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 occurring 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 intracellulary 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. Intracellularly, 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.


B. Citable abstract:

PAPERS CITING THE PUBLICATIONS ON WHICH THE DISSERTATION IS BASED:
Papers citing Article 1.:
Ko KM, Yiu HY.: Schisandrin B modulates the ischemia-reperfusion induced changes in non-enzymatic antioxidant levels in isolated-perfused rat hearts. MOL CELL BIOCHEM 220: 141-147, 2001

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 glutathione 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:**


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 indenoindo le 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$_2$O$_2$ 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 caused by H$_2$O$_2$. The protective effect of this antioxidant was further evidenced by the inhibition of LDH release and accumulation of TBARS after H$_2$O$_2$ 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 °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 frequency 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$_2$O) and temperature (37 °C) were kept constant.
In experiments II and IV left ventricular systolic (LVSP) and end-diastolic pressures (LVEDP) were measured. Coronary flow (CF) was measured by timed collections of coronary effluent. The hearts were perfused with Krebs-Henseleit buffer containing 95% O₂ and 5% CO₂ for 25 min. At the end of perfusion, the heart was frozen and stored at –80°C until 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 freeze-clamped with liquid nitrogen at the end of the experiments (60 min), and stored at –80°C until analyzed for LDH activity. Hemodynamic data from the freeze-clamped hearts are not included. 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 takes place.

Functional changes following global ischemia

Thirty minutes of global, normothermic ischemia impaired left ventricular function as indicated by a marked, sustained increase of LVEDP, and a decrease of LVDP and CF during reperfusion. Severe arrhythmias occurred early in reperfusion in all groups. Only hearts that met the following criteria at the end of stabilization 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 II and IV, global ischemia was induced by clamping the inflow tubing for 30 minutes, followed by 20 minutes of reperfusion. 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 manifested as microvascular spasm, extravascular compression due to interstitial edema, and endothelial swelling.

Preparation for Electron Microscopy

At the end of experiments hearts were perfusion-fixed with McDowell’s fixative for 10 min at room temperature and at a perfusion pressure of 60 cm H₂O. Only the LV was used for morphometry. The volume of the ventricular myocardium was measured by liquid displacement and expressed in µm³. The LV was 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 (32 mm x 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. The perfusions were placed in 1% aqueous O₂O₂ for 2 hours. After a subsequent wash in buffer the pieces were dehydrated in a
chosen as a convenient and reproducible model of generating injury by exogenous free radicals. H$_2$O$_2$ 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$_2$O$_2$ may also have direct cardiodepressive effects. In our experiments a 10 min perfusion with H$_2$O$_2$ resulted in a reversible, non-lethal model of injury and does not produce long-lasting injury. The left ventricular dysfunction was partly reversible, the diastolic dysfunction being more long-lasting.

Compound H290/51 was added to the culture medium for the 6 days of culturing at a final concentration of 10$^{-6}$ mol/l in 0.1% ethanol. The medium was changed every 2 days. Similar medium changes were performed in the control group. The ischemia-induced ultrastructural damage was severe in control hearts (Group A), evident as interstitial and cellular edema, disruption of sarcomerism, 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.

**Experimental groups**

- **Group I**: H$_2$O$_2$ 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)

**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$^{-6}$ mol/l. LDH leakage was assessed from samples taken 1, 2, 3 and 4 h after reoxygenation.

**Studies with isolated rat hearts** (Study II)

Isolated rat hearts subjected to oxidant stress by hydrogen peroxide were studied 7 days after reoxygenation. H$_2$O$_2$ was given for 10 min, followed by 50 min recovery (n = 9). H$_2$O$_2$-induced increase in TBARS demonstrates that H290/51 did inhibit lipid peroxidation in the present model. The delayed administration of H290/51 following H$_2$O$_2$ also resulted in a reduction, albeit less pronounced, of oxidant injury. This suggests that lipid peroxidation occurred even after the end of H$_2$O$_2$-administration. The ischemia-induced ultrastructural damage was severe in control hearts (Group A), evident as interstitial and cellular edema, disruption of sarcomerism, 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 at the end of ischemia. A relatively greater interstitial volume due to edema in the control group may explain this.
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_{2}O_{2} perfusion

LVEDP increased significantly already after 5 minutes of H_{2}O_{2} administration (group I) and remained elevated throughout the experiment. H_{2}O_{2} (group I) reduced LVDP then it gradually recovered by the end of reperfusion. H_{2}O_{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_{2}O_{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_{2}O_{2} confirmed that oxidative stress and lipid peroxidation occurred. H_{2}O_{2} was