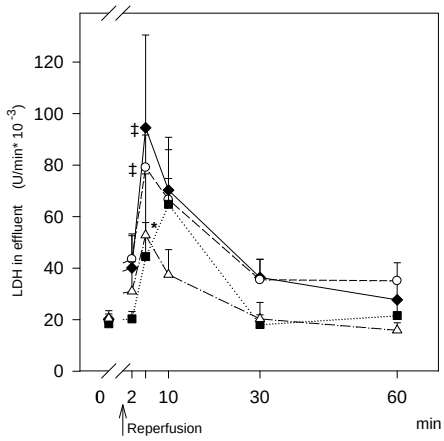

Fig. 12. Lactate dehydrogenase activity in the coronary effluent of isolated, Langendorff-perfused rat hearts subjected to 30 min global ischemia followed by 60 min reperfusion. Group 1: Ischemic controls (◆-◆). Group 2: Vehicle added throughout perfusion (○ - ○). Group 3: Vehicle added before ischemia, and H290/51 added during reperfusion (■ - ■). Group 4: H290/51 added throughout the experiments (△-△). n=10 in all groups, values are mean ± SEM. ‡ denotes p<0.05 compared to initial value, * denotes p<0.05 compared to controls.

Tissue content of TBARS

The group given vehicle only had the highest tissue content of TBARS at baseline (n.s.). There was no increase in TBARS after ischemia in group 1 compared to baseline. However, H290/51 reduced TBARS during reperfusion as compared to group 1 (at 40 and 60 min, p<0.004 and 0.001, group 4).

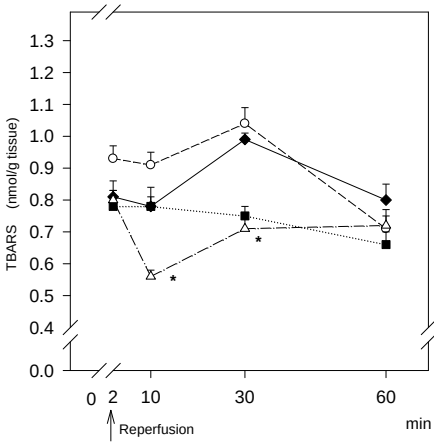

Fig. 13. Thiobarbituric acid-reactive subastances (TBARS) content in freeze-clamped isolated, Langendorff perfused rat hearts subjected to 30 min global ischemia followed by 60 min reperfusion. Group 1: Ischemic controls (◆-◆). Group 2: Vehicle added throughout perfusion (○ - ○). Group 3: Vehicle added before ischemia, and H290/51 added during reperfusion (■ - ■). Group 4: H290/51 added throughout the experiments (△-△). n=10 in all groups, values are mean ± SEM. * denotes p<0.05 compared to controls.

DISCUSSION

Does reperfusion injury exist?

The importance of reperfusion injury is often debated and somewhat controversial. There are numerous findings consistent with the occurrence of necrosis during reperfusion (Röth et al., 1987; Matsumura et al., 1998; Becker et al., 1999; Bolli, 1998). Some investigators, however, could not demonstrate the extension of necrosis on reperfusion by electron microscopy (Ganz et al., 1990) and attributed cell death predominantly to ischemia and not to reperfusion (Klein et al., 1996). Hence, it is not settled whether reversibly injured myocytes, potentially viable at the time of reperfusion, die as a consequence of reperfusion (Ferrari, 1997). There are arguments that the major part of myocardial injury occurs during ischemia, even if manifesting itself both histologically and functionally only after reperfusion (Robicsek and Schaper, 1997). The apparent controversy can, however, be resolved in part by making a distinction between reversible and irreversible reperfusion injury. There is a bulk of evidence supporting reversible reperfusion injury. On the other hand, the existence of lethal reperfusion injury remains highly controversial since there are not enough conclusive data available from reproducible, controlled experiments. Most investigators seem to agree, however, that reperfusion injury is a true entity and that there are sufficient theoretical reasons suggesting the existence of lethal reperfusion injury (Ferrari, 1998).

