OXYGEN FREE RADICALS IN MYOCARDIAL ISCHEMIA AND REPERFUSION

OXYGEN FREE RADICALS IN MYOCARDIAL ISCHEMIA AND REPERFUSION

VRIJE ZUURSTOF RADICALEN IN HART ISCHEMIE EN REPERFUSIE

PROEFSCHRIFT

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Voor mijn ouders
Voor Terry en Gayle

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CHAPTER I

I. INTRODUCTION

I.I. OXYGEN FREE RADICALS

A free radical is by definition an atom or a molecule with one or more unpaired electron(s) in its outer orbital (1,2). Normally, a chemical compound contains two electrons in its outer orbital which spin in opposite directions. Free radicals are very reactive in order to restore the situation of paired electrons. Molecular oxygen (O_2) is a radical itself because it contains two unpaired electrons in its outer orbitals ("biradical"). The word "free" in free radicals originates from a controversy at the end of the last century about whether free radicals could exist in a free form. After the discovery of their actual "free" existence the terms "free radical" and "radical" have become synonyms (3). So in theory, an atom or a molecule will become a free radical either by gaining an additional electron or by loosing an electron.

In properly functioning mitochondria, oxygen is reduced tetravalently by the enzyme cytochrome c oxidase. Oxygen bound to this enzyme takes up four electrons at the same time by which it reduces to water (Fig.1). This reduction is coupled to the formation of adenosine triphosphate (ATP) which is an indispensible energy source for many cellular processes. This tetravalent reduction is harmless to the cell as long as no intermediate oxygen radicals are formed. If oxygen is reduced in univalent steps, three types of reactive intermediates can be formed (Fig.1). The addition of a single electron results in the formation of the superoxide anion ($^{\cdot}O_2^-$); divalent reduction results in the formation of hydrogenperoxide (H_2O_2) and trivalent reduction in the formation of the hydroxyl radical ($^{\cdot}OH$). Superoxide and the hydroxyl radical are by definition free radicals as they possess an unpaired electron in their outer orbitals. Hydrogen-peroxide, although a strong oxidant, is not a free radical because all its electrons are paired.

TETRAVALENT REDUCTION OF OXYGEN

$$O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$$

UNIVALENT REDUCTION OF OXYGEN

$$O_2 + e^- \longrightarrow {}^{\cdot}O_2^- \quad \text{(superoxide)}$$

$$^{\cdot}O_2^- + e^- + 2H^+ \longrightarrow H_2O_2 \quad \text{(hydrogenperoxide)}$$

$$H_2O_2 + e^- + H^+ \longrightarrow H_2O + OH^{\cdot} \quad \text{(hydroxyl radical)}$$

Fig.1. Oxygen free radicals formed during univalent oxygen reduction.

The toxicity of superoxide in biological systems, as e.g. heart tissue, is regarded not very high (4). Nonetheless, $^{\cdot}O_2^-$ can react with a variety of substrates in which it can act as a reductant or an oxidant. For example, superoxide is able to reduce ferricytochrome c or transition-metal complexes and superoxide is able to oxidize alpha-tocopherol, catecholamines or ascorbic acid (5-7). The protonated form of superoxide, $HO_2^{\cdot}$, is a stronger oxidant than superoxide and also reacts with fatty acids and amino acids (8-9). Hydrogen peroxide reacts only slowly with organic substrates but is able to generate hydroxyl radicals ($^{\cdot}OH$) in the presence of transition metals such as iron or copper (10-11). The hydroxyl radical is regarded the most toxic oxidant, as it is able to react with almost any molecule. Because of this high reactivity, hydroxyl radicals are able to destroy substrates like DNA, proteins or lipids, which accounts for their toxicity in vivo (12-13).

I.II. CELLULAR DEFENSE MECHANISMS AGAINST OXYGEN FREE RADICALS

Minor free radical concentrations are considered harmless for the cell because relatively large amounts of protective enzymes and free radical scavengers are present in the cell (5,14). Under physiological conditions, small amounts of free radicals are continuously formed. In normal phagocytosis, oxygen free radicals are used to destroy invading microorganisms. This process, which is called "the respiratory burst", is due to activation of the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. A proper function of this enzyme is essential to overcome infections, as substantiated by genetic disorders of NADPH oxidase (chronic granulomatous disease, 15). Other physiological sources of free radicals are the enzyme cytochrome P-450 in the liver (16), which is used to detoxify certain substrates or drugs, and the "univalent leak" from mitochondria (17). Because of the physiological functions, oxygen free radicals are considered essential for one's well-being. Only excess formation of free radicals, outrunning the defense capacities, is considered to be toxic. For this reason, good health is preserved by a correct balance between free radical formation and free radical defense mechanisms (Yin-Yang principle, 18).

The protection against oxygen free radicals depends partially on specific enzymes and partially on free radical scavengers (Fig.2). Mitochondria as well as cytosol contain considerable amounts of the enzymes superoxide dismutase, catalase and glutathione peroxidase. Superoxide dismutase neutralizes superoxide to hydrogen peroxide whereas hydrogen peroxide is neutralized by catalase and/or glutathione peroxidase to water (1,19-21). Further cellular protection against free radical attack is obtained from substances that are able to "scavenge" or neutralize free radical reactivity (free radical scavengers, Fig.2).

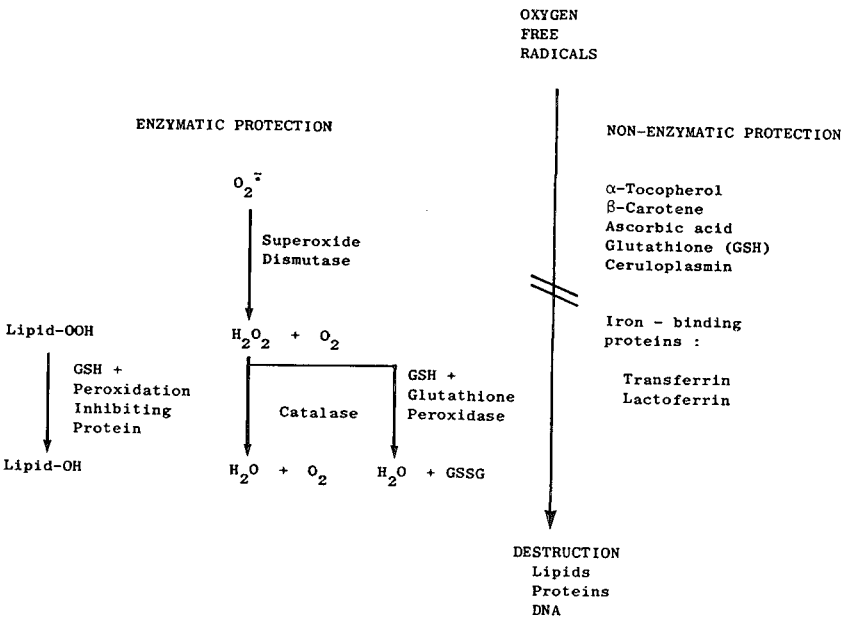

Fig.2. Enzymatic and non-enzymatic protection against oxygen free radicals.

Alpha-tocopherol (vitamin E) and beta-carotenes are important scavengers which are localized in the cellular membrane (22). This is not surprising, in view of the fact that plasma membranes are built up by phospholipids which contain large amounts of unsaturated fatty acyl chains, that are highly vulnerable to peroxidation by free radicals. Reduced glutathione (GSH) is shown in Figure 2, as it is an essential cofactor for the enzymatic action of glutathione peroxidase. Ferritin, transferrin, lactoferrin and ceruloplasmin are summarized because they are able to prevent hydroxyl radical formation from hydrogen peroxide (Haber-Weiss reaction, see chapter I.VI.). Under physiological conditions, iron is stored in ferritin in which it is unable to catalyze the

formation of these radicals. Under pathological conditions, as e.g. inflammation, iron can be released from ferritin by superoxide (73) which is potentially toxic. The toxicity is controlled by the proteins transferrin, lactoferrin and ceruloplasmin. Transferrin and lactoferrin have a high affinity for iron. After binding of iron to these proteins, iron is no longer available to catalyze hydroxyl radical formation. The acute phase reactant, ceruloplasmin, which concentration increases during inflammation, possesses ferro-oxidase activity (23) responsible for the conversion of ferro-iron to ferric-iron. As a result, hydroxyl radical formation will be hampered because its generation from hydrogen peroxide is catalyzed by ferro-iron (Haber-Weiss reaction [10], see chapter I.VI.).

I.III. EVIDENCE OF OXYGEN-DERIVED FREE RADICAL INVOLVEMENT IN (POST)-ISCHEMIC MYOCARDIAL INJURY

The concept of oxygen-derived free radical (ODFR) involvement in the pathogenesis of myocardial infarction has obtained much attention over the past few years. The phenomenon "reperfusion injury" or "reoxygenation injury" was first described by Hearse et al. in 1973 (24,25). He found that readmission of oxygen to ischemic or anoxic hearts is responsible for a dramatic increase of tissue injury and rhythmic disturbances. This phenomenon, which is also called the "oxygen paradox", is associated with a decline in antioxidant defenses as measured by decreased activities of superoxide dismutase and glutathione peroxidase in hypoxic hearts (26). As a result of these early findings, oxygen free radicals were associated for the first time with post-ischemic reperfusion injury.

Several studies have subsequently demonstrated protective effects of superoxide dismutase (SOD) and catalase during ischemia and reperfusion (27-30). Even administration of SOD at the onset of post-ischemic reflow turned out to protect isolated rabbit hearts (30).

In several studies, the effects of free radical generating systems on cardiac function and ultrastructure were examined. Burton et al. (31) generated superoxide in rabbit hearts by adding purine and xanthine oxidase to the perfusion medium. Hydroxyl radicals were generated by concomitant administration of iron. Using electron microscopy, myocardial alterations were found, which appeared quite similar to the alterations observed as a result of ischemia. In addition, experiments in our laboratory have shown hypercontraction and calcium-overload in rat hearts that were perfused with cumene-hydroperoxide, which is a chemical inducer of lipid peroxidation by free radical action (32). These results are quite comparable to the alterations observed as the result of transient ischemia.

Further evidence in support of the free radical hypothesis is based on the production and release of malondialdehyde (MDA), which is one of the endproducts of

free radical mediated lipid peroxidation. Guarnieri et al. (26) showed an enhanced release of this aldehyde during reperfusion of hypoxic rat hearts. A good correlation was found between the extent of MDA release and the total amount of hypoxia, indicating the occurrence of reoxygenation dependent lipid peroxidation in rat hearts. Moreover, Barsacchi et al. (33) observed a burst of free radical induced chemiluminescence in hypoxic hearts after reoxygenation. In this study, glutathione depleted hearts were compared to control hearts. Larger amounts of chemiluminescence were found in the glutathione depleted hearts compared to the control hearts.

The above mentioned studies provide accumulating evidence of free radical generation during ischemia and reperfusion. However, all this evidence is indirect as free radicals itself are not detected. In this regard, the studies of Zweier et al. and Blasig et al. (34,35), in 1987, are of special interest because they reported the direct identification of free radicals in rat myocardium during ischemia and reperfusion with electron spin resonance spectroscopy (ESR). These findings, however, need to be analyzed carefully since: 1) artifactual radicals may disturb proper interpretation of the signals (36), and since 2) recent studies in our laboratory have shown that the ESR signals do not likely originate from oxygen free radicals, although, they are reproducible and coupled to ischemia and reperfusion (chapter V, 37).

I.IV. POSSIBLE FREE RADICAL SOURCES DURING ISCHEMIA AND REPERFUSION

Many potential sources of free radicals generated during ischemia and/or reperfusion have been described in the literature. Until now, the actual source(s) is(are) not known. It might well be possible that several sources, at the same time, contribute to free radical generation. In searching the precise origin of free radials, progress is probably hampered by the fact that free radical sources are different in the various species or models of ischemia used. Four, most likely alternative sources of free radical generation are discussed here.

1) Xanthine dehydrogenase/oxidase.

A possible source of free radical generation during ischemia and reperfusion is the enzyme xanthine dehydrogenase (20). McCord suggested in 1984 that xanthine dehydrogenase (XDH), present in myocardial tissue of several species, is converted during ischemia to xanthine oxidase (XO) (Fig.3). This conversion probably involves activation of calcium-dependent proteases. Hypoxanthine, the main substrate for this enzyme and breakdown product of ATP, accumulates during ischemia in the myocardium. Upon reperfusion, the presence of oxygen will initiate the conversion of

hypoxanthine to xanthine by xanthine oxidase. This reaction is accompanied by the production of superoxide (Fig.3).

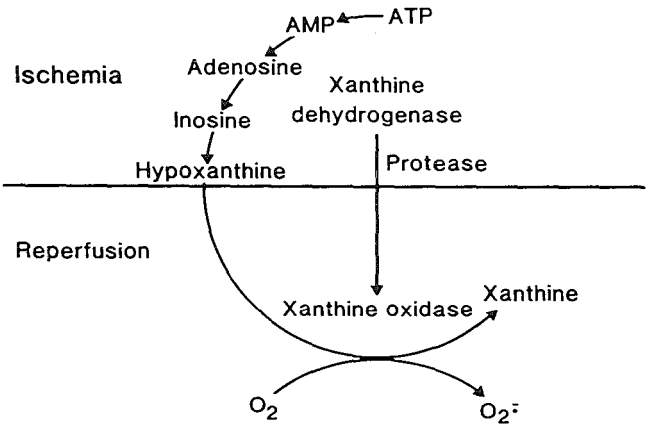

Fig.3. Possible mechanism of oxygen free radical generation during post-ischemic reperfusion. As a result of ischemia ATP is catabolized to hypoxanthine and xanthine dehydrogenase to xanthine oxidase (XO). Upon reintroduction of oxygen hypoxanthine is converted by XO to xanthine in which superoxide is formed.

In support of this hypothesis are several well documented studies in which protection was reported with xanthine oxidase inhibitors (allopurinol and oxypurinol, 38-40). The validity of this hypothesis is strongly debated, at the moment, as it is discredited by reports that human hearts as well as pig and rabbit hearts do not contain XDH or XO (41,42). A recent study contradicts these findings as activities of XDH and XO were reported in human and rabbit hearts (43). The authors (43) suggested the differences to result from a cytosolic inhibitor of XDH/XO present in human and rabbit heart tissue.

2) Activated leukocytes.

Activation of leukocytes will result in the formation of a variety of mediators capable to induce tissue injury. Among them are proteolytic enzymes, leukotrienes,

prostaglandins and activated species of oxygen (44). Macrophages and polymorphonuclear cells (PMN) are able to produce superoxide and hydrogen peroxide (45). After stimulation of these cells membrane bound NADPH-oxidase is activated which results in the formation of these reactive compounds. The extent of leukocyte activation during myocardial ischemia and its implications to myocardial injury is subject of many investigations at this moment. Romsom et al. (46) reported 40% reduction of canine infarct size, if the animals were depleted from neutrophils during ischemia and reperfusion. The same results were reported by Engler et al. (47), who measured in dogs enhanced recovery of post-ischemic contractile function after reducing the number of circulating granulocytes by leukopak filtration. Gillespie et al. (48) studied the effects of neutrophils on Langendorff perfused rabbit hearts. Neutrophil activation by phorbol myristate acetate (PMA), in this study, resulted in higher coronary flow resistance and lower left ventricular developed pressure. The interpretation of these results, however, is delicate for reasons that PMA itself, at very low (nanomolar) concentrations, is able to induce the same effects. Unpublished experiments in our laboratory indicate that it is impossible to wash away PMA from neutrophils without disturbing their viability. The observed differences, published by Gillespie, might therefore be a direct effect of PMA itself.

Although, neutrophil depletion during ischemia and reperfusion can be beneficial to the heart, it has to be emphasized that isolated hearts, perfused without any blood cell, are still sensitive to reperfusion injury. In addition, these hearts can be protected by SOD, catalase and free radical scavengers (27-30). So neutrophil dependent free radical generation cannot solely be responsible for tissue injury during post-ischemic reperfusion. Only an additive contribution to tissue damage after invasion of the injured myocardium possibly occurs.

3) Mitochondria.

As mentioned above, mitochondria catalyse the tetravalent reduction of oxygen to water. Proper mitochondrial function might be altered as a result of ischemia leading to univalent reduction and oxygen free radical generation. VandePlassche and Borgers (49) reported the formation of hydrogen peroxide by nicotinamide adenine dinucleotide (NADH) dehydrogenase during ischemia in dogs. Using electron microscopy, they visualized this peroxide in mitochondria of ischemic myocardium in clear contrast to control myocardium. Considerable amounts were detected in the cytosolic compartment as well, which indicates a possible diffusion of this peroxide into the cytosol. Superoxide is thought to be formed by NADH dehydrogenase (50), and its dismutation product, hydrogen peroxide, will be formed by SOD present in mitochondria and the cytosol. In order to substantiate a role of this particular mechanism in the pathogenesis of myocardial ischemic injury, further experiments are needed.

4) Arachidonic acid.

Several studies have reported an increased release of arachidonic acid from cardiac tissue as a result of ischemia (51,52). Arachidonic acid can be metabolized either by lipoxygenase or cyclooxygenase which results in the formation of respectively leukotrienes or prostaglandins. These metabolic pathways of arachidonic acid are accompanied by the generation of free radical species (44). The extent and influence of these radicals on heart function during ischemia and reperfusion is not known. Because of the simultaneous production of free radicals and leukotrienes/prostaglandins by these metabolic pathways, it is difficult to differentiate free radical action from the effects of leukotrienes and/or prostaglandins. Experiments using specific cyclooxygenase inhibitors like aspirin, indomethacin and naproxen have shown no protection of ischemic heart tissue (53-55). In contrast, promising effects of specific lipoxygenase inhibitors during heart ischemia were reported (56,57), which indicates a possible role of lipoxygenase metabolites in ischemic cardiac necrosis. Since leukotrienes are strong neutrophil activators, the protective properties of these inhibitors can be explained by a decreased activation and accumulation of these cells in the ischemic myocardium. Reduction of free radical by-products of leukotriene synthesis, however, might well be a good alternative mechanism.

Experiments investigating the effects of lipoxygenase inhibitors on isolated heart preparations, in the absence of neutrophils, are presently undertaken in our laboratory. Preliminary results have indeed shown protection of isolated hearts by lipoxygenase inhibitors. These findings suggest a neutrophil independent mechanism of ischemic protection by lipoxygenase inhibitors.

I.V. MYOCARDIAL ISCHEMIA AND LIPID PEROXIDATION

Formation of hydroxyl radicals in biological tissues is generally accepted to induce peroxidation of polyunsaturated fatty acids (PUFAs), present in membrane phospholipids (58-61). Initiation of this process results in a cascade of peroxidation reactions, which propagation is dependent on the presence of transition metals such as iron. The cascade is responsible for the destruction of PUFAs and will result in the formation of aldehydes, alkyl radicals, peroxides, pentane, ethane and malondialdehyde (MDA, Fig.4). Pentane and ethane are considered good parameters for lipid peroxidation, as they are stable and relatively easy to measure in the expired air of the body. The lipid radicals $R^{\cdot}$, $RO^{\cdot}$ and $ROO^{\cdot}$ themselves, are capable to induce a new cascade of lipid peroxidation reactions which accounts for the ongoing chain reactions. The occurrence of lipid peroxidation in biological tissues is therefore considered highly autocatalytic. The peroxidation cascade will continue to proceed unless the hydrogen atom, that is involved in the formation of lipid hydroperoxide (ROOH), is supplied by a non-PUFA molecule.

From these hydrogen donors or antioxidants (QH, Fig. 4), free radicals take the hydrogen atom forming a scavenger radical. As shown in figure 4, the antioxidant GSH will be oxidized to the glutathione radical (GS•). Two glutathione radicals will form GSSG (oxidized glutathione). Reduced GSH can be regenerated by the enzyme glutathione reductase (Gred) in the presence of NADPH.

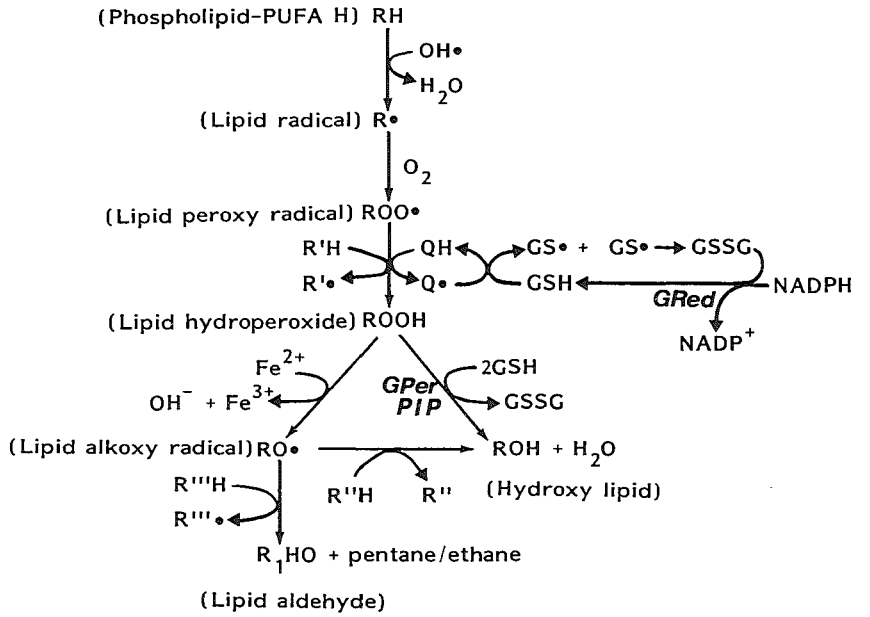

Fig.4. Reaction mechanism of hydroxyl radical induced lipid peroxidation in biomembranes: effects of antioxidant enzymes and free radical scavengers. GPer: glutathione peroxidase, Gred: glutathione reductase, PIP: peroxidation inhibiting protein, GSH: reduced glutathione, GSSG: oxidized glutathione, QH: lipid-radical scavenger, (•): free electron, RH, R'H, R"H, R'"H: PUFA chains.

Membrane phospholipid peroxidation is considered deleterious for proper cell functioning. Altered membrane fluidity (as a result of PUFA destruction), increased membrane permeability, and destruction of transmembrane proteins, all contribute to these injuring effects. (62,63). Moreover, enhanced membrane destruction can be

induced because of the higher affinity of phospholipase A and C for peroxidized phospholipids (64). For these reasons, an imbalance between free radical mediated lipid peroxidation and antioxidant capacity will result in the destruction of membrane integrity. This ultimately creates a situation in which the cell is unable to maintain its ionic homeostases which is incompatible to cellular life.

I.VI. THE ROLE OF IRON IN MEDIATING INCREASED CARDIAC INJURY DURING ISCHEMIA AND REPERFUSION

At least three possible aspects of iron are known, in which this cation increases oxidative stress. As already mentioned, iron facilitates the decomposition of lipid peroxides (65) which produces several cytotoxic substances such as aldehydes, peroxides, ethane and pentane. Secondly, iron is involved in the nonenzymic oxidation of catecholamines, a reaction that is accompanied by the generation of superoxide and/or hydrogen peroxide (66). A third and perhaps most important aspect of iron is the capacity of this transition metal to catalyze the formation of highly reactive hydroxyl radicals from superoxide and hydrogen peroxide (modified Haber-Weiss or Fenton reaction, 10).

$$\cdot O_2^- + H_2O_2 \xrightarrow{Fe} O_2 + OH^- + OH\cdot$$

The hydroxyl radical, as described above, is highly toxic for biological tissue because it induces lipid peroxidation and destruction of many biological substrates (5,14,19,20, chapter IV). In order to substantiate the role of iron in the reperfusion syndrome, rats were iron-loaded and the hearts perfused under anoxic and reperfusion conditions (67) and compared with control hearts. The results of this study are described in chapter II (68). Chapter III describes the effects of two recently developed iron-chelators (69) on heart function during ischemia and reperfusion (70).

Bernier et al. (71) and Ambrosio et al. (72) reported protection of transient ischemic hearts by the iron-chelator deferoxamine which might be explained by impairing hydroxyl radical formation in the Haber-Weiss reaction. Normally, most of the iron, present in myocardial cells, is stored in ferritin in which location it is unable to catalyze hydroxyl radical formation. In this regard, the findings of Biemond et al. (73) are important because they have shown that superoxide is able to mobilize iron from ferritin. Superoxide-dependent iron release from ferritin so creates an optimal situation in which

lipid peroxidation occurs and might therefore be an important mechanism in the pathogenesis of the reperfusion syndrome.

I.VII. EVIDENCE OF FREE RADICAL GENERATION IN ENDOTHELIAL CELLS

The occurrence of specific endothelial cell damage during myocardial ischemia was reported by Hülsmann and Dubelaar (74) in 1987. In this study, early endothelial cell damage was measured by a relative increment of lactate dehydrogenase release to creatine kinase release. Ratych et al. (75) reported in the same year, free radical generation upon reperfusion in isolated endothelial cells in the absence of neutrophils or parenchymal cells. They were able to protect these endothelial cells after 45 minutes of anoxia by superoxide dismutase, catalase and allopurinol. In 1988, Zweier et al. (76) directly measured free radicals in isolated endothelial cells, during reoxygenation, using electron spin resonance spectroscopy (ESR) and the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). All these reports suggest central involvement of endothelial cells in the reperfusion syndrome.

More evidence in support of this hypothesis are studies that show protection of ischemic heart tissue by superoxide dismutase (SOD) (29,30,39,77). One cannot expect a rapid penetration of this relatively large molecule (Mw 30,000) into myocardial cells after short incubation periods. So protection by SOD, e.g. administered solely during reperfusion (30), might well be explained by scavenging superoxide in the vascular lumen and/or on the vascular wall. A reasonable origin of superoxide will then be the endothelial cell, especially, since the enzymes xanthine dehydrogenase/oxidase, lipoxygenase and cyclooxygenase are located in these cells (78). In chapter IV, an iron-localization study in rat hearts after a short period of iron-load is described (79). In this study, iron was found to be predominantly stored in endothelial cells and pericytes. These findings further suggest free radical generation in endothelial cells as discussed in chapter IV.

