IRON AND THE OXYGEN PARADOX IN ISCHEMIC HEARTS.

IJzer en de Zuurstof Paradox in het Ischemische Hart

PROEFSCHRIFT

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CONTENTS

CHAPTER ONE

ISCHEMIA AND REPERFUSION

Introduction.

Blue - green algae caused the accumulation of oxygen in the terrestrial atmosphere by starting photosynthesis. Surrounding life forms and the algae themselves were forced to adapt to this oxidising agent and developed defences against the reduced forms of oxygen: superoxide, hydrogen peroxide and the hydroxyl radical (1,2). These defences allowed the evolution of aerobic metabolism in which carbohydrates created in photosynthesis are oxidised by molecular oxygen. In present day aerobic metabolism superoxide and hydrogen peroxide are continuously generated and even used in biosynthesis, intercellular signalling and in the defense against invading microorganisms. However, the reality of oxygen toxicity remains and is illustrated by a number of diseases in which the balance between generation and degradation is thought to be disturbed (3,4,5). This thesis deals one of those situations, myocardial ischemia and reperfusion, as a part of the continuing research to clarify the underlying principles of oxygen toxicity.

Reperfusion: expression of ischemic damage or reperfusion injury ?

Blockade of a part of the blood flow to the heart or the brain is a major cause of morbidity and mortality in western society. The lack of circulation causes complete or partial deprivation of oxygen leading to eventual necrosis of the affected part of the organ. Because functional recovery depends on the duration of ischemia the techniques to open up the occluded arteries and restore oxygen supply before irreversible damage occurs have reduced mortality over the recent years. Unfortunately, it is now evident that restoration of the circulation is not without danger and contributes to the tissue damage and organ disfunction. This is known as reperfusion injury (6,7,8,9).

The earliest observation of cardiac reperfusion injury was the induction of ventricle fibrillation by reopening the occluded coronary artery in the dog heart (10). A newer finding is that after a short period of ischemia prolonged myocardial dysfunction occurs during reperfusion. No real tissue injury occurs because the phenomenon is completely reversible by post-extra systollic potentiation or by adrenergic stimulation (11). Therefore, it is known as myocardial stunning (12). Both are potentially life threatening because of the loss of pump function. The reperfusion induced arrhythmias have been extensively studied both in vivo and in isolated hearts (13). These may be caused by disturbances of lipid metabolism, Ca^{2+} overload, increase in cAMP, stimulation of adrenergic receptors and oxygen radical production. The proposed causes behind myocardial stunning (14) include a reduction in the ability to resynthesize ATP, altered calcium concentrations, impairment of the sarcoplasmatic reticulum and oxygen radicals. Another classical finding was that in the early minutes of reoxygenation of rat hearts, that had been perfused with hypoxic buffer, massive enzyme release occurred which did not occur when the hypoxia was continued (7,15). Apparently, the reintroduction of oxygen which is necessary for functional recovery injured these hearts. This effect could have been caused by the accelerated expression of injury already present or by new injury caused by reoxygenation. Lethal cell injury occurring during reperfusion could be caused by calcium overload, mitochondrial injury and oxygen radicals.

The existence of reperfusion injury, defined as "de novo" injury caused by reperfusion, has been questioned (16) because most successful "anti-reperfusion-injury-

interventions" are started during or even before ischemia (6). The only validation of the existence of reperfusion injury would be a beneficial intervention that is started after ischemia. A small number of studies have indeed documented such an intervention (17,18). The failure in other studies could be explained if events causing the injury occurs extremely early in reperfusion. The earliest, well documented event during reoxygenation is the formation of oxygen radicals. This has been measured directly by electron spin resonance techniques using both in vivo (19,20) preparations or isolated hearts (21,22). Garlick et al. (22) showed that if ischemic hearts were reperfused in the absence of oxygen no radical production occurs. Pre-ischemic interventions limiting the production or toxicity of reactive oxygen species during reperfusion are beneficial in most experimental studies (20). Collectively, these studies have provided evidence for the oxygen paradox some ten years after it was first proposed: the reintroduction of oxygen in ischemic tissue causes the generation of reactive oxygen species that contribute to tissue damage (3,23,24,25,26).

Reactive oxygen species and radicals

A free radical is any molecular species that contains one or more unpaired electrons. Following this definition molecular oxygen in its ground state is a radical since it has two unpaired electrons each located in one of the π^* antibonding orbitals. Both unpaired electrons have parallel spins so that all reactions involving molecular oxygen are spin restricted causing slow reaction rates. Removal of this restriction leads to the formation of "reactive oxygen species", not all of which are radicals.

The spin restriction can be circumvented in three different ways. Electrons can be moved to another orbital or forced to change spin by ionizing radiation. Thus singlet oxygen states can be formed (27,28). A second way is the univalent reduction of molecular oxygen (Figure 1). Addition of the first electron leads to the formation of the superoxide radical. Here one antibonding orbital is filled with two paired electrons while the remaining orbital is occupied by the single electron so that the reactions of this molecule are not spin restricted. In addition the molecule gains a negative charge. Further reduction leads to the formation of the peroxide ion in which both antibonding orbitals are occupied by two paired electrons. This is the conjugated base of hydrogen

peroxide. It is not a radical but a stable compound although more reactive than molecular oxygen because the oxygen - oxygen bond is weakened. Addition of the third electron breaks the oxygen - oxygen bond and leads to the formation of the hydroxyl radical and one hydroxide ion. Addition of the fourth electron then reduces the hydroxyl radical to the second hydroxide ion completing the reduction of oxygen to two water molecules.

Superoxide, in aqueous solutions is both a reducing and oxidizing agent of moderate reactivity (29). It spontaneously reacts to form hydrogen peroxide and molecular oxygen at a rate of 5×10^5 $M^{-1}s^{-1}$ at physiological pH (30). The protonated form of superoxide, the hydroperoxyl radical, is known to be a more aggressive oxidant which can cause the peroxidation of lipids (31,32). Hydrogen peroxide is a stable compound of limited reactivity and no further reduction will take place in the absence of a suitable catalyst.

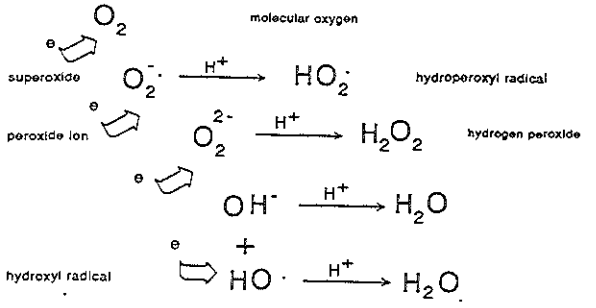

Figure 1.
Scheme of the univalent reduction of molecular oxygen leading to superoxide, hydrogen peroxide and the hydroxyl radical. Only the formation of hydrogen peroxide from superoxide will proceed uncatalysed.

The third way to overcome the spin restrictions is the formation of a complex with a transition metal that has the ability to accept and donate single electrons due to the presence of unpaired electrons in the d-orbitals (33). This distinctive property makes

transition metals extremely good catalysts of radical reactions and essential to for the formation the hydroxyl radical (3,26,33,34,35,36).

The reactions in which the hydroxyl radical can be formed involve the redox cycling of a transition metal together with hydrogen peroxide and superoxide (figure 2). The reactions will proceed in the presence of mosttransition metals. In vivo this will be iron since this is the most abundant in biological tissue.

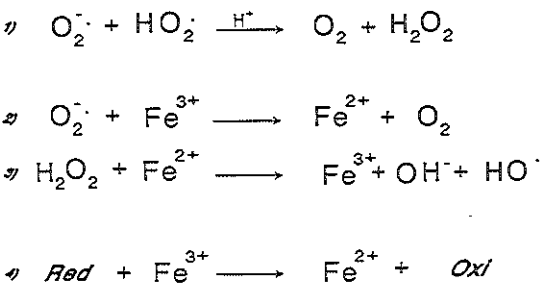

Figure 2.
Hydroxyl radical formation in the presence of catalytic iron. Reaction 1) is the dismutation of superoxide generating hydrogen peroxide. Reactions 2) and 3) are known as the Haber-Weiss reactions in which the redox cycling of iron occurs. Reaction 3) is known as the Fenton reaction. Reaction 4) stresses that superoxide can be replaced by other reducing agents to replenish ferrous iron needed in the Fenton reaction.

Reaction 1) is the spontaneous dismutation of superoxide generating hydrogen peroxide. Reactions 2) and 3) are known as the Haber-Weiss (37) reactions in which the redox cycling of iron occurs. In addition to the hydroxyl radical iron containing species such as the ferryl ion (FeO^{2+}) result from this reaction, which are of similar reactivity (33,38). The ferric iron may also be reduced by other reducing agents (39) (reaction 4) which will enhance the yield of hydroxyl radicals.

Due to the poor solubility of ferric hydroxide the Fe^{3+} ion concentration will be extremely low under physiological pH and these reactions will not proceed (3,33). Only in the presence of a suitable chelator that keeps the iron in solution can it participate in these reactions. The chelator must allow one-electron transfer so at least one coor-

dination site must be occupied by an easily displaceable ligand (40). Therefore the nature of the complex determines why some iron chelators enhance and others completely prevent iron driven radical reactions (41).

The hydroxyl radical is an extremely oxidizing agent which will abstract protons from proteins and lipids at a rate of 10^9 $M^{-1}s^{-1}$ (42,5). For instance, reaction with a poly unsaturated lipid will generate an carbon centred radical which can react with molecular oxygen to form the lipid peroxyl radical (figure 3). In this way a chain reaction can be initiated in which one hydroxyl radical causes the oxidation of a number of fatty acid molecules. The chain is terminated when two radicals meet.

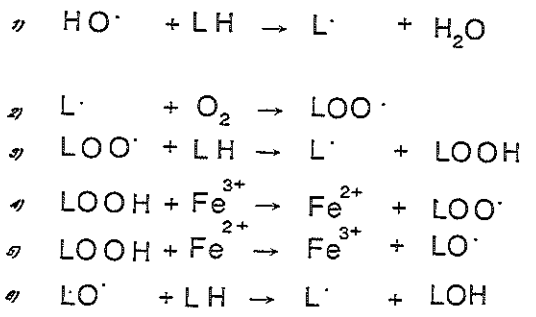

Figure 3. Initiation (reaction 1) and propagation reactions 2 - 6 of the chain reactions causing lipid peroxidation. Termination of the reactions occurs when two radicals meet.

Here another important role of iron is presented. Next to its part in the initiation of the chain it is a good catalyst of the propagation. Iron reacts with lipid peroxides (ROOH) present in the membranes to form lipid peroxyl (ROO.) and lipid alkoxyl (RO.) radicals (43) that again may perpetuate the chain reaction.

Sources of oxygen radicals.

All aerobic life forms use oxygen through the stepwise enzymatic reductions of the molecule using transition metals to overcome the spin restrictions (44). This causes a constant generation of superoxide and hydrogen peroxide from different sources, which may be either accidental through leakage or intentional to produce substrate for further reactions (1,4). Some of these metabolic pathways contribute to radical generation during reperfusion.

Mitochondrial respiration is one of the main sources of reactive oxygen in normal metabolism (45). In the final step of the mitochondrial respiratory chain the tetravalent reduction of molecular oxygen takes place in one step: four electrons from four cytochrome c molecules are transferred to molecular oxygen to generate two water molecules (46). Separate from leakage from respiration mitochondria generate superoxide through an NADH oxidase that may exist on the mitochondrial outer membrane (47, 48). Mitochondrial generation of reactive oxygen is enhanced by ischemia and reperfusion (47,49,50).

The peroxisomal ß-oxidation accounts for an important part of the fatty acid oxidation and a constant source of hydrogen peroxide (143,144) in the peroxisomes.

Prostaglandins and leukotrienes are extremely potent local hormones generated by the oxidation of arachidonic acid through the cyclooxygenase or lipoxygenase pathway, respectively. Both pathways involve lipid peroxides as intermediates and are stimulated by either free arachidonic acid, superoxide, hydrogen peroxide and lipid peroxides (51) and have been shown to generate reactive oxygen species (52). Arachidonic acid is the major unsaturated fatty acid in cardiac membranes (53). Ischemia causes an elevation of Ca^{2+} and an increase in free arachidonic acid (54) making these pathways a likely source of oxygen radicals during reperfusion (52,55,56,57).