Updated view on ROS

It has recently become evident that ROS are more than simply cellular toxicants and that they may be important modulators of cellular gene expression patterns and also act as signal transduction molecules. Cellular H_2O_2 was transiently increased upon activation of platelet-derived growth factor (Sundaresan et al., 1995). ROS can activate different cellular signalling molecules and pathways, such as Ca^{2+} and protein tyrosine kinases. When cells are activated by extracellular stimuli, the cells produce ROS, which in turn stimulate other cellular signalling pathways, indicating that ROS act as second messengers (Kamata and Hirata, 1999). There is also a role of ROS in the induction of apoptosis. ROS can induce activation of caspases, which in turn cause cell death (Ceselli et al., 2001).

Methodological considerations

Pharmacological interventions and surgical procedures for the treatment of myocardial ischemia followed by reperfusion must rely on proper knowledge of the underlying pathophysiological mechanisms. The recommended therapy should be based on appropriately designed and conducted clinical trials. Nevertheless, much basic pathophysiological insight originates from animal experiments. Hence, animal models will continue to be of fundamental importance for the development of new treatment modalities. A major factor in an experimental model is the animal species used. Rats, dogs and pigs are most frequently used. Dogs are increasingly more difficult to obtain because of ethical concerns and high costs. The other disadvantage of dogs is the difference in coronary structure, that is, in contrast to humans, dogs have a substantial collateral circulation. In our experiments we used rats, which are very suitable if a large number of animals are required, mainly for *ex vivo* preparations. The availability of genetically homogeneous strains ensures reproducibility. These animals are easy to handle and inexpensive. The use of pigs is more relevant when a large animal is needed or when *in vivo* experimental conditions are needed.

Isolated hearts

In studies II through IV we employed the Langendorff model. It is a stable, easy to control preparation, and technically simple thus enabling several sets of experiments to be performed. A major advantage of the isolated heart model is that global ischemia results in a homogenous tissue response that enables the use of homogenized tissue for biochemical analyses. It should be noted, however, that the perfusion solution is lacking blood cells, plasma proteins, growth factors and this *per se* can lead to changes in heart function independent of ischemia-induced damages. The organ is also denervated and without hormonal stimuli. However, the isolated rat heart is a suitable model for screening drugs. Due to its advantages this model has become a first-choice model for studies of the biochemical and cellular consequences of myocardial ischemia-reperfusion. An intraventricular balloon was inserted for pressure readings in studies II and IV. This, however, may cause perfusion defects in the papillary muscle and eventually necrosis and therefore hemodynamic measurements were not employed in the experiment aimed at ultrastructural analysis (Study III).

Exogenous H_2O_2 as a model of oxidative injury

H_2O_2 was chosen as a convenient and reproducible model of generating injury by exogenous free radicals. H_2O_2 is inexpensive, easy to store and add to the perfusate. Increased postischemic tissue levels of H_2O_2 have been measured in isolated rat (Brown et al., 1989), and rabbit hearts (Shlafer et al., 1990). The levels of H_2O_2 correlated to depression of cardiac performance. Addition of exogenous H_2O_2 to mammalian cardiomyocytes in vitro produced a lethal peroxidative membrane injury (Janero et al., 1991). Infusion of H_2O_2 into rabbits caused a rapid irreversible deterioration of cardiac function (Ambrosio et al, 1992.). The exact mechanism of H_2O_2-induced injury is not completely elucidated. H_2O_2 may have direct cardiodepressive effects (Valen, 1993; Takemura, 1993). H_2O_2 is also the source of the highly toxic hydroxyl radical in the Haber-Weiss reaction catalyzed by intracellular or membrane bound transition metals, which initiates lipid peroxidation (Cochrane, 1991). The feasibility of perfusing isolated rat hearts with hydrogen peroxide to study the effects caused by reactive oxygen species has been tested in our laboratory (Skjelbakken, 1996). In that study H_2O_2 rather than the hydroxyl radical was found to be the main injurious ROS in this model. In our experiments a 10 min perfusion with H_2O_2 was employed to obtain a reversible, non-lethal model with marked cardiodepression. The length of administration of H_2O_2 and its concentration was selected on previous experiments in our laboratory where these parameters were necessary to achieve the required cardiac dysfunction (Valen, 1993).