I.VIII. FREE RADICALS AND CORONARY VASODILATION

The integrity of vascular endothelium is increasingly recognized to be important in the regulation of vascular tone (80,81). For example, vasorelaxation induced by cholinergic agonists, the calcium ionophore A23187, histamine, serotonin and bradykinin, is dependent on an intact endothelium which is capable to secrete a humoral factor known as the "endothelium derived relaxant factor" (EDRF, first described by Furchgott and Zawadzky in 1980, 82). The chemical identity of EDRF has recently been clarified and represents nitric-oxide (NO, 83). EDRF exerts its biological activity by activating the enzyme guanylate cyclase in vascular smooth muscle cells, which induces the formation

of cyclic guanosine mono phosphate (cGMP, 84,85). cGMP is a well known trigger of vasodilation, probably by activating the plasmamembrane Ca^{2+} pump, which lowers intracellular Ca^{2+} levels (84). Besides EDRF, two classes of agents are known which elevate cGMP levels in vascular smooth muscle cells:

1) Nitroso-compounds, e.g. sodiumnitroprusside (SNP), which stimulates soluble (cytosolic) guanylate cyclase, presumably by interacting with a hemegroup in this enzyme (86).
2) The atrial natriuretic factor (ANF) and atriopeptides (containing the active site of ANF), which activate the membrane bound form of guanylate cyclase that is directly coupled to the ANF-receptor (87).

Endothelium dependent relaxation, by EDRF, is presently subject to many investigations as it might be an important factor in controlling vascular tone. Recently, EDRF was even hypothesized to be involved in essential hypertension and vasospasms encountered in atherosclerotic vessels (80-82).

Earlier studies in our laboratory have shown that cumene hydroperoxide (CUM) and 4-hydroxy-2,3-nonenal (HN) are able to induce strong coronary vasodilations in Langendorff perfused rat hearts (88). CUM is a good model compound to mimic lipid peroxidation, as induced by oxygen-derived free radicals. It is capable to initiate an immediate oxidative stress in heart tissue, probably by the generation of hydroxyl radicals ($OH^{\cdot}$;88). HN has received particular attention because this aldehyde was found to be formed in rather high quantities during lipid peroxidation (89). Formation of peroxides or aldehydes during ischemia or post-ischemic reperfusion might well be responsible for generalized post-ischemic hyperaemia. This condition, as e.g. encountered after prolonged aortic or cardiac surgery, can become critical if it induces massive vasodilation, hypotension or shock (90,91).

In a study, described in chapter VI, the vasodilatory effects of CUM and HN were investigated in order to establish the mechanism of vasodilation by these compounds and lipid peroxidation in general.

I.IX. OTHER FREE RADICAL RELATED DISEASES

Oxygen free radicals have been implicated to be involved in various clinical conditions. Among them are inflammatory diseases, such as, rheumatoid arthritis and autoimmune diseases, radiation injury, cigarette-smoke effects, atherosclerosis, Parkinson disease, retrolental fibroplasia, carcinogenesis, porphyria, iron-overload and aging (for reviews see 5,14,18). Four of these clinical conditions are discussed here.

1) Rheumatoid arthritis.

There is substantial evidence suggesting oxygen free radical involvement in the pathogenesis of rheumatoid arthritis (RA, 93). As a result of the activation of granulocytes and macrophages, e.g. encountered during RA, large amounts of superoxide and hydrogen peroxide are produced (45). In patients with RA, administration of superoxide dismutase, either locally or systemically, was found to depress the disease (94,95). Moreover, malondialdehyde, an endproduct of lipid peroxidation, is detectable in the synovial fluid of these patients (96). Finally, drugs commonly used in RA have been reported to interfere with superoxide. D-penicillamine possesses SOD activity (97) whereas gold compounds and non-steroidal anti-inflammatory drugs inhibit superoxide production by reducing NADPH-oxidase activity in granulocytes (98,99,104).

2) Atherosclerosis.

Several studies have suggested the involvement of oxygen free radicals in the development of atherosclerosis (100). Low density lipoproteins (LDL), associated with atherosclerosis, are known to be sensitive to peroxidation by oxygen free radicals. LDL, modified by these radicals, was found to be toxic to cultured endothelial cells (100). Loss of endothelial cell integrity, which attracts platelets and macrophages from the blood, are relevant early events in promoting atherosclerotic plaque formation. Whether oxidized LDL has the same effect in vivo needs to be further investigated since blood serum itself is protective (102).

3) Carcinogenesis.

In carcinogenesis, an initiation stage and an promotion stage can be distinguished. In the initiation stage a chemical, physical or biologic agent directly induces an alteration in the molecular structure of DNA. This alteration, if not repaired, is followed by a promotion stage in which the expression of the genes that regulate cell growth is changed. Oxygen free radicals have been reported to be possibly involved in cancer development (103). This suggestion is based on studies in which activated phagocytes, producing oxygen free radicals, were found to induce mutations in bacteria and in cultured mammalian cells (105). In vivo, chronic inflammation is known to be associated with a higher risk of carcinogenesis. In addition, exogenous radiation, a condition known to induce free radical generation, is responsible for an increased incidence of cancer development. So, free radical generation in biological tissues, which induces DNA destruction, is potentially carcinogenic.

4) Aging.

The hypothesis of free radical involvement in aging, suggests, that free radicals produced during normal metabolism, damage DNA and other molecules which leads to degeneration, malignancies and death. The hypothesis is based on the following. The age pigment lipofuscin is thought to be formed as a result of lipid peroxidation and accumulates in tissues with aging (106). In vitro experiments have shown a correlation between lipid peroxidation and the formation of fluorescent lipofusin (101). Long-lived species have a lower metabolic rate and are therefore likely to produce fewer oxygen free radicals (107). The amount of SOD among the various species seems to be correlated with life-span, in a way that the highest SOD level is encountered in animals with the longest life-span (107). As a consequence, this hypothesis suggests that free radical scavengers and antioxidants might prevent aging. A study with multi-doses vitamin E (108) showed increased mortality among "self-selected" participants of 65 years and older consuming the vitamin. The authors suggested this result to originate form selection of the participants rather than from vitamin E toxicity. At present, an association between life-span and antioxidant intake has never been positively demonstrated.

I.X. CLINICAL RELEVANCE AND THE AIM OF THE STUDY

In patients with acute myocardial infarction, death normally results from rhythmic disturbances or pump failure. Over the past 3 decades, the mortality rate from this disease has been substantially reduced predominantly by vigorous treatment of dysrhythmias using modern monitoring techniques. These achievements are in contrast with the mortality rate from mechanical failure (manifested by cardiogenic shock, pulmonary edema or chronic heart failure) which has not been reduced in the same period (109). Pump failure in patients with acute myocardial infarction is known to be closely correlated with the extent of tissue necrosis (110). Therefore, any therapeutic strategy which enables a decrease of myocardial necrosis can be expected to be extremely useful. Not only, immediate cardiogenic shock but also chronic heart failure can be treated with more success if patients prevail a greater quantity of viable myocardium. In addition, prevention of ischemia-induced myocardial injury during heart surgery and heart transplantation is of fundamental importance for ultimate success. Because of these considerations, experiments were designed (this thesis) in isolated rat hearts to seek for ischemic protection. Since current knowledge strongly suggests oxygen free radical involvement in post-ischemic cardiac injury, attention was focussed on the involvement of these radicals in the pathogenesis of myocardial infarction.

CHAPTER II

IRON-LOAD INCREASES THE SUSCEPTIBILITY OF RAT HEARTS TO OXYGEN REPERFUSION DAMAGE.

PROTECTION BY THE ANTIOXIDANT (+)-CYANIDANOL-3 AND DEFEROXAMINE.

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ABSTRACT

To investigate whether iron is involved in the reperfusion syndrome by aggravating free radical injury, the hearts from iron-loaded and control rats were perfused under normoxic, anoxic, and reperfusion conditions. Normoxic perfusion revealed no change in coronary flow, contractility or lactate dehydrogenase (LDH) release between these two groups. Under anoxic and reperfusion conditions, however, we found a significant increase of ventricular fibrillation (VF) (56 % vs 0 % , $p < 0.01$, n=9), a significant lower recovery of contractility (21 ± 7.4 % vs 81 ± 6.6 %, mean and SEM, $p < 0.001$), and a significant increase of LDH release (667 ± 142 mU vs 268 ± 37 mU LDH/min/g wet weight, mean ± SEM $p < 0.05$). Administration of either 20 uM of the antioxidant (+)-cyanidanol-3 or 50 uM of the iron-chelator deferoxamine totally prevented the generation of VF and normalized contractility to control levels in the iron-loaded group. Moreover, 20 uM (+)-cyanidanol-3 significantly lowered LDH release in this period (312 ± 67 mU), whereas deferoxamine had no protective effect on this LDH release (1,494 ± 288 mU). Normal hearts appeared to be protected by 20 uM (+)-cyanidanol-3 as well. In this group, (n=6), a significantly higher recovery of contractility (97.1 ± 3.2 % vs 81 ± 6.6 %, $p < 0.05$) and a significantly lower release of LDH (110 ± 27 mU vs 268 ± 37 mU $p < 0.05$) was found compared with the control group (n=9). No difference in superoxide dismutase or glutathione peroxidase activity was found between both groups. It is concluded that: 1) iron-loaded rat hearts are more susceptible to anoxia and oxygen reperfusion damage; 2) iron-load itself, under normoxic conditions does not seem to be harmful; 3) the antioxidant (+)-cyanidanol-3 is able to protect normal as well as iron-loaded rat hearts against anoxic and reperfusion damage. We suggest that iron plays an important role in the occurrence of tissue damage and ventricular fibrillation during anoxia and reperfusion, probably through the formation of hydroxyl radicals and/or perferryl oxide.

INTRODUCTION

Several recent investigations have reported that oxygen-derived free radicals might play an important role in the pathogenesis of ischemic and postischemic (reperfusion) cardiac injury (1-3). Indirect evidence supporting this hypothesis is based on studies that have reported protective effects of free radical scavengers, such as superoxide dismutase (SOD), catalase, mannitol and allopurinol (4-7), on heart tissue during ischemia and reperfusion. Further evidence is based on the formation of malondialdehyde, an end-product of lipid peroxidation, which parallels postischemic reperfusion damage (1). Moreover, oxygen free radicals have recently been identified, directly, in (post)ischemic cardiac tissue using electron-spin-resonance techniques (8). The free radical superoxide and hydrogen peroxide, which are thought to be formed during ischemia and reperfusion

(1-3,8) are, however, not very toxic for myocardial tissue in the absence of a transition metal (9). Their reactivity is low and they can easily be converted by SOD, catalase and glutathione peroxidase. In the presence of catalytic amounts of transition metals such as iron, however, superoxide and hydrogen-peroxide can be transformed into a highly reactive hydroxyl-radical, $OH^{\cdot}$. (Haber-Weiss reaction, 10).

$$^{\cdot}O_2^- + H_2O_2 \xrightarrow{Fe} O_2 + OH^- + OH^{\cdot}$$

The hydroxyl radical is very toxic for biological tissue because it reacts immediately with lipids, proteins, or DNA and destroys their molecular structure. Reaction with poly-unsaturated fatty acids, for example, can easily induce lipid peroxidation which might lead to membrane damage and cell death (11). Almost all cellular iron is located in ferritin. Recently, we have shown that superoxide is able to mobilize iron from ferritin and that ferritin can be the physiological iron donor for the initiation of lipid peroxidation (12). In the present study, we investigated whether an iron-dependent mechanism of cell damage might be responsible for the reperfusion syndrome. Therefore, the amount of total iron in rat hearts (stored in ferritin) was increased by injecting the rats once a week with imferon (iron-dextran) for a period of six weeks and by adding an iron supplement ($FeSO_4$) to their food. After this treatment, we studied these hearts under normoxic, anoxic and reperfusion conditions by measuring contractility (apex-displacement), coronary flow, and LDH release. To substantiate the hypothesis that iron and free radicals are involved in the reperfusion syndrome, the effects of the iron chelator deferoxamine and the antioxidant (+)-cyanidanol-3 (13,14) were studied. The antioxidant, (+)-cyanidanol-3, is of special interest because this drug has already been used in clinical trials for treating alcoholics. Finally, we measured SOD and glutathione peroxidase activities to exclude a possible iron-dependent impairment of the defense system against free radicals.

MATERIALS AND METHODS

Animals.

Thirty male Wistar rats, 12 weeks old, were randomly divided into 2 groups, to obtain an iron-loaded group (15 animals) and a control-group (15 animals). Iron-load was accomplished by injecting 0.5 ml imferon (iron-dextran, 50 mg Fe/ml; Finsons, Leusden, The Netherlands) in the animals' gluteus muscles once a week for a period of six weeks and by adding a supplement ($FeSO_4.7H_2O$; 7.5 mg Fe/g standard food) to their

food during this period. The injections were administered after a brief anesthesia with di-ethylether. The control group was injected with dextran (10 % solution) under the same conditions. No differences in body weight or behavior between the two groups during this period were observed. The perfusion experiments were performed 1 week after the last injection, at which time body weight was 200-250 g.

Perfusion protocol.

The hearts were perfused according to Langendorff after using di-ethylether again as an anesthetic. The perfusion was carried out at 37° C and pH 7.4. We used a modified Tyrode's buffer containing 128 mM NaCL, 4.7 mM KCL, 1.3 mM $CaCL_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCL_2$ and 11 mM glucose saturated with 95 % O_2 and 5 % CO_2. Perfusion pressure was held constant at 80 cm H_2O. Coronary flow was measured indirectly in the aorta as a retrograde aortic flow. (transflow 601 system, Skalar, Delft, Holland). We measured contractility as apex displacement with a Harvard heart-smooth muscle transducer (model 386, Edenbridge, UK) that was hooked to the apex of the heart. Contractility was calculated by multiplying heart frequency (beats/minute) by amplitude (mm). After 15 minutes of initial perfusion, just before starting anoxic perfusion, coronary flow and contractility were measured and defined as 100 %. Anoxia was induced by perfusing the hearts under the same conditions with Tyrode's buffer saturated with 95 % N_2 and 5 % CO_2. After 45 minutes of anoxia, we reperfused with the oxygen saturated Tyrode's buffer for a period of 20 minutes. The two compounds (50 uM deferoxamine or 20 uM cyanidanol) were administered throughout the whole experiment, which took 80 minutes.

Chemicals and determinations.

Deferoxamine (desferal) was purchased from Ciba-Geigy, Switzerland, and (+)-cyanidanol-3 [(+)-catechin] was purchased from Zyma, Nyon, Switzerland. Both compounds were dissolved in Tyrode's buffer. The cyanidanol-containing buffer was protected against light by covering the perfusion apparatus with aluminum-foil. All other chemicals used were analytical grade. SOD activity was determined using the cytochrome c reduction inhibition method of McCord (15). The SOD concentration was expressed in microgram per milligram protein with bovine erythrocyte SOD (lot no. 10594720-11, Boehringer, Mannheim, FRG) as a standard. Glutathione peroxidase activity was determined according to the method of Paglia and Valentine (16). One unit corresponds to one micromole NADPH oxidation per minute. LDH was measured with a Boehringer commercial kit. Total iron was determined, after acidic destruction of the heart, by adding 10 mM Ferene-S and using an extinction coefficient, at 595 nm, of 35,500/M/cm (17).

Statistical analysis.

Data are presented as the mean ± SEM. All comparisons were made with one-way analysis of variance, except the occurrence of ventricular fibrillation which was compared with Fisher's exact test for dichotomy. $p < 0.05$ was considered to be significant.

RESULTS

Effects of iron-load on contractility, coronary flow and LDH release.

During the initial equilibration period no differences were measured in contractility (Fig.1), flow (Fig.2) or LDH release (Fig.3) between the iron-loaded (n=9) and control group (n=9). In the iron-loaded group, six rat hearts developed ventricular

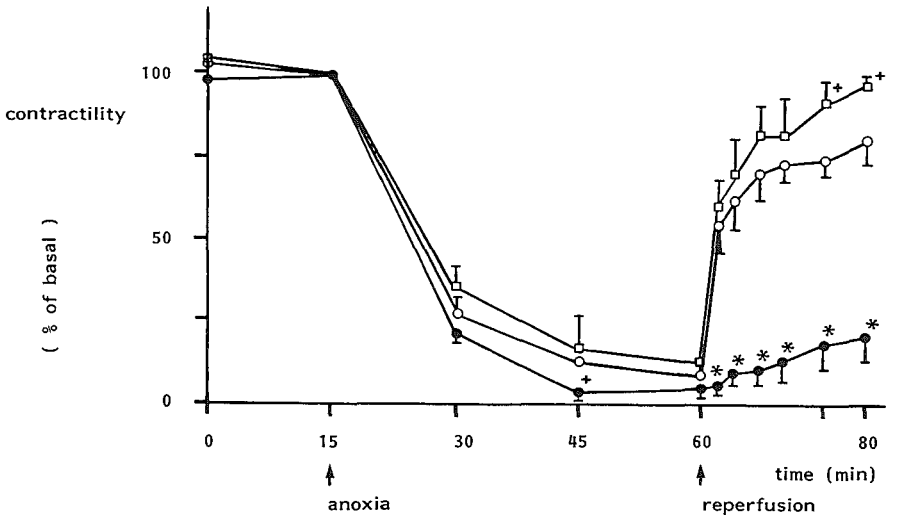

*Fig.1. Effects of anoxia and reperfusion on the contractility of iron-loaded rat hearts (•, n=9), control hearts (○,n=9) and control hearts in the presence of 20 uM (+)-cyanidanol-3 (□,n=6), expressed as percentage of the pre-anoxic (basal) value. Recovery of contractility during reperfusion was significantly lower in the iron-loaded group (at 80 minutes : 21.0 ± 7.4 % mean ± SEM, * p < 0.001) and significantly higher in the (+)-cyanidanol-3 protected group (97.1 ± 3.2, + p < 0.05) compared with the control group (81.0 ± 6.6 %).*

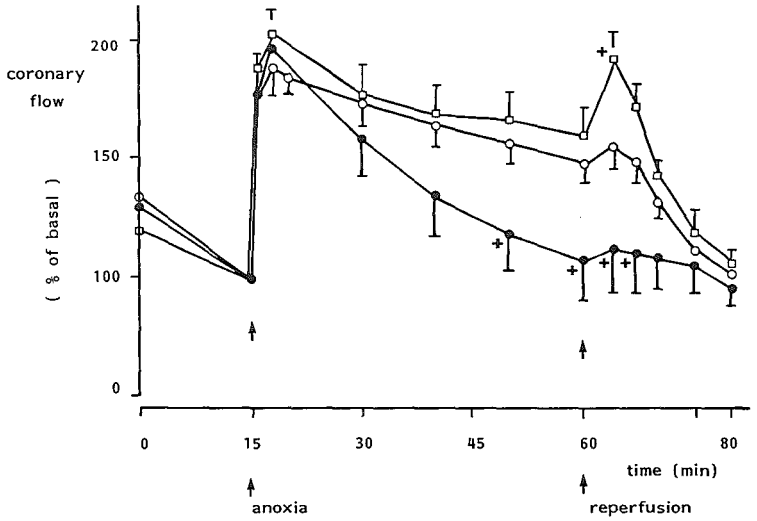

Fig.2. Coronary flow during anoxia and reperfusion of iron-loaded rat hearts (•,n=9), control hearts (○,n=9) and control hearts in the presence of (+)-cyanidanol-3 (20 uM) (□,n=6), expressed as percentage of pre-anoxic (basal) value. 100 % equals a coronary flow of 9.8 ± 0.7 ml/ min. (mean ± SEM) in the iron-loaded group, 9.7 ± 0.6 ml/ min. in the control group and 9.2 ± 1.2 in the (+)-cyanidanol-3 protected group. + p < 0.05 and significantly different from the control group.

fibrillation (VF) during anoxia or reperfusion (56 %), whereas no VF was found in the control hearts ($p < 0.01$ Fisher's exact test). These VFs started in five hearts at 21, 24, 32, 33 and 36 minutes of anoxic perfusion and in one heart after 12 minutes of reperfusion. Only two hearts (32 and 36 minutes) spontaneously recovered from fibrillation during early reperfusion at 3- and 1- minute reperfusion times, respectively. Contractility was regarded zero during VF (Fig.1). After 20 minutes' reperfusion, contractility returned to 81 ± 6.6 % in the control group and 21 ± 7.4 % in the iron-loaded group (n=9, mean ± SEM, $p < 0.001$) (Fig.1). If the hearts, which were fibrillating throughout reperfusion, are not taken into account, the contractility is restored to 41 ± 6.1 % (n=5, mean ± SEM, $p < 0.01$ vs control, n=9), and if all fibrillating hearts including those that recovered during early reperfusion are not taken into account, contractility is restored to 47.3 ± 4.9 % (n=3, mean ± SEM, $p < 0.05$ vs control n=9, figures not shown). Coronary flow revealed no difference between the iron

and control group in the first 10 minutes of anoxia (Fig.2). After these 10 minutes, however, the flow in the iron-loaded group dropped compared with the control group. After 30 minutes of anoxia and during early reperfusion, this drop becomes significantly different compared with the control group ($p < 0.05$). If all fibrillating hearts are not taken into account, no difference in coronary flow is found between these two groups (figures not shown), so VF itself in the iron-loaded group might well have been responsible for this drop in coronary flow.

LDH release in the effluent during reperfusion was significantly higher in the iron-loaded group ($p < 0.05$ Fig.3). After two minutes reperfusion, LDH release was maximal in both groups and 268 ± 37 mU/min/g wet weight in the control group (n=9) versus 667 ± 142 mU in the iron-loaded group (mean ± SEM, n=9 , $p < 0.05$). If all fibrillating hearts again were not taken into account, LDH release remained significantly different from control group (795.5 ± 124.9 mU, n=3, figures not shown).

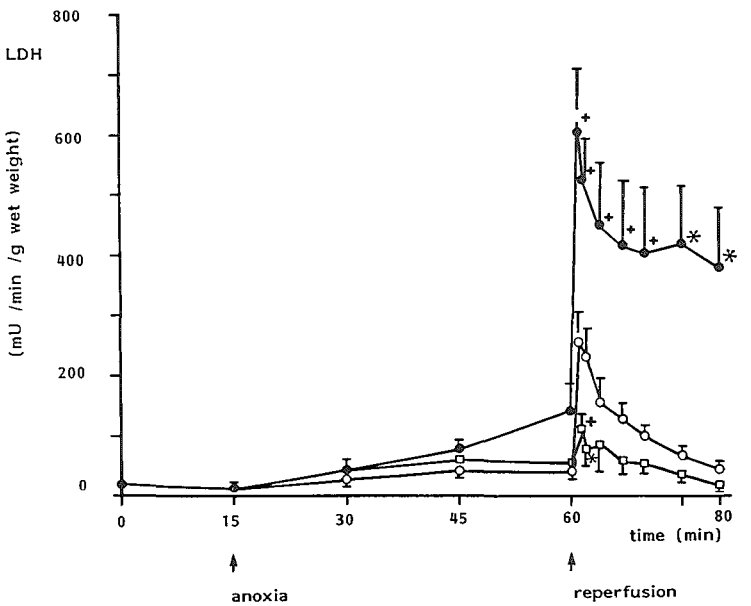

*Fig.3. LDH release during anoxia and reperfusion measured in heart effluents of iron-loaded rat hearts (•,n=9), control hearts (○,n=9) and control hearts in the presence of (+)-cyanidanol-3 (20 uM) (□ ,n=6). LDH release during reperfusion was significantly higher in the iron-loaded group and significantly lower in the (+)-cyanidanol group compared with the control group. * p < 0.01 and + p < 0.05.*

Effects of (+)-cyanidanol-3 and deferoxamine on iron-loaded rat hearts, during anoxia and reperfusion.

Administration of (+)-cyanidanol-3 (20 uM, n=3) to the perfusion fluid completely protected the iron-loaded rat hearts during anoxia and reperfusion. We found no VF, whereas 56 % was found in the unprotected iron-loaded group. In the reperfusion period, contractility was restored to control levels (87.7 ± 9.0 %) and significantly different from the unprotected iron-loaded hearts (21 ± 7.4 %, $p < 0.001$, mean ± SEM, Fig.4). Perfusion with (+)-cyanidanol-3 showed a complete normalization of LDH release to control levels (312.3 ± 117.0 vs 268.0 ± 37.0 mU), which is also significantly lower compared with the unprotected iron-loaded group (667 ± 142, Fig.6). Coronary flow in the iron-loaded hearts in the presence of cyanidanol, revealed a pattern comparable with control hearts (Fig.2 and 5).