The autooxidation of catecholamines generates superoxide (58,59). Because noradrenaline release has been seen during reperfusion this may contribute to radical formation as well.

The endothelium derived relaxing factor nitric oxide (NO, EDRF) is also a radical (60) and reacts with superoxide. A balanced production of both radicals by the endothelium would be a way of regulating vascular tone (61,62). On the other hand the

reaction between nitric oxide and superoxide has been proposed to generate peroxynitrite (63), which is converted to the hydroxyl radical (64) which could induce endothelial damage (65). Activation of granulocytes leads to a sudden increase in oxygen consumption and of the hexose mono phosphate shunt. More than 90 % of the oxygen consumed in this "respiratory burst" is converted to superoxide through the NAD(P)H oxidase (66). The superoxide is converted to hydrogen peroxide and this is used by the myeloperoxidase to generate the very toxic hypochlorous acid (67) wielded to kill bacteria. Granulocytes accumulate in the infarcted area after in vivo ischemia and may be a major source of reactive oxygen even early in reperfusion (68).

The conversion of xanthine dehydrogenase to xanthine oxidase caused by ischemia has been documented in ischemic rat hearts and livers (69,70). Xanthine oxidase generates superoxide in the reaction with hypoxanthine and xanthine. Due to the breakdown of ATP both substrates accumulate during ischemia and with the reintroduction of oxygen a burst of superoxide production can occur.

Toxicity of oxygen radicals.

Oxygen radicals can affect essential cellular functions (71). The lipid bilayer of cellular membranes are susceptible to oxygen radicals through lipid peroxidation as presented in figure 3. This not only causes changes in membrane fluidity but also produces the potentially toxic ROH species such as hydroxy-nonenal (72). DNA strand breaks can be induced by oxygen radicals. Exposure of proteins to free radical generating systems may lead to denaturation and cross linking due to modification of amino acids. The conformational changes may then enhance the susceptibility to proteases and consequently to a loss of biological function (73,74). A direct effect of oxidative stress can be found on the membrane proteins that regulate the intracellular concentration of various ions (75). A disturbance of intracellular Ca^{2+} could lead to activation of proteases and thus to damage. Phosphorylation of ADP, both by glycolysis and oxidative phosphorylation can be inhibited by hydrogen peroxide (76). Mitochondrial function can be inhibited by superoxide in vitro (77). The function of a whole organ will suffer if enough cells are affected.

Organ function will directly be affected by the reaction of superoxide with nitric oxide which may interact with vascular tone and affect perfusion pressure. In addition superoxide, hydrogen peroxide and chemotactic lipid peroxides induce leucocyte adhesion to the endothelium (78,79,80) contributing to the infiltration that is seen eventually in the infarcted area.

Protection against free radicals.

Detoxification of oxygen radicals occurs through an array of systems at different levels (81) (figure 4). The dismutation reaction of superoxide is speeded up tenthousand fold by the enzyme superoxide dismutase to yield hydrogen peroxide and molecular oxygen. The hydrogen peroxide is degraded by catalase to yield water and oxygen. Glutathione peroxidase removes hydrogen peroxide at the expense of reduced glutathione. The same enzyme can catch up with the first breach in the defence system by detoxifying lipid peroxides in a similar reaction. In addition a membrane bound version of this enzyme exists, that reduces phospholipid hydroperoxides inside the cellular membranes (82). This line of defence strongly depends on the availability of GSH.

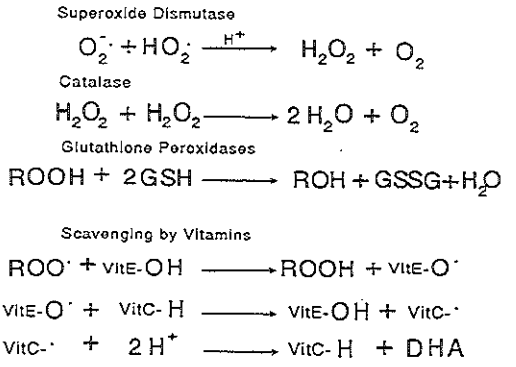

Figure 4.
Cellular defence against free radicals. Reactions catalysed by superoxide dismutase, catalase and glutathione peroxidase. Non enzymatic removal occurs through a number of chain breaking scavengers. Here the concerted action of vitamin E and vitamin C is presented. The end product of the ROO radical is the non toxic dehydroascorbic acid (DHA)

Therefore all systems responsible for the reduction of GSSG are essential, including NADPH formation, GSSG reductase and GSSG transport from the cell.

Non-enzymatic antioxidant systems include alpha tocopherol (vitamin E), ascorbate (vitamin C) and urate as direct scavengers and chain breakers. However, the product of this reaction is itself a radical, although less reactive, and must be removed. It has been proposed that the vitamin E radical reacts with ascorbic acid to yield dehydroascorbic acid (81).

Recently Winterbourne (83) proposed a cycle in which radicals react directly with oxygen to yield superoxide or through GSH to yield superoxide and GSSG. The hypothesis was put forward that in the absence of SOD, superoxide would continue the cycle with GSH to yield hydrogen peroxide and GSSG. Removal of superoxide from the cycle by SOD would reduce the production of hydrogen peroxide and GSSG. In this way only one enzyme would be necessary to remove a whole range of radicals.

Possibly the most efficient form of defence is the prevention of hydroxyl radical generation by limiting the availability of catalytic transition metals. To this end copper is stored and transported in ceruloplasmin (84) and iron in the transport protein transferrin and the intracellular storage protein ferritin (85).

Iron and the oxygen paradox.

The role of iron as the catalytic transition metal in post ischemic free radical toxicity is substantiated by a wide variety of experiments (3). Indirect evidence comes from studies in which iron chelators that inhibit in vitro lipid peroxidation were shown to attenuate reperfusion injury in different experimental models (6,86). The iron chelator deferoxamine forms a hexadentate complex with iron that prevents one electron reactions of the transition metal (40). Deferoxamine treatment has been shown to decrease hydroxyl radical production (87) and preserve membrane phospholipids in post ischemic rat hearts (88). In vivo studies using dogs (89) and pigs (90) have confirmed the beneficial effects in whole animals. Smith et al. (91) have shown that the protective action of deferoxamine does indeed depend on iron chelation since the effect is abolished when the same amount of its iron containing counterpart ferrioxamine is used. More direct evidence for the important role of iron comes from iron loading of

endothelial cells (92) and from the increased sensitivity towards a mild anoxic insult of hearts from iron overloaded rats (93).

Although the importance of iron during post ischemic radical toxicity is well established it is unclear where this catalytic iron comes from or in which form it is present. One possibility is that the oxidation of deoxymyoglobin to ferrylmyoglobin by hydrogen peroxide (94) which can participate in the redox cycling (95). Under physiological conditions iron is transported in transferrin and stored in ferritin, proteins which inhibit the reaction of iron with reduced oxygen metabolites (85) unless the iron is liberated from these proteins. Only a very small amount is thought to be present in a low molecular weight pool (97,98,99) in which the iron is chelated to small molecules such as ATP and AMP (99). This form has been shown to catalyse hydroxyl radical formation and lipid peroxidation (100,101). Evidence has been presented that iron is delocalised into this pool during ischemia in dog heart (102), gerbil brain (103) and rat kidneys (104).

A method to determine the amount of catalytic iron during ischemia and reperfusion would allow a direct evaluation between the extent of damage and the amount of catalytic iron in the LMW pool. In the present thesis such a method was developed to systematically investigate the interaction of superoxide, hydrogen peroxide and iron during reperfusion of the ischemic rat heart.

Experimental model.

Current literature uses the term "ischemia" for a variety of experimentally created situations that are very different in severity. In no-flow ischemia, which in cardiac terms is a complete cessation of coronary flow, all the residual oxygen will be consumed and metabolites cannot be removed and accumulate. Perfusion with buffer, equilibrated against nitrogen, causes oxygen deprivation but not metabolite accumulation. This, henceforth called anoxia, usually is a mild insult (93) compared to ischemia. A completely different model is the occlusion of the left anterior descending artery, (LAD) which should be referred to as partial ischemia. In terms of tissue damage this is a mild insult, however functional recovery is severely hindered due to ventricle fibrillation. The

work presented in this thesis was all performed in the isolated rat heart perfused according to Langendorff (105).

CHAPTER TWO

CONTRADICTORY EFFECTS OF SUPEROXIDE DISMUTASE AFTER GLOBAL OR REGIONAL ISCHEMIA IN THE ISOLATED RAT HEART

Adapted from: Arthur Voogd, Wim Sluiter, Johan F. Koster: Contradictory Effects of Superoxide Dismutase after Global or Regional Ischemia in the Isolated Rat Heart. Free Radicals in Biology & Medicine 1991;11:71-75

ABSTRACT

The effect of superoxide dismutase was investigated in two different models of ischemia and reperfusion in the isolated rat heart; global and regional ischemia. The results of this comparison show that reperfusion arrhythmias after ten and fifteen minutes of regional ischemia, induced by occlusion of the left coronary artery, can be prevented by SOD confirming the results of other investigators. Paradoxally SOD was without effect after ten minutes of global ischemia, obtained by stopping coronary flow completely. After fifteen minutes of global ischemia, SOD induced ventricle fibrillation. Apparently the effect of SOD depends on the model of ischemia and reperfusion that is used.

INTRODUCTION

Reperfusion of ischemic tissue with normoxic medium is accompanied by the formation of oxygen derived free radicals (ODFR) causing additional damage to the post-ischemic tissue. The phenomenon is known as the oxygen paradox or the reperfusion syndrome [1, 2]. Recently ODFR were shown to occur in perfusate of reperfused rabbit [3] and rat [4] hearts using the spin trap agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). In these studies the enzymatic activity of superoxide dismutase (SOD, EC 1.15.1.1) was shown to diminish the DMPO-OH signal. This predicts a protective effect of SOD in ischemia reperfusion experiments. Effects of the enzyme have been measured in a variety of models of ischemia and reperfusion with ambiguous results. Unambiguous protection has been found in the isolated rat heart after occlusion of the left coronary artery [5]. In this model a ligature is placed around the left coronary artery so that regional ischemia can be induced. This induces ventricle fibrillation upon reperfusion in control hearts [6]. A protective effect in the treatment group is measured as the decrease in the incidence and/or duration of the arrhythmias. Measurement of other parameters to assess protection may lead to biased conclusions because ventricle fibrillation as such may cause tissue damage.

In the global ischemia model the heart is made ischemic by stopping coronary flow completely. Usually no severe arrhythmias are induced in control hearts upon reperfusion which allows
reproducible measurements of contractility and biochemical parameters. This model has been used in our laboratory extensively for other studies [7]. When it was used to study ischemia/reperfusion induced free radical generation in the isolated rat heart a deleterious effect of SOD was found.

In order to determine if the effect of SOD depends on the way ischemia is applied, these two models of myocardial infarction were compared in the Langendorff rat heart.

MATERIALS AND METHODS.

Animals and perfusion protocols.

Twelve week old male Wistar rats were used and the hearts were perfused according to Langendorff. The hearts were perfused with tyrode buffer (pH = 7.4 and 37 °C) containing 128 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$ and 11 mM glucose. The buffer was saturated with 95% O_2 and 5% CO_2. Bovine erythrocyte superoxide dismutase (EC 1.15.1.1* was obtained from Boehringer Mannheim and dissolved (30 mg/L, 9 x 104 Units/L) in tyrode buffer.

Global Ischemia.

After stabilization by perfusion with tyrode for fifteen minutes, explanted hearts were perfused for ten minutes with tyrode (controls, n=6) or with tyrode containing 30 mg/L SOD (treated, n=6). The hearts were then subjected to global ischemia by closing the valve for ten or fifteen minutes. During the ischemic period the hearts were submerged in warm tyrode which was bubbled through with 95% N_2 and 5% CO_2. Control hearts were reperfused with tyrode only while treated hearts were reperfused with tyrode containing 30 mg/L SOD. Ten or fifteen minutes of global ischemia is known to induce damage that is severe enough to allow reproducible detection a protective effect [7].

