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

Ph.D. Thesis

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## INTRODUCTION

Coronary artery disease is the principle cause of mortality, morbidity and early disability in the industrialised world. Acute coronary syndromes 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. The major goal of treatment is to restore blood flow to the jeopardised myocardial area. Although an absolute pre-requisite for tissue survival, it is widely accepted that reperfusion may increase injury over that sustained during ischemia. Thus, paradoxically 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. Reperfusion injury may have major clinical implications in such frequent clinical conditions as angina or in procedures performed to recover vascular patency like thrombolysis, angioplasty and cardiac surgery. 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.

### Role of reactive oxygen substances (ROS)

ROS are important mediators of myocardial ischemia-reperfusion injury. 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. In the heart ROS can be produced in the sarcoplasmic reticulum, the mitochondria, the vascular endothelium and by aggregating neutrophils. A number of sites, at which ROS may be produced, are intracellularily located, thus not easily available to exogenous antioxidant compounds. 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. 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. Membrane abnormalities may cause a serious defect in calcium regulation and cellular volume, inducing mitochondrial dysfunction, calcium overload and cellular swelling. ROS can also damage nucleic acids causing mutations. These injuries can eventually lead to cell death. ROS also increase leukocyte activation, chemotaxis, and leukocyte-endothelial adherence after ischemia-reperfusion.



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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. ROS production increases during ischemia, and subsequently there is a burst in production during reperfusion. Severe ischemia induces a reduction in the tissue concentration of various scavenger compounds that protect against oxygen toxicity. Lipid peroxidation is detected through the estimation of malondialdehyde (MDA). 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. Under physiological conditions the tissue concentration of free radicals is maintained by a system of enzymatic and non-enzymatic antioxidants. The enzymatic defence predominates within the cell. Superoxide dismutase (SOD) enzymatically protects against $\cdot O_2^-$. $H_2O_2$ is enzymatically scavenged by gluthathione peroxidase or catalase. Ischemia causes a decrease in mitochondrial glutathione peroxidase activity, thus detoxification of $H_2O_2$ is impaired. There is no physiological scavenger against $\cdot OH$, and the defence is directed against preventing its formation. Extracellular defence is mainly exerted through metal binding proteins such as transferrin and ceruloplasmin. Among the non-enzymatic antioxidants, $\alpha$-tocopherol (Vitamin E) is the most efficient in the lipid phase. It is mainly present in the plasma, low-density lipoprotein particles and the cell membrane. The myocardium has a high concentration of Vitamin E. It has recently been shown that plasma Vitamin E levels correlate well with coronary endothelial function. Other important endogenous antioxidants are Vitamin C and ubiquinone.

### Lipid peroxidation

Lipid peroxidation has been suggested to be a main mechanism of oxidative injury. Inhibition of lipid peroxidation has been shown to be protective against reperfusion-induced myocardial damage. *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. The disadvantage of Vitamin E is that its extreme lipophilicity causes a slow availability to cardiomyocytes, making it less feasible in acute ischemic situations. Treatment with Vitamin E just prior to the ischemia-reperfusion episode is of limited value only, and effective pretreatment needs large doses employed. Hence, other drugs should be investigated with similarity in action to Vitamin E but with a more favourable pharmacokinetic profile.

A number of antioxidants have been tested for their possible protective effect in ischemia-reperfusion injury of the heart. Experimentally, SOD and catalase have

gained the most attention as of possible value in the treatment of reperfusion injury. The results are far from consistent, ranging from a marked protection to no protective effects at all. 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.

H290/51

Indenoindoles have previously been shown to inhibit lipid peroxidation in several test systems. A novel indenoindole derivative, H290/51 (cis-5, 5a, 6,10,b-tetrahydro-9-methoxy-7-methylindeno [2,1-b] indole) is a low molecular weight (288 MW) inhibitor of lipid peroxidation. H290/51 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. The lipophilicity of H290/51 is considerably lower than that of vitamin E while it is a more potent inhibitor of lipid peroxidation. 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 give effective protection to jeopardised subcellular compartments and protect the myocardium from ischemia-reperfusion injury.