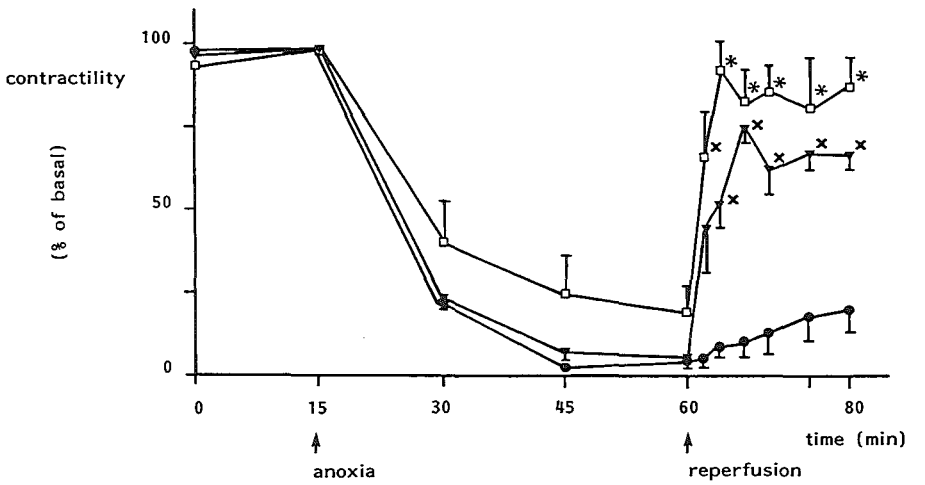

*Fig.4. Protective effects of (+)-cyanidanol-3 (20 uM) and deferoxamine (50 uM) on the contractility of iron-loaded rat hearts during anoxia and reperfusion. Iron-loaded group (•,n=9), iron-loaded group + 20 uM (+)-cyanidanol-3 (□,n=3), iron-loaded group + 50 uM deferoxamine (▼,n=3). Contractility was restored significantly higher in the (+)-cyanidanol-3 and deferoxamine iron-loaded group compared with the unprotected iron-loaded group (* $p < 0.01$ and + $p < 0.05$).*

Administration of deferoxamine (50 uM, n=3) protected the iron loaded hearts against VF. Also, no VF was found in this group compared with 56 % in the unprotected iron-loaded group. During reperfusion contractility was restored to 68.0 ± 6.1 % (Fig.4). Although this recovery was not as high as in the cyanidanol (87.7 %) or control group (81 %), it was significantly higher than in the unprotected iron-loaded group ($p < 0.01$, Fig.4). Measurements of coronary flow, which are exhibited in Fig.5, during anoxia with deferoxamine show no drop in coronary flow, which was seen with iron-load alone. In the presence of deferoxamine, a higher LDH release was found during early reperfusion (Fig.6). After 10 minutes of reperfusion, however, LDH release became lower compared with the iron-loaded group although still higher than in the presence of (+)-cyanidanol-3. Deferoxamine did not influence the LDH assay. At the moment, we do not have an explanation for this initial release of LDH and, as far as we know, this has never been reported before.

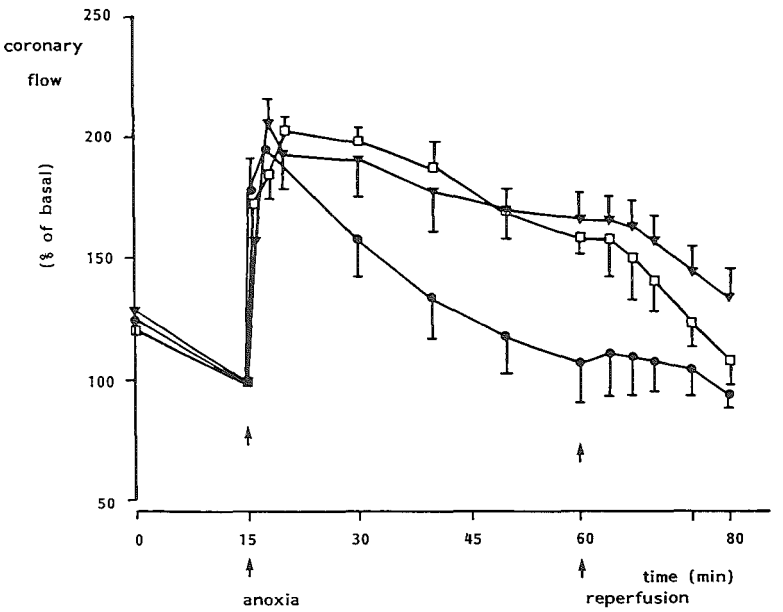

Fig.5. Effects of (+)-cyanidanol-3 (20 uM) (□,n=3) and deferoxamine (50 uM) (▼,n=3) on the coronary flow of iron-loaded rat hearts during anoxia and reperfusion in relation to unprotected iron-loaded rat hearts (●,n=9). 100 % coronary flow equals 9.8 ± 0.7 ml/ min. in the unprotected iron-loaded group, 9.3 ± 0.5 in the (+)-cyanidanol-3 group and 9.1 ± 0.2 ml/ minute in the deferoxamine group.

Effects of (+)-cyanidanol-3 and deferoxamine on normal rat hearts during anoxia and reperfusion.

The protective effects of deferoxamine on reperfusion injury in normal heart tissue have been reported extensively in the literature (18,19). As far as we know, no reports have been presented on the effects of (+)-cyanidanol-3 on normal heart tissue during anoxia and reperfusion. We, therefore, studied these effects more extensively. The antioxidant (+)-cyanidanol-3 (20 uM) protected normal rat hearts under the same conditions against anoxic and reperfusion injury. During anoxia, contractility was the same in the (+)-cyanidanol-3 protected (n=6) and control group (n=9) (Fig.1).

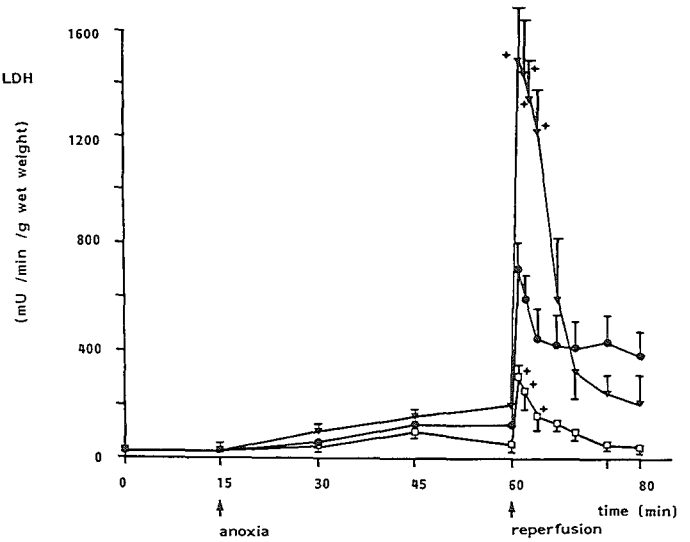

Fig.6. LDH release during anoxia and reperfusion in iron-loaded rat hearts. Iron-loaded group (●,n=9), iron-loaded group + 20 uM (+)-cyanidanol-3 (□,n=3), iron-loaded group + 50 uM deferoxamine (▼,n=3). Release of LDH in (+)-cyanidanol-3 group during reperfusion was significantly lower compared to the unprotected iron-loaded group, whereas administration of deferoxamine revealed a significant higher LDH release. (+ $p < 0.05$).

After 15 minutes of reperfusion, however, a significantly higher recovery of contractility with 20 uM (+)-cyanidanol-3 was found. (97 ± 3.2 vs 81 ± 6.6 Fig.1.). During early reperfusion, contractility recovered more rapidly and with less rhythmic

disturbances in the (+)-cyanidanol-3 group. Also, LDH release during reperfusion was significantly lower in the (+)-cyanidanol-3 protected group (110 ± 27 vs 268 ± 37 $p < 0.05$, Fig.3), whereas coronary flow was significantly higher during early reperfusion (Fig.2). This might be related to contractility, which is restored earlier and with less rhythmic disturbances in these hearts.

Measurements of total iron and activities of SOD and glutathione peroxidase.

Table 1, clearly reveals that administration of iron to the rats results in an increment of total iron content in the heart tissue. To exclude the possibility that the mechanism of anoxic and reperfusion injury is due to an impaired defense system against free radicals, SOD and glutathione peroxidase activities were measured. Table 1, clearly shows that the activities of these two enzymes are equal in all four groups.

Table 1. Total iron content and SOD and glutathione peroxidase activities in control and iron-loaded rat hearts after reperfusion.

	Total iron (μg/g heart)	Superoxide dismutase (μg/mg protein)	Glutathione peroxidase (mg/mg protein)
Control ($n=9$)	50.8 ± 11.0	1.77 ± 0.30	285.9 ± 26.4
Iron-loaded ($n=9$)	112.4 ± 24.1*	1.85 ± 0.28	270.5 ± 24.5
Iron-loaded plus 50 μM deferoxamine ($n=3$)	107.2 ± 22.7†	2.26 ± 0.21	267.5 ± 8.0
Iron-loaded plus 20 μM cyanidanol ($n=3$)	108.6 ± 17.0†	2.20 ± 0.11	285.3 ± 39.3

*$p<0.01$, †$p<0.05$.

DISCUSSION

The mechanism by which free radicals might be formed in anoxic, ischemic, or post-ischemic heart tissue is poorly understood. Several hypothesis have been postulated in the literature. McCord suggested in 1985 that xanthine dehydrogenase is converted during ischemia to xanthine oxidase by calcium activated proteases (2). The formation of this enzyme might be responsible for the generation of superoxide, $\cdot O_2^-$, which is directly formed when hypoxanthine is converted by this enzyme. Others have

demonstrated a minor role of this mechanism in rabbits, because of the absence of this enzyme in this animal (20). They have suggested that the ability of allopurinol, an inhibitor of xanthine oxidase, to protect rabbit hearts during ischemia must be due to a mechanism other than inhibition of this enzyme. Other sources of free radicals generated in ischemia in vivo might be polymorphonuclear cells and monocytes (21,22), activated under ischemic conditions, auto-oxidation of catecholamines (23), or formation of free radicals in impaired mitochondria (24). Experiments in vitro have shown that the toxicity of superoxide and hydrogen peroxide is lagerly diminished for biological tissues in the absence of iron (9). In the presence of iron, however, these radicals can be transformed into highly reactive hydroxyl radicals, $OH^{\cdot}$ (Haber-Weiss reaction, 10) which are regarded to be very toxic for biological tissues. Furthermore, it was shown that ferritin, the iron storage protein present in every cell, can be the free iron donor (Fe^{2+}) during $^{\cdot}O_2^-$generation (25). Our experiments show that iron-load itself, over a six week period, is not toxic. We found no differences in body weight or behavior between iron-loaded and control animals; moreover, under normoxic perfusion conditions contractility, flow and LDH release were the same in both groups. During anoxia and reperfusion, however, iron-loaded rat hearts turned out to be much more vulnerable, measured by a significant increase of VF, lower recovery of contractility and higher release of LDH. The iron-loaded rat hearts were protected with the antioxidant (+)-cyanidanol-3 and the iron-chelator deferoxamine, although LDH release with deferoxamine was higher compared to control levels and in contrast with protecting effects on VF and contractility. Because of equal SOD and glutathione peroxidase activities in both groups, it can be concluded that the occurring damage is not due to an impaired defense mechanism against free radicals but probably to a larger supply of $OH^{\cdot}$ radicals formed in the presence of increased iron amounts. During anoxia, mainly contractility is disturbed, as shown by a significant increase of VF, whereas during reperfusion cell necrosis becomes more prominent as shown by a higher LDH release and lower recovery of contractility. Protection of normal as well as iron-loaded rat hearts during anoxia and reperfusion by (+)-cyanidanol-3 has never been reported before and might be a promising progress in establishing protective measures in ischemia. Although, we cannot exclude that on the long term the storage of iron damages the cell directly, it is shown by our data that iron-loaded rat hearts are much more susceptible during anoxia and reperfusion. This gives strong evidence that besides the generation of free radicals, the availability of transition metals, such as iron, is indispensable for mediating anoxic or ischemic damage. The use of iron chelators which cannot induce lipid peroxidation (26), might therefore be a promising approach in preventing ischemic myocardial injury.

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CHAPTER III

PREVENTION OF POST-ISCHEMIC CARDIAC INJURY BY THE ORALLY ACTIVE IRON-CHELATOR 1,2-DIMETHYL-3-HYDROXY-4-PYRIDONE (L1) AND THE ANTIOXIDANT (+)-CYANIDANOL-3.

EVIDENCE OF IRON-MEDIATED FENTON-CHEMISTRY IN POST-ISCHEMIC CARDIAC INJURY.

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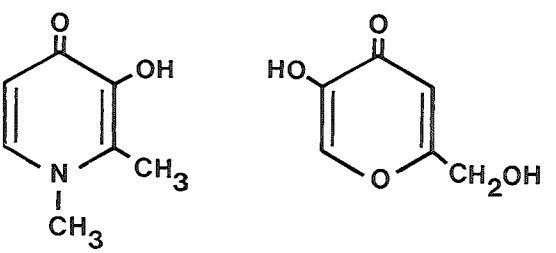
ABSTRACT

In this study, we investigated the role of oxygen-derived free radicals (ODFR) and iron in mediating myocardial injury during ischemia and reperfusion. Iron is of special interest because it may enhance tissue injury during ischemia and reperfusion by catalyzing the formation of highly reactive hydroxyl radicals (by modified Haber-Weiss or Fenton reactions). Rat hearts, perfused by the Langendorff method, were subjected to global ischemia (15 minutes at 37° C) and reperfusion. The effects of two iron-chelators, 1,2-dimethyl-3-hydroxy-4-pyridone (L1) and 5-hydroxy-2-hydroxymethyl-4-pyrone (kojic acid) and one antioxidant, (+)-cyanidanol-3, on contractility, coronary flow, lactate dehydrogenase (LDH) release and lactate production were studied. The combination of these iron-chelators is of special importance because L1 is known to prevent lipid peroxidation, induced by ADP/Fe^{3+} and NADPH in microsomes, in contrast to kojic acid. We found significant protection of contractility (apex-displacement) during reperfusion with 50 uM L1, 20 uM (+)-cyanidanol-3 ($p < 0.01$, $n=6$), whereas no protection was found with 50 uM kojic acid ($n=6$). Measurements of LDH release during reperfusion showed a protective pattern, similar to that found for heart contractility, although 50 uM kojic acid showed a significantly lower LDH release during the first ten minutes of reperfusion. No differences in coronary resistance or lactate release were found between the various groups. Our findings indicate that iron and ODFR are important in the pathogenesis of post-ischemic reperfusion injury, probably because of the formation of hydroxyl radicals. During heart ischemia, administration of the orally active iron-chelator L1 or the antioxidant (+)-cyanidanol-3 may be a promising approach in establishing post-ischemic cardiac protection.

INTRODUCTION

A growing amount of evidence indicates that oxygen-derived free radicals (ODFR) are important in the pathogenesis of cardiac tissue destruction during ischemia and reperfusion (1-4). Most of the evidence is indirect and based on studies that have reported protective effects of superoxide dismutase (SOD), catalase, mannitol or other free radical scavengers (5-8). Recently, ODFR have been directly identified in ischemic and post-ischemic cardiac tissue using electron-spin-resonance techniques (9-10). The actual source of ODFR in ischemic heart tissue is still not known, but several sources have been postulated in the literature, for example, the mitochondrial electron transport chain (11); the enzyme xanthine oxidase (2); activated polymorphonuclear cells (12-13); auto-oxidation of catecholamines (14); and the biosynthesis of prostaglandins (15). Generation during ischemia and reperfusion of superoxide ($^{\cdot}O_2^-$) or its dismutated product, hydrogen peroxide (H_2O_2), is toxic for cardiac tissue (16,17). This toxicity, however, can be markedly increased when a transition metal is present that can catalyze

hydroxyl radical formation from superoxide and H_2O_2 (Haber-Weiss reaction 18,19). Iron is considered the most important transition metal present in cardiac tissue. Normally, all iron is stored in ferritin in which it is unable to catalyze hydroxyl radical formation. In earlier studies, we showed that superoxide mobilizes iron from ferritin (20). Liberation of iron during ischemia by this mechanism may well be responsible for the formation of hydroxyl radicals and enhanced cardiac injury. Because of their high reactivity, hydroxyl radicals are regarded to be very toxic for biological tissues. Upon generation, they react rapidly with various molecules such as lipids, proteins, or DNA, thereby destroying their molecular structure. Moreover, hydroxyl radicals can initiate lipid peroxidation that may lead to membrane damage and cell death (21). To substantiate the role of hydroxyl radicals in mediating ischemic and post-ischemic cardiac injury, we perfused rat hearts under normoxic and post-ischemic reperfusion conditions with the antioxidant (+)-cyanidanol-3, and with the iron-chelators L1 and kojic acid. The naturally occurring flavonoid (+)-cyanidanol-3 is a relatively non-toxic compound with an oral LD_{50} in the rat of more than 16g/kg (22). This flavonoid interacts powerfully with many free radical generating systems, in which it neutralizes superoxide and hydroxyl radicals (22-24). For this reason, (+)-cyanidanol-3 has been used in clinical trials to treat patients with acute viral hepatitis (25). The iron-chelators L1 and kojic acid were used in the present investigation because of the contrasting effects of both compounds on lipid peroxidation induced by ADP/Fe^{3+} and NADPH in microsomes (26). Mostert et al. showed that iron complexed to L1 is unable to catalyze lipid peroxidation in microsomes in contrast to iron complexed to kojic acid. These two iron-chelators are part of a new, recently developed generation of iron-chelators that form water soluble, colored complexes with iron at physiological pH (27-29). The molecular structures are presented in Figure 1. Three molecules of each chelator form a complex with one iron atom. The iron binding constants of L1 and kojic acid are 10^{35} and 10^{27} respectively (30).



1,2-dimethyl-3-hydroxy-4-pyridone

(L1)

5-hydroxy-2-hydroxymethyl-4-pyrone

(kojic acid)

Figure 1. Molecular structure of the two iron chelators used in this study.

METHODS

Animals and perfusion protocol.

Twenty-seven male Wistar rats that were 20 weeks of age (body weight between 200 and 250 g) were divided into five groups: control group (n=6), cyanidanol group (n=6), two groups for iron-chelation with L1 (n=6) and kojic acid (n=6), and one group for combined perfusions with L1 plus cyanidanol (n=3). After a short anesthesia with di-ethylether, the hearts were quickly removed from the body and placed in ice-cold Tyrode's buffer to stop contractility. Immediately after cessation of contractility, the hearts were cannulated in the aorta and perfused by the method of Langendorff (31). The perfusions were carried out at 37° C and pH 7.4, with a modified Tyrode's buffer, containing 128 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$ and 11 mM glucose saturated with 95 % O_2 and 5 % CO_2. At the end of an initial equilibration period of 15 minutes, contractility and coronary flow were defined 100 %. Samples of the perfusate were collected during this stabilization period and during reperfusion to determine LDH and lactate-release. Total ischemia (15 minutes) was induced by closing the tap between the perfusion apparatus and the heart. Tissue temperature was maintained in this period by immersing the hearts in Tyrode's buffer of 37°C. We saturated this buffer with 95 % N_2 and 5 % CO_2 to minimize oxygen diffusion at the epicardial surface. The hearts were reperfused for 15 minutes with oxygenated perfusate. During ischemia, contractility was not measured to avoid technical complications. The hearts were perfused, apart from the ischemic period, with a constant pressure of 80 cm H_2O. Coronary flow was measured indirectly in the aorta, as a retrograde aortic flow. (Transflow 601 system, Skalar, Delft, The Netherlands). Contractility was recorded as apex displacement using a Harvard heart/smooth muscle transducer (Type 386, Edenbridge U.K.). A total load of 1 gram was hooked to the apex of the heart (32). We defined contractility as heart frequency (beats/min) multiplied by amplitude (mm). This product was considered a valid parameter for heart function since Stam and DeJong (33) reported a good correlation between this index and left ventricular systolic pressure in rat hearts. All compounds tested were administered throughout the whole experiment. Based on dose-dependent experiments, we decided to perfuse with 50 uM concentrations of L1 and kojic acid. This concentration had no influence on contractility or coronary resistance and is sufficient to chelate all possible free iron.

Chemicals and determinations.

(+)-Cyanidanol-3 was obtained from Zyma Nyon, Switzerland. and 5-hydroxy-2-hydroxymethyl-4-pyrone (kojic acid) from Aldrich (Gillingham, UK.). 1,2-Dimethyl-3-

hydroxy-4-pyridone (L1) was prepared by the method of Kontoghiorghes (34). These compounds were dissolved in Tyrode's buffer to obtain 50 uM solutions of L1 and kojic acid and a 20 uM solution of cyanidanol. The cyanidanol-containing buffer was protected against light by covering the perfusion apparatus with aluminum-foil. Lactate was measured by the method of Hohorst (35) and LDH was measured with a Boehringer commercial kit (Mannheim, FRG). All other chemicals were of analytical grade.

Statistical analysis.

Data are presented as the mean ± SEM, unless indicated otherwise. All comparisons were made with analysis of variance according to Bonferroni (36). Significance was considered at $p < 0.05$.

RESULTS

Effects of cyanidanol, L1 and kojic acid on contractility, during normoxia and reperfusion.

Throughout the experiment, the hearts were perfused with either 50 uM L1, 50 uM kojic acid, 20 uM cyanidanol or the combination of 50 uM L1 and 20 uM cyanidanol and the effects upon contractile function were compared with control hearts (Figures 2 and 3). As mentioned in "Methods" these concentrations were derived from dose-dependent experiments and had no influence on heart contractility. After 15 minutes of normal perfusion, contractility was 12,365 ± 4,013 for control hearts, 12,018 ± 2,259 for cyanidanol hearts, 12,468 ± 3,644 for L1 hearts, 12,891 ± 3,253 for kojic acid hearts and 12,247 ± 765 mm/min for L1 plus cyanidanol hearts (mean ± SD, n=6; except the L1 plus cyanidanol group, which was n=3). After 15 minutes of total ischemia, during reperfusion, contractility was restored significantly higher in the L1 group compared with the control group ($p < 0.01$, n=6; Figure 2). Kojic acid showed, in clear contrast to L1, no protective effect on contractility in this period (Figure 2). Figure 3 shows contractility of control hearts in relation to cyanidanol and L1 plus cyanidanol perfused hearts. Also, 20 uM cyanidanol was able to protect rat heart contractility during reperfusion ($p < 0.01$ at 15 minutes reperfusion, n=6) though to an extent lower than did 50 uM L1 (60.6 ± 7.1% vs. 66.2 ± 5.8%, mean ± SEM). We found no additional protective effect with combined perfusions of L1 and cyanidanol on contractility during reperfusion (67.7 ± 8.4%, n=3; NS compared with the control group, n=6). All hearts showed no contractility at the end of the ischemic period, and without exception, all started to beat spontaneously during reperfusion. No ventricular fibrillation was encountered in any heart.

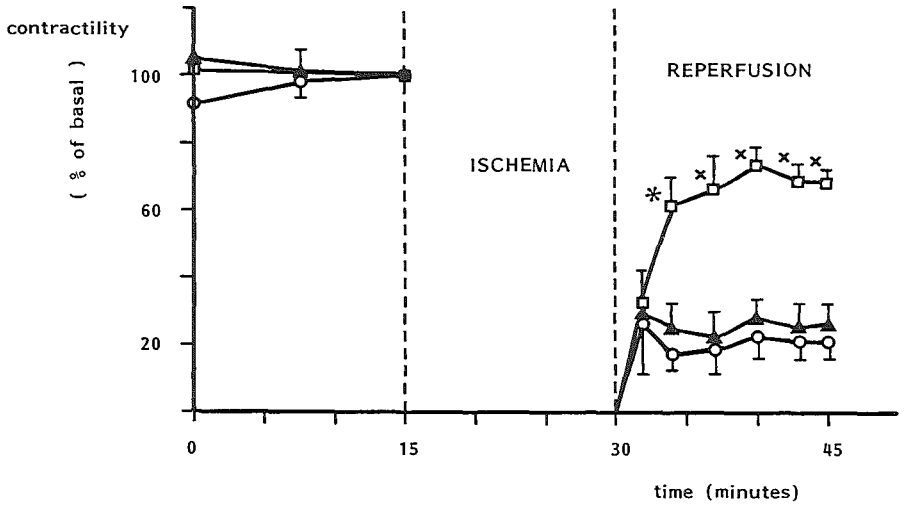


*Fig.2. Plot of contractility during normoxia (0-15 min.) and reperfusion of control hearts (○) and hearts perfused with 50 uM L1 (□) or 50 uM kojic acid (▲). At 15 minutes contractility was defined 100 % and 12,365 ± 4,013 for control hearts, 12,468 ± 3,644 for hearts perfused with L1 and 12,891 ± 3,253 for hearts perfused with kojic acid (mean ± SD). During reperfusion, contractility was restored significantly higher in the L1 group compared with the control group (66.2 ± 5.8% versus 20.9 ± 4.6% at 45 minutes, mean ± SEM, * = $p < 0.05$, x = $p < 0.01$, n=6), whereas no differences were found between control hearts and hearts perfused with kojic acid.*

Effects of cyanidanol, L1 and kojic acid on LDH release, lactate release and coronary flow during reperfusion.