Regional Ischemia.

Occlusion of the left coronary artery was performed as described by Bernier et. al. [5]. After a stabilization period of fifteen minutes the hearts were perfused with tyrode only (controls, n=7) or with tyrode containing SOD (treated, n=7) for ten minutes. Then the ligature was closed and released ten or fifteen minutes later. These ischemic periods have been shown to induce maximal incidence of ventricle fibrillation in control hearts [8].

Functional parameters.

Lactate dehydrogenase (LDH) release, as a measure of tissue damage, and coronary flow were measured as described earlier [7]. Apex displacement was detected

with a smooth muscle transducer. Cardiac work, expressed as contractility, was calculated as the product of amplitude and frequency, which were recorded every thirty seconds. Ventricle fibrillation (VF) was scored visually at time points indicated. A heart was considered to show VF when apex amplitude was less then 5% of the pre-ischemic value and when the ventricle trembled at the same time. A time point was considered positive when the heart fibrillated during the entire period since the previous point. Sustained VF is defined as VF that does not stop within five minutes after reperfusion [5].

Statistics.

All data are presented as means ± SEM. To evaluate differences between groups, n-way Analysis of variance was performed on the data using the Stata release 2.0. (Computing Resource Centre, LA, California). To analyze the effect of fibrillation on LDH release fibrillation score was entered in the data and assigned the value of "0" for pre-fibrillation, "1" for fibrillating and "2" for post-fibrillation.

RESULTS

To examine whether SOD has a different effect in two models of ischemia reperfusion, treatment with the antioxidant enzyme was first tested in isolated rat hearts undergoing global ischemia. After ten minutes ischemia the control and the protected hearts return to respectively 32.1 ± 6.8 and 33.2 ± 8.1 percent of pre-ischemic contractility at twenty minutes of reperfusion. No ventricle fibrillation was observed in these groups. SOD infusion had no effect on LDH release or coronary flow after ten minutes of ischemia. The peak in LDH release, expressed as units per minute per gram wet weight (U/min/gr ww) fell at twelve minutes after reperfusion in controls and SOD hearts and reached 0.196 ± 0.012 and 0.182 ± 0.019 U/min/gr ww. respectively (not shown).

After a period of fifteen minutes of global, warm, ischemia transient VF occurs in three hearts in the control group (table 1). In the SOD group VF occurs in all hearts in the first four minutes of reperfusion. The hearts were reperfused for twenty five minutes and at that point all had stopped fibrillating. Contractility measured

Table 1. Fibrillation score of individual hearts after 15 minutes global ischemia.

	Minute after reperfusion										
	1	2	3	4	5	7	10	12	15	20	25
Controls											
1	0	0	0	0	0	1	2	2	2	2	2
2	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	1	1	1	1	2	2	2
10	0	0	0	0	0	1	2	2	2	2	2
13	0	0	0	0	0	0	0	0	0	0	0
SOD											
3	1	1	1	1	1	1	1	2	2	2	2
4	1	1	1	1	1	1	1	1	1	2	2
7	1	1	1	1	1	2	2	2	2	2	2
11	1	1	1	1	2	2	2	2	2	2	2
12	1	1	1	1	1	1	2	2	2	2	2
14	1	1	1	1	1	1	1	1	2	2	2

A heart scores "0" when it does not show VF, "1" when it does and "2" when it has stopped.

at twenty five minutes after reperfusion was significantly higher in the control group (20.7 ± 5.50% in controls and 8.0 ± 3.0% in SOD group.) The incidence of VF precludes a comparison of contractility values of both groups before this point, since fibrillating hearts do not contract.

The amount of LDH released after fifteen minutes global ischemia (figure 1, panel a.) was the same in both groups during the first twelve minutes of reperfusion. Thereafter, LDH release in the SOD group was higher than in the control group. Analysis of variance, performed to find out which parameters contribute to the LDH release, shows that LDH release is significant only on time ($p<0.01$) and on the fibrillation score ($p<0.01$) of the individual hearts but not on SOD treatment ($p<0.966$). In figure 1, panel b it is shown that LDH release is equal for fibrilation scores "0" and "1". The hearts that have reverted to a normal rythm, score "2", release twice as much LDH. The differences between the controls and the SOD treated hearts are not significant

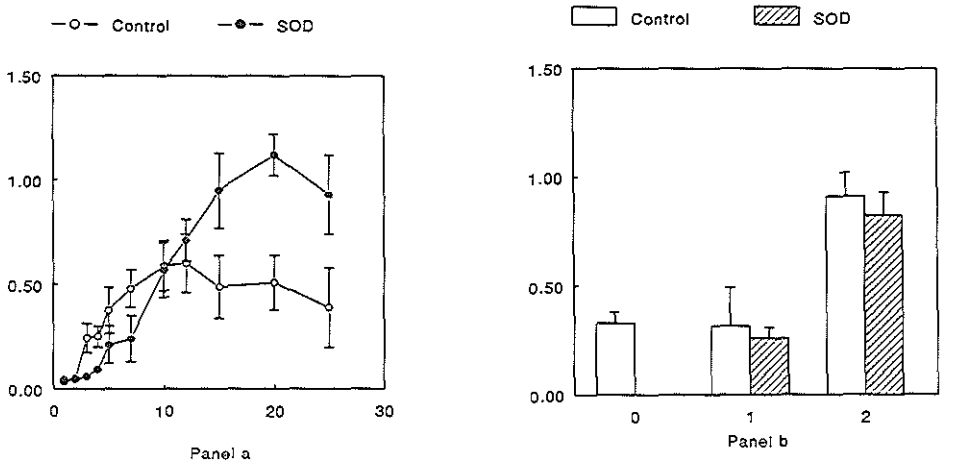

Figure 1.
LDH release after fifteen minutes of global ischemia.
LDH is expressed in units per minute per gram wet weight.
panel a. Time course (minutes) of LDH release (mean ± SEM, n=6) after reperfusion.
panel b. LDH release (mean ± SEM) by fibrilation score (see table 1) after fifteen minutes of global ischemia. Differences between controls and SOD treated hearts were not significant.

To study the effect of SOD on the incidence of ventricle fibrillation induced by regional ischemia, control and SOD hearts were subjected to either ten or fifteen minutes occlusion of the left coronary artery. The number of hearts showing ventricle fibrillation at the time points after reperfusion is presented in table 2. After a ten minutes occlusion of the coronary artery sustained VF, defined as continuous fibrilation in the first five minutes of reperfusion, occurred in five out of seven control hearts while only one out of seven SOD treated hearts showed sustained VF. The incidence of sustained VF after fifteen minutes coronary artery occlusion was five out of seven in the control group and three out of seven in the SOD group.

Table 2.
Incidence of ventricle fibrilation after regional Ischemia

		Minute after reperfusion						
		1	2	3	4	5	7	10
10 Minute Regional Ischemia (n = 7)								
Controls		7	7	7	6	5	5	4
SOD protected	4	3	3	2	1	1	1	
15 minute Regional Ischemia (n = 7)								
Controls		7	6	5	5	5	5	5
SOD protected	5	6	3	3	3	3	3	

Presented are the number of hearts that showed VF during the entire period from the previous to the indicated time point, n = 7 in all groups.

DISCUSSION

The results of the present study show that SOD decreases the incidence of ventricle fibrillation after a ten or fifteen minute occlusion of the left coronary artery. This is in agreement with the results of other investigators. [5, 8, 9] In contrast, after ten minutes global ischemia SOD has no effect, while the enzyme induces ventricle fibrillation after fifteen minutes global ischemia.

Regional ischemia, which causes heterogeneous damage to the heart, is thought to induce arrhythmias because conduction is impaired to a different extent along the myocardium [6]. Therefore, the protective effect of SOD in the regional ischemia model is explained by scavenging superoxide generated upon reperfusion.

In global ischemia conduction is probably diminished equally along the myocardium and generally no arrhythmias are induced by reperfusion in the control situation. The question arises by which mechanism SOD induces ventricle fibrillation in these hearts. The result of the present study show that SOD treatment does not cause tissue damage as measured by LDH release. Therefore, it is likely that the enzyme causes the VF by a physiologic mechanism such as the release of arrhythmogenic substances. Histamine has been shown to enhance hypoxia induced ventricle fibrillation in isolated rat hearts [11]. It is conceivable that the hydrogenperoxide that is generated from superoxide by SOD in the treated hearts triggers histamine release [12] from the resident cardiac mast cells [13]. There may be two explanations why no VF is observed after ten minutes of global ischemia. One is that histamine did not induce arrhythmias in normoxic control hearts in the study of Dai [11], but histamine did significantly shorten the time to onset of VF during anoxic perfusion by thirty minutes compared to controls. Allthough the time course of anoxic perfusions cannot simply be compared with global ischemia, this does show that a minimum period of oxygen deprivation is essential to make the heart susceptible to this effect. Another is that the amount of superoxide generated upon reperfusion depends on the duration of the preceding ischemia [10]. Therefore, in the present experiments the ten minute period could be too short because the hearts are not yet susceptible or too little superoxide is generated upon reperfusion.

Multiple interacting triggers, among these oxygen free radical-dependent and calcium-dependent, for reperfusion arrhythmias have been proposed [8]. These authors suggested that the dominance of one factor over another is determined by the duration

of the regional ischemia. The results of the present study suggest that there is also a balance between several superoxide dependent mechanisms contributing to the arrhythmias. The way the preceding ischemia was induced apparently tips the scales of this balance. Further experiments concerning mediator release and the effect of other experimental conditions will be necessary to elucidate the mechanisms behind the different role of the superoxide radical in these two models.

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CHAPTER THREE

IRON ACCUMULATION IN THE ENDOTHELIUM OF IRON-LOADED RAT HEARTS

Lack of morphological damage after reoxygenation

Adapted from: Arthur Voogd, Antonius M.M. van der Kraaij, Maud I. Cleton, Henk G. van Eijk, Catharina E. Essed, and Johan F. Koster: Iron Accumulation in the Endothelium of Iron-loaded Rat Hearts. Manuscript in preparation.

ABSTRACT

Hearts from iron-loaded rats are very sensitive to reoxygenation damage after forty five minutes of anoxic perfusion. Non iron-loaded control hearts and hearts perfused with (+)-cyanidanol-3 or deferoxamine are virtually unharmed by this insult. In the present study the cellular location of the administrated iron was investigated by light microscopy and Electron Spectroscopic Imaging. The results show that cardiac iron accumulation after short term in vivo iron-loading occurs mainly in the endothelial cells of the capillaries. No additional iron was found in cardiomyocytes. Some iron accumulated in pericytes and in the perivascular space. Subsequently, iron-loaded and control hearts were subjected to anoxia and reoxygenation and studied by electron microscopy. In contrast to the dramatic functional deterioration iron-loaded hearts showed essentially healthy morphology after anoxia and reoxygenation. The only indication of injury is found in the form of limited blebbing of the endothelial cells of the iron-loaded hearts five and ten minutes after reoxygenation. This was not seen in iron-loaded hearts treated with desferrioxamine and (+)-cyanidanol-3 or in non iron-loaded hearts.

INTRODUCTION

Restoration of the oxygen supply to ischemic myocardium 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 (4,7,16), a reduction in cardiac function (19,24) and the occurrence of rhythm disturbances such as ventricular fibrillation (2,9). These adverse effects of coronary flow restoration, usually termed "reoxygenation injury" or "the oxygen paradox" (15,6) are thought to be caused by oxygen-derived free radicals, the hydroxyl radical ($^{\cdot}OH$) in particular. (1,8,22)

In a previous study from our laboratory (23) it was shown that iron-loaded rat hearts are extremely sensitive to reoxygenation injury after a period of forty five minutes of anoxic perfusion compared control hearts. A part of the results of that study is summarized in figure 1. The increased sensitivity of iron-loaded hearts is demonstrated by a lower return of contractility upon reoxygenation. Non iron-loaded hearts regain almost all (80 %) of their pre-anoxic contractility. This effect of iron-loading could be prevented by the administration of either the free radical scavenger (+)-cyanidanol-3 or the iron chelator desferrioxamine.