## 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 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



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## PUBLICATIONS ON WHICH THE DISSERTATION IS BASED:

### A. Articles:

I. 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.

II. 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-

predominantly in the first 30-60 seconds of reperfusion. 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 a novel low molecular weight indenoindole derivative with balanced water-soluble and lipophilic properties. 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 of the ultrastructure after global normothermic ischemia 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.

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, thus our studies may provide an experimental basis for a possible practical application.



## MATERIALS AND METHODS

(abbreviated, for a detailed description see the dissertation and attached publications)

### Animals

The experiments were performed on cardiac myocyte cultures (heart cells were obtained from neonatal male Sprague Dawley rats) and isolated rat hearts.

### Experimental setup

#### *Cultured myocytes*

Cardiac ventricles were isolated, cut into small pieces, and placed into Hanks solution free of $Ca^{2+}$ and $Mg^{2+}$ but containing 0.35 g/l sodium carbonate. The cells were dispersed in the same solution, but supplemented with collagenase at 37 $^{o}$C for 10 min and centrifuged at 160 g for 5 min. Subsequently, five serial 20-min 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 cells, they were replated twice for 30 and 90 min. The myocytes were plated on 35 mm plastic culture dishes at a density of approximately 9 x $10^5$ cells/ml (1.8 x $10^6$ cells/dish). The cells were cultured for 6 days in an atmosphere of 95% $O_2$ and 5% $CO_2$. During this period the cells were beating spontaneously at a frequncy of 20-40 beats/min. The medium was changed each day. Before starting the experiments, the medium was 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 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% $CO_2$). Cell damage was assessed by estimating the leakage of LDH to the medium by serial analysis in aliquots of 100 µl.

#### *Isolated rat heart*

Hearts were obtained from rats anesthetized with ether. After a rapid excision, they were immediately immersed into ice-cold Krebs-Henseleit buffer during preparation. The ascending aorta was cannulated for retrograde perfusion as a modified Langendorff preparation. Perfusion pressure (100 cm $H_2O$) and temperature (37 $^{o}$C) 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 and bubbled with a gas mixture containing 95% $O_2$ and 5% $CO_2$. . CF was measured every 2.5 min. 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 min reperfusion aliquots of coronary effluent were collected in precooled tubes, rapidly frozen, and stored at –80 $^{o}$C 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 $^{o}$C before analysis of tissue contents of 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 stabilization. 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 $H_2O$. 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. All the 1 mm thick slices were then placed at random under a regularly perforated plexiglass 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. Using a biopsy needle, one biopsy was taken at every second perforation. 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



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. H290/51 had anti-ischemic rather than anti-reperfusion effects from a morphological and ultrastructural point of view. Possibly the present concentration of H290/51 was able to quench the ROS during ischemia, but was not capable to counteract the burst during reperfusion.

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. In our experiments the protection afforded by H290/51 was effected 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, sustained increase of LVEDP, and a decrease of LVDP and CF during reperfusion. Severe arrhythmias occurred early in reperfusion in all groups. Concurrently LDH release was increased, indicating myocyte damage. The gradual decrease in CF was greater following ischemia/reperfusion in the control group indicating development of a no-reflow phenomenon. The exact mechanism of no-reflow implies many pathways, and 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.

H290/51 given throughout perfusion inhibited both the biochemical and functional injury induced by ischemia-reperfusion. The increase of LVEDP was attenuated and significantly less severe arrhythmia occurred in hearts treated with H290/51. LVDP was significantly higher if H290/51 was given throughout perfusion (group 4), but not if administered only during reperfusion. Addition of H290/51 (group 4) significantly attenuated the ischemia-induced decrease in CF. This was less pronouncedly attenuated by the delayed administration of H290/51 (group 3). H290/51 throughout perfusion (group 4) inhibited the ischemia-induced LDH release and a delayed administration of H290/51 (group 3) resulted in an intermediate course between group 4 and groups 1. H290/51 reduced TBARS during reperfusion as compared to group 1.

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

chosen as a convenient and reproducible model of generating injury by exogenous free radicals. $H_2O_2$ is 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. $H_2O_2$ may also have direct cardiodepressive effects. In our experiments a 10 min perfusion with $H_2O_2$ resulted in a reversible, non-lethal model with marked cardiodepression, similar to post-ischemic myocardial stunning in humans. A corresponding functional deterioration, was also observed after global ischemia-reperfusion in Study IV, indicating a role of ROS in ischemia-reperfusion injury. The left ventricular dysfunction was partly reversible, the diastolic dysfunction being more long-lasting.