LDH release in the effluent was significantly higher in the control group compared with the L1 group after 7 minutes of reperfusion ($p < 0.05$, n=6; Figure 4). The highest level of LDH release in the L1 group occurred between 5 and 7 minutes of reperfusion. LDH release in the control group was maximal after 10 minutes reperfusion. Perfusions with kojic acid resulted in a significantly lower LDH release at 7 and 10 minutes reperfusion compared with control hearts (Figure 4). After 15 minutes of reperfusion, LDH release in this group was still increasing to become no longer significantly different from control levels. We do not have an good explanation for this

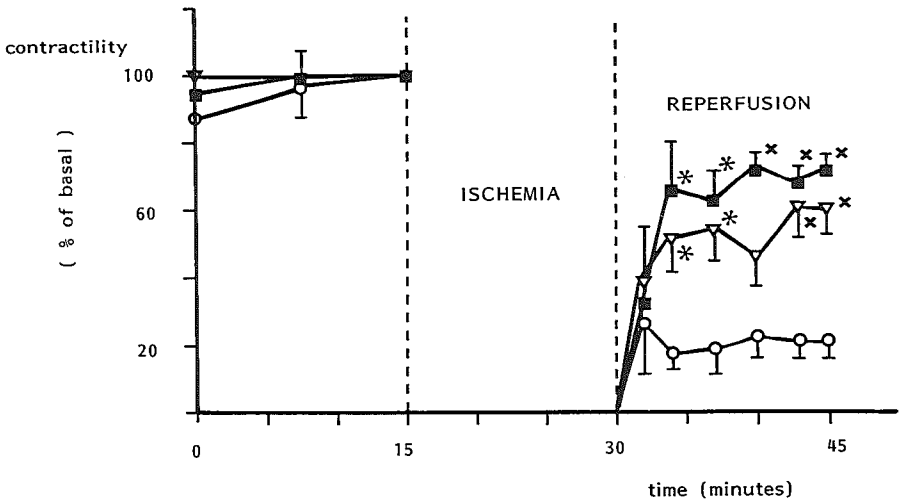


*Fig.3. Contractility during normoxia (0-15 min.) and reperfusion of control hearts (○) and hearts perfused with 20 uM cyanidanol (▽) or 20 uM cyanidanol plus 50 uM L1 (■). After 15 minutes contractility was defined 100% and 12,365 ± 4,013 for control hearts, 12,018 ± 2,259 for cyanidanol hearts and 12,247 ± 765 for L1+cyanidanol hearts (mean ± SD). During reperfusion contractility was restored significantly higher in the cyanidanol group (60.6 ± 7.1 %, n=6) and cyanidanol+L1 group (67.7 ± 8.4 %, n=3) compared with the control group (20.9 ± 4.6 %, n=6, mean ± SEM, * = $p < 0.05$, x = $p < 0.01$).*

delayed LDH release because no differences in the coronary flow were measured between the various groups (Table 1) and because contractility in kojic acid hearts remained stable at the time of the elevated LDH release. Figure 5 shows LDH release in cyanidanol and L1 plus cyanidanol perfused rat hearts in relation to control hearts. A significantly lower LDH release with cyanidanol alone was found compared to control levels ($p < 0.05$, n=6). Reperfusion in the presence of the combination of cyanidanol plus L1 showed a comparable protection, indicating that no additional effect occurred. To assure that a comparable ischemia was induced under the various conditions, lactate release was measured. More than 80% of total lactate release, measured during reperfusion, was released in the fist five minutes of this period. Figure 6 shows this release. No differences were encountered among the five groups, which indicates that the amount of ischemia was the same in all five groups. Finally, coronary flow during

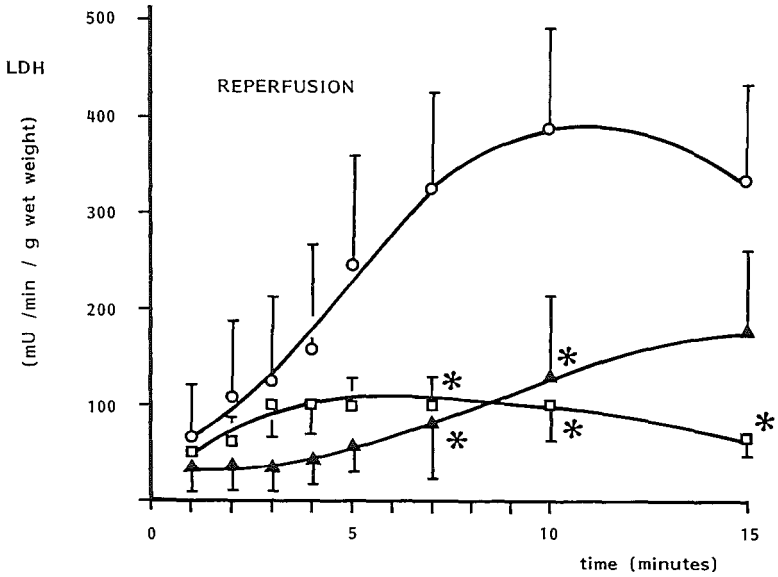


*Fig.4. LDH release during reperfusion measured in heart effluents of control hearts (○, n=6), hearts perfused with 50 uM L1 (□, n=6) and hearts perfused with 50 uM kojic acid (▲, n=6). LDH release was significantly lower in the L1 group, after 7 minutes reperfusion, and in the kojic acid group, at 7 and 10 minutes, compared with the control group (* = $p < 0.05$).*

Tabel 1. Coronary flow during post-ischemic reperfusion as percentage of preischemic level (100%).

Time (min)	Control (*n*=6)	Cyanidanol (*n*=6)	L1 (*n*=6)	Kojic acid (*n*=6)	Cyanidanol+L1 (*n*=3)
2	93±8.9	77±7.5	80±18.9	95±24.0	97±13.5
4	119±18.9	134±23.5	151±36.4	135±19.6	138±14.5
7	141±26.3	169±17.7	157±37.9	158±22.0	158±14.8
10	142±23.8	157±20.9	148±29.5	157±7.3	141±19.8
13	124±21.3	122±24.4	130±36.2	133±16.1	122±19.3
14	119±19.8	114±24.7	124±33.7	129±12.5	117±16.1
15	111±19.0	110±24.5	119±32.0	122±13.3	112±16.1

Data are mean±SD.

reperfusion is displayed in Table 1. A comparable pattern was found during reperfusion in the five separate groups, suggesting that the compounds of interest, as well as contractility or cellular necrosis, had no influence on post-ischemic coronary resistance in our perfusion experiments.

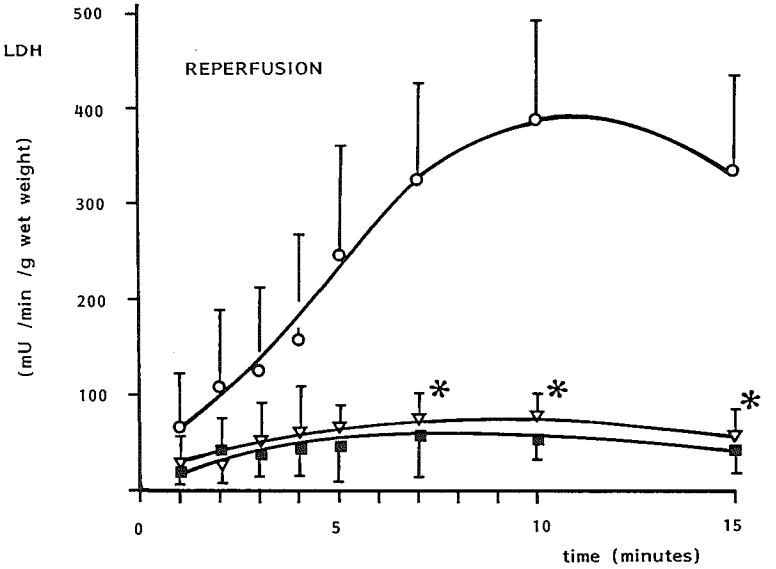


*Fig.5. LDH release during reperfusion measured in heart effluents of control hearts (○, n=6), hearts perfused with 20 uM cyanidanol (▽, n=6) and with 20 uM cyanidanol + 50 uM L1 (■, n=3). LDH release was significantly lower in the cyanidanol group after 7 minutes reperfusion (* = p < 0.05), and the same as the LDH release in the cyanidanol + L1 group.*

DISCUSSION

In this study, we found that administration of the iron chelator 1,2-dimethyl-3-hydroxy-4-pyridone (L1) and the antioxidant (+)-cyanidanol-3 protect post-ischemic cardiac tissue. Heart perfusions with these substances resulted in a higher recovery of contractility and a lower release of LDH during post-ischemic reperfusion. Moreover, the administration of a second iron chelator, 5-hydroxy-2-hydroxymethyl-4-pyrone (kojic acid), had no effect under the same conditions. The differences observed between these iron-chelators can be explained by the opposite effects each has in catalyzing the modified Haber-Weiss or Fenton reactions (18-19). The property of L1 to inhibit

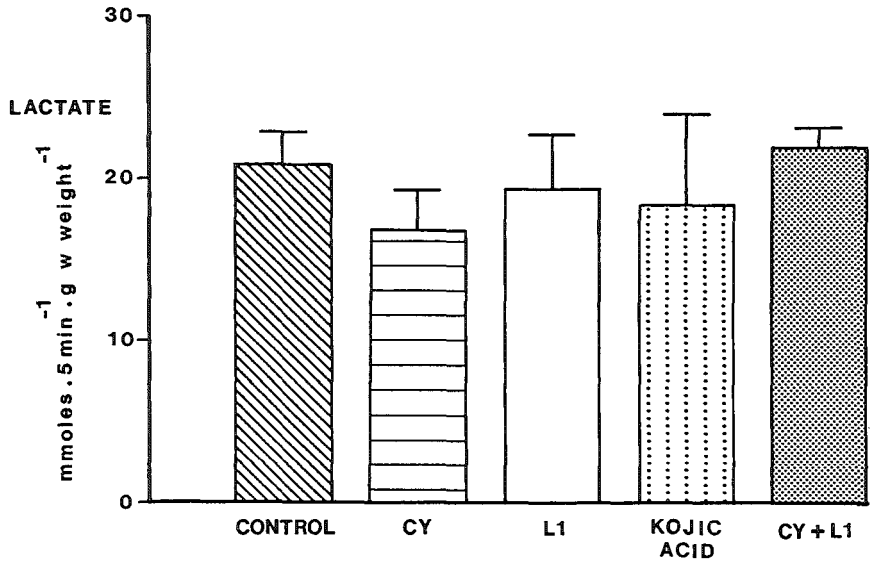


Fig.6. Lactate production during the first five minutes of reperfusion in control hearts, hearts perfused with 20 uM cyanidanol (cy), 50 uM L1 (L1), 50 uM kojic acid, and 20 uM cyanidanol + 50 uM L1 (cy + L1). No significant differences were observed, indicating that the amount of ischemia was comparable.

hydroxyl radical formation in vitro, may well be responsible for the observed heart protection during post-ischemic reperfusion. Our findings that equal concentrations of another iron-chelator, kojic acid, with structural similarities to L1, had no protective effect, can be explained by the fact that iron complexed to kojic acid is still able to catalyze hydroxyl radical formation (26). The contrast between these two chelators strengthens our suggestion that the binding of iron is responsible for post-ischemic protection and not a possible scavenging capacity.

Protection of heart function and tissue necrosis by the antioxidant (+)-cyanidanol-3 is consistent with the hypothesis that oxygen-derived free radicals are involved in the pathogenesis of myocardial injury during ischemia and reperfusion (1-4). The capacity of (+)-cyanidanol-3 to neutralize superoxide (22) may well account for the protection observed. It must be emphasized, however, that by scavenging superoxide, iron is not released from ferritin by this radical. It is therefore not surprising that by using the combination of (+)-cyanidanol-3 and L1, no additional protection is seen.

The results of the present study are consistent with experiments in which protection with another iron-chelator, deferoxamine, was reported (37-38). Badylac et

al. (37) showed protection of rat hearts by deferoxamine after 60 minutes of total ischemia. In that study, they reported significant lower creatine phosphokinase release and less coronary resistance during reperfusion. Unfortunately, no parameter for heart function was assessed in this study. We found, using a similar Langendorff model, after 60 minutes of total ischemia at 37°C, no restoration of heart function at all in rat hearts. We have estimated that the point of no return of heart function as a result of ischemia under these conditions is between 20 and 30 minutes (unpublished observations). In our opinion, 60 minutes of total ischemia at 37°C is too long to establish possible cardiac protection by drugs. Further protective evidence of deferoxamine was reported by Ambrosio et al. (38). They found that administration of deferoxamine at the time of post-ischemic reflow was able to protect rabbit hearts after 30 minutes of total ischemia (37°C). A higher recovery of myocardial function and less LDH release was observed in this study. These results are in contrast to the findings of Myers et al. (39), who reported no protection of deferoxamine in rabbit hearts after two hours of global ischemia at 27° C. In this study, they hypothesized that iron-catalyzed hydroxyl radical formation can still be important because intracellular iron pools may be inaccessible to deferoxamine.

In several studies performed by Hearse et al. (40,41), 83% ventricular fibrillation (VF) was encountered in control rat hearts after 10 minutes of ischemia. In the present study, we found no VF in any heart. This marked difference may be explained by the fact that regional ischemia was established by Hearse et al. in contrast to total ischemia in our experiments. Generation of reentry circuits is known to be responsible for the induction of VF (for review, see Manning and Hearse, 42). Heterogeneity of tissue injury is a critical progenitor of these reentry circuits. Our contrasting findings may therefore be explained by the use of different forms of ischemia.

As far as we know, no studies have been published reporting (post)-ischemic cardiac protection with iron-chelators other than deferoxamine. Considering the contrasting results about the effectiveness of deferoxamine administration, our findings are important in establishing the role of iron during ischemia and reperfusion. In recent experiments in our laboratory, we showed that iron-loaded rat hearts are more susceptible to reperfusion injury after 45 minutes of anoxia (43). This study indicates that iron-loading itself in cardiac tissue can accelerate tissue destruction during anoxia and reperfusion probably by the formation of hydroxyl radicals. In the present study, we have shown that ischemic rat hearts can be protected during post-ischemic reperfusion by the administration of the iron-chelator L1 and the antioxidant (+)-cyanidanol-3. Moreover, we found that the iron chelator kojic acid, the complex of which is unable to block hydroxyl radical formation in vitro (26), had no protective effect. For these reasons, we conclude that iron and ODFR are important in mediating post-ischemic cardiac injury probably because of the formation of hydroxyl radicals. The protection achieved by the orally active iron-chelator L1, which has already been used in clinical trials to treat patients with beta-thalassaemia (28), may be a promising and easily assessable approach in establishing (post)-ischemic cardiac protection in patients in the near future.

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CHAPTER IV

SPECIFIC IRON-ACCUMULATION IN MYOCARDIAL ENDOTHELIAL CELLS AND PERICYTES AFTER SHORT TERM IRON-LOADING.

EVIDENCE FOR REPERFUSION INJURY TO BE INITIATED IN VASCULAR TISSUE.

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(submitted)

ABSTRACT

The distribution of iron in rat hearts after a short period of iron-load was investigated by light microscopy, electron spectrocopic imaging (ESI) and electron energy loss spectroscopy (EELS). Iron was found to be abundantly present in endothelial cells and pericytes, sparsely present in some macrophages located in the perivascular space but not present in cardiomyocytes themselves. This observation is important in clarifying the site of free radical toxicity in the heart as a result of ischemia and post-ischemic reperfusion. Earlier experiments in our laboratory have shown that a standardized period of six weeks of iron-load is responsible for a significant increase of susceptibility of rat hearts to reperfusion injury. Generation of superoxide ($^{\cdot}O_2^-$) and its dismutated product hydrogenperoxide is regarded to be associated with reperfusion injury. In the presence of iron-catalysts, these radicals are known to induce highly oxidizing hydroxyl radicals (modified Haber-Weiss or Fenton reaction). Because of their high reactivity, these latter radicals can be expected to combine "site specific" i.e. at the place where iron-catalysts are situated. The increased susceptibility of iron-loaded rat hearts to reperfusion injury, together with the present observation of specific iron-storage in vascular endothelial cells and pericytes, strongly indicate free radical toxicity to be initiated in these cells. We therefore suggest reperfusion injury to be initially a vascular problem associated with oxygen-derived free radical generation in endothelial cells and/or pericytes.

INTRODUCTION

Termination of myocardial ischemia by restoration of the coronary flow is essential to achieve recovery of the heart. This restoration, however, is also responsible for increased ischemic damage as demonstrated by a rise in tissue necrosis (1-3), a reduction in cardiac function (4,5), and the occurrence of rhythmic disturbances as e.g. ventricular fibrillation (6,7). These adverse effects of coronary flow restoration are usually termed "reperfusion injury" or "the oxygen paradox" (8,9). Many investigators are presently studying this phenomenon as myocardial reperfusion has become a regular clinical event which can be established by coronary vasodilators, angioplasty or thrombolytic therapy (streptokinase or tissue plasminogen activator). Recently, oxygen-derived free radicals, and more specifically hydroxyl radicals ($^{\cdot}OH$), have been implicated to be responsible for reperfusion injury (10,11).

In a previous study (12), we have found that iron-loaded rat hearts are much more sensitive to reoxygenation injury after a period of 45 minutes of anoxic perfusion than control hearts. This increased sensitivity was demonstrated by a significant increase of ventricular fibrillation and lactate dehydrogenase release (LDH) and by a significant decrease of heart function. We proposed that this phenomenon is associated with the generation of hydroxyl radicals, because we were able to reverse this iron-load effect by

administration of either the free radical scavenger, (+)-cyanidanol-3, or the iron-chelator deferoxamine (12). In iron-loaded rats, the total amount of iron is doubled in the heart as a result of our standardized iron-administration technique (12). The present study was developed to investigate the site of iron-storage in these hearts using light- and electron microscopy and electron energy loss spectroscopy (EELS). The reason to focus on specific iron-localization is the increasing amount of evidence suggesting reperfusion injury to be initiated in endothelial cells rather than in cardiomyocytes themselves (13-17).

METHODS

Animals.

At the age of twelve weeks, three male Wistar rats were iron-loaded by injecting 0.5 ml Imferon (iron-dextran, 50 mg Fe/ml; Fisons, Leusden, The Netherlands) in the gluteus muscles once a week for a period of six weeks, and by adding a supplement of iron ($FeSO_4.7H_2O$; 7.5 mg Fe/g standard food) to their food during this period (12). The injections were administered after a brief anesthesia with di-ethylether. Control rats were injected under the same conditions with a 10 % dextran solution (n=3). No differences were observed in body weight or behavior between the two groups. After the last Imferon injection the rats were rested for a period of two weeks, at which time their body weight was between 200-250 g.

Perfusion protocol, tissue fixation and electron microscopy.

After these two weeks, the animals were again anesthetized with di-ethylether to remove the hearts which were directly cannulated in the aorta and perfused retrogradely according to Langendorff (18). The perfusions were carried out at 37° C and pH 7.4. using a modified Tyrode's buffer, containing 128 mM NaCL, 4.7 mM KCL, 1.3 mM $CaCL_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1.0 mM $MgCL_2$ and 11 mM glucose saturated with 95 % O_2 and 5 % CO_2. Perfusion pressure was held constant at 80 cm H_2O. After a perfusion period of 15 minutes, the hearts were fixed by perfusing them with 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for a period of 5 minutes. The hearts were kept in this solution for two hours (20°C). The tissue was postfixed in 1% OsO_4 in 0.1 M cacodylate buffer for an additional 2 hr at 4°C. After dehydration in ethanol (ten minutes incubation in respectively 70%, 80%, 90% and 96% ethanol) and incubation in 99% propylenoxide for one hour, the hearts were embedded in Epon 812 (Shell, The Netherlands). Ultrathin sections of

approximately 60 nm were examined without additional heavy metal staining in a Zeiss EM 902 transmission electron microscope. This microscope is equipped with an integrated electron energy spectrometer which allows: 1) high resolution imaging with mono-energetic electrons (Electron Spectroscopic Imaging, ESI) and 2) specific element detection by measuring element-specific energy absorption (Electron Energy Loss Spectroscopy, EELS, 19). Light microscopic sections were stained for iron according to Perls (20).

RESULTS

Light microscopy.

After staining with Perls' prussian blue, iron can be detected in light microscopic sections as blue granular deposits. In black and white photographs, these deposits are visible as black spots. Fig.1 shows three examples of a section through the left ventricular muscle of iron-loaded rat hearts. Throughout the heart muscle, iron appeared to be mainly stored in the capillary wall. Two examples of this particular location are shown in the upper two parts of Fig.1. No iron-deposits (black spots) can be seen in the heart muscle itself. In contrast to the abundant iron-storage in the capillaries, iron was rarely detected in arteries, arterioles or veins, venules. The lower part of Fig.1 displays two arterioles with iron stored in the perivascular space. These iron-deposits are presumably located in macrophages around these vessels. Although this site of iron-storage is evident, it was far less frequently seen than iron-deposits in the capillary wall. Notice again that no iron is visible in the heart muscle itself.

Transmission electron spectroscopy.

Fig.2 is composed of three ESI-micrographs in which the middle part displays a cross section through a capillary vessel in the left ventricular muscle. The luminary side of the vessel is indicated with an "L". The marked parts of the capillary wall were further magnified and are displayed in the upper and lower part of the figure. High density particles with a diameter of about 6 nm can be clearly seen in this figure and probably represent ferritin molecules. In the upper micrograph, an endothelial cell (E) contains hardly any of these particles whereas an underlying pericyte (P) is packed with them (arrow). The lower micrograph shows another endothelial cell, characterized by its pinocytotic vesicles (PV), with similar high density particles stored in lysosomes (L) and in the cytosolic compartment.

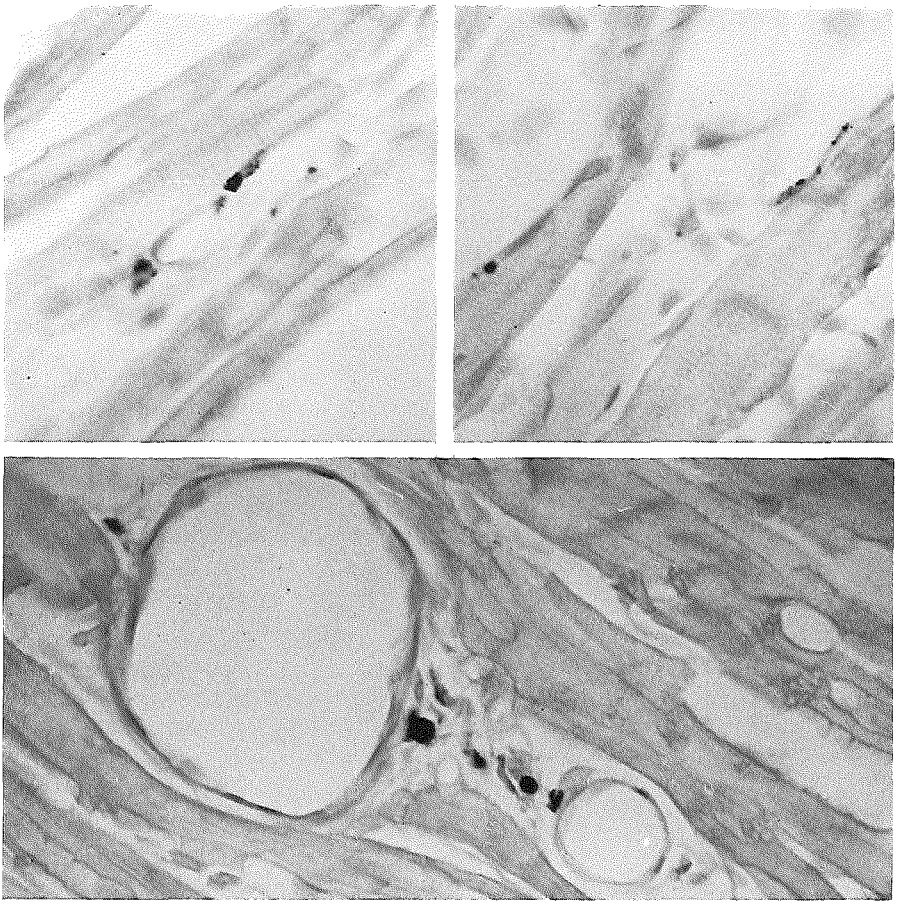

Fig.1. Light microscopic sections through left ventricular heart muscle of iron-loaded rat hearts. The sections were stained with Perls' prussian blue (black spots) and nuclear fast red (160x). In the upper two pictures iron-deposits (black spots) in the wall of two capillaries can be seen. In the lower part two arterioles are visible with iron (black spots) located in macrophages around the arterioles. Notice that no iron is detectable in cardiomyocytes themselves throughout this figure.

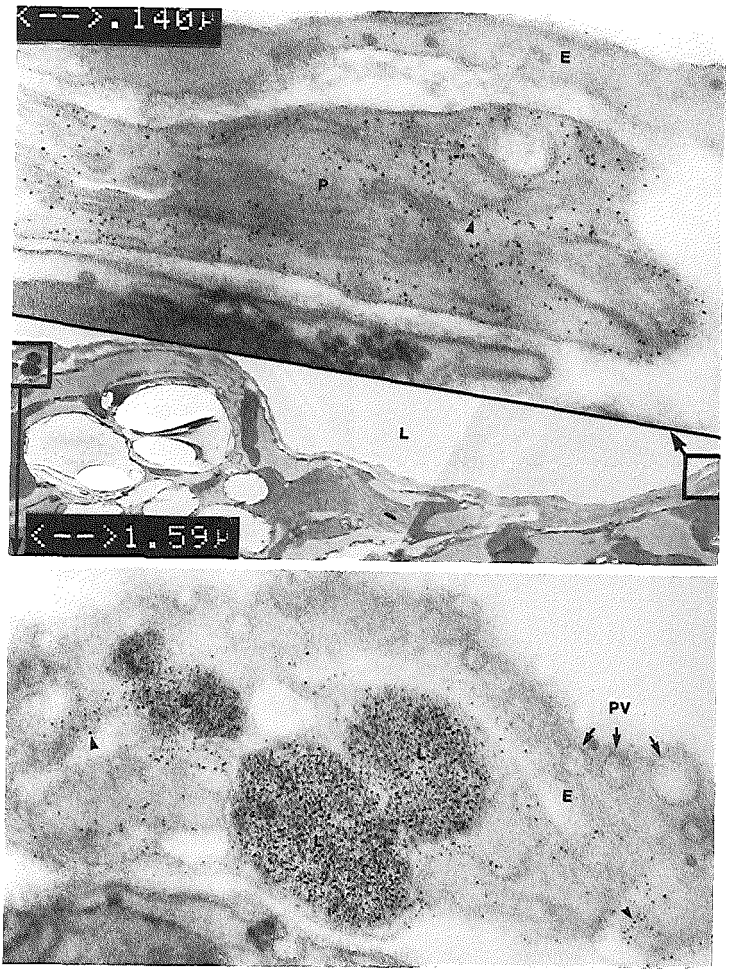

Fig.2. Transmission electron microscopy of left ventricular heart muscle. The upper and lower micrograph are magnifications (50,000x) of the squares in the middle section (4,400x). In the upper micrograph a pericyte (P) with high density particles (arrows) is clearly visible together with an endothelial cell containing some of these particles. The lower micrograph shows an endothelial cell (E), as characterized by its pinocytotic vesicles (PV), packed with high density particles in the cytosol and in lysosomes (L). "L" in the middle part indicates the vascular lumen. The particles were identified as iron-deposits using electron energy loss spectroscopy (see text).