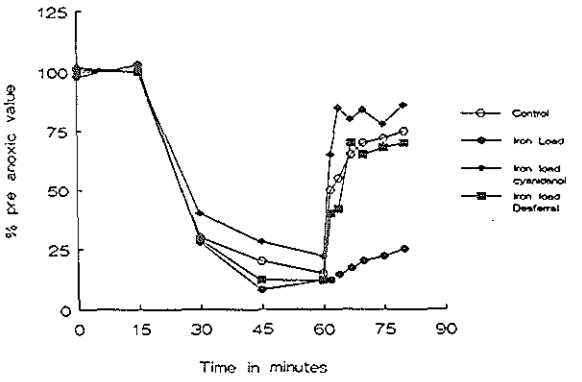

Figure 1.
Results of studies into the effect of in vivo iron-loading on contractility of isolated rat hearts, expressed as % of pre-anoxic value, during anoxia and reoxygenation. Anoxia starts at t=15 and reoxygenation starts at t=60 (23).

Iancu et al. (11) have studied the distribution pattern of iron in rats fed an iron-carbonyl supplemented diet for 4 to 15 months. They found that after this longterm iron-loading protocol cardiomyocytes contained little extra iron whereas numerous ferritin particles were visible in cardiac endothelial cells. Since hydroxyl radicals are formed through Fenton chemistry after the liberation of iron from ferritin by superoxide (3), this could imply that the increased susceptibility of iron loaded-hearts to reoxygenation damage may be caused by endothelial cell injury. This, putative, central role of the endothelial cells is supported by evidence from various studies (10,20,25,21). However, our short term iron-loading protocol differs substantially from that of Iancu (11), which might lead to a different cardiac location. The aim of this study was to determine the cardiac location of iron after our short term iron-loading protocol and to investigate whether the functional loss could be related to morphological evidence of injury in the endothelial layer.

MATERIALS AND METHODS

Localisation of Iron.

In order to evaluate the cardiac location of iron three iron-loaded and three non iron-loaded hearts were perfused for fifteen minutes and then fixed for examination. From every heart six ultrathin section were studied by Electron Spectroscopic Imaging (ESI) and ten light microscopic sections were stained for iron according to Perls (17).

Anoxia and reoxygenation.

Four groups of four hearts were used: non iron-loaded control hearts, iron-loaded hearts, iron-loaded hearts protected with desferrioxamine and iron-loaded hearts with (+)-cyanidanol-3. The protected hearts were perfused with tyrode containing 20 μM (+)-cyanidanol-3 or 50 μM deferoxamine during the entire experiment. In order to visualize the emergence of morphologic damage in the early phase of reoxygenation the hearts were fixed either immediately after the period of anoxia or at one, five and ten minutes after reoxygenation. One non iron-loaded and one iron-loaded heart were perfused with normoxic tyrode for one hour to serve as reference.

Animals and iron-loading.

Eighteen male Wistar rats twelve weeks of age were iron-loaded by injecting 0.5 ml Imferon (iron-dextran, 50 mg Fe/ml; Fisons, Leusden, The Netherlands) in the gluteus muscles, after a brief anaesthesia with di-ethylether, 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 (23). Control rats were injected under the same conditions with a 10 % dextran solution (n=8). After the last Imferon injection the rats were rested for a period of two weeks at which time their body weight was between 240 - 330 g. No differences were observed in body weight or behaviour between the two groups.

Perfusion protocol, tissue fixation and electron microscopy.

I. Localisation of Iron

The animals were anaesthetized with di-ethylether to remove the hearts which were directly cannulated in the aorta and perfused retrogradely according to Langendorff (13). 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. Hearts were fixed by perfusion with 2 % paraformaldehyd and 1 % glutaraldehyde in 0.1 M cacodylate buffer, pH=7,4, for a period of fifteen 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). Six ultrathin sections of approximately 60 nm were taken from each heart and 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). Ten light microscopic sections were taken from each heart and stained for iron according to Perls (17)

II. Anoxia - reoxygenation.

Hearts were prepared and perfused for fifteen minutes as described above. After this stabilisation period the hearts were perfused with anoxic tyrode buffer containing 11 mM glucose for forty-five minutes. The buffer was made anoxic by saturation with 95 % N_2 and 5 % CO_2 for at least one hour. The hearts were reoxygenated by changing to the normoxic tyrode buffer and fixed as described above at the indicated times.

Three tissue samples were studied from each heart. The tissue was washed in cold buffer and then post fixed in 2 % osmium tetroxide. After a second series of washings in buffer and dehydration in graded alcohol, the samples were embedded in Epon 812. Sections of 1 μM thin were cut and stained with toluidine blue for light microscopy. Ultra thin

sections (30 - 40 nm) were prepared from artifact free areas and stained with uranyl acetate and lead citrate prior to examination in a Zeiss EM 902 transmission electron microscope.

The number of capillaries screened under EM was counted per heart and scored for blebbing. Blebbing is a local outpouching of the cel membrane caused by destabilisation of lipids (18). This leads to a disturbance of the cellular osmotic balance and is considered evidence of severe loss of membrane integrity.

III. Quantification of iron deposits.

Iron deposits were quantified by light microscopy in 5 μm coupes stained for Iron according to Perls. After staining with Perls' prussian blue iron shows as blue granular deposits. In black and white photographs these deposits are visible as black spots. In fields of 0,0325 mm^2 the number of capillaries with and without iron deposits was counted. At least ten fields were counted per heart.

Chemicals

Deferoxamine Mesylate (desferral) was purchased from Ciba-Geigy, Switzerland and (+)-cyanidanol-3 from Zyma, Nyon, Switzerland. Cyanidanol buffer was protected from light by wrapping the perfusion apparatus in aluminium foil.

RESULTS

Light microscopy.

Examination of the left ventricular muscle of iron-loaded rat hearts showed that iron, visible as black spots, was stored in the capillary wall or to a lesser extent in the perivascular space (fig. 2, upper panels). This observation was highly consistent for all iron-loaded rat hearts. No iron deposits (black spots) were ever encountered in any section in the heart muscle itself. In contrast to the abundant iron-storage in the capillaries iron was rarely detected in arteries, arterioles or veins and in venules. The lower part of figure 2 displays two arterioles with iron stored in the perivascular space.

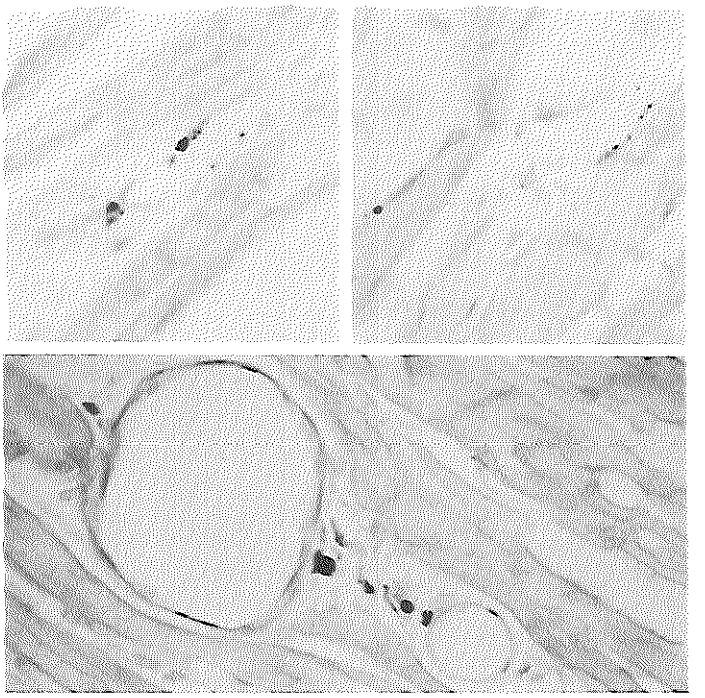

Figure 2.
Light microscopic sections through left ventricular heart muscle of iron-loaded rat hearts. The sections were stained for iron with Perl's prussian blue (black spots) and nuclear fast red (160x). In the upper two pictures iron-deposits (black spots) in the wall of two capillaries can be seen. In the lower part two arterioles are visible with iron (black spots) located around the arterioles. Notice that no iron is detectable in cardiomyocytes themselves throughout this figure.

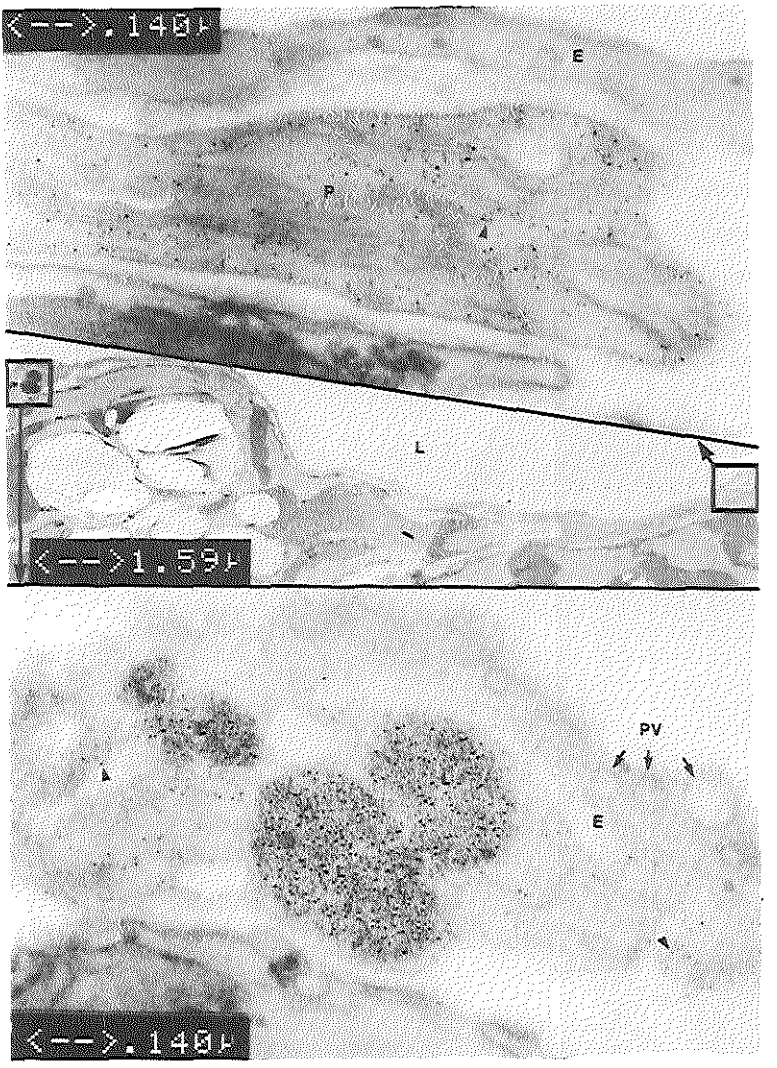

Figure 3.
Transmission electron microscopy of left ventricular heart muscle. The upper and lower micrograph are magnifications (50,000x) of the squares in the middle section (4,400x). In the upper micrograph a pericyte (P) with high density particles (arrows) is clearly visible together with an endothelial 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).

Note that no iron is visible in the cardiomyocytes. The average number of capillaries was 998 ± 110 per mm^2 of which 86 ± 12 contained iron deposits. No iron deposits were encountered in the non iron-loaded control hearts.

Transmission electron spectroscopy.

To study the nature and intracellular location of the iron deposits observed by light microscopy, hearts were prepared for Electron Microscopy. ESI of the iron-loaded hearts (fig. 3) showed high density particles with a diameter of about 6 nm and these were shown to contain iron by electron energy loss spectroscopy (M.I. Cleton et al., Manuscript in preparation). Based on the size and the iron content these structures represent ferritin particles. An endothelial cell (E), shown in the upper micrograph contains hardly any of these particles whereas an underlying pericyte (P) is packed with them (arrow). The lower micrograph shows another endothelial cell with similar high density particles stored in lysosomes (L) and in the cytosolic compartment. In endothelial cells lysosomal iron-storage was generally more evident than in pericytes, whereas in cardiomyocytes no iron-deposits were encountered. In three hearts of non iron-loaded rats no iron-deposits could be detected in any cell type (results not shown).