Administration of H290/51 protected against the cardiac injury induced by exogenous ROS. H290/51 attenuated the rise in LVEDP caused by $H_2O_2$ and LVEDP normalized by the end of the experiments. Addition of H290/51 to the perfusate after the end of $H_2O_2$ perfusion (group V) resulted in an intermediate course as compared to group I and group IV. As the $H_2O_2$ -induced 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, but not when it was given after $H_2O_2$ perfusion (group V). 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. 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*

The ischemia-induced ultrastructural damage was severe in control hearts (Group A), evident as interstitial and cellular edema, disruption of sarcolemma, swollen mitochondria and myofilament disruption. In hearts treated with H290/51 (Group B) the ischemic damage was less apparent, the structure remained fairly normal. Most ischemic changes persisted after reperfusion. There were no signs of progressive damage during reperfusion. Interstitial edema tended to increase in the control group. The increasing edema during reperfusion may be the continuation of an ischemic injury and does *per se* suggest that reperfusion did take place.

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. H290/51 attenuated the ischemia-induced increase in the relative volume of cytosol within the cardiomyocytes (($V_v$ cell edema/myocyte). 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.



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.

### *Stereology*

Volume fractions were estimated using the point counting method (Weibel). 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 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 $5 \times 10^{-6}$ mol/l in 0.1% ethanol. The medium was subjected to 1 h hypoxia. LDH leakage was assessed from samples taken after 1, 2, 3 and 4 h after reoxygenation.

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

Group I: $H_2O_2$ was given for 10 min, followed by 50 min recovery (n = 9)
Group II: Vehicle of H290/51 was given to the buffer for 10 min (n = 9)
Group III: H290/51 was added for 10 min (n = 8)
Group IV: Like in group I, but H290/51 was added from time 0 onwards (n = 12)
Group V: Like in group IV, but H290/51 was added from 10 min 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: vehicle added throughout 25 min stabilisation and reperfusion (n = 18)
Group 3: like in group 2, but H290/51 added throughout reperfusion (n = 21)
Group 4: H290/51 added throughout both stabilization and reperfusion (n = 13)

## Biochemistry

LDH activity in the coronary effluent and cell medium was measured with a commercial reagent kit (Boehringer-Mannheim, Germany). 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 h hypoxia.

TBARS. Myocardial formation of TBARS was quantified using an automated continuous-flow technique.

## 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 evaluated by the 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 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 AND DISCUSSION

The importance of reperfusion injury is often debated and somewhat controversial. It is not settled whether reversibly injured myocytes, potentially viable at the time of reperfusion, die as a consequence of reperfusion. There are arguments that the major part of myocardial injury occurs during ischemia, even if manifesting itself only after reperfusion. The apparent controversy can be resolved in part by making a distinction between reversible and irreversible reperfusion injury. There is a bulk of evidence supporting reversible reperfusion injury. Most investigators seem to agree, however, that reperfusion injury is a true entity.

## *TBARS*

In Studies II and IV we employed TBARS as a nonspecific marker of abnormal tissue oxidation. Although TBARS did not increase during reperfusion after global ischemia (Study IV), 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). Studies on accumulation of lipid peroxidation products in ischemic-reperfused hearts have yielded conflicting results. TBARS are usually regarded as being representative 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. It is possible that the antioxidant defense may be able to quench the peroxidation chain reaction before reaching the end product.

## *Hypoxia-reoxygenation injury of isolated cardiomyocytes*

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.

## *$H_2O_2$ perfusion*

LVEDP increased significantly already after 5 minutes of $H_2O_2$ administration (group I) and remained elevated throughout the experiment. $H_2O_2$ (group I) reduced LVDP then it gradually recovered by the end of reperfusion. $H_2O_2$ (group I) increased CF it remained higher than the basal value throughout the observation period. HR was not altered during the observation period. After 10 min perfusion with $H_2O_2$ (group I), LDH activity was similar to controls. However, LDH increased during recovery, indicating myocyte damage. An increase of TBARS in the hearts after perfusion for 10 minutes with $H_2O_2$ confirmed that oxidative stress and lipid peroxidation occurred. $H_2O_2$ was