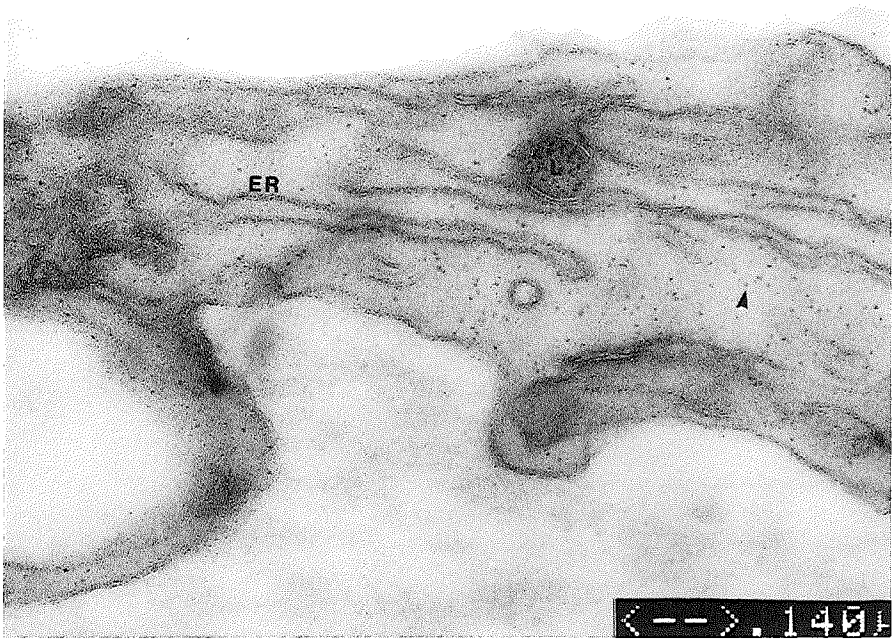

Fig.3. Typical iron-localization encountered in pericytes of iron-loaded rat hearts with high density particles in the cytosolic compartment and in a lysosome (L), (50,000x). ER: endoplasmatic reticulum.

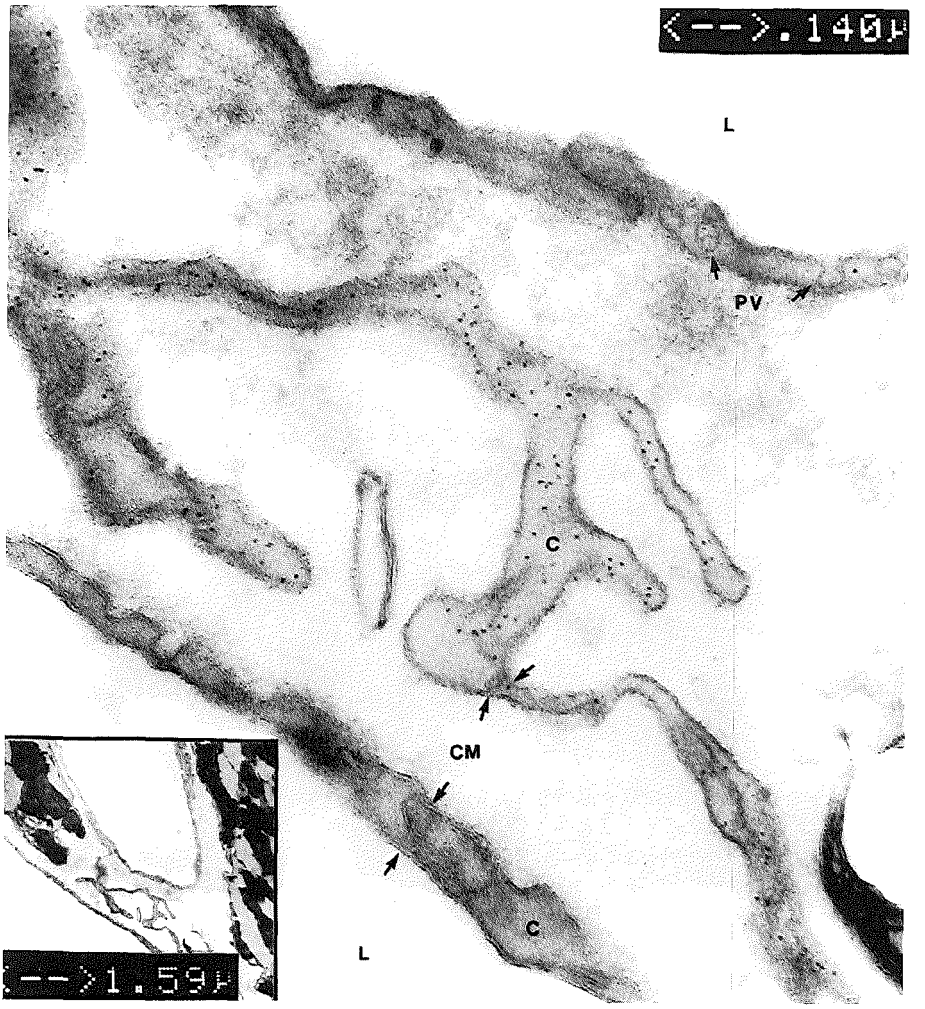

Fig.4. Perpendicular section through (a) folded endothelial cell(s) (50,000x). The inserted photo represents the same section at lower magnification (4,400x). C: cytosol, PV: pinocytotic vesicles, CM: cellular membranes. High density particles identified as iron-deposits with the EELS technique are clearly visible in the cytosol of the middle cell.

Fig. 3 and 4 show other examples of the presence of ferritin-like particles in iron-loaded rat heart capillaries. This particular site of iron-storage was encountered throughout the left and right ventricular muscles of all iron-loaded rat hearts. Fig.3 displays a pericyte with high density particles in the cytosolic compartment and in a lysosome (L). Fig.4 is a perpendicular section through presumably (a) folded endothelial cell(s). The micrograph is a further magnification of a part of the inserted picture in which two capillary lumina can be detected. The endothelial cell(s) is (are) again characterized by its (their) pinocytotic vesicles (PV). Iron is clearly visible in the cytosolic compartment (C). The cellular membranes are indicated (CM). In endothelial cells lysosomal iron-storage was generally more evident than in pericytes, whereas in cardiomyocytes no iron-deposits were ever encountered. The results were compared with unloaded rat hearts in which no iron-deposits were detected in any cell type (results not shown).

Electron Energy Loss Spectroscopy (EELS).

The EELS technique allows characterization of elements by recording element-specific energy-loss in eV (1eV $\approx$ 1.602 x 10^{-19} Joule). Upon varying transmission-energy and measuring energy-loss (absorbtion) by the section, a spectrum can be recorded which is specific for the elements in the section (19). In various places, the ferritin-like particles encountered in endothelial cells and pericytes of iron-loaded rat hearts were analyzed with EELS and appeared to be iron-deposits without any exception. An example of a relative absorption spectrum originating from these particles is displayed in Fig 5. The two peaks around 715 eV and 725 eV are known to be typical for iron (19). This in agreement with our morphological finding that the 6nm particles are indeed ferritin.

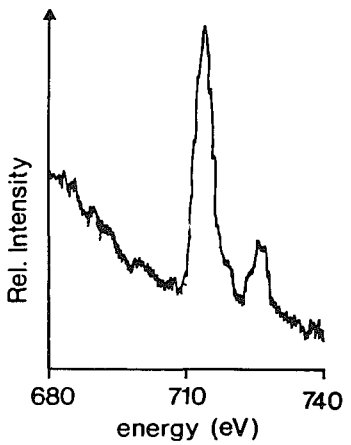

Fig.5. Example of Electron Energy Loss Spectroscopy (EELS) of high density particles encountered in iron-loaded rat hearts. The relative absorbtion spectrum of these particles is displayed here. This curve with high intensities around 715 and 725 eV is known to be typical for iron (see text).

DISCUSSION

Oxygen-derived free radicals have been implicated to be responsible for myocardial post-ischemic reperfusion injury (8,9). Among these radicals, the hydroxyl radical ($^{\cdot}OH$) is regarded the most oxidizing species, which is capable to induce severe cytotoxicity. Hydroxyl radicals can be generated in vivo from the less toxic oxygen radical superoxide ($^{\cdot}O_2^-$) and hydrogenperoxide in the presence of metal-catalysts (modified Haber-Weiss reaction or Fenton reaction (21)).

$$^{\cdot}O_2^- + H_2O_2 \xrightarrow{Fe} {}^{\cdot}OH + OH^- + O_2$$

The best candidate (metal) present in heart tissue to catalyze $^{\cdot}OH$ formation is iron although to a lesser extent copper could be an alternative (22). There is substantial evidence which indicates the involvement of hydroxyl radicals in the reperfusion syndrome (10,11,23-25). This evidence is mainly based on studies in which post-ischemic cardiac protection was reported by administration of the iron chelator deferoxamine (3,11,12) or by administration of the putative hydroxyl radical scavengers dimethylthiourea and mannitol (26). In a recent study (23) from our laboratory, we found that the iron-chelator 1,2-dimethyl-3-hydroxy-4-pyridone (L1) is able to prevent post-ischemic cardiac injury as well, in contrast to the structurally related iron-chelator 5-hydroxy-2-hydroxymethyl-4-pyrone (kojic acid). The inability of the latter chelator to mediate protection was anticipated since this chelator is known not to prevent lipid peroxidation in microsomes (induced by ADP/Fe^{3+} and NADPH) in contrast to L1 (27).

The reactivity of hydroxyl radicals is extremely high and they can be expected to combine directly with all kinds of neighboring molecules (28). This implicates that $^{\cdot}O_2^-$ and/or H_2O_2 toxicity, mediated through $^{\cdot}OH$ radicals, acts "site specific" i.e. at the site where catalytical iron-complexes are located. It is therefore conceivable that if iron is attached to e.g. DNA it will induce specific damage to this molecule whereas iron-attachment to membranes will induce lipid peroxidation (10,28). In earlier experiments (12), we measured the total amount of iron in iron-loaded and control hearts with ferene-S after acidic destruction of the heart. Iron was found to be doubled as a result of our iron-load technique (50.8 ± 11.0 versus 112.4 ± 24.1, n=9). This extra amount of iron is apparently stored in capillary endothelial cells and to a lesser extent in pericytes and some macrophages located in the perivascular space, whereas no iron is stored in cardiomyocytes themselves. This observation strongly indicates free radical generation in endothelial cells and/or pericytes or macrophages.

The high density particles seen with electron microscopy most likely represent

ferritin molecules which are about 6 nm in size. Ferritin is an iron-storage protein which is capable to store maximally 4,500 atoms of ferric iron per molecule (29). Interestingly, iron atoms were found to be released from ferritin by the oxygen radical superoxide (30). So, whatever the source of superoxide and hydrogenperoxide as a result of ischemia/anoxia and reperfusion, increased toxicity induced by iron can be expected to occur at the site where iron-complexes (ferritin) are located. Little is known about aspecific intracellular iron-binding. The existence of a low molecular weight pool in the cell has been described and is probably built up by iron bound to ATP/ADP, free aminoacids (glycine and cysteine), citric acid and aspartic acid (31). Iron bound to these molecules still catalyzes the Haber-Weiss reaction in contrast to iron bound to ferritin. We do not know whether this low molecular weight pool is of any importance in the reperfusion syndrome nor do we know whether this iron-pool is changed as a result of the presently undertaken iron-load strategy.

The possibility of reperfusion injury to be initiated in vascular tissue has been suggested before (17). Superoxide dismutase (SOD), administered during reperfusion, has repeatedly been reported to be protective (3-5). One can not expect a rapid penetration of this relatively large SOD molecule (Mw 30,000) into myocardial cells. So protection by SOD, administered during reperfusion, might well be explained by scavenging superoxide in the vascular space and/or vascular tissue. A reasonable origin of superoxide generation in isolated heart preparations devoid of circulating leukocytes is therefore the endothelial cell. More evidence in support of this view was suggested by Hülsmann and Dubelaar (13), who found early damage of endothelial cells in rat hearts during ischemia measured by a relative increment of lactate dehydrogenase release to creatine kinase release. Ratych et al. (15) reported free radical generation at reperfusion in isolated endothelial cells in the absence of neutrophils or parenchymal cells. They were able to protect reoxygenated endothelial cells after 45 minutes of anoxia with SOD, catalase or allopurinol. In addition, Zweier et al. (16) directly measured free radicals in isolated endothelial cells during reoxygenation using electron paramagnetic resonance spectroscopy (EPR) and the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). All these reports suggest a central role for endothelial cells in the reperfusion syndrome.

The mechanism by which oxygen-derived free radicals are formed as a result of ischemia and/or reperfusion is not known. McCord suggested in 1985 the conversion of the enzyme xanthine dehydrogenase (XDH) to xanthine oxidase (XO) during ischemia (8). XO is capable to produce superoxide upon catalyzing the breakdown of hypoxanthine to uric acid. The validity of this hypothesis is strongly debated at the moment as it is discredited by reports that human hearts as well as pig and rabbit hearts do not contain XDH or XO (32,33). A recent study contradicts these findings as activities of XDH and XO were found in human and rabbit hearts (34). The authors (34) suggested these differences to result from a cytosolic inhibitor of XDH/XO present in these hearts. Among other possible sources of oxygen free radical generation in the heart, activation of lipoxygenase could be an alternative, especially since this enzyme is

widely present in endothelial cells and its substrate arachidonic acid accumulates in the heart during ischemia (35). Studies with specific lipoxygenase inhibitors have indeed shown protective effects of these compounds during post-ischemic reperfusion (36,37). Whether this effect is a result of specific lipoxygenase inactivation or of aspecific free radical scavenging needs to be further investigated.

In conclusion, we report specific iron-storage in vascular endothelial cells, pericytes and some macrophages located in the perivascular space as a result of short term iron-loading. Since iron accumulation in rat hearts is accompanied by an increased susceptibility of these hearts to reperfusion injury (12) the present observations strongly indicate reperfusion injury to be initiated in vascular tissue.

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CHAPTER V

REAPPRAISAL OF THE EPR SIGNALS IN (POST)-ISCHEMIC CARDIAC TISSUE.

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ABSTRACT

The present study was designed to measure directly oxygen-derived free radicals in (post)-ischemic or (post)-anoxic rat hearts using electron paramagnetic resonance (EPR) spectroscopy. Twenty-two rat hearts were rapidly freeze-clamped at 77 K under normoxic, anoxic, ischemic or reperfusion conditions. The samples were measured at three different temperatures (13 K, 77 K and 115 K) and several microwave powers and compared with isolated rat heart mitochondria. Samples were prepared both by grinding and as tissue cuts. The two preparation techniques gave identical EPR results, which excludes the occurrence of grinding artifacts. No free radical signals linked to reperfusion injury were detected. Several electron transfer centers known in the mitochondrial respiratory chain were measured. The signals previously assigned to post-ischemic reperfusion injury were found to originate from electron transfer centers of the respiratory chain, predominantly the iron-sulfur cluster S-1 in succinate dehydrogenase. The differences in signal intensity between normoxic, ischemic and reperfused hearts were found to result from the different redox stages of these centers under the various conditions tested. These findings do not necessarily imply that oxygen-derived free radicals are not formed in cardiac tissue during (post)-ischemic reperfusion. The constitutive background of paramagnetism from the respiratory chain, however, seriously hampers the direct detection of comparatively low concentrations of free radicals in cardiac tissue. It is therefore expedient to focus future experiments in this field on the use of spin-trapping agents.

INTRODUCTION

The hypothesis that oxygen-derived free radicals (ODFR) are responsible for (post)-ischemic myocardial injury has obtained much attention over the past few years [1,2]. Not only myocardial necrosis but also reperfusion induced arrhythmias [3], ventricular fibrillation [4] and post-ischemic coronary vasodilation [5] are now thought to be related with ODFR generation. Evidence in support of this free radical theory is mainly based on experiments in which protection of (post)-ischemic cardiac tissue was reported with superoxide dismutase [6,7], catalase [8,9], free radical scavengers (e.g. mannitol, vitamin E, [10,11]), and drugs that are able to block free radical generation (allopurinol, oxypurinol, deferoxamine, [12-15]). This evidence, however, is all indirect, since ODFR themselves were not measured in these studies. In order to obtain more direct information about ODFR involvement in (post)-ischemic cardiac injury, electron paramagnetic resonance spectroscopy (EPR) has recently been introduced in this field [16,17].

In the present study we investigated freeze clamped rat hearts which were perfused under normoxic, anoxic, ischemic, and reperfusion conditions. The study was

especially designed to answer the following questions : 1) are the published EPR signals [17-19] in (post)-ischemic cardiac tissue reproducible; 2) do these signals originate from oxygen-derived free radicals; 3) to what extent are we able to measure electron transfer centers in cardiac tissue using EPR; 4) do these signals interfere with the signals originating from ODFR; and 5) are there any signals artifactually induced in the myocardium as a result of the pulverization process under liquid nitrogen.

We here report that the originally published EPR signals in (post)-ischemic myocardial tissue [17-19] are reproducible and not artifactually induced, however, our interpretation of these signals is drastically different. The signals are found to originate from electron-transfer centers (mainly iron-sulfur clusters) which are located in respiratory chain complexes, specifically, NADH dehydrogenase and succinate dehydrogenase. The published differences [17-19] in signal intensity between normoxic, ischemic/anoxic and reperfused hearts are found to result from signal saturation and/or from different redox stages of these enzymes as a direct response to the presence or absence of oxygen.

MATERIALS AND METHODS

Perfusion protocol.

22 male Wistar rats, body weight between 200 and 250 g, were used in the present study. After a short anesthesia with di-ethylether, the hearts were quickly removed from the body and put into ice-cold Tyrode's buffer. Within one minute after this procedure the hearts were cannulated in the aorta and perfused retrogradely according to Langendorff [20]. The perfusions were carried out at 37° C and pH 7.4, using a modified Tyrode's buffer, containing 128 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$ and 11 mM glucose saturated with 95 % O_2 and 5 % CO_2. Perfusions were performed with a constant pressure of 80 cm H_2O. At the end of an initial equilibration period of 15 minutes, in which the hearts were stabilized, total ischemia, anoxia or control perfusions were started for 15, 30 or 45 minutes (13 rat hearts in all; see Table I for the exact conditions used). Tissue temperature during total ischemia was maintained by immersing the hearts in Tyrode's buffer of 37°C, saturated with 95 % N_2 and 5 % CO_2. Anoxia was induced by perfusing the hearts under the same conditions with Tyrode's buffer saturated with 95 % N_2 and 5 % CO_2. Nine separate hearts were reperfused for 20 seconds after a period of 15, 30 or 45 minutes of ischemia or anoxia (see Table I).

Sample preparation.

At the end of each perfusion experiment the hearts were rapidly frozen using Wollenberger tongs precooled in liquid nitrogen (77 K, [21]). Directly after this procedure samples were prepared under liquid nitrogen using two different techniques. The first method involved grinding of the hearts, at 77 K, by means of a mortar and pestle of porcelain. The second procedure was performed by cutting the hearts under liquid nitrogen into pieces with the same mortar and a razor-sharp 1 cm chisel. After these procedures the samples were transferred to EPR tubes under liquid nitrogen. Heart mitochondria were prepared according to [22].

EPR spectroscopy.

Electron Paramagnetic Resonance spectroscopy was done with a Bruker (ED 200 D) EPR spectrometer. The microwave frequency was measured with a Systron Donner frequency counter (model 1292 A). The dc magnetic field was measured with an AEG Kernresonanz Magnetfeldmesser (type GA-EPR 11/21-02). The field was modulated with a frequency of 100 kHz and an amplitude of 6.3 Gauss. Heart samples were measured at nitrogen flow temperatures (115 K), using a Bruker (ER 4111 VT) variable temperature unit, and at helium flow temperatures (13-77 K) using a home build cryostat, in which the temperature was measured by means of a calibrated 5 kOhm Allen-Bradley carbon resistor.
All data were collected as 1024-point spectra using an Olivetti M24 PC with software written in Asyst. This program allowed us to perform signal averaging, baseline correction, frequency alignment and signal integrations.

RESULTS

EPR spectroscopy at 13 K.

In Fig.1, we show an EPR spectrum at 13 K of a pulverized, freeze-clamped rat heart after 30 minutes ischemia and 20 seconds reperfusion. Under these conditions three types of signals can be observed : 1) metal-related signals, 2) radical signals and 3) a doublet signal.

1) Metal-related signals : the concentration of the respiratory chain in this sample is so high that the obtainable EPR spectrum is almost the same in quality as those of purified enzymes from the respiratory chain. The EPR properties of these enzymes have been documented in great detail in the literature [23-35]. With this information it is straight-forward to identify in Fig.1 the electron transfer centers (iron-sulfur clusters) N1,

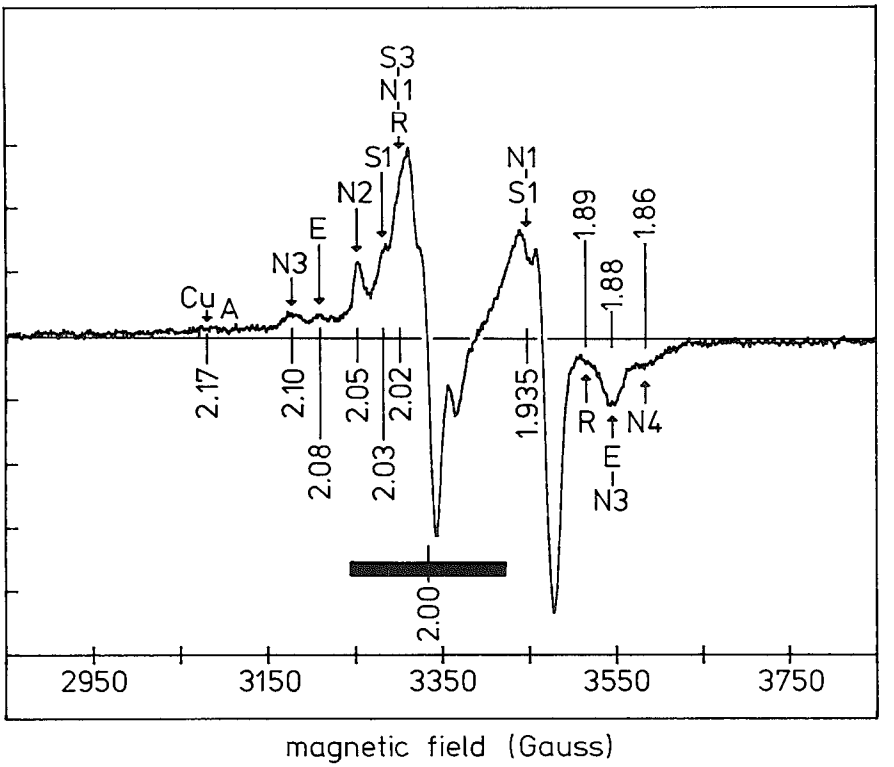

Fig.1. Overview of EPR detectable redox centers in whole-heart tissue. The heart was subjected to 30 min. of global ischemia followed by 20 s. of aerobic reperfusion. After freeze-clamping and pulverization at 77 K a 4 times averaged EPR spectrum was taken at 13 K with a microwave power of 3.2 mW and a microwave frequency of 9.33 GHz. The centers N-1 to N-4 are iron-sulfur clusters in NADH dehydrogenase; S-1 and S-3 are iron-sulfur clusters in succinate dehydrogenase; E is the iron-sulfur cluster in ETF dehydrogenase; R is the iron-sulfur cluster in the Rieske protein; and Cu_A is one of the copper ions in cytochrome c oxidase (23-35). Selected g values are also given. The solid bar indicates the limited field range used in previous EPR studies on perfused hearts (17-19).

N2, N3 and N4 known in NADH dehydrogenase [23-26], S1 and S3 in succinate dehydrogenase [27-30], E in ETF dehydrogenase [31], R in the Rieske protein [32,33], and copper in cytochrome c oxidase (Cu_A, [34,35]). Center S-2 from succinate dehydrogenase, is the only non-detectable iron-sulfur (Fe/S) cluster in Fig.1 because of its extreme spectral width [30].

2) Radical signals : a clear isotropic signal with a g-value close to 2.00 can be detected in Fig.1. This signal is thought to originate from coenzyme Q_{10} and the flavins FMN (flavin mononucleotide) in NADH dehydrogenase and FAD (flavin adenine dinucleotide) in succinate dehydrogenase [23-35]. The semiquinone states (half-reduced or single-electron reduced states) of these compounds are all paramagnetic. FAD in succinate dehydrogenase in its semiquinone form gives rise to a well developed radical signal at g=2.005 with a "peak-to-peak" width of the signal of 12 Gauss [36]. The situation for coenzyme Q_{10} is more complicated as there appears to be two different forms of coenzyme Q_{10} that give rise to two slightly different isotropic resonances. One is located at g=2.005 and the other at g=2.006 with a "peak to peak" width of respectively 10 and 8.3 Gauss [37]. A radical from FMN in NADH dehydrogenase has thus far never been positively identified. The semiquinone EPR signals from the respiratory chain account for the isotropic signal observed here and in previous studies [16-19] at g≈2.00. The slight broadening of this signal observed in ischemic samples (see e.g. Fig.5C) indicates that the spectrum comes mainly from the FAD radical (12 Gauss width) while the spectrum from normoxic heart is dominated by coenzyme Q_{10} (10 and 8.3 Gauss width). This conclusion is in agreement with earlier reports of Baker et al. [18].

3) The doublet signal : as shown in Fig.1 the isotropic signal at g≈2.00 is flanked by two sharp, unidentified signals (at g≈2.01 and at g=1.985). We further label these signals "center C" after Zweier et al. and Nakazawa et al. [17,19]. Zweier et al. ascribed center C to a nitrogen-centred free radical associated with reperfusion injury. We will discuss this center below.

EPR signals in ischemic/anoxic and reperfused hearts at 13 K.

EPR detectable responses of heart tissue under normoxic, ischemic and reperfusion conditions are presented in Fig.2. For comparison a spectrum of isolated, resting mitochondria is also shown. This latter spectrum is dominated by a nearly isotropic signal of the oxidized center S-3 from succinate dehydrogenase (g=2.015). The respiratory chain in these mitochondria is almost completely oxidized as only a trace of reduced Fe/S clusters is detectable around g=1.93. Note that the line at g=1.985 of the unidentified center C [17,19] is also detectable in these mitochondria. The normoxic or control heart sample represents an intermediate redox situation in which oxidized S-3 is still easy to detect. In this control spectrum an enhanced reduction of Fe/S clusters from NADH dehydrogenase and succinate dehydrogenase can be seen around g=1.93.