TBARS

In Studies II and IV we employed TBARS as a nonspecific marker of abnormal tissue oxidation. Although TBARS did not increase during reperfusion, the tissue contents were lower during reperfusion in hearts perfused with H290/51. However, in isolated rat hearts injured by hydrogen peroxide for 10 minutes (Study II), significant functional and biochemical injury occurred concomitant with increased levels of TBARS in the heart. Adding H290/51 to the perfusate attenuated both functional and biochemical injury, and inhibited accumulation of TBARS (Study II).

TBARS are usually regarded as being representative of MDA, despite of well-known limitations as a measure of lipid peroxidation (Esterbauer et al, 1991). Additionally,

production of TBARS may not necessarily correlate with functional impairment after reoxygenation (Salaris and Babbs, 1988). Studies on possible accumulation of lipid peroxidation products in heart tissue and effluent from ischemic-reperfused hearts have yielded highly conflicting results. Generation of ROS without concomitant detection of MDA has been found in cardiac tissue. In a model of 10 minutes of normothermic ischemia to isolated rat hearts, ROS were measured directly with electron paramagnetic resonance spectroscopy, but no evidence of lipid peroxidation was found (Monpoil and Rochette, 1988). In another work, isolated rat hearts were exposed to exogenous free radicals in the perfusate, or subjected to ischemia for 60 minutes (Ballagi-Pordany, 1991). Only the group with the most severe radical-induced injury had increased tissue levels of MDA. MDA is an end product of lipid peroxidation, and not all free radical induced injury may lead to accumulation of end products of lipid peroxidation. No release of MDA was observed from the cardioplegic-reperfused human heart (Valen et al, 1994). It may be that the antioxidant defense was able to quench the peroxidation chain reaction before reaching the end product. Thus, only an extreme oxidant stress may be able to significantly increase the end products of lipid peroxidation.

H290/51

In the present experiments we studied the effect of H290/51, an indenoindole type compound, on ischemia-reperfusion injury in different experimental settings. Indenoindole compounds have previously been shown to inhibit lipid peroxidation in several test systems (Shertzer and Sainsbury, 1991; Shertzer and Sainsbury, 1988; Westerlund et al, 1996). As H290/51 is a small molecule with water-soluble and lipophilic properties, it will easily and rapidly get access to the intracellular sites of ROS generation, lipid peroxidation, and oxidant injury. These properties make H290/51 an interesting compound with clinical potential.

H290/51 was originally regarded as an inhibitor of lipid peroxidation only. However, it has other actions as well not related to its antioxidant properties. It inhibits upregulation of nNOS during heat stress (Alm et al. 2000) , and in the same model attenuates upregulation of 72 kD heat shock protein (Hedlund et al., 1999). These observations may indicate properties of H290/51 beyond inhibition of lipid peroxidaton. Vitamin E also has properties other than as an antioxidant only. It may inhibit atherogenesis through several other mechanisms at the

molecular and cellular levels (Meydani, 2001). Vitamin E can inhibit smooth muscle cell proliferation and growth during atherosclerosis through inhibition of protein kinase C activity (Boscoboinik et al., 1994). Vitamin E was shown to inhibit production of chemokines by the endothelium. The modulation of adhesion molecules appears to be through inhibition of ROS-sensitive signalling pathways (Wu et al., 1999).

Discussion of results

Myocytes subjected to hypoxia/reoxygenation

Reoxygenation of isolated myocytes after 1 hour of hypoxia resulted in a time-related injury evidenced by LDH leakage. H290/51 almost completely prevented this injury at a concentration of 10^{-6} mol/l. This concentration was therefore selected in further experiments with isolated rat hearts. Our findings were similar to that of the effect of Vitamin E reported in corresponding experimental conditions (Ek et al., 1994).