Morphological findings in the reoxygenated hearts.

To examine by electron microscopy the nature and the location of the reoxygenation injury, control and iron-loaded rat hearts were subjected to fourty five minutes of anoxic perfusion and fixed either immediately or at one, five and ten minutes after reoxygenation. Eight additional iron-loaded rat hearts were subjected to the same experiment in the presence of either (+)-cyanidanol-3 or desferrioxamine. Immediately after the anoxic period the cell membranes of the endothelial cells and pericytes of iron-loaded, unprotected hearts are intact (figure 4, panel a.) The myocardium shows no signs of deterioration: sarcomeres are intact and mitochondria show tightly packed cristae. The number of capillaries (nc) screened under EM in this heart was 360. Panel b of figure 4 shows a capillary of the iron-loaded heart one minute after reoxygenation. Iron is visible in a lysosome of the endothelial cell. No blebbed endothelial cells were found in the screened capillaries (nc=1556). Five minutes after reoxygenation focal endothelial "blebbing" is visible, representing an extensive outpouching of the cell

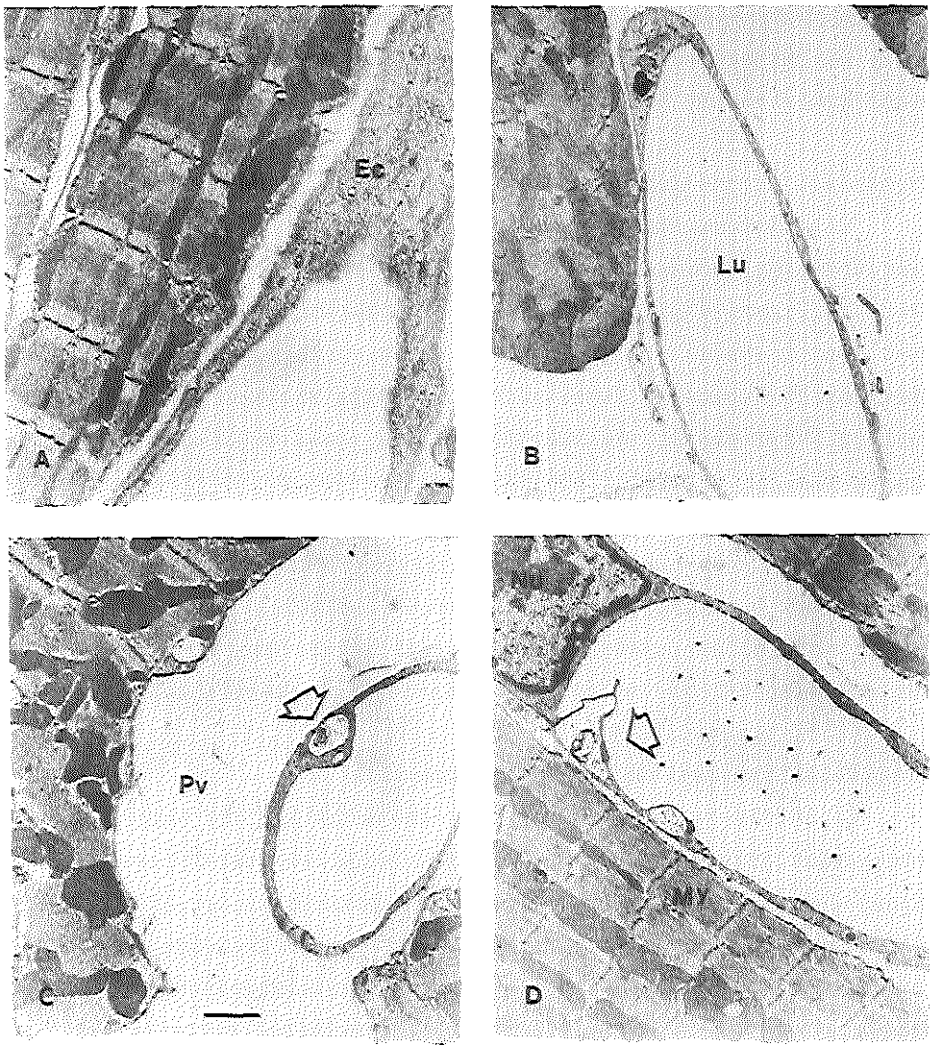

Figure 4.
Effect of 45 minutes anoxic perfusion and reoxygenation in unprotected, iron-loaded rat hearts. All heart were subjected to 45 minutes anoxic perfusion. The hearts were fixed by perfusion with 0,1 M Cacodylate, pH = 7.4, containing 1% paraformaldehyde and 2 % glutaraldehyde. These hearts were fixed immediately (a), at 1 minute (b), 5 minutes (c) or ten minutes (d) after reoxygenation Ec=endothelial cell, Pe=pericyte, My=cardiomyocyte, Pv=perivascular space, Mi= mitochondrion, Nu=nucleus, Fe=iron, Lu=lumen of the capillary, Sr=sarcoplasmatic recticulum.

membrane with local intracellular hydrops (figure 4, panel c, arrow). Here a remnant of a membranous structure seems to surround an iron particle. Myocardial tissue shows no evident signs of damage apart from occasional dilation of sarcoplasmatic recticulum vesicles. However, only two of the 813 capillaries that were screened were found to contain blebbed endothelial cells. After ten minutes reoxygenation the endothelial cell shown (fig. 4, panel d) displays extensive blebbing. However, again only two endothelial cells of 1004 capillaries show this extensive blebbing. Note that the cardiomyocytes and the endothelial cell nucleus appear intact. In the non iron-loaded control hearts no blebbing was found at all (nc=2555 in four hearts). No blebbing or other signs of injury to endothelial cells and myocardial tissue were ever encountered in the capillaries screened in the four hearts treated with desferrioxamine (nc=1154) or (+)-cyanidanol-3 (nc=2450).

DISCUSSION

The results of this study show that iron accumulates in endothelial cells, pericytes and in the perivascular space but not in cardiomyocytes. Link et al. (14) showed that isolated myocytes accumulate iron after administration of iron citrate but not of iron transferrin. The rats used in our study were iron-loaded by addition of iron sulphate to their food and by weekly iron dextran injections. Apparently the short term iron-loading protocol used here leads to essentially the same cardiac iron distribution as the long term protocol of Iancu et al.(11). In earlier experiments (23) we measured the total amount of iron in iron-loaded and control hearts. Iron was found to be doubled as a result of the iron-loading regimen (50.8 ± 11.0 versus 112.4 ± 24.1 μg/gm wet weight, n=9). In view of the predominantly endothelial localisation of the iron after iron-loading, the amount of iron inside the endothelial cells must have increased much more than twofold. Therefore, it seems likely that, in these hearts, the endothelial cells are more vulnerable to anoxia and reoxygenation because ferritin can be an iron donor for superoxide driven lipid peroxidation (12). Because the endothelium performs a dynamic role in regulating a selective permeability and with that the nourishment of the surrounding myocardial tissue (5) disruption of these regulatory functions could explain loss of cardiac function.

Contrary to our expectations, based on the functional data presented in figure 1, we found no morphological evidence of injury in the iron-loaded, unprotected hearts after reoxygenation other than some endothelial blebbing as presented in figure 4. Although blebbing reflects severe membrane disturbances and has been shown to be associated with irreversible damage to the myocardium (18) the number of blebbed cells is too small to conclude that this is a morphological feature of the reoxygenation injury. Taken together the results of the present study show that, in spite of the endothelial accumulation of iron, the dramatic functional impairment of iron-loaded hearts is not reflected in morphological evidence of damage to the endothelium. Based on these findings it has to be concluded that a more subtle mechanism causes the loss of function. It remains to be elucidated whether or not this is confined to a specific cell type.

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CHAPTER FOUR

LOW MOLECULAR WEIGHT IRON AND THE OXYGEN PARADOX IN ISOLATED RAT HEARTS

Adapted from: Arthur Voogd, Wim Sluiter, Henk G. van Eijk and Johan F. Koster. Low Molecular Weight Iron and the Oxygen Paradox in Isolated Rat Hearts. Journal of Clinical Investigation 1992;90:2050-2055*

ABSTRACT

Little is known about changes in the amount of iron in the intracellular low molecular weight pool, which catalyses the Fenton reactions during reperfusion after ischemia. In this study a new approach is presented to measure low molecular weight iron and it is applied to normal hearts during ischemia and to iron-loaded hearts during anoxia and reoxygenation. The results of this study show that 1) during ischemia in normal hearts a progressive 30-fold increase occurs in low molecular weight iron after 45 minutes of ischemia, while 2) during 45 minutes of anoxic perfusion the low molecular weight iron does not increase. This means that the reductive release from the storage protein ferritin is greatly enhanced by the acidification that occurs during ischemia. 3) Anoxic perfusion of iron-loaded hearts does increase low molecular weight iron and there is a further increase upon reoxygenation, which is prevented by (+)-cyanidanol-3. Based on these findings it is concluded that oxygen deprivation enhances the susceptibility of rat hearts to oxygen radicals by increasing the amount of catalytic, ferrous iron in the low molecular weight pool.

INTRODUCTION

The generation of oxygen-centred free radicals is thought to contribute to the exacerbation of tissue injury upon restoration of the oxygen supply to ischemic organs (1,2). This hypothesis is based on protective effects of the addition of scavengers of superoxide, hydrogen peroxide and the hydroxyl radical (3,4,5,6,7). Direct measurements of the radical adducts of spintrap agents have since confirmed that these radicals are generated (8,9). However, neither superoxide nor hydrogen peroxide are very toxic in themselves but in the presence of a transition metal, such as iron or copper, the very toxic hydroxyl radical or ferryl ion is formed (10). The significance of iron to ischemia and reperfusion is supported by studies in which iron chelation protects post-ischemic tissue (11,12,13). In addition to the circumstantial evidence based on iron chelation, we have shown that the hearts from iron-loaded rats are very sensitive to reoxygenation after a mild anoxic insult (14).

Under normal conditions only a minute quantity of the intracellular iron is present in a low molecular weight pool. This iron is bound to weak chelators such as AMP and ATP (15) and in this form iron is able to catalyze hydroxyl radical formation (16,17). The bulk of the intracellular iron exists as $Fe(OH)_3$ stored in ferritin and as haem-iron in a variety of proteins. This iron is not available to catalyze hydroxyl radical formation. It has been shown that superoxide can mobilize iron from ferritin and that this iron can catalyze hydroxyl radical formation (18,19). In addition, other reducing agents like reduced flavins can cause the reductive release of iron from ferritin (20,21). This makes ferritin a potentially hazardous biomolecule under pathological conditions when sufficient reducing equivalents are available. It is therefore important to investigate whether there is a rise in low molecular weight iron during ischemia or anoxia and after reoxygenation to appreciate the contribution to tissue injury under these conditions.

There are reports in which an increase in low molecular weight iron has been found during ischemia. However, both Holt et al. (22) and Komora et al. (23) have neglected the possibility that iron was released from ferritin during their procedures to isolate the low molecular weight pool. Healing et al. (24) have realized the possibility but argue that since the iron they measure is chelatable, it must have been loosely bound and therefore potentially catalytic.

The purpose of this study is to present an alternative procedure to measure low molecular weight iron which circumvents the drawbacks mentioned above. Using this method, we have investigated the changes in the low molecular weight iron pool during no-flow ischemia which causes a profound acidosis (pH < 6) and during anoxic perfusion where the cellular pH remains constant. In addition we have extended this study to hearts from iron-loaded rats. These hearts, which contain twice as much total iron as control hearts, were shown to be very sensitive to anoxia and reoxygenation (14). The iron in these hearts is predominantly located in the endothelium but the dramatic loss of contractility that occurs could not be correlated with morphological indications of injury in endothelial cells. In order to evaluate the role of low molecular weight iron in these hearts we have measured the size of the low molecular weight pool during anoxia and reoxygenation.