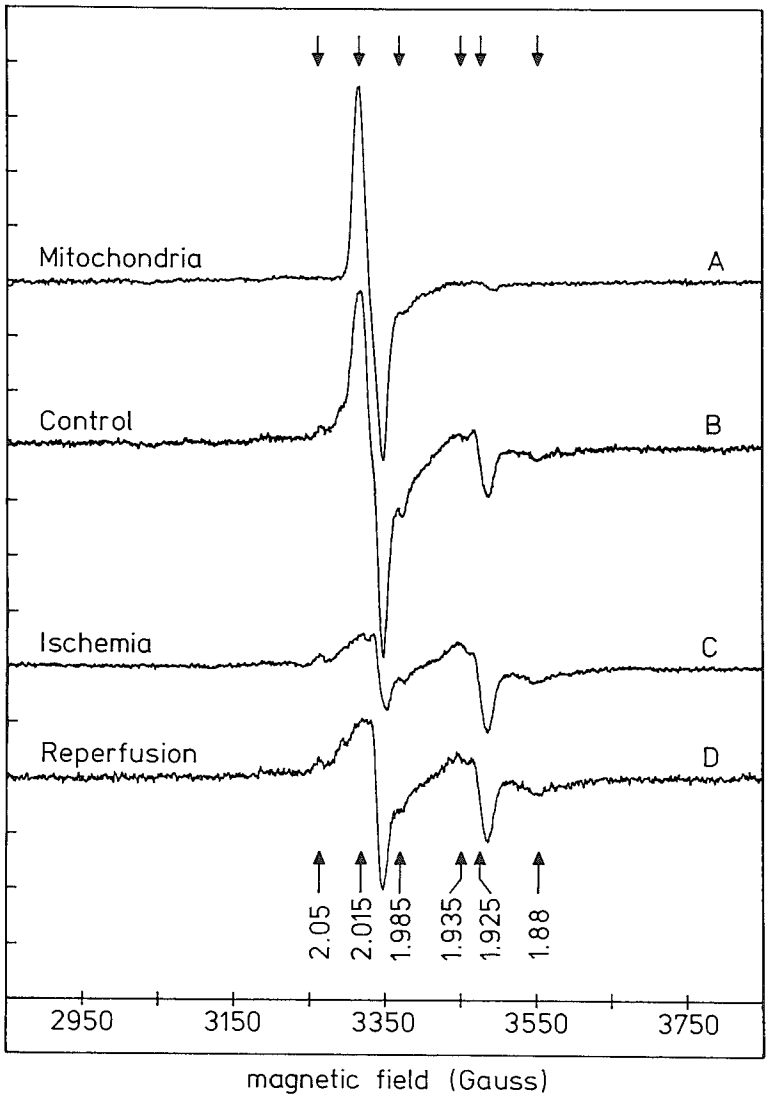

Fig.2. Low temperature (T=13 K) EPR spectra as a function of perfusion condition. Trace A, isolated mitochondria in air; trace B, heart subjected to 30 min. of aerobic perfusion; trace C, heart subjected to 30 min. of global ischemia; trace D, heart subjected to 30 min. of global ischemia followed by 20 s. of aerobic reperfusion. The six arrows and g values indicate the redox centers quantified in Table I. EPR conditions: microwave power, 3.2. mW; microwave frequency, 9.35 GHz.

The six arrows and g-values indicate the Fe/S clusters and center C as they were quantified in Table I (see below). The ischemic heart sample is characterized by a further reduced respiratory chain which is especially evident by a decrease of the S-3 signal (g=2.015). 20 seconds reperfusion appears to induce only minor changes in the reduction state. As already said, the line at g=1.985 of the unidentified center C is easily detectable. The relative quantification (% amplitude) of this signal, however, is difficult as it is always on the ascending slope of the stronger isotropic signal. Because of its possible importance, being previously reported to represent a nitrogen-centered free radical associated with reperfusion injury [17], the amplitude of this signal was semi-quantitatively measured on a scale of four gradations from 0 to +++. The gradation of +++ corresponds to a well-developed resonance as in traces B and C of Fig.2. The weaker signals of traces A and D would be labeled ++. The symbol + is used when the signal is just barely detectable as an inflection on the ascending slope.

The results of these quantifications together with the amplitudes of the electron transfer centers in all 22 hearts at 13 K are presented in Table I. The columns are arranged according to an increasing resonance field corresponding to the arrows in Fig.2 from left to right. The amplitudes are proportional to the concentration of the centers as indicated on top of each column (see also Fig.1). All amplitudes have been normalized by putting the average amplitude at each g-value in the three control samples equal to 1.0. In this Table it can been seen that center C is present in either normoxic, anoxic, ischemic or reperfused hearts. For this reason we conclude, in contrast to Zweier et al. [17], that this signal is not associated with reperfusion injury.

From the Fe/S clusters listed in Table I only S-3 is paramagnetic in its oxidized state. All other centers in this Table I are paramagnetic in their reduced state. The amplitudes in Table I at g=2.05 (center N-2), g=1.925 (sum of amplitudes from centers N-2 and N-4) and g=1.88 (centers N-3 and E) are remarkably invariant to changes in the perfusion condition. Apparently the steady-state reduction level of the NADH dehydrogenase and the ETF dehydrogenase are rather insensitive to the oxygenation history of the heart. Although the reduction level of center N-1 in NADH dehydrogenase cannot be measured separately in these experiments, it is reasonable to assume that this center behaves in concert with the other three centers (N-2, N-3 and N-4) in the enzyme. Therefore, the changes at g=1.935 (N-1/S-1) are mainly due to a change of center S-1 from succinate dehydrogenase.

When the heart is made ischemic or anoxic the S-1 signal (g=1.935) responds with an extensive increase in amplitude, i.e. reduction. This response is parallelled by a decrease, of comparable extent, of the signal at g=2.015 from center S-3 (i.e. also a reduction of this iron-sulfur cluster). Both centers appear to only slightly reoxidize over a period of 20 s. of aerobic reperfusion. The extent of reduction appears to be dependent on the time of ischemia. This is especially evident in the signal from S-3, which is the strongest and, therefore, the most accurately measurable signal. This time dependency in ischemic samples is reflected in its reoxidation response to reperfusion. The S-3 signals in the anoxic samples were found to be somewhat smaller compared to

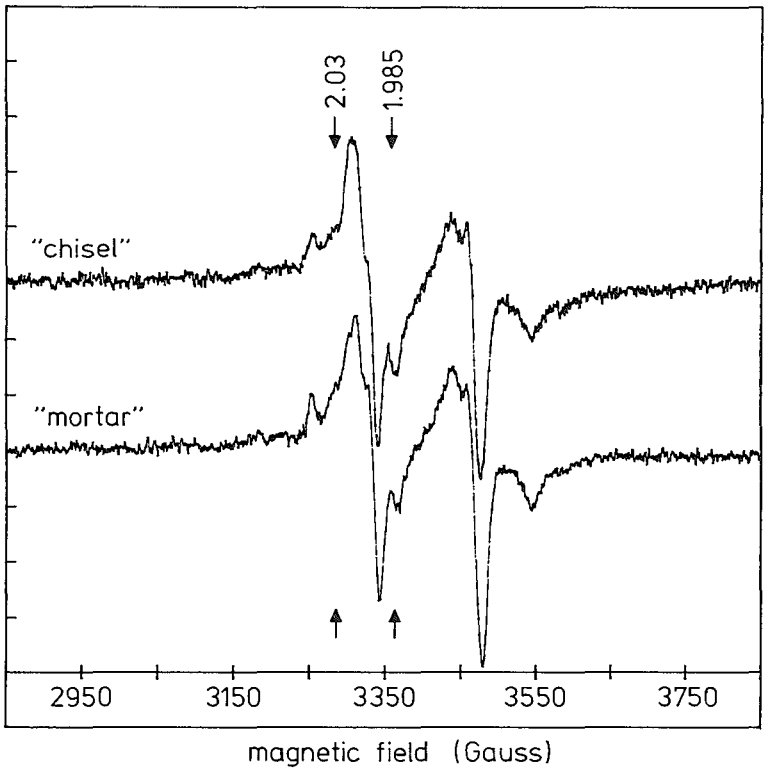

Fig.3. A comparison of low-temperature EPR data from samples prepared by different techniques. A heart was subjected to 30 min. of global ischemia followed by 20 s. of aerobic reperfusion. The trace "chisel" is from the one half of the freeze-clamped heart cut with a chisel into millimeter-sized pieces. The trace "mortar" is from the other half of the heart ground to a fine powder in a mortar. EPR conditions were as for Fig. 1.

the ischemic signals and not time dependent. This difference might be related to the fact that rat hearts during anoxic perfusion are still beating in contrast to ischemic hearts [14]. This can imply that during anoxic perfusion a higher degree of reduction is reached.

In summary, the reduction level of iron-sulfur clusters in the enzyme succinate dehydrogenase is strongly correlated with the perfusion history of the heart. The amplitude of the signal from center C is not correlated to the perfusion condition. It is clear that the signal intensity of center C in reperfusion samples is not higher than those in control samples (Table I).

Independence of the EPR spectroscopy on sample preparation technique.

The relevance of the original observations by Zweier et al. [17] was recently discredited by Baker et al. [18] and Nakazawa et al. [19] as due to an artifact of sample preparation. The latter two groups claimed that two out of the three EPR signals were absent when the freeze-clamped heart was not pulverized in a mortar, but rather cut into pieces with a spatula [18] or a chisel [19]. We have prepared several heart samples by grinding one half and chiseling the other half at 77 K. Between these pair samples we have not found significant differences in their EPR spectra either at 13 K or at 77 K. In Fig.3 an example is shown of a low-temperature EPR spectrum from pair samples prepared from a reperfused heart after 30 min. ischemia. Note especially that the signal amplitudes at g=2.03-2.04 and at g=1.985, previously assigned to artifacts of grinding [18,19], are not significantly different for the two samples.

EPR spectroscopy at 77 K.

Fig.4 shows data of a warming-up experiment of normoxic sample measured at two microwave powers (0.5 and 13 mW). The experiment confirms previous observations [17,19] that the unidentified center C (i.e. the two satellite lines flanking the central radical line at g=2.005 in the control spectrum) is unstable towards warming-up for 1 min. to -80°C; also a peak at g=2.03 is unstable towards 30 min. warming at -20°C. However, this experiment tells that both the isotropic radical at g=2.005 and the center C saturate much more readily than the other observable signals. In fact at powers where the line at g=2.03 is readily detectable (upper two spectra at high power), the isotropic radical and center C are partially saturated (smaller compared to the low power situation). For this reason we conclude that difference spectra previously used to prove ODFR generation in post-ischemic cardiac tissue are difficult to interpret [17,19]. For the same reason we criticize the previous conclusions that center C is either a triplet spectrum with 25 Gauss splitting [17,19] or a doublet spectrum with 50 Gauss splitting ([18], see discussion).

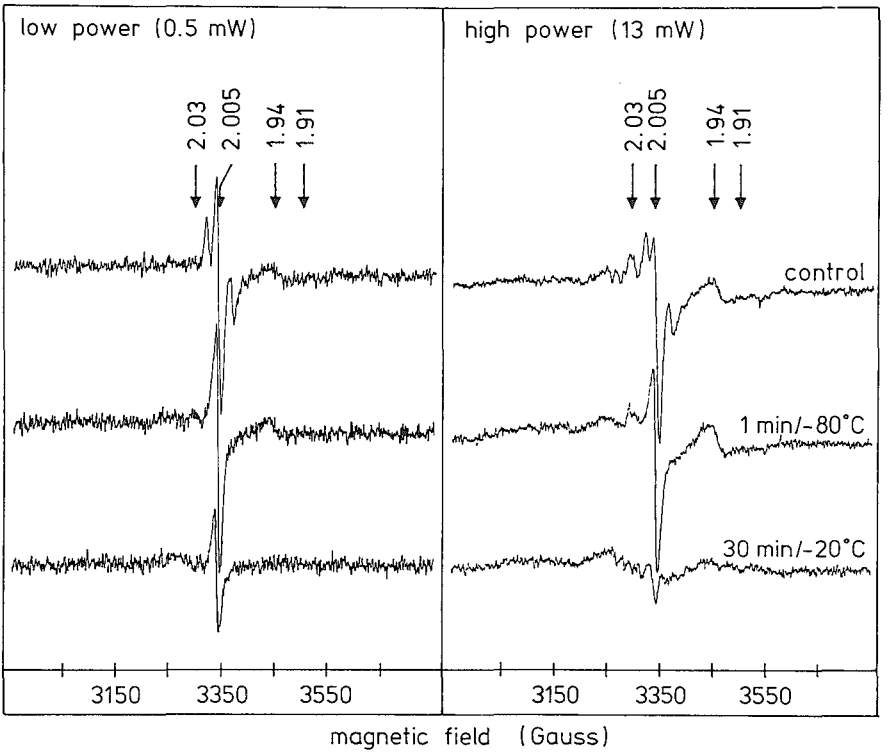

Fig.4. Warming-up experiment to stepwise abolish EPR signals in perfused heart. The EPR was monitored at a temperature of 77 K and at two different microwave power levels. The heart was subjected to 30 min. of normoxic perfusion, freeze-clamped and pulverized, before being measured (upper traces). After this the sample was warmed up to -80°C for 1 min., and re-cooled to 77 K (middle traces). Finally the sample was warmed up to -20°C for 30 min. and re-cooled to 77 K (lower traces). EPR conditions: all traces are an average of 4 scans; microwave frequency, 9.37 GHz; microwave power, as indicated.

It is also clear from Fig.4, when comparing the low and high power spectra and the post-annealing spectra, that the peak at g=2.03 is not linked to the strong line at g=2.005 as claimed by Zweier et al. and Nakazawa et al. It can be seen, however, from this figure that the peak at g=2.03 goes together with the signals at g=1.94 and g=1.91. This conclusion destroys the basis for the previous assignment that the signal at g=2.03 is part of an axial spectrum from an oxygen-centered radical [17,19]. We found that the signal at g=2.03 is part of the spectrum from the iron-sulfur cluster S-1 in succinate dehydrogenase. This cluster is known to generate an EPR signal with three g-values at 2.03, 1.94 and 1.91 [28]. In the high-power spectra of Fig.4 we also see a weak, very broad peak at g≈2.17 (3085 Gauss) and a corresponding broad derivative-shaped feature around g≈2.03 characteristic for the CuA center in cytochrome c oxidase [34,35].

EPR spectroscopy and saturation characteristics at 115 K

All three previous studies [17-19], carried out at 77 K, report an increase of the isotropic signal at g=2.005 in reperfused hearts compared to the control hearts. We have confirmed these observations at 77 K (data not shown). In view of the above noted problem of partial power saturation of this signal at 77 K we have measured the samples under definitely non-saturating conditions (115 K and microwave power of 0.05 mW).

In Fig.5, we first show in panel A that detection of the signals in control hearts is still possible at this "high" temperature of 115 K using a microwave power of 80 mW. The signal from center S-1 can be readily detected (g=2.03, 1.94 and 1.91) in these control hearts. The isotropic signal at g=2.005 and the signal from center C are extensively reduced in amplitude by microwave power saturation. In panel B the peak-to-peak amplitude of the isotropic signal is plotted as a function of the microwave power. It is clear from this graph that even at the elevated temperature of 115 K only power levels below 0.2 mW will not induce saturation. In panel C, we show EPR spectra at the definitely non-saturation condition of 115 K and 0.05 mW. Evidently, the previously found apparent increase of the isotropic radical signal [17-19] in reperfused hearts at 77 K was not encountered under the present conditions. We therefore conclude that the earlier published, reperfusion dependent increase of the isotropic signal [17-19] is a result of microwave power saturation.

More data concerning different heart samples measured under these non-saturating conditions are shown in Table II. The amplitudes of the isotropic radical signal at g=2.005 in ten heart samples were again normalized with respect to control levels. This Table clearly shows that the signal intensity is halved upon deprivation of oxygen. Reperfusion induces only a small recovery of the signal. The line width in the control sample is approximately 9 Gauss and increases maximally 2-3 Gauss upon ischemia/anoxia or reperfusion (Fig.5). Even corrected for this broadening the intensities (i.e. concentrations) never significantly exceeded those of the normoxic samples, which contrasts the findings published in references [17-19].

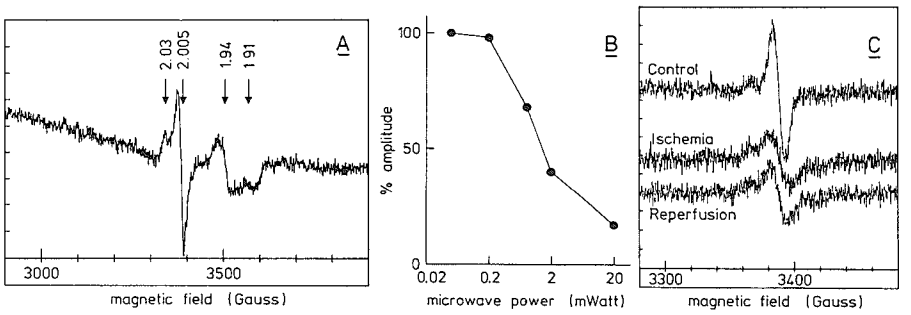

Fig.5. EPR spectroscopy of whole-heart samples at a temperature of 115 K. Panel A, high-power (80 mW) spectrum showing, in addition to the radical line at g=2.005, the complete spectrum (i.e. g=2.03, 1.94 and 1.91) of iron-sulfur cluster S-1 in a control heart (30 min. normoxic perfusion). The spectrum is an average of 4 scans corrected for an average of 4 baseline scans. Panel B, power plot of the radical line at g=2.005 (see text). Panel C, low-power (0.05 mW) spectra of the radical line at g=2.005 in a control heart (30 min. normoxic perfusion), a heart subjected to 30 min. of global ischemia, and a heart subjected to 20 s. of aerobic reperfusion after 30 min. of ischemia. For all spectra the microwave frequency was 9.50 GHz.

DISCUSSION

The proposal that ODFR are involved in (post)-ischemic myocardial injury [38], combined with the notion that EPR spectroscopy can be used to detect these radicals, has in recent years encouraged several groups to use EPR spectroscopy in attempts to gain insight into the molecular mechanisms underlying ischemic injury. Zweier et al. [17] were the first to claim the direct measurement of three different radical species, among which an oxygen-centered one, generated upon reperfusion. Subsequently, Baker et al. [18] and Nakazawa et al. [19] claimed that two of the three signals, reported by Zweier et al. [17], including the one attributed to an oxygen-centered radical, are artifacts due to sample-preparation. Baker et al., however, repeated the claim that reperfused myocardium contained an increased amount of radicals. In a recent letter by Baker et al. this increase in radical concentration is no longer mentioned [39]. In the same paper an EPR signal in ischemic hearts is ascribed to reduction of an iron-sulfur center in a mitochondrial dehydrogenase, and this leads the authors to conjecture that the respiratory chain of intact mitochondria might contribute to "oxy-radical production during reperfusion".

✚ TABLE I.

RELATIVE EPR AMPLITUDES AT A TEMPERATURE OF 13 K FOR THE REDOX CENTERS IN EACH OF THE 22 WHOLE-HEART PREPARATIONS AT THE VARIOUS EXPERIMENTAL CONDITIONS.

	N-2 (2.05)*	S-3 (2.015)	C (1.985)	N-1/S-1 (1.935)	N-2/N-4 (1.925)	N-3/E (1.88)
Control:						
15 min	0.89	1.03	+++	1.39	0.87	0.90
30 min	1.19	0.97	++	0.96	1.28	0.99
45 min	0.92	1.00	+++	0.65	0.85	1.12
Ischemia:						
15 min	1.02	0.48	0	4.13	1.59	1.43
30 min	1.02	0.30	0	3.78	1.62	1.34
30 min	0.66	0.23	+++	3.61	1.23	1.11
30 min	0.90	0.27	+	4.13	1.23	1.06
45 min	1.02	0.16	+	4.96	1.37	1.06
Anoxia:						
15 min	0.89	0.23	+++	3.09	1.15	0.91
30 min	0.97	0.22	+++	4.22	1.17	1.09
30 min	0.75	0.24	++	5.30	1.09	0.95
30 min	1.00	0.20	++	4.35	1.21	1.01
45 min	0.80	0.20	+++	3.74	0.93	0.94
Ischemia						
15 min + reperfusion**	0.93	0.55	+++	1.78	1.10	0.80
30 min + "	0.93	0.39	+	1.52	0.98	0.70
30 min + "	0.95	0.27	+	3.83	1.20	0.99
30 min + "	1.02	0.42	++	2.74	1.29	1.18
45 min + "	1.01	0.20	+	3.62	1.44	1.18
Anoxia:						
15 min + reperfusion**	0.97	0.30	+++	2.65	0.93	0.93
30 min + "	0.96	0.24	++	2.09	1.08	0.85
30 min + "	1.09	0.20	++	4.26	1.14	1.06
45 min + "	1.01	0.16	++	3.35	0.79	0.60

Note:- amplitudes were normalized by putting the average amplitude of the control samples equal to 1.00.
- * : g-value at which the amplitude is measured (cf. Fig. 2).
- ** : reperfusion time : 20 seconds.
- Iron-sulfur clusters in NADH dehydrogenase (N-1 to N-4), in succinate dehydrogenase (S-1 to S-3) and in ETF dehydrogenase (E). C is "Center C" (see text).

✚ TABLE II.

PEAK-TO-PEAK AMPLITUDE OF THE ISOTROPIC RADICAL SIGNAL AT g=2.005 AS A FUNCTION OF PERFUSION CONDITION IN 10 HEART SAMPLES. T=115 K; MICROWAVE POWER 0.05 mW.

normoxia 30 min.	0.89
normoxia 30 min.	1.11
ischemia 30 min.	0.46
ischemia 30 min.	0.43
anoxia 30 min.	0.49
anoxia 30 min.	0.56
Ischemia (30 min.) + reperfusion (20 s)	0.35
Ischemia (30 min.) + reperfusion (20 s)	0.54
anoxia (30 min.) + reperfusion (20 s)	0.56
anoxia (30 min.) + reperfusion (20 s)	0.75

Note : amplitudes were normalized by putting the average amplitude of the control samples equal to 1.00.

In the present study we have repeated the experiments of Zweier et al. [17], Baker et al. [18], and Nakazawa et al. [19] at 77 K. We have extended our measurements to lower (13 K) and higher (115 K) temperatures, we have studied a wide magnetic-field scan range and varied the microwave power extensively. All the signals we measure, however, in normoxic, ischemic, anoxic and reperfused hearts can be ascribed to components intrinsic to the mitochondrial respiratory chain and not to potentially dangerous radicals.

Zweier et al. [17] reported two signals to be related to aerobic reperfusion. Firstly, they observed an axial signal (i.e. two g-values, at g=2.033 and g=2.005) which the authors assigned to superoxide or an alkyl peroxide radical. They claim that resistance to microwave power saturation and instability above 100 K are properties of these oxygen-centered radicals. However, here they misread the literature [40,41], since Copeland [40] reports that alkyl peroxide radicals can withstand annealing for 10 min. at 193 K and no power saturation data whatsoever were reported in this study. In addition, Knowles et al. [41] report that superoxide is stable at 123 K and that it can be recognized by its ease of saturation. On the basis of our multi-temperature and multi-microwave power study we can now reassign this "oxygen-radical" axial signal (g=2.033 and g=2.005, [17]) to originate partly from center S-1 from succinate dehydrogenase (three g-values at g=2.03, g=1.94 and g=1.91, [28]) and partly from the saturated isotropic signal at g=2.005 at 77 K.

The second signal reported by Zweier et al. [17], "center C", was interpreted as an isotropic triplet from a nitrogen-centered free radical of unknown identity. Baker et al. [18] reread the signal as an isotropic doublet from a carbon-centered radical, possibly a lipid radical. In the present study, we have made several observations that may help in identifying this elusive center. The middle part of the signal from center C cannot be defined because it is overshadowed by the isotropic signal from semiquinone and the strong signal of S-3 at low temperatures. The two outermost features of the signal ($g \approx 2.01$ and g=1.985) have a positive absorption shape and a negative absorption shape, respectively, which is characteristic for a transition metal. The amplitude of center C in our hands definitely does not peak in reperfused heart samples, but was shown to be present under a variety of conditions. This means that the signal is not related to reperfusion injury. The apparent g-values together with the observed slow relaxation is reminiscent of molybdenum [42,43]. Since the high field line of this center, at g=1.985, was also encountered in this study in mitochondria, we surveyed the literature for more data. We found that a similar signal was detected as early as 1971 in membrane fragments from mitochondria [23]. This group of workers later suggested this signal to originate from the QH2:ferrocytochrome c oxidoreductase complex [44], however, this signal is also observable in published spectra from the NADH:Q oxidoreductase complex [24]. A similar "triplet-pattern" has recently been observed in the bacterium Paracoccus denitrificans [45] which has a respiratory chain that is quite similar to the mammalian respiratory chain.

In summary, "center C" appears to be an intrinsic component of the mitochondrial

respiratory chain. Its chemical identity remains at present obscure. It may be a radical or a biradical related to coenzyme Q_{10} [46] or the transition metal molybdenum.

Finally, EPR spectroscopy in cardiac tissue detects a variety of compounds from the mitochondrial respiratory chain, which respond to the varying perfusion conditions (ischemia, anoxia, reperfusion). Free radicals linked to reperfusion injury were not detected. All the signals previously assigned to post-ischemic reperfusion injury can be explained to originate from electron-transfer centers in the mitochondrial respiratory chain and/or from signal saturation. This conclusion does not necessarily imply that ODFR are not formed during post-ischemic reperfusion in cardiac tissue. There is still much indirect evidence indicating free radical involvement [1-15]. Moreover, ODFR have recently been detected with EPR spectroscopy in perfusates of reperfused rat hearts as adducts of the spin-traps DMPO (5,5'-dimethyl-1-pyrroline-N-oxide, [16,47]) or PBN (N-tert-butyl-alpha-phenylnitrone, [48]). Recently, we have confirmed these observations using DMPO (unpublished). Since we show in this study that the constitutive background of paramagnetism from the respiratory chain seriously hampers the detection of relatively low concentrations of free radicals in cardiac tissue, it is expedient to focus future experiments in this field on the use of spin trapping agents.