H_2O_2 perfusion

Exposure to exogenous H_2O_2 impaired left ventricular function evident as a marked elevation of LVEDP and a decrease in LVDP (Study II). Similar functional deterioration occurs in post-ischemic myocardial stunning (Bolli, 1998). The observed left ventricular dysfunction was partly reversible, the diastolic dysfunction being more long-lasting. H_2O_2 induced a sustained increase in CF. Concomitant with the functional changes LDH release occurred, indicating myocyte damage. The observed effects of H_2O_2 are in agreement with previous studies (Valen et al., 1993; Lesnefsky, 1991). The increase of TBARS in the hearts after perfusion with H_2O_2 confirmed that oxidative stress and lipid peroxidation occurred. Administration of H290/51 attenuated the cardiac injury induced by exogenous ROS. H290/51 inhibited the rise in LVEDP caused by H_2O_2 and LVEDP normalized by the end of the experiments in contrast to the group given only H_2O_2 where LVEDP was increased throughout the observation period. As the decrease in LVSP and LVDP was not influenced it appears that H290/51 attenuated ROS-induced diastolic, but not systolic dysfunction. In addition H290/51 inhibited LDH release after H_2O_2 exposure. Consequently H290/51 reduced both biochemical and functional injury induced by H_2O_2. Inhibition of H_2O_2-induced increase in TBARS demonstrates that H290/51 did inhibit lipid peroxidation in the present model of cardiac injury by exogenous

ROS. The delayed administration of H290/51 following H_2O_2 also resulted in a reduction, albeit less pronounced, of oxidant injury. This suggests that lipid peroxidation occurred even after the end of H_2O_2 administration.

Ultrastructural alterations after global ischemia

Ischemia and reperfusion caused interstitial and cellular edema, disruption of sarcolemma, swollen mitochondria and myofilament disruption. These findings are in agreement with earlier studies (Hearse, 1998; Jennings and Ganote1994; Schaper, 1986). Quantitative stereological morphometry suggested that the hearts treated with H290/51 had less injury than control hearts. After ischemia the increased interstitial volume and the increased volume fractions of both interstitium and myocytes were inhibited by H290/51. This demonstrates that both extra- and intracellular edema after ischemia was inhibited or reduced by this drug. In addition the volume fraction capillary/myocardium was less in the control group at the end of ischemia. A relatively greater interstitial volume due to edema in the control group may explain this. In explaining volume fraction differences it should be noted, however, that volume fractions are given as ratios. An altered volume fraction may be due to an altered numerator or an altered reference volume.

Surprisingly the differences between groups occurred at the end of ischemia, but no difference was found during reperfusion, although several changes compared to baseline were observed. One can speculate if some differences during reperfusion would have become apparent if the number of hearts had been increased or if the time point(s) for evaluation had been different. On the basis of our data we can only state that in the present study no tendency towards any difference between groups is evident in the reperfusion period. Consequently, from a morphological and ultrastructural point of view H290/51 has anti-ischemic rather than anti-reperfusion effects.

Another explanation of why the antioxidant was effective during ischemia is the fact that production of ROS takes place already during ischemia. This phenomenon was measured by electron paramagnetic resonance spectroscopy (Zweier, 1987) and recently with a fluorescent probe method (Becker et al., 1999). If there is a progressive ROS generation during ischemia,

it is evident why lipid peroxidation inhibitor H290/51 was more effective when given already during ischemia and not only from the start of reperfusion in Study IV. Possibly in Study III the present concentration of H290/51 was able to quench the ROS during ischemia, but was not capable to counteract the burst during reperfusion. This concentration was, however, protective against reoxygenation injury in cultured myocytes (Study I). The same concentration in Study IV resulted in attenuation of ischemia-reperfusion injury measured both functionally and biochemically.

In the present study the morphological alterations were surprisingly few, except for an increase of mitochondrial, interstitial, and the left ventricular volume indicating intra- and extracellular edema, with the ultrastructure becoming more normal during reperfusion. The increasing edema may be the continuation of an ischemic injury and does *per se* suggest that reperfusion did take place.

Ultrastructural changes of the mitochondria are a crucial step in the development of ROS-induced myocardial damage (Hegstad et al., 1994; Lucas and Szweda, 1998). H290/51 did not influence the appearance of mitochondria after ischemia. V_v(alt mito/myocard) remained similar in both groups at the end of 20 minutes of reperfusion. The absolute volume of mitochondria, however, was increased only in the group without H290/51, but only during reperfusion. The increase of V_v mito/myocyte observed in the untreated group both after ischemia and reperfusion indicated that mitochondria were swollen above the extent of the general myocardial swelling in this group. Others (Ytrehus, 1987) have reported results similar to our findings. In these experiments supplementation of superoxide dismutase and catalase to isolated rat hearts subjected to ischemia - reperfusion resulted in a better preserved sarcolemmal integrity. However, no differences were detected in mitochondrial swelling. A parallel effect was observed after reperfusion of dog skeletal muscle rendered ischemic (Ytrehus et al., 1995). It is possible that in our experiments the protection afforded by H290/51 was similar, i.e. a protection on cellular membranes and sarcolemmal integrity, but less on mitochondria.