MATERIALS AND METHODS

Animals and perfusion protocol.

Twelve to fourteen week old male Wistar rats were used. After a brief anaesthesia with diethyl ether the hearts were excised and placed in ice cold tyrode buffer. The excised hearts were cannulated through the aorta and perfused retrogradely according to Langendorff (25). 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.

Iron overloading.

Iron overload in male Wistar rats, twelve weeks of age, was achieved by injection of 0.5 ml Imferon (iron-dextran, 50 mg Fe/ml; Fisons, Leusden, The Netherlands) in the gluteus muscles, after a brief anaesthesia with di-ethylether, 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 (14).

Ischemia and Anoxia.

Hearts were always perfused with normoxic tyrode for at least 15 minutes before either ischemia or anoxia was induced. Warm, no-flow ischemia was induced by stopping coronary flow completely while the heart was submerged in tyrode buffer saturated with 95 % N_2 and 5 % CO_2 and kept at 37° C. Reperfusion is achieved by restoring coronary flow. Anoxia was caused by perfusing the hearts with tyrode containing glucose (11 mM) and saturated with 95 % N_2 and 5 % CO_2. Reoxygenation is achieved by changing to the normoxic tyrode.

Low molecular weight iron measurements.

The hexadentate (26) iron chelator deferoxamine forms a 1:1 complex (HDFX-Fe, $K_{as}=10^{31}$ or H_2DFX-Fe Kas$=10^{21}$, (27)) with iron which is known as ferrioxamine. Therefore there is a linear relation between the ratio ferrioxamine/deferoxamine and original iron concentration after addition of excess deferoxamine to iron containing

solutions or tissue homogenates. The very high stability of the complex ensures that all iron is extracted from the weaker intracellular chelators. In this study iron was measured as the ratio of ferrioxamine to deferoxamine formed in incubations at 37 °C of 10,000 x g supernatant of heart homogenates with 2 mM deferoxamine as described by Gower et al. (28). In this procedure all the iron in the low molecular weight pool is chelated within one hour. However, it also leads to the removal of iron from ferritin which is linear in time over at least two hours (29). To obtain the actual amount of low molecular weight iron we have followed the increase in ferrioxamine in these incubations in time by measuring ferrioxamine/deferoxamine after 5, 30, 60, 90 and 120 minutes. Next, we have subtracted the linear increase between 60 and 120 minutes from the 60 minutes value.

Tissue preparation and ferrioxamine/deferoxamine determination.

Immediately after the experiment rat hearts were blotted dry, weighed and homogenized to a 20 % w/v homogenate in 100 mM Tris/HCl pH 7.4. Homogenate was centrifuged at 10,000 x g for 15 minutes and 1 ml samples were taken from the supernatant and brought to 2 mM deferoxamine by adding 100 μl 22 mM deferoxamine and incubated at 37 °C. Incubations were stopped by passing the sample through a SEP PAK C18 cartridge (Millipore Corp., Milford, MA) pre-eluted with 5 ml methanol followed by 5 ml distilled water, on which ferrioxamine and deferoxamine are retained. After application of the sample the cartridge was washed with 5 ml distilled water and deferoxamine and ferrioxamine were eluted with two ml methanol and the eluate was applied to the HPLC column.

HPLC Analysis.

HPLC was performed with dual LKB 2150 pumps, controlled through the LKB 2152 LC controller equipped with automatic sample injector. Effluent from the column was passed through two LKB 2151 variable wavelength detectors to allow simultaneous detection of ferrioxamine at 430 nm and deferoxamine at 229 nm. Pump heads and high pressure tubing are all made of titanium. The stationary phase is a 250 x 4 mm id Merk RP18 stainless steel cartridge. The mobile phase consisted of 88 % 20 mM Na_2HPO4/NaH_2PO_4, 2 mM Na EDTA, 1 M ammonium acetate (pH 6.6) and 12 %

acetonitrile. All reagents were of HPLC grade and buffers were filtered and degassed before use. Recovery of deferoxamine and ferrioxamine peaks was measured using standard dilutions in methanol and always exceeded 75 %. The relation between the ratio of ferrioxamine/deferoxamine and the amount of iron was calculated from standards incubated and extracted as the samples.

Experimental Design.

a. Ischemia and reperfusion.

The effect of ischemia on low molecular weight iron was determined in hearts that were subjected to warm ischemia for 15, 30 and 45 minutes. Control hearts were perfused with normoxic buffer for sixty minutes. To investigate the effect of reperfusion on the low molecular weight iron pool, hearts were subjected to 30 minutes ischemia and low molecular weight iron was determined after 20 minutes reperfusion. The influence of superoxide or iron chelation on low molecular weight iron during reperfusion was determined in two groups of hearts subjected to 30 minutes ischemia and 20 minutes reperfusion in the presence of 30 mg/l SOD or 50 μM deferoxamine.

b. Anoxia and reoxygenation in iron-loaded rat hearts.

To evaluate the effect of anoxia and reoxygenation on the size of the low molecular weight iron pool in iron-loaded hearts, low molecular weight iron was analyzed in normoxic hearts (n=3), hearts that were perfused with anoxic buffer for forty five minutes (n=3) and not reoxygenated or reoxygenated for 10 (n=3) minutes. In order to evaluate the effect of iron chelation and antioxidant intervention under those conditions these experiments were performed with tyrode only or with tyrode containing 50 μM deferoxamine or 20 μM (+)-cyanidanol-3 which has been shown to scavenge both superoxide and the hydroxyl radical (30).

Statistical evaluation.

All low molecular weight measurements are expressed as nmoles per gram wet weight and presented as means ± SEM. Intergroup differences were evaluated by oneway analysis of variance with the Bonferroni option using STATA release 2 (Computing Resource Center, Los Angeles, CA).

Chemicals.

Deferoxamine Methanesulfonate (Desferrioxamine Mesylate, Desferral) was purchased from Ciba-Geigy, Switzerland and (+)-cyanidanol-3 from Zyma, Nyon, Switzerland. (+)-cyanidanol-3 buffer was protected from light by wrapping the perfusion apparatus in aluminium foil. Bovine erythrocyte Cu-Zn SOD (3000 U/mg) was purchased from Sigma.

RESULTS

Low molecular weight iron determinations.

The standard curve (fig. 1) relating the quantity of iron to the ferrioxamine/deferoxamine ratio follows a linear relation with $R^2=0.970$ ($p < 0.001$). Contaminating iron from the chemicals used, determined by extrapolation of this curve to ferrioxamine/deferoxamine=0, was consistently low at 2.1 ± 0.2 nmole/ml (n=3 separate curves). Both slope and background were similar to those presented by Gower et al. (28). To measure the low molecular weight iron pool the 10.000 x g supernatant

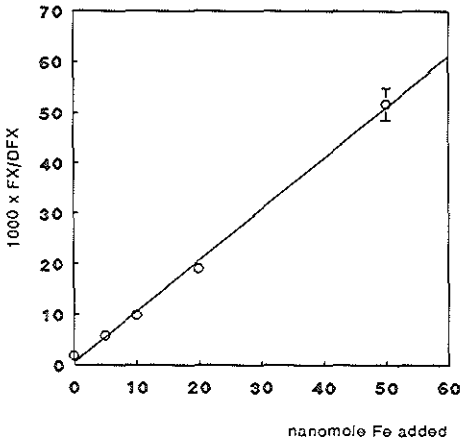

Figure 1.
Standard curve.
Relationship between the added amount of iron and ferrioxamine/deferoxamine ratio of standard samples extracted and incubated as the experimental samples. Means ± SEM of triplicate determinations of one set of samples. Linear regression of these data shows $R^2=0.970$, $p<0.001$. Background iron is calculated by extrapolation to ferrioxamine/deferoxamine = 0.

was incubated with deferoxamine, aliquots are drawn at different time points and the ratio ferrioxamine/deferoxamine was determined. Initially a rapid rise was found that turns into a linear increase after one hour (fig. 2). The initial rate of iron chelation was lower in the normoxic hearts than in hearts that had been ischemic for forty-five minutes. After one hour both curves have levelled off to a constant rate of ferrioxamine formation. In order to evaluate the slope of both curves regression analysis was

performed for the time points 60, 90 and 120 using time and ischemia (0 or 1) as independent variables. This showed that the data can be described by the linear increases as indicated in figure two. Inclusion of the interaction-term (time x ischemia)

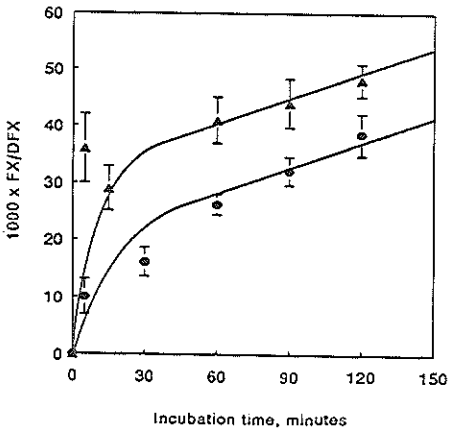

Figure 2.
Ferrioxamine formation during incubation of supernatants.
Effect of the duration of incubation on the ferrioxamine/deferoxamine ratio of 1 ml supernatant of normoxic rat hearts (circles) or rat hearts subjected to forty-five minutes ischemia (triangles) with 2 mM deferoxamine. Aliquots of 1 ml from supernatants of individual hearts (n=3) were incubated during the times indicated and processed as described under Materials and Methods. Regression analysis using time and ischemia (0 or 1) as independent variables results in the equation: ratio=0.147 x time + 12.0 x ischemia +19.4. (R^2=0.624) Inclusion of the interaction term time x ischemia showed a non-significant contribution indicating that there is no significant difference in the slope of both curves. Each point represents the mean ± SEM.

in the regression analysis showed a non significant contribution, indicating that there is no significant difference in the slope of these curves. No attempt was made to perform non-linear regression on the earlier data points. This result fits the observations that all the iron in the low molecular weight pool is chelated within one hour (28) and that the release of iron from ferritin is linear over at least two hours (29). Therefore, to obtain the actual low molecular weight iron in individual hearts, we correct for this ferritin derived iron by subtracting the amount of iron released between one and two hours from the amount measured at one hour.

The effect of ischemia and reperfusion.

To evaluate the effect of ischemia on the low molecular weight iron pool rat hearts were subjected to warm ischemia for 15, 30 and 45 minutes. The results (fig. 3) show a progressive increase in low molecular weight iron due to ischemia from 2.1 ± 4.8 nmole per gram wet weight in normoxic hearts to 54.2 ± 4.1 nmole per gram wet weight after forty five minutes of ischemia ($R^2=0.660$, $p<0.001$).

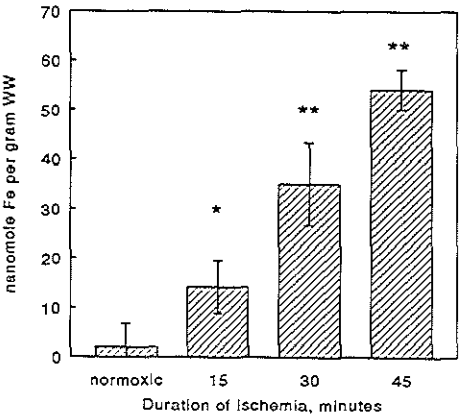

Figure 3.
Low molecular weight Iron and Ischemia.
Effect of the duration of warm no-flow ischemia on low molecular weight iron content of rat hearts. Normoxic hearts were perfused with normoxic tyrode for 60 minutes. ferrioxamine/deferoxamine ratio was determined in supernatants after 60, 90 and 120 minutes and low molecular weight calculated as described under Materials and Methods. Each bar represents mean ± SEM, n=6. * denotes p=0.09, ** denotes p < 0.01 vs normoxic controls. Linear regression shows a significant increase in time $p < 0.001$, $R^2=0.660$.