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CHAPTER VI

CUMENE-HYDROPEROXIDE, A LIPID PEROXIDATION INDUCING AGENT, AND 4-HYDROXY-2,3-NONENAL, A LIPID PEROXIDATION PRODUCT, CAUSE CORONARY VASODILATION IN PERFUSED RAT HEARTS BY A CYCLIC NUCLEOTIDE INDEPENDENT MECHANISM.

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(submitted)

ABSTRACT

In the present study, we investigated whether cumene-hydroperoxide (CUM), a substance known to induce lipid peroxidation through free radical action, and 4-hydroxy-2,3-nonenal (HN), a major aldehyde formed in the process of lipid peroxidation, induce coronary vasodilation in Langendorff perfused rat hearts by changing cyclic-nucleotide levels. Both compounds showed a dose dependent and reversible increase of coronary flow which was comparable to the effect of sodiumnitroprusside (NP). With NP we found a good correlation between the extent of vasodilation and total heart cGMP levels. Vasodilation induced by CUM or HN was not accompanied by a rise in total heart cGMP or cAMP levels. Isoprenaline was used as a positive control for cAMP. In order to obtain more information about the precise localization of cGMP in the heart a new cGMP antiserum was used. We clearly found cGMP immuno-staining in coronary vascular smooth muscle after vasodilation with NP, whereas no cGMP immunostaining was found in vascular smooth muscle after vasodilation with CUM or HN. Our data indicate that CUM and HN, can provoke reversible coronary vasodilation in isolated perfused rat hearts by a cyclic nucleotide independent mechanism.

INTRODUCTION

Prolonged perfusion of heart tissue under ischemic or anoxic conditions will inevitably cause dysfunction of the heart. The only way to preserve the heart under these conditions is to reperfuse it with oxygen. This is, however, not without danger. Many recent investigations have shown that reperfusion itself is responsible for the induction of additional damage to these hearts (1,2). There is a growing amount of evidence which now indicates the involvement of oxygen-derived free radicals (ODFR) in this phenomenon (reperfusion syndrome, oxygen-paradox 2,3). Generation of ODFR in biological tissue is regarded highly toxic because they are able to destroy molecules such as proteins, DNA and lipids. In addition, ODFR can initiate lipid peroxidation, a chain reaction that is responsible for membrane destruction and cell death (4). We became interested in studying the effects of cumene-hydroperoxide (CUM) and 4-hydroxy-2,3-nonenal (HN) on heart tissue because these two substances are known to be associated with the process of lipid peroxidation. CUM is able to induce lipid peroxidation in biological tissue through free radical action (5,6), whereas HN is considered a main endproduct of this process (7). Earlier studies in our laboratory have shown considerable vaso-reactive properties (5) of these two substances. Micro-molar concentrations of CUM or HN are able to induce strong vasodilations of coronary arteries in Langendorff perfused rat hearts. In the present study, we further investigated this effect, with special emphasis to the question whether these vasodilations are mediated by a change in cyclic nucleotide levels as proposed e.g. for isoprenaline (cAMP, 8), sodiumnitroprusside

(cGMP, 9) or the endothelium derived relaxant factor (EDRF, cGMP 9,10).

METHODS

Determination of flow, contractility and cyclic nucleotide levels in the isolated rat hearts.

The hearts were obtained from male Wistar rats (body weight between 200 and 250g). After a short anesthesia with di-ethylether, the hearts were quickly removed from the body, and perfused retrogradely according to Langendorff (11). The perfusions were carried out at 37° C and pH 7.4 using a modified Tyrode's buffer which contained 128 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$ and 11 mM glucose saturated with 95% O_2 and 5% CO_2. During the experiments the hearts were paced at 300 pulses/min (2 ms duration with 0.2 mA), except for the experiment in which the effects of NP, CUM and HN on heart contractility were investigated (Fig.1). Perfusion pressure was held constant at 90 cm H_2O. Coronary flow was measured indirectly in the aorta, as a retrograde aortic flow. (transflow 601 system, Skalar, Delft, Holland). Contractility was measured as apex-displacement by using a Hewlett-Packard transducer (7-DCDT-100). The perfusion system consisted of three circuits allowing rapid switch from one perfusate to an other. Compounds of interest were tested after an initial equilibration period of at least 15 minutes in which a constant flow, defined as 100% in the figures, was achieved. We perfused the hearts with different concentrations of CUM, HN or NP as indicated in the figures. After a perfusion period of exactly 2 minutes with the compound of interest, the hearts were rapidly frozen using Wollenberger tongs precooled in liquid nitrogen (12) for cGMP and cAMP determinations. A perfusion period of two minutes allowed us to achieve a reproducible and stable coronary flow elevation which was correlated with total heart cGMP and cAMP levels. The frozen hearts were homogenized in liquid nitrogen in the presence of 4 ml $HClO_4$ (5%) with a mortar. This procedure allows complete deactivation of phosphodiesterases which is necessary to stabilize cyclic nucleotide levels. The recovery of cAMP or cGMP by this procedure was determined from the addition of standard amounts of exogenous cAMP or cGMP and calculated more than 90%. After thawing, the homogenate was centrifuged for 20 minutes at 4° C and 10,000 g. The supernatant was collected and its pH adjusted to 7.4 using KOH. After 30 minutes, a second centrifugation step was performed (5 min. at 10,000 g) to remove the precipitate: $KClO_4$. cGMP and cAMP levels were determined in 50 or 100 ul samples of the supernatant by using a radioimmunoassay for cGMP and a binding assay for cAMP (cAMP and cGMP commercial kits AMERSHAM). All chemicals used were analytical grade. Cumene-hydroperoxide was purchased from Sigma (USA) and 4-hydroxy-2,3-nonenal from Prof. Esterbauer, University of Graz, Austria. Results are presented as mean values ± SEM. Comparisons were made with Student's t-test. $p < 0.05$ was considered significant.

Immunocytochemistry of cGMP in formaldehyde-fixed rat hearts.

Antibodies against cGMP were raised in rabbits, by injecting these animals with a cGMP-protein conjugate, which was prepared by coupling cGMP to bovine thyroglobulin using formaldehyde. Note that this antibody is different from the one used in the RIA assay. Details of this procedure and the specificity of this antibody have been described in a study by DeVente et al in 1987 (13). As fast fixation of cGMP is a prerequisite for successful application of immunocytochemistry of cyclic nucleotides, the following protocol was adopted. The coronary flow of Langendorff perfused rat hearts was elevated as described above, with 100 uM CUM, 100 uM HN or 5 uM NP. After two minutes the perfusion medium was switched to 4% freshly depolymerized paraformaldehyde in Tyrode's buffer (pH 7.4). The hearts were kept in this solution for one hour at 0°C before being washed in 0.1 M phosphate buffer (pH 7.4) containing 5% sucrose. After this procedure the hearts were frozen in Tissue-Tek-compound, using CO_2. Sections were made on a cryostat chuck of 35 um and thawed onto chrome-alum coated glass slides. We incubated these sections overnight on ice with the primary cGMP-antibody, diluted 1:300 with 0.1 M phosphate buffer (pH 7.4) containing 0.1% Triton-X100. cGMP-immune-reactivity was visualized using a fluorescein-isothiocyanate coated secondary sheep-anti-rabbit antibody. No immunostaining was observed using a non-immune serum, or by using a antiserum which had been pre-incubated with the cGMP-formaldehyde-thyroglobulin conjugate.

RESULTS

Effects of CUM and HN on coronary flow.

As shown in Fig.1, administration of 50 uM CUM or 50 uM HN for a period of two minutes caused an instantaneous increase of the coronary flow in Langendorff perfused rat hearts. After washing out CUM or HN the flow returned to its original value. Re-addition of 200 uM CUM or HN resulted in a second and higher flow increase which again returned to baseline upon perfusion in the absence of these compounds. These results are comparable with the effects of sodiumnitroprusside (NP) although at a fifty times lower concentration of this compound. The results suggest that the vasodilatory action of CUM and HN are reversible and dose dependent. Fig.1 also shows the effects of NP, CUM and HN on heart contractility. In order to establish a proper test condition, the hearts were not paced during this particular experiment in contrast to the other experiments. Contractility is shown just before administration of 1 uM NP, 50 uM CUM or 50 uM HN and just after two minutes perfusion with these compounds. Fig.1 shows that these compounds had no major effects on heart contractility. Figure 2 demonstrates that the flow elevations provoked by CUM or HN are dose dependent and

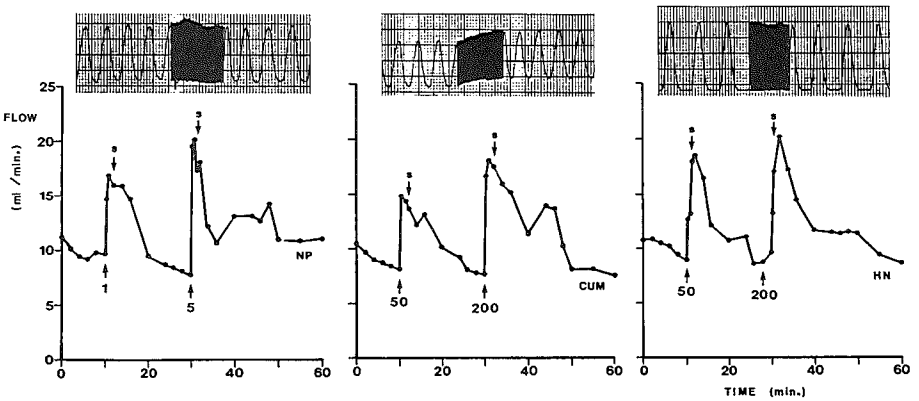

Fig.1. Reversability of coronary flow. Three rat hearts were perfused two periods of two minutes with either sodiumnitroprusside (NP, 1 and 5 uM), cumene-hydroperoxide (CUM, 50 and 200 uM) or 4-hydroxy-2,3-nonenal (HN, 50 or 200 uM). Perfusion start together with concentrations added are indicated. After "s" perfusion with these compounds was stopped and replaced by standard perfusion buffer. Cardiac contractility is shown just before and just after administration of 1 uM NP, 50 uM CUM and 50 uM HN (paper speed 25 mm/sec). Notice that no major contractility disturbances were encountered.

quite comparable to the vasodilatory action of NP. The maximal elevation of coronary flow measured with CUM was 200%, with HN 220% and with NP 185%. However, the concentration needed for this maximal response was lower in the case of NP (5 uM) compared to the other two compounds (200 uM). During the experiments, as shown in Fig.1, contractility was recorded and used as a parameter for heart function. CUM, HN and NP caused an instantaneous elevation of the coronary flow always within two minutes. In this period, no major disturbances in heart contractility were encountered, suggesting that changes in contractility itself are not responsible for the observed flow differences.

Measurements of cyclic nucleotide levels in perfused rat hearts.

A possible activation of guanylate cyclase or adenylate cyclase in response to CUM or HN was investigated by measuring the cGMP and cAMP levels in freeze-

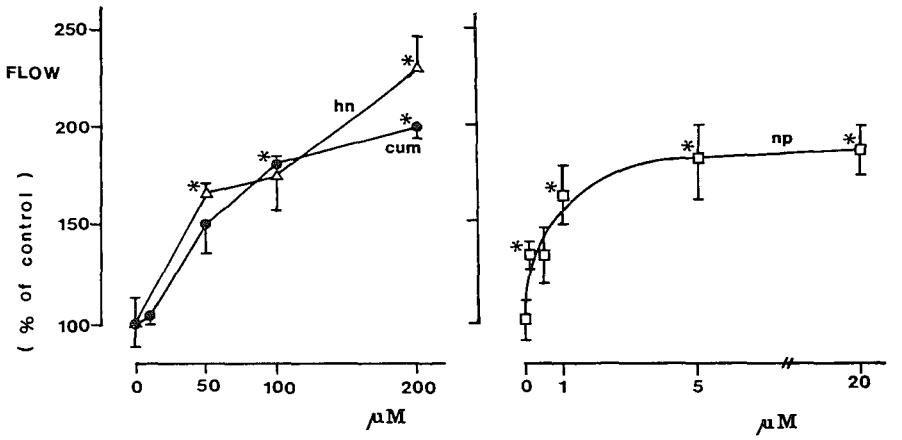

*Fig.2. Coronary flow in relation to perfusions with several concentrations of sodiumnitroprusside (np), cumene-hydroperoxide (cum) and 4-hydroxy-2,3-nonenal (hn) (n=2). 100 % coronary flow equals 10.6 ± 1.8 ml/min. (n=2). * = $p < 0.05$ and significantly different from control level.*

clamped rat hearts after two minutes perfusion with the compound of interest. We found that HN at concentrations that caused an increase of coronary flow, was unable to change cGMP levels in the perfused rat hearts (Fig.3 and 4). Unless the coronary flow was elevated by 200 uM CUM, a significant decrease of cGMP compared to control levels was observed (Fig.3). In order to introduce a positive control for the cGMP measurements several rat hearts were perfused with NP which is known to mediate vasodilation by elevating smooth muscle cGMP levels (14,15). We found that a ten fold lower concentration of NP caused a twenty fold increase of tissue cGMP levels (Fig.3). Moreover, a clear correlation was found between total heart cGMP levels and the increase of coronary flow at suboptimal doses of NP (Fig.4). Since we were able to measure a good correlation between total heart cGMP concentrations and coronary flow increment by NP, we provisionally conclude that coronary vasodilation induced by CUM or HN is not mediated by cGMP. CUM and HN always induced coronary flow elevation within two minutes. Because of this short perfusion time and the fact that these substances had no influence on heart contractility we, tentatively, do not expect them to have a major impact on cyclic nucleotide levels of the myocardial cells. Further evidence for this assumption is described in the immunocytochemical part of this paper (Fig.7). The differences in total heart cyclic nucleotide levels therefore likely reflect cyclic nucleotide levels in the vessel wall. The effects of CUM and HN on cAMP levels are

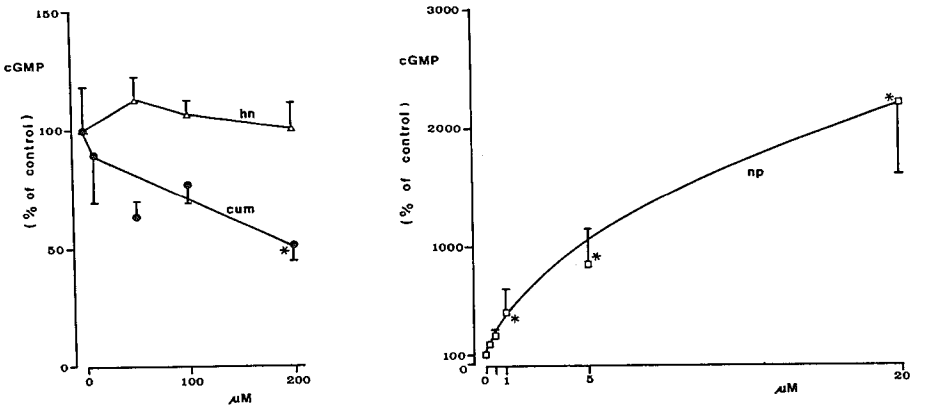

*Fig.3. cGMP levels measured in total rat hearts in relation to perfusion with various concentrations of sodiumnitroprusside (np), cumene-hydroperoxide (cum) and 4-hydroxy-2,3-nonenal (hn) (n=2). The means and SEM at each concentration reflects two separate experiments. * = p < 0.05 and significantly different from control (100 %) level. 100 % value equals 10.2 ± 2.1 pMoles cGMP/100 mg protein (n=2).*

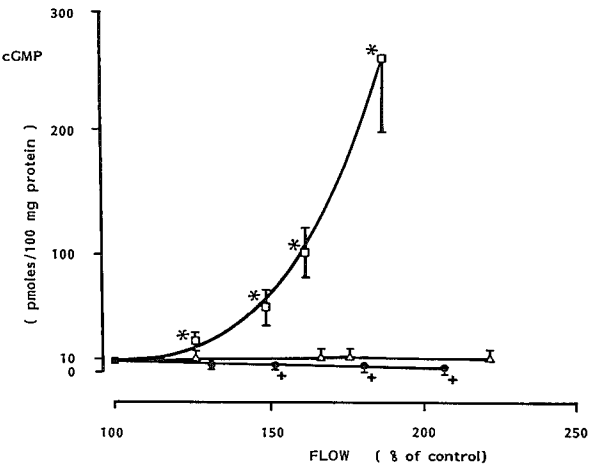

*Fig.4. cGMP levels in total rat hearts after perfusion with various concentrations of sodiumnitroprusside (□), cumene-hydroperoxide (•) and 4-hydroxy-2,3-nonenal (△) (n=2) in relation to coronary flow. Means and SEM are shown. Significant differences from control (100%) levels: * = p < 0.01, + = p < 0.05. 100% of coronary flow equals 10.6 ± 1.8 ml/minute (n=2). 100% of cGMP equals 10.2 ± 2.1 pMoles /100 mg protein (n=2).*

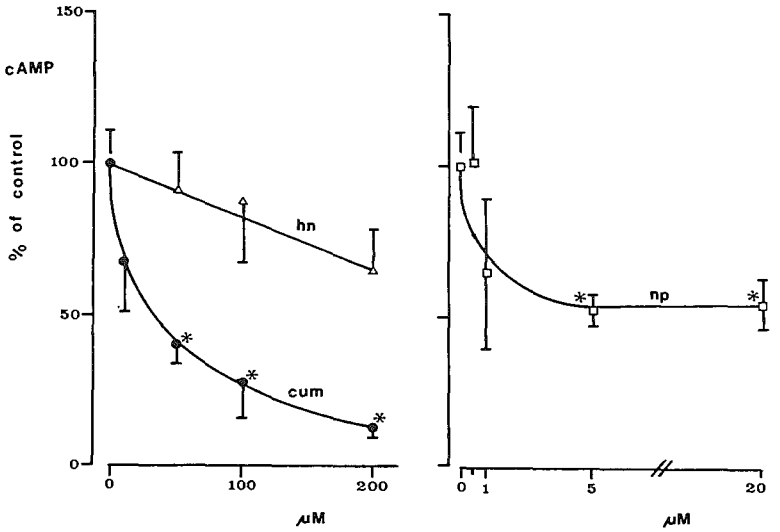

*Fig.5. cAMP levels measured in total rat hearts in relation to perfusion with various concentrations sodiumnitroprusside (np), cumene-hydroperoxide (cum) and 4-hydroxy-2,3-nonenal (hn) (n=2). The means and SEM are shown each time of two separate experiments. Significant differences: * = p < 0.05. 100% value equals 232.2 ± 24.7 pMoles cAMP/100 mg protein (n=2).*

visualized in Fig.5. CUM and NP caused a significant reduction of tissue cAMP levels to 50% of control levels, while HN provoked a smaller decrease to about 75%. Plotting cAMP levels against coronary flow revealed Fig.6. This figure clearly shows that rat coronary vasodilation induced by CUM, HN or NP is not associated with a rise in cAMP levels. The effects of two minutes perfusion with 10^{-7} M isoprenaline on cAMP levels in the heart are shown in Table I. Isoprenaline was used to check the methodology of the cAMP determinations. As shown in Table I, isoprenaline induced an increase of total heart cAMP levels.

Immunocytochemistry of cGMP.

As shown in Fig. 3 and 4, perfusions with CUM or HN did not elevate total heart cGMP levels. However, in these figures the concentration is expressed as pmoles/mg protein. Due to the fact that most of the protein is coming from the myocardial cells it

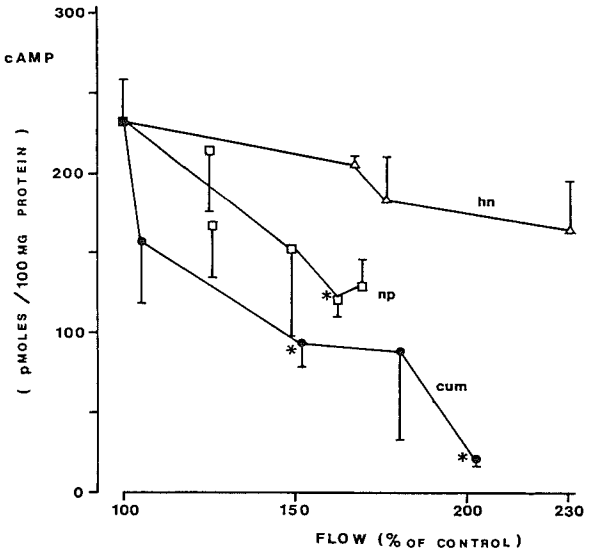

*Fig.6. cAMP levels in total rat hearts after perfusion with various concentrations of sodiumnitroprusside (np), cumene-hydroperoxide (cum) and 4-hydroxy-2,3-nonenal (hn) (n=2) in relation to coronary flow. Means and SEM are shown. * = p < 0.05 and significantly different rom control levels. 100% of coronary flow equals 10.6 ± 1.8 ml/minute. 100% of cAMP equals 232.2 ± 24.7 pMoles/100 mg protein (n=2).*

is possible to miss a rise in cGMP in the smooth muscle cells. To circumvent this possible error we performed immunocytochemistry of cGMP. Figure 7, shows cross-sections of isolated rat hearts after perfusion with 5 uM NP, 100 uM CUM or 100 uM HN compared with an undilated control heart. A coronary artery is clearly visible in all four pictures. No cGMP immunostaining was found in the vessel wall of the control heart, whereas clear cGMP staining was found in the smooth muscle cells of the coronary arteries perfused with 5 uM NP. A coronary flow elevation of 100 % induced by 100 uM CUM or 100 uM HN was not accompanied by cGMP immunostaining in the vessel wall. We found no cGMP staining ever in the cardiac muscle itself under the four conditions tested, which is in agreement with our earlier statement that because of the short perfusion time and the absence of contractility disturbances CUM and HN had no major impact on cardiac muscle cyclic nucleotide levels. The results in Fig.7, therefore clearly reflect cGMP measurements performed in the total heart homogenates (Fig.3).

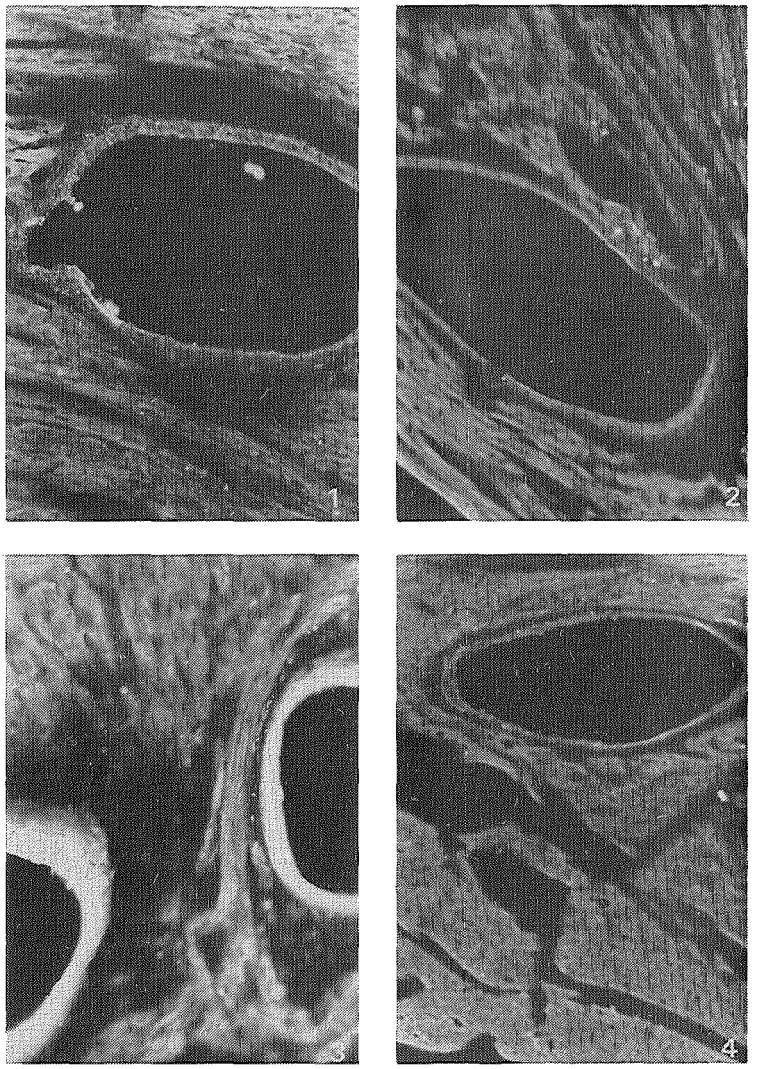

Fig.7. Photomicrograph of transversal sections of coronary arteries in Langendorff perfused rat hearts, after incubation with antibodies against cGMP. Intracellular cGMP immmunoreactivity is clearly visible in arterial vascular smooth muscle cells of the heart perfused with 5uM NP (3), whereas no cGMP immmunoreactivity was found in arterial vascular smooth muscle cells of a control heart (1) or the hearts perfused with 100uM HN (2) or 100uM CUM (4). No immune-reactivity was ever encountered in the cardiac muscle itself.