Functional changes following global ischemia

Thirty minutes of global, normothermic ischemia impaired left ventricular function evident as a marked increase of LVEDP, and a decrease of LVDP and CF during reperfusion. Concurrently LDH release increased, indicating myocyte damage. The decrease in CF was more marked following ischemia/reperfusion in the control group indicating development of a no-reflow phenomenon. The exact mechanism of no-reflow implies many pathways, and only a part of these has been clarified. A major role of ROS is assumed through the induction of microvascular damage. The vascular injury may be manifest as microvascular spasm, extravascular compression due to interstitial edema, and endothelial swelling (Eeckhout et al., 2001). H290/51 given throughout perfusion inhibited both the biochemical and functional injury induced by ischemia-reperfusion. This is in accordance with a study performed in the working rat heart model, where the effect of H290/51 on ischemia-reperfusion injury after cold cardioplegic arrest was investigated (Wang et al., 1995). In this study, however, H290/51 was given only together with cardioplegia infusion at the onset of ischemia. The difference in our study was that with preischemic administration, H290/51 provided a more marked functional and biochemical protection compared to its effect when it was given only during reperfusion. Oxidant injury with production of ROS probably takes place predominantly in the first 30-60 seconds of reperfusion (Bolli, 1992). Ideally H290/51 should be at the site of ROS production before reperfusion starts for maximal effect, and this may explain that H290/51 during reperfusion only had less protective effect. However, we cannot exclude a possible direct anti-ischemic effect of H290/51 in addition to anti-reperfusion effects.

CONCLUSIONS

The effects of an inhibitor of lipid peroxidation on different settings of ischemia-reperfusion injury were tested. The antioxidant selected for the experiments H290/51, is an indenoindole derivative. H290/51 was able to inhibit LDH leakage from cultured myocytes subjected to hypoxia/reoxygenation indicating a protective effect against oxidative injury. This effect was dose-dependent. Oxidative injury was generated by exogenous H_2O_2 perfusion in an isolated rat heart model resulting in a largely reversible functional and biochemical impairment of cardiac function (Study II). H290/51 attenuated diastolic dysfunction as it inhibited the increase in LVEDP casued by H_2O_2. The protective effect of this antioxidant was further evidenced by the inhibition of LDH release and accumulation of TBARS after H_2O_2 exposure.

The effect of H290/51 on ischemia/reperfusion injury was studied in two experiments. Morphological alterations after global normothermic ischemia in the ultrastructure were evaluated by quantitative stereological morphometry and by qualitative histological examination in the isolated rat heart. H290/51 administration resulted in a better preserved sarcolemmal integrity and cellular volume control. The morphological difference was already present during ischemia but vanished during reperfusion. The protective effects of H290/51 on myocardial ultrastructure during ischemia could form the basis for the better hemodynamic function of treated hearts in the reperfusion period in Study IV.

Inhibition of lipid peroxidation after global normothermic ischemia resulted in a higher LVDP and less increased LVEDP (Study IV). The compound also inhibited malignant arrhythmias, attenuated the ischemia-induced decrease of CF. Biochemically, H290/51 inhibited the increased LDH release. Its effect was less evident when it was administered only from the start of reperfusion.

Extrapolation of our in vitro findings to in vivo or clinical practice requires caution. But *in vivo* applicability of this compound was recently demonstrated by Shimizu et al (Shimizu et al., 1998) in pigs. H290/51 reduced infarct size induced by occlusion of left anterior descending artery and improved left ventricular functional recovery, supporting our conclusions from *in vitro* and *ex vivo* experiments.

Our data suggest that to some extent the antioxidant properties can be effective not only during reperfusion, but already during ischemia. A better preserved functional and morphological status of the myocardium during ischemia may yield a better capacity to survive reperfusion injury. The ability to rapidly reach the intracellular sites where ischemia/reperfusion injury and ROS generation takes place, makes H290/51 an interesting substance for clinical testing.

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