To investigate the effect of reperfusion in the presence or absence of SOD or deferoxamine on the size of the low molecular weight iron pool, hearts were subjected to 30 minutes ischemia followed by 20 minutes reperfusion. Addition of SOD does not affect low molecular weight iron during reperfusion, while iron chelation results in a tremendous decrease in low molecular weight iron (fig. 4). Both the SOD group and the control group were lower than the ischemic group but the difference does not reach significance levels. The chelation of the iron into ferrioxamine probably facilitates the wash-out from the cells.

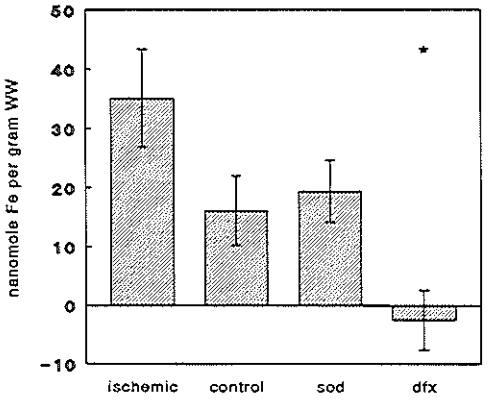

Figure 4.
Low molecular weight iron and reperfusion.
Effect of 30 minutes ischemia (ischemic) and 20 minutes reperfusion after 30 minutes ischemia on low molecular weight iron in hearts perfused in the absence (control) or in the presence of SOD (sod) or deferoxamine (dfx). Bars represent means ± SEM, n = 6 for ischemic, n = 3 for control, sod and dfx. * denotes $p < 0.05$ compared to ischemic hearts.

The effect of anoxia.

Anoxia differs substantially from no-flow ischemia in that, although there is oxygen depletion, the coronary bed is still perfused. This means that there still is supply of glucose and removal of metabolites and acidosis is prevented. This makes the latter a mild insult (14). To evaluate the effect of anoxia, the size of the low molecular weight iron pool was compared in hearts that were perfused for 45 minutes under normoxic or anoxic conditions. Anoxic perfusion for 45 minutes does not lead to an increase in the low molecular weight iron content. (fig. 5, left part).

Earlier observations from our laboratory have shown that normal rat hearts, perfused with anoxic buffer for 45 minutes, regain 80 % of pre-anoxic contractility upon reoxygenation (14) whereas normal hearts subjected to 15 minutes of ischemia regain only 40 % of pre-ischemic contractility (12) upon reperfusion. From the results described above it is clear that ischemia leads to a tremendous increase in low molecular weight iron and to severe damage, while anoxia leads to little or no increase in low molecular weight iron and the consequences are mild. Iron-loaded hearts however, regain only 20 % of pre-anoxic contractility after 45 minutes of anoxic perfusion (14). Therefore we

have measured the amount of low molecular weight iron in iron-loaded hearts. Normoxic iron-loaded hearts have a higher low molecular weight iron pool than normal hearts (fig. 5). In contrast to normal hearts the size of this low molecular weight pool increases dramatically during anoxia.

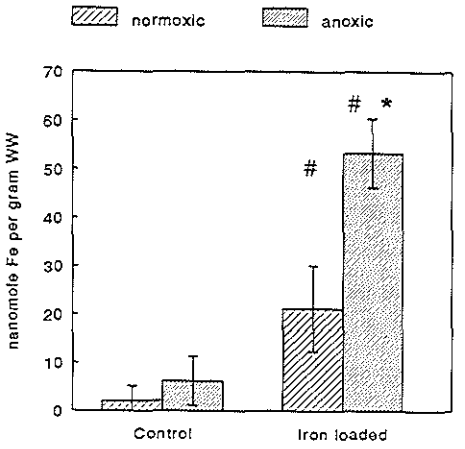

Figure 5.
Low molecular weight iron and anoxia.
Effect of 45 minutes of anoxic perfusion on low molecular weight iron of normal (control) and iron-loaded rat hearts compared with hearts that were perfused with normoxic tyrode for 60 minutes. Each bar represents the mean ± SEM. (n=6 in non iron-loaded hearts and, n=3 in the iron-loaded hearts, * denotes $p < 0.05$ vs normoxic value, # $p < 0.05$ vs non iron-loaded).

Effect of iron chelation and antioxidant protection in iron-loaded hearts.

The dramatic loss of function of iron-loaded hearts during anoxia and reoxygenation could be prevented by perfusion with either (+)-cyanidanol or deferoxamine (14). Therefore, the size of the low molecular weight iron pool during

anoxia and reoxygenation of iron-loaded rat hearts was evaluated under these conditions in a separate experiment.

The size of the low molecular weight iron pool in normoxic iron-loaded rat hearts amounted to 15.4 ± 6.1 nmole per gram wet weight (fig. 6, control). Perfusion with anoxic buffer led to the expected increase of low molecular weight iron. Upon reoxygenation a further increase was found. The low molecular weight pool in iron-loaded hearts is unaffected by normoxic perfusion with 20 μM (+)-cyanidanol-3 for one hour and during anoxic perfusion the same increase was found as in control hearts (fig. 6, cyanidanol). However, upon reoxygenation no further increase was found. Normoxic perfusion with 50 μM deferoxamine for one hour led to a very small low molecular weight iron pool (fig 6, deferoxamine). This could be due to washout from ferrioxamine from the heart. Anoxic perfusion in the presence of deferoxamine again increased low molecular weight iron and upon reoxygenation there is a further increase of low molecular weight iron.

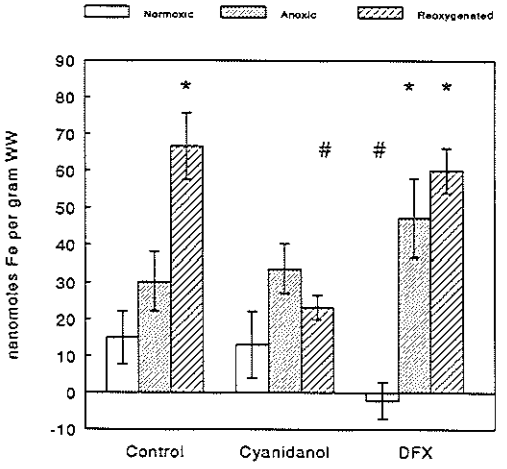

Figure 6.
Low molecular weight iron during anoxia and reoxygenation in iron-loaded rat hearts.
Effect 60 minutes normoxic perfusion (normoxic), 45 minutes anoxic perfusion (anoxic) and reoxygenation after 45 minutes anoxia for 10 minutes (reoxygenation) in the absence (control) or presence of 20 μM (+)-cyanidanol (cyanidanol) or 50 μM deferoxamine. Each bar represents the mean ± SEM (n=3). * denotes $p < 0.05$ vs normoxic hearts in that group, # denotes $p < 0.05$ vs untreated control.

DISCUSSION

In the present paper we have presented a method to determine the size of the intracellular low molecular weight iron pool and applied this method to ischemic or anoxic rat hearts. The chelated iron in normoxic rat hearts as measured in sixty minute incubations by Gower et al. was 15.44 ± 6.37 nmoles per gram wet weight. In our hands the sixty minute incubations yielded 21.6 ± 3.6 (n=6) nmoles chelated iron per gram wet weight. Following our correction for ferritin derived iron the low molecular weight pool contains 2.1 ± 4.8 nmole per gram wet weight. This figure means that the low molecular weight pool in normoxic rat hearts is so small that it escapes more exact measurement. However, in hearts that had been ischemic the bi-phasic timecurve of iron chelation from the supernatant shows a faster initial phase while there is no significant difference between normoxic and ischemic hearts in the slope after one hour. Thus correction for this ferritin derived iron leads to a higher value for the low molecular weight pool.

Our results show that the amount of low molecular weight iron increases dramatically during ischemia from 2.1 ± 4.8 to 54.2 ± 4.1 nmoles per gram wet weight. Healing et al. (24) have studied the amount of iron in rabbit kidney homogenates after cold ischemia by measuring ferrioxamine/deferoxamine ratios in homogenates in 60 minute incubations. It was shown that the amount of iron increases from approximately 15 nmoles in non-ischemic control to 60 nmoles per gram tissue after seventy-two hours of cold ischemia. However, these authors did not correct for the iron released from ferritin that has occurred during this incubation. Others (22) showed that the low molecular weight iron in ischemic and non-ischemic myocardium after a two hour in vivo coronary artery occlusion in dogs increased from 130.6 to 183.2 nmoles per gram tissue. In dog brain an increase in low molecular weight iron from 90.5 to 370.4 (nmoles/gram wet weight) was found after cerebral ischemia induced by cardiac arrest (23). No measurements of total iron were presented in those studies, but mammalian tissue, except liver and spleen, contains approximately 1000 nmole iron per gram wet weight (20, 35). Recalculation of the above results would imply that in mongrel dog brain and heart 10 % of the total iron is in the low molecular weight iron pool under normoxic conditions. This is very high regarding other estimates in which the low molecular weight iron pools amounts to only 0.2 % (28) or 3 % (33) of the total iron. Possibly these high

values can be explained by the fact that during ultrafiltration the homogenate was not buffered and contained only EDTA, which may have led to acidification. Since the iron release from ferritin depends on reducing equivalents and is enhanced by acidification (20,21), additional iron release may have occurred during those incubations.

We have shown that ischemia causes a progressive increase in low molecular weight iron while during anoxia low molecular weight iron does not increase in non iron-loaded hearts. To release iron from ferritin a reduction to Fe(II) must take place and the ferrous iron must be chelated (20,21). One possible mechanism is a superoxide dependent reduction. However, the iron released through this mechanism has been shown not to exceed 1.5 mol iron per mol ferritin (32). Furthermore, it is unlikely that superoxide is produced during ischemia and we found no significant difference in the amount of low molecular weight iron in hearts subjected to ischemia and reperfusion with or without SOD. Funk et al. (20) have studied the reductive mobilisation of iron from isolated horse spleen ferritin. Here it was shown that reduced FMN induces the release 1200 Fe atoms per ferritin molecule within 3 minutes at pH 7. At pH 5.1 this was increased to 1900 atoms Fe. Ischemia causes a drop in intracellular pH to 5.9 (34) and even to 5.7 after 20 five minutes (31). Thus, the reducing equivalents present in ischemic tissue and the acidification will facilitate the reductive release of iron from ferritin. During anoxic perfusion coronary flow is maintained allowing the supply of glucose and the efflux of metabolites. The metabolic consequence is that acidosis is prevented. Therefore, reducing equivalents will be present during anoxia but no acidification occurs. It has to be concluded that the acidification contributes tremendously to the mobilisation of iron from ferritin.

The increment of low molecular weight iron during reoxygenation of iron-loaded hearts must have an other mechanism than the increase due to reducing agents during anoxia. This is substantiated by our finding that low molecular weight iron in iron-loaded hearts increased during anoxic perfusion in the presence of (+)-cyanidanol-3, while the additional increase during reoxygenation was prevented by (+)-cyanidanol (fig. 6, cyanidanol). This implies that the increase of low molecular weight iron in iron-loaded hearts during reoxygenation is due to superoxide.

Normoxic iron-loaded hearts perfused with deferoxamine for one hour show a very small low molecular weight pool. This is probably due to a loss of ferrioxamine to the

perfusate which will deplete the low molecular weight pool first. An increase occurs during anoxia because apparently the reductive release of iron exceeds the washout effect. Ten minutes after reoxygenation low molecular weight iron in these hearts was higher than in the (+)-cyanidanol-3 hearts. Again, this could be caused by superoxide production since this is not prevented by deferoxamine.

Normoxic iron-loaded rat hearts have a higher low molecular weight iron content compared to the non iron-loaded hearts. This is without apparent effect on the physiological parameters such as coronary flow or contractility (14). Due to the presence of oxygen the iron in the low molecular weight iron pool in normoxic iron-loaded hearts is in the ferric state, in which it will not catalyze the formation of hydroxyl radicals directly. In contrast, the low molecular weight iron in anoxic or ischemic hearts must be ferrous iron. Moreover, we have shown here that the amount of low molecular weight ferrous iron is increased by ischemia or anoxia. This will directly form hydroxyl radicals together with the hydrogen peroxide which arises from the dismutation of superoxide that is generated upon reoxygenation. Indeed, the effect of reoxygenation after the anoxic insult is quite dramatic in the iron-loaded hearts and this could be prevented by perfusion with (+)-cyanidanol-3 (14). Despite the increase in low molecular weight iron the iron-loaded hearts perfused with deferoxamine recover to 80 % of pre-anoxic contractility (14) which shows that the low molecular weight iron in these hearts is not in a form that catalyses the Fenton reaction because it is chelated as ferrioxamine (17).

Taken together our results show that the low molecular weight iron pool increases during oxygen deprivation and that this release is due to the reducing equivalents generated in the absence of oxygen. The drop in pH that occurs in ischemic hearts dramatically increases this reductive iron release. Furthermore, free radicals generated during reoxygenation release additional iron. From these results, it can be concluded that ischemia increases the susceptibility to the oxygen radicals generated during reoxygenation by increasing the amount of catalytic, ferrous iron in the low molecular weight pool.

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CHAPTER FIVE

THE INCREASED SUSCEPTIBILITY TO HYDROGEN PEROXIDE OF THE (POST-)ISCHEMIC RAT HEART IS ASSOCIATED WITH THE MAGNITUDE OF THE LOW MOLECULAR WEIGHT IRON POOL.

Adapted from: Arthur Voogd, Wim Sluiter and Johan F. Koster: The increased Susceptibility to Hydrogen Peroxide of the (post-)ischemic Rat Heart is Associated with the Magnitude of the Low Molecular Weight Iron Pool. Free Radical Biology & Medicine, submitted

ABSTRACT

Recently we have shown that intracellular low molecular weight (LMW) iron increases during ischemia. It is hypothesized that this increase in LMW iron during ischemia underlies the reported hydrogen peroxide toxicity towards ischemic hearts. To study this, we subjected rat hearts to reperfusion with anoxic buffer containing 10 μM hydrogen peroxide for seven minutes after fifteen minutes ischemia. Upon reoxygenation cardiac function recovered to only 47±6% of the pre-ischemic value. Hearts subjected to the same protocol but preperfused with deferoxamine before ischemia or reperfused without hydrogen peroxide recovered to 78±8% and 80±7%, respectively. Immediate reoxygenation after ischemia led to only 45±6% recovery of function. During ischemia LMW iron increased from 49±45 to 183±45 pmole/mg protein ($p<0.05$) and decreased to 58±38 pmole/mg protein ($p<0.05$) during the subsequent anoxic perfusion. Rat hearts preloaded with deferoxamine showed a slightly higher LMW iron content than normal (85±23 and 49±45 pmole/mg protein, respectively; n.s.) but ischemia did not lead to an increase of LMW iron in those hearts (136±42 pmole/mg protein after 15 min of ischemia; n.s.). No significant changes were found in reduced and oxidized glutathione content and glutathione peroxidase or catalase activities under those conditions. Our results indicate that hydrogen peroxide toxicity is determined by the amount of catalytic iron in the LMW pool and not by a decrease in antioxidant defense capacity to hydrogen peroxide.

INTRODUCTION

Over the recent years it has been well established that reoxygenation of ischemic tissue leads to the generation of reactive oxygen species (ROS) [1,2]. It is likely that those ROS contribute to the reperfusion syndrome by damaging proteins, causing breakage of DNA strands, and initiating lipid peroxidation [2,3,4]. In the presence of a catalytic transition metal such as iron the toxicity of the ROS is tremendously increased by the conversion of hydrogen peroxide to the highly reactive hydroxyl radical [5].

Under physiological conditions iron is stored or contained in proteins in a non-catalytic form [6]. Only a very small amount is thought to be present in a low molecular weight (LMW) pool [7,8] in a form that has been shown to catalyze hydroxyl radical formation [9,10,11,12]. In a recent study from our laboratory [13] it has been shown that intracellular LMW iron increases dramatically during ischemia in isolated rat hearts. In vitro, iron can be released from ferritin through a reductive mechanism and this release is enhanced by a lower pH [14]. Therefore, it has been proposed [13] that this reallocation of iron during ischemia is caused by reducing equivalents arising during ischemia and that this is greatly enhanced by the acidification that occurs in the ischemic hearts [15].

The role of iron in post-ischemic free radical toxicity has been substantiated by studies in which iron chelators that inhibit in vitro lipid peroxidation, attenuate reperfusion injury in a variety of experimental animal models [16,17,18,19]. However, this has never been studied in relation to the amount of catalytic iron in post ischemic tissue. Shattock et al. [20] have shown that ischemic rat hearts were much more sensitive to hydrogen peroxide than normoxic hearts. This raised the question whether the rise in the intracellular LMW iron pool during ischemia as shown by us underlies the increased susceptibility towards hydrogen peroxide.

To investigate this we have studied hydrogen peroxide toxicity in post-ischemic rat hearts in relation to the LMW iron pool and the hydrogen peroxide defense system.

METHODS AND MATERIALS

Animals and perfusion protocol.

Twelve to fourteen week old male Wistar rats were used. After a brief anaesthesia with diethyl ether the hearts were excised and placed in ice cold Tyrode buffer. The hearts were cannulated through the aorta and perfused retrogradely according to Langendorff [21]. Perfusions were carried out at 37 °C with Tyrode buffer containing 128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$ and 11 mM glucose, pH 7.4. The buffer was saturated with 95% O_2 and 5% CO_2. Tyrode buffer was made anoxic by saturation with 95% N_2 and 5% CO_2 for at least one hour. Perfusion pressure was held constant at 80 cm water pressure.

Experimental design.

To assess hydrogen peroxide toxicity in the absence of superoxide, hearts were subjected to fifteen minutes of warm no flow ischemia and reperfused with anoxic buffer either with or without 10 μM hydrogen peroxide for seven minutes. After this anoxic reperfusion the hearts were perfused with oxygenated Tyrode buffer to assess functional recovery. The effect of iron chelation on this insult was investigated by preloading hearts with 50 μM deferoxamine during a ten minute perfusion immediately before ischemia and addition of 10 μM hydrogen peroxide during the anoxic reperfusion period. During ischemia, the hearts were submerged in warm (37 °C) Tyrode buffer which was gassed with 95% N_2 and 5% CO_2. To assess hydrogen peroxide toxicity directly after anoxia, hearts were subjected to fifteen minutes of anoxic perfusion and then switched to anoxic buffer containing 10 μM hydrogen peroxide for seven minutes and compared with hearts that were perfused with anoxic buffer for twenty-two minutes.

Catalase, glutathione peroxidase, reduced glutathione, total glutathione and LMW iron were determined in separate groups of hearts subjected to the appropriate perfusion protocol.

Functional parameters.

Lactate dehydrogenase (LDH) release in the coronary effluent, as a measure of tissue damage, and coronary flow were determined as described earlier [18]. Apex

displacement was detected with a smooth muscle transducer. Contractility, as a measure of cardiac work, was calculated as the product of apex-amplitude and apex-frequency, which were recorded every thirty seconds. Data aquisition was started after the hearts had shown a stable contractility for ten minutes. The mean value of the contractility of each heart during ten minutes before ischemia was set at 100% and subsequent performance expressed as percentage of pre-ischemic contractility.

Antioxidant, antioxidant enzymes and LMW iron determinations.

After the apropriate perfusions hearts were immediately homogenized to a 10 % homogenate in ice cold 100 mM Tris/HCl pH 7.4. The homogenate was centrifuged at 10.000 g for fifteen minutes.

Reduced glutathione was determined after protein precipitation by 5 % trichloric acid of the resulting supernatant by reduction of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) measured as absorbance at 412 nm. Total glutathione was determined by the reduction of DTNB in the presence of NADPH and glutathione reductase [22].

Glutathione peroxidase was determined as described elsewhere [23] and expressed as units/mg protein (1 unit = 1 μmole NADPH/min). Protein concentration of the supernatant was determined using Biuret reagens with bovine serum albumin as standard. Catalase activity was assessed as the disappearance of hydrogen peroxide as determined at 240 nm at a hydrogen peroxide concentration between 7 and 9 mM and expressed as μmoles per min.

LMW iron was determined in the supernatant as described in detail elsewhere [13].

Statistics.

All data are presented as means ± standard error of the mean (SEM). To evaluate differences between groups, n-way analysis of variance was performed on the data using the Stata release 2.0. (Computing Resource Centre, LA, California).

Chemicals.

Unstabilised hydrogen peroxide was obtained from Merck (MOS selectipure, 12341) to avoid confounding effects of stabiliser substances. Deferoxamine Mesylate (deferoxamine) was obtained from Sigma.

RESULTS

Functional Recovery and Tissue Damage.

To study the toxicity of hydrogen peroxide on the rat heart the effect of this compound on cardiac function and tissue damage was established under various conditions.

ISCHEMIA:Immediate reoxygenation of ischemic hearts impaired cardiac function (45±6% recovery; Fig. 1a), while a seven-minute period of anoxic perfusion preceding reoxygenation led to a much better (80±7%) restoration of contractility (Fig. 1a). However, if 10 μM hydrogen peroxide was administered during this anoxic perfusion a recovery of only 47±6% was found (Fig. 1b). To investigate whether this effect of hydrogen peroxide is mediated by an iron dependent mechanism, the hearts were perfused with 50 μM deferoxamine for ten minutes before ischemia. Under those conditions the hydrogen peroxide included in the anoxic buffer did not significantly decrease the recovery of the contractility (78±9%, Fig. 1b).

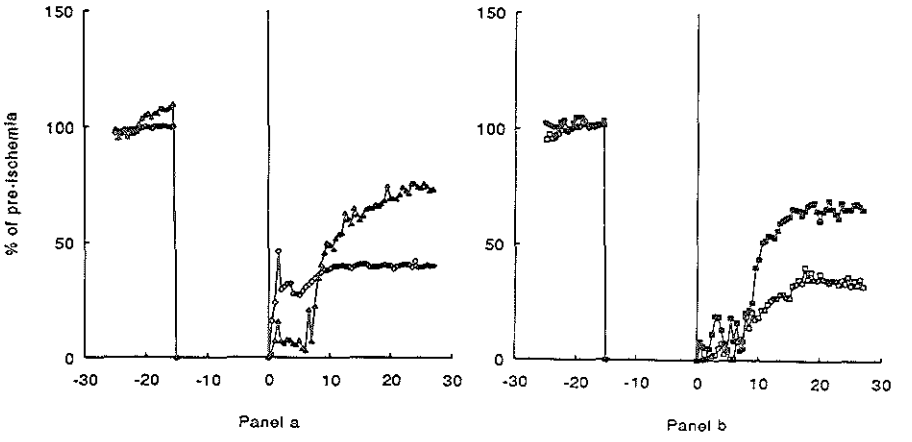

Figure 1.

Panel a. Effect of immediate reoxygenation on the recovery of contractility of hearts subjected to fifteen minutes of ischemia (open circles) and the protective effect of a period of seven minutes of anoxic reperfusion preceding reoxygenation (open triangles).

Panel b. Effect of 10 µM hydrogen peroxide administered during anoxic reperfusion after ischemia on the recovery of function upon reoxygenation (open squares) and the protective effect of preloading with deferoxamine (closed squares)

Contractility is expressed as percentage of the pre-ischemic value. A period of fifteen minutes of ischemia preceded reperfusion which starts at time zero. Standard errors are omitted for clarity. (n=6 hearts in each group).

The susceptibility of the Langendorff rat heart to hydrogen peroxide was also studied by the release of LDH in the coronary effluent as a parameter of tissue damage. Analysis of variance showed that hearts, reoxygenated immediately after fifteen minutes of ischemia released more LDH than hearts that were reperfused for the first seven min after ischemia with anoxic buffer and then reoxygenated (Fig. 2a; $p<0.01$). However, if the anoxic buffer contained 10 μM hydrogen peroxide the LDH in the coronary effluent increased significantly (Fig. 2b; $p<0.01$ compared to anoxia without hydrogen peroxide, Fig. 2a.). The increased release of LDH was prevented by preloading of the hearts with deferoxamine (Fig. 2b; $p< 0.01$, for the difference in the absence of deferoxamine).