Table I. cAMP levels measured in control rat hearts and hearts perfused for two minutes with 10^{-7} M isoprenaline.

	cAMP pMoles/100mg protein (mean ± sd)
control hearts (n=2)	232.2 ± 24.7
isoprenaline 10^{-7} M (n=2)	850.3 ± 154.7

DISCUSSION

Earlier studies in our laboratory have shown that cumene hydroperoxide (CUM) and 4-hydroxy-2,3-nonenal (HN) are able to induce strong coronary vasodilations in Langendorff perfused rat hearts (5). These two substances are both associated with lipid peroxidation, a process which might be initiated in cardiac tissue as a result of post-ischemic reperfusion (1-4). CUM can be regarded a good model compound to mimic lipid peroxidation, because it is able to initiate an immediate oxidative stress in the heart by generating hydroxyl radicals (OH;5). These radicals will react with the polyunsaturated fatty acids located in membrane phospholipids, in which they form a new generation of lipid-radicals (lipid peroxidation intermediates). These radicals will induce a new cascade of peroxidation reactions. These ongoing reactions are highly autocatalytic and will ultimately result into membrane destruction and cell death (16,17). The peroxidation cascade can be interrupted if a radical reacts with an antioxidant as e.g. vitamin E or reduced glutathione (GSH). As a result of lipid peroxidation, a great diversity of aldehydes are formed among which malondialdehyde and HN. Recently, HN has received particular attention since it is formed in rather high quantities during lipid peroxidation (18,19). Formation of peroxides or aldehydes during ischemia and/or post-ischemic reperfusion might well be responsible for generalized post-ischemic vasodilation (hyperaemia). This condition, as e.g. encountered after prolonged aortic or cardiac surgery, may induce massive vasodilation, hypotension or shock (20,21).

In the present study, we investigated the vasodilatory effects of CUM and HN. We were especially interested whether these vasodilations are mediated by a cyclic nucleotide dependent mechanism. Our data indicate that CUM and HN are able to double the coronary flow without elevating cGMP or cAMP levels. Perfusions with

isoprenaline induced an elevation of cAMP to 370% of control levels. Sodium-nitroprusside (NP) induced coronary vasodilations that were properly correlated with cGMP levels. These findings indicate that if coronary vasodilation induced by CUM or HN was mediated by cGMP or cAMP we would have detected an increase of these cyclic nucleotides in the heart homogenates. In addition, we found no cGMP immunostaining in arterial coronary smooth muscle cells with CUM or HN in contrast to NP. We therefore conclude that rat coronary vasodilation induced by CUM or HN is not mediated by a rise in cyclic nucleotide levels. Since lipid peroxidation might be expected to occur in heart tissue during post-ischemic reperfusion (4) and since this process is associated with coronary vasodilation (post-ischemic hyperaemia, 22), the vasodilatory effects of CUM and HN might well be mediated by a similar mechanism as post-ischemic vasodilations. It is therefore important to further investigate this particular mechanism of coronary vasodilation.

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CHAPTER VII

GENERAL DISCUSSION

Oxygen-derived free radicals (ODFR) are increasingly recognized to be responsible for post-ischemic reperfusion injury in cardiac tissue (1,5,14). Not only myocardial necrosis but also rhythmic disturbances (ventricular fibrillation, 71) and post-ischemic coronary vasodilation, are now thought to be associated with ODFR generation. Evidence in support of this "free radical" hypothesis is predominantly based on experiments in which lipid peroxidation was found during ischemia and reperfusion (59), and on experiments in which the hearts of laboratory-animals were protected using free radical scavengers or antioxidants (26-30). Superoxide dismutase (SOD) and catalase, enzymes which are normally present in the myocardium, have repeatedly been reported to reduce post-ischemic myocardial injury (27-30). In addition, substances that interfere with oxygen free radical toxicity such as mannitol, alpha-tocopherol (vitamin E), allopurinol and deferoxamine were reported to be protective (38-40, 72).

Despite this accumulating evidence, the involvement of oxygen free radicals in (post)-ischemic myocardial injury is still controversial. This can be explained by the fact that oxygen free radicals are highly reactive which is responsible for a very transient existence in vivo. As a result, the direct detection of oxygen free radicals in cardiac tissue is extremely difficult. In addition, the literature is not conclusive as several studies have not confirmed protection by oxygen free radical scavengers (2,14,20). Because of these considerations experiments were designed (this thesis) to elucidate the exact role of oxygen free radicals in myocardial ischemia and post-ischemic reperfusion.

1) Iron and post-ischemic reperfusion injury.

In the first study, described in chapter II (68), the influence of iron in mediating reperfusion injury was investigated. In theory, superoxide is thought to be initially formed during post-ischemic reperfusion (7,20). Superoxide itself is not very toxic because its reactivity is low and it will be rapidly neutralized, in vivo, by SOD to hydrogen peroxide. In the presence of iron, however, hydrogen peroxide can be converted into highly reactive hydroxyl radicals (modified Haber-Weiss or Fenton reaction) which in turn are good candidates to induce post-ischemic reperfusion injury. In order to investigate whether "Fenton chemistry" can actually occur in heart tissue, rats were iron-loaded for a period of six weeks. The hearts of these animals were perfused under anoxic and reperfusion conditions and turned out to be much more sensitive to reperfusion injury than control (unloaded) hearts. This was measured by a significant lower recovery of contractility, a significant higher LDH release and the occurrence of

significantly more ventricular fibrillation. The iron-loaded hearts could be protected with the anti-oxidant (+)-cyanidanol-3 and the iron-chelator deferoxamine which strongly indicates the involvement of free radicals, as well as, iron in the reperfusion syndrome. Because SOD and glutathione peroxidase activities in both iron-loaded and control hearts are equal, it can be concluded that the occurring damage is not due to an impaired defense mechanisms against free radicals but most likely to an increased generation of hydroxyl radicals formed in the presence of increased iron amounts. So, the results described in chapter II give strong evidence that the availability of transition metals, such as iron, aggravate anoxic or ischemic damage.

In chapter III, a study is described in which two newly developed iron-chelators were tested for (post)-ischemic cardiac protection. One iron-chelator, 1,2-dimethyl-3-hydroxy-4-pyridone (L1), induced protection whereas the second iron chelator, 5-hydroxy-2-hydroxymethyl-4-pyrone (kojic acid), had no effect. The differences between these two structurally related iron-chelators can be explained by their opposite effects in catalyzing hydroxyl radical formation in the Haber-Weiss reaction (69). Iron bound to L1 does not catalyze hydroxyl radical formation in contrast to iron bound to kojic acid. The results of this study further strengthen our suggestion that iron excess may aggravate the reperfusion syndrome. Since the orally active iron-chelator L1 is about to be registered as a drug, our results might open a promising and easy assessable approach in establishing (post)-ischemic cardiac protection in the near future.

2) Free radical generation in endothelial cells.

In chapter IV, the distribution of iron in rat hearts after a short period of iron-load was investigated by light microscopy, electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS). Iron was found to be abundantly present in endothelial cells and pericytes, sparsely present in some macrophages located in the perivascular space but not present in cardiomyocytes themselves. This observation is important in clarifying the site of free radical toxicity in the heart as a result of ischemia and post-ischemic reperfusion. Because of their high reactivity, hydroxyl radicals can be expected to combine "site specific" i.e. at the place where iron-catalysts are situated (5,13,14). For this reason, specific iron-storage in vascular endothelial cells and pericytes strongly indicates hydroxyl radical formation in these cells and not in cardiomyocytes. The results of the studies, described in chapter II, III, IV, therefore indicate reperfusion injury to be initially a vascular problem associated with hydroxyl radical formation in endothelial cells and/or pericytes.

This hypothesis is consistent with experiments in which protection with the iron-chelator, deferoxamine, was reported (38,72). Badylac et al. (38) showed protection of rat hearts by deferoxamine after 60 minutes of total ischemia. Moreover, Ambrosio et al. (72) reported protection by deferoxamine administered at the onset of post-ischemic reflow. The results contrast the findings of Myers et al. (39) who reported no protection

by deferoxamine in rabbit hearts after two hours of global ischemia at 27° C. In that study it was hypothesized that iron-catalyzed hydroxyl radical formation can still be important for reasons that intracellular iron pools may be inaccessible to deferoxamine.

The possibility of reperfusion injury to be initiated in vascular tissue has been suggested before (75). Superoxide dismutase (SOD) has repeatedly been reported to be protective (28-30). One can not expect a rapid penetration of this large molecule (Mw 30,000) into myocardial cells after relatively short administration periods. So protection by SOD may well be explained by scavenging superoxide in the vascular space and/or in vascular tissue. More evidence in support of this view was reported by Hülsmann et al. (74), who found early damage of endothelial cells in rat hearts during ischemia. In addition, Ratych et al. (75) reported free radical generation during reperfusion in isolated endothelial cells in the absence of neutrophils or parenchymal cells. They were able to protect reoxygenated endothelial cells after 45 minutes of anoxia with SOD, catalase or allopurinol. This may well be result of superoxide and/or hydrogen peroxide release from endothelial cells, because SOD and catalase are unable to penetrate the endothelial cells. Zweier et al. (76) directly measured free radicals in isolated endothelial cells during reoxygenation using electron paramagnetic resonance spectroscopy (EPR) and the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO).

So at present, a reasonable origin of oxygen free radical generation in isolated heart preparations devoid of circulating leukocytes are the endothelial cells. The mechanism of oxygen free radical formation during ischemia and/or reperfusion is not known. McCord suggested in 1985 the conversion of the enzyme xanthine dehydrogenase (XDH) to xanthine oxidase (XO) during ischemia (20). XO is known to produce superoxide upon catalyzing the breakdown of hypoxanthine to uric acid. The validity of this hypothesis is strongly debated at the moment as it has been reported that healthy human hearts as well as pig and rabbit hearts do not contain XDH or XO (41,42). A recent study contradicts these findings as activities of XDH and XO were reported in human and rabbit hearts (43). The authors (43) suggested the differences to result from a cytosolic inhibitor of XDH/XO present in human and rabbit heart tissue. However, diseased hearts, or hearts with leukocytes attached to their endothelium may be expected to contain XDH/XO as white blood cells do contain these enzymes (115).

Among other possible sources of oxygen free radical generation in the heart activation of lipoxygenase could be an alternative, especially since this enzyme is widely present in endothelial cells and its substrate arachidonic acid accumulates in the heart during ischemia (51). Studies with specific lipoxygenase inhibitors have indeed shown protective effects of these compounds. Whether this effect is a result of specific lipoxygenase inactivation or of aspecific free radical scavenging needs to be further investigated.

Normally, most of the iron, present in cells, is stored in ferritin in which it is unable to catalyze hydroxyl radical formation. In this regard the findings of Biemond et al. (73) are important because they have shown that superoxide is able to mobilize iron from ferritin. Superoxide-dependent iron release from ferritin so creates an optimal

situation in which lipid peroxidation occurs and might therefore be an important mechanism in the pathogenesis of the reperfusion syndrome.

3) EPR-spectroscopy.

In chapter IV, a study is described in which oxygen-derived free radicals in (post)-ischemic and (post)-anoxic rat hearts were measured using electron paramagnetic resonance (EPR) spectroscopy. No free radical signals linked to reperfusion injury were detected in this study. The results contrast the experiments of Blasig et al. (35) and Zweier et al. (36) who, in 1987, were the first to claim the direct detection of oxygen free radicals in post-ischemic cardiac tissue using EPR. The signals described by Blasig et al. and Zweier et al. were found to be fully reproducible in our study but we interpret them completely different. We found the signals to originate partially from signal saturation and partially from electron transfer centers located in the respiratory chain, predominantly the iron-sulfur cluster S-1 in succinate dehydrogenase. The differences in signal intensity between normoxic, ischemic and reperfused hearts are a result of the different redox stages of these centers under the various perfusion conditions tested. These findings are quite important since the study of Zweier et al. (34) is widely used as a definite prove of oxygen free radical involvement in the reperfusion syndrome. Our findings do not necessarily imply that oxygen-derived free radicals are not formed in cardiac tissue during (post)-ischemic reperfusion. There is still much indirect evidence indicating free radical involvement (1,5,7), and oxygen free radicals have recently been detected with EPR spectroscopy in perfusates of reperfused rat hearts as adducts of the spin-traps DMPO (5,5'-dimethyl-1-pyrroline-N-oxide, 111) or PBN (N-tert-butyl-alpha-phenylnitrone, 112). The constitutive background of paramagnetism from the respiratory chain, however, seriously hampers the direct detection of comparatively low concentrations of free radicals in cardiac tissue.

4) Oxygen free radicals and coronary vasodilation.

In a study, described in chapter VI, the vasodilatory effects of cumene hydroperoxide (CUM) and 4-hydroxy-2,3-nonenal (HN) were investigated in order to understand the mechanism of vasodilation by these compounds and lipid peroxidation in general. Earlier studies in our laboratory have shown that CUM and HN are able to induce strong coronary vasodilations in Langendorff perfused rat hearts (88). CUM is a compound known to induce lipid peroxidation in biological tissue, whereas HN is known to be formed in rather high quantities as a result of lipid peroxidation (89). We were interested whether these vasodilations are mediated by cyclic nucleotides, particularly cGMP, as this is the second messenger of the endothelial derived relaxant factor (EDRF, 113). Our study indicate no involvement of cGMP or cAMP (114) in this

type of vasodilation. In low flow ischemia or hypoperfusion, oxygen free radicals may indeed be involved. Hülsmann and Dubelaar (74) arrived at this conclusion as during hypoperfusion of Langendorff rat hearts, cardiac function was lost, in which SOD or vasoxine, an alpha-adrenergic vasoconstrictor, afforded protection. Vasodilation, as a result of ischemia or hypoperfusion, is a useful physiological phenomenon. However, in the presence of an obstruction (e.g. atherosclerosis), vasodilation is harmful as it further decreases perfusion pressure in the obstructed area, leading to a decline in ATP synthesis (116). Since lipid peroxidation may be expected in heart tissue during hypoperfusion or post-ischemic reperfusion, its role and mechanism of action in vasodilation and hypotension needs further clarification.

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SUMMARY

Over the past 3 decades, the mortality rate from myocardial infarction has been substantially reduced predominantly by vigorous treatment of arrhythmias in coronary care units. At present, further reductions are hampered since the treatment of heart failure (cardiogenic shock, pulmonary edema) has not been analogously improved. Heart failure in acute myocardial infarction is known to be closely correlated with the extent of tissue necrosis. Therefore, any therapeutic strategy which enables a decrease of myocardial necrosis can be expected to be extremely useful. Against this background, experiments were designed to seek for ischemic protection. Since oxygen free radicals are thought to be involved in myocardial infarction, the role of these radicals in the pathogenesis of this disease was investigated.

At first, (chapter II) the influence of iron was studied. From in vitro experiments, iron is known to increase oxygen free radical toxicity by catalyzing hydroxyl radical formation from hydrogen peroxide. A possible role of this mechanism in vivo was investigated by increasing the amount of iron in the heart. Rats were iron-loaded for a period of six weeks, after which their hearts were perfused under normoxic, anoxic and reoxygenation conditions (with or without oxygen). The hearts were found to be extremely vulnerable to anoxia and reperfusion in contrast to control (unloaded) hearts. This was measured by a significantly lower recovery of heart function, a significantly higher lactate dehydrogenase release (LDH) and the occurrence of significantly more ventricular fibrillation. The iron-loaded hearts could be totally protected by the anti-oxidant (+)-cyanidanol-3 and the iron-chelator deferoxamine. Anti-oxidant levels in the hearts (superoxide dismutase and glutathione peroxidase) were not changed as a result of iron-load. These findings strongly indicate the involvement of iron and hydroxyl radicals in the pathogenesis of myocardial infarction. This has two consequences for achieving ischemic cardiac protection. Firstly, oxygen free radical generation itself has to be reduced and secondly, iron must be kept unavailable to catalyze hydroxyl radical formation.

In order to further substantiate the involvement of iron, two recently developed iron-chelators were tested for (post)-ischemic cardiac protection in control hearts (chapter III). One iron-chelator, 1,2-dimethyl-3-hydroxy-4-pyridone (L1), induced significant protection whereas the second iron-chelator, 5-hydroxy-2-hydroxymethyl-4-pyrone (kojic acid), had no effect. The differences between these two iron-chelators can be explained by their opposite effects in suppressing hydroxyl radical formation in vitro. Iron bound to L1 is unavailable for catalysis, in contrast to iron bound to kojic acid. The results of this study indicate that protection is mediated by iron-chelation and not by aspecific scavenging of free radicals since kojic acid, which is structurally related to L1, had no effect. The results strengthen our suggestion that iron and hydroxyl radicals are involved in (post)-ischemic cardiac injury. Since the orally active iron-chelator L1 is about to be registered as a drug (for the treatment of hemochromatosis), our results might open a promising and entirely new approach in establishing (post)-ischemic

cardiac protection in patients.

The distribution of iron in rat hearts after iron-load was investigated by light- and electron microscopy (chapter IV). Iron was found to be abundantly stored in endothelial cells and pericytes but not in cardiomyocytes. This observation is important in clarifying the site of hydroxyl radical generation in the heart. The increased sensitivity of iron-loaded hearts to reperfusion injury together with specific iron-storage in endothelial cells and pericytes strongly indicates hydroxyl radical formation in or near these cells. Therefore, we suggest reperfusion injury to be initially a vascular problem associated with oxygen free radical generation in endothelial cells and/or pericytes.

In order to measure oxygen free radicals directly in the heart, electron paramagnetic resonance (EPR) spectroscopy was performed (chapter V). Rat hearts were perfused under normoxic, anoxic, ischemic and reperfusion conditions and measured at 13 and 77 Kelvin. No free radical signals linked to ischemia, anoxia or reperfusion were detected. Signals previously assigned to oxygen free radicals were found to represent signals from the iron-sulfur cluster S-1 in succinate dehydrogenase. Our findings with EPR spectroscopy do not necessarily imply that oxygen free radicals are not formed during myocardial infarction. Much indirect evidence still indicates free radical involvement. The physiological paramagnetism of the respiratory chain, however, makes the direct detection of oxygen free radicals in heart tissue impossible.

In chapter VI, we investigated whether cumene-hydroperoxide (CUM), a substance known to induce lipid peroxidation and 4-hydroxy-2,3-nonenal (HN), a major aldehyde formed in the process of lipid peroxidation, induce coronary vasodilation in rat hearts by changing cyclic-nucleotide levels. Both compounds showed a dose dependent and reversible increase of coronary flow which was comparable to the effect of sodiumnitroprusside (NP). With NP we found a good correlation between the extent of vasodilation and total heart cGMP levels. Vasodilation induced by CUM or HN was not accompanied by a rise in total heart cGMP or cAMP levels. Since lipid peroxidation might be expected in heart tissue during post-ischemic reperfusion and since this is associated with vasodilation, hypotension and shock the exact mechanism of this type of vasodilation needs further investigation.

SAMENVATTING

Het onderzoek in dit proefschrift bestudeerd de rol van "vrije zuurstof radicalen" in het ontstaan van weefselbeschadiging tijdens het hartinfarct. Sterfte aan hart- en vaatziekten is nog altijd doodsoorzaak nummer één in de westerse wereld. Hoewel deze sterfte duidelijk is teruggelopen in de afgelopen 30 jaar, door intensieve behandeling van hartritme-stoornissen in zg "coronary care units", is de therapie van hartzwakte (asthma cardiale, cardiale shock, long oedeem) a.g.v. weefsel-versterf tijdens het infarct niet duidelijk verbeterd. Om deze reden is elke vorm van behandeling, die weefsel-versterf kan voorkomen, uitermate nuttig voor een betere behandeling van het infarct en het uiteindelijke herstel van de patient. Tegen deze achtergrond zijn we vier jaar geleden ons onderzoek gestart. Aangezien vrije zuurstof radicalen mogelijk verantwoordelijk zijn voor weefsel-versterf, hebben we ons onderzoek specifiek gericht op de rol van deze verbindingen in het hartinfarct.

Allereerst zijn we begonnen met een studie naar de rol van ijzer. De resultaten van dit onderzoek zijn neergeschreven in hoofdstuk II. In theorie zijn er twee radicaal verbindingen van zuurstof bekend nl. superoxide ($^{\bullet}O_2^-$) en het hydroxyl radicaal ($^{\bullet}OH$). Deze verbindingen zijn uiterst reactief en derhalve vrijwel onmogelijk om te meten. Er zijn diverse ontstaanswijzen van zuurstof radicalen beschreven. Al deze beschrijvingen gaan uit van de primaire vorming van superoxide. Nu is algemeen bekend dat superoxide zelf niet erg toxisch is voor hartweefsel daar het gemakkelijk geneutraliseerd kan worden. In aanwezigheid van ijzer echter, kan superoxide omgezet worden in het uiterst toxische hydroxyl radicaal. In een poging dit mechanisme nader te onderzoeken, werden ratten kortdurend beladen met ijzer. De harten van deze beesten bleken veel gevoeliger voor een infarct dan controle harten. Dit kwam tot uiting in meer ritme-stoornissen, meer weefsel-versterf en lager funktie herstel tijdens reperfusie. De grotere gevoeligheid bleek volledig te kunnen worden opgeheven door toediening van enerzijds een "radicaal-vanger" (cyanidanol) en anderzijds een ijzer-chelator (deferoxamine). Op basis van deze resultaten concluderen wij dat zuurstof radicalen (met name het hydroxyl radicaal) en ijzer van belang zijn tijdens het hartinfarct.

Om de rol van ijzer nader te bestuderen werd een tweede onderzoek opgestart waarvan de resutaten neergeschreven zijn in hoofdstuk III. De essentie van dit onderzoek berust op het feit dat we de beschikking kregen over twee ijzer-chelatoren, met een vergelijkbare moleculaire struktuur, waarvan de ene ijzer-chelator (L1), in een reageerbuis, wel hydroxyl radicaal vorming kan blokkeren in tegenstelling tot de tweede ijzer-chelator (kojic-acid). Met de "hydroxyl radicaal blokkerende" ijzer-chelator vonden we een duidelijke bescherming van hartweefsel, tijdens het infarct, in tegenstelling tot de tweede chelator die geen effect sorteerde. Deze resultaten bevestigen de rol van ijzer en hydroxyl radicalen tijdens het hartinfarct. Dit heeft rechtstreeks consequenties voor preventie van weefsel-versterf tijdens het infarct. Behoudens het neutraliseren van superoxide dienen maatregelen genomen te worden die de schadelijke werking van ijzer te niet doen.

De verdeling van ijzer in ratte harten na ijzer-belading werd bestudeerd in hoofdstuk IV. We vonden dat ijzer voornamelijk opgeslagen wordt in bloedvaten en niet in de hartspier zelf. Deze bevinding is van belang voor het ontrafelen van de bron van zuurstof radicalen tijdens het hartinfarct. De toegenomen gevoeligheid van ijzer-beladen ratte harten tijdens een infarct en de aanwezigheid van ijzer in de bloedvatwand, suggereert dat zuurstof radicalen tijdens het hartinfarct worden gevormd in de bloedvatwand.

Met behulp van electron spin resonantie hebben we vervolgens geprobeerd vrije zuurstof radicalen rechtstreeks te meten in hartweefsel tijdens een infarct (hoofdstuk V). De signalen die voorheen door Amerikaanse en Japanse onderzoekers aan zuurstof radicalen waren toegeschreven, bleken zeer wel reproduceerbaar maar niet afkomstig van vrije zuurstof radicalen. De signalen vinden hun oorsprong in ijzer-zwavel clusters van eiwitten uit de ademhalingsketen. Deze "electron transfer centers" reageren op de verschillende zuurstof concentraties (redox toestanden) in het hart voor, tijdens en na het infarct. De bevindingen in deze studie houden niet automatisch in dat vrije zuurstof radicalen niet gevormd kunnen worden in hartweefsel tijdens het infarct. De sterke paramagnetische achtergrond van de ademhalingsketen maakt hun direkte detectie echter vrijwel zeker onmogelijk.

In hoofdstuk VI wordt tenslotte een studie beschreven waarin de vaatverwijding van kransslagaders bestudeerd wordt. Vaatverwijding tijdens een infarct is op zich een gunstig fenomeen, tenzij het voorbij een obstructie (bv aderverkalking) plaatsvindt. Vanuit fysiologisch oogpunt is dit ongunstig daar de perfusie-druk in de achterliggende hartspier hierdoor afneemt. Vrije zuurstof radicalen en een infarct zijn beide instaat vaatverwijding te induceren. Met behulp van chemische verbindingen, die radicalen kunnen opwekken in hartweefsel, hebben we vaatverwijdingen gegenereerd. Deze verwijdingen bleken niet gerelateerd aan "second messenger" niveaus van cGMP of cAMP, wat de bekende mediatoren zijn van vaatverwijding. Het werkingsmechanisme van deze verbindingen, alsook een mogelijk vergelijkbaar werkingsmechanisme van vaatverwijding tijdens het infarct, blijft daarom vooralsnog onopgehelderd.

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CURRICULUM VITAE.

De schrijver van dit proefschrift werd geboren op 23 mei 1958 te 's-Hertogenbosch. Na het behalen van het Atheneum B diploma aan het Mgr. Frencken College te Oosterhout (NBr) werd in 1977 met de studie geneeskunde begonnen aan de Erasmus Universiteit te Rotterdam. Tijdens deze studie werd als keuze-praktikant onderzoek gedaan naar de farmacologische aspecten van de calcium antagonist nifedipine (adalat) op de afdeling Experimentele Cardiologie (Dr. P. Verdouw). In 1984 werd het artsexamen afgelegd. Aansluitend werd een militaire dienstplicht van anderhalf jaar vervuld op de Palm-kazerne te Bussum, alwaar hij werkzaam was als onderdeels-arts. Vanaf 1 januari 1986 tot op heden is de schrijver werkzaam als assistent in opleiding op de afdeling Biochemie I van de Medische Faculteit Rotterdam, onder leiding van Prof. Dr. J.F. Koster, alwaar het hier gepresenteerde onderzoek werd uitgevoerd.

LIST OF PUBLICATIONS

1. Iron-load increases the susceptibility of rat hearts to oxygen reperfusion damage. Protection by the antioxidant (+)-cyanidanol-3 and deferoxamine.
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   (submitted).

9. Cumene-hydroperoxide, a lipid peroxidation inducing agent, and 4- hydroxy-2,3-nonenal, a lipid peroxidation product, cause coronary vasodilation in perfused rat hearts by a cyclic nucleotide independent mechanism.
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(submitted).

10. Rat heart perfusion as model system for enzyme replacement therapy in glycogenosis type II.
A.T. van der Ploeg, A.M.M. van der Kraaij, R. Willemsen, M.C.B. Loonen, A.J.J. Reuser, J.F. Koster.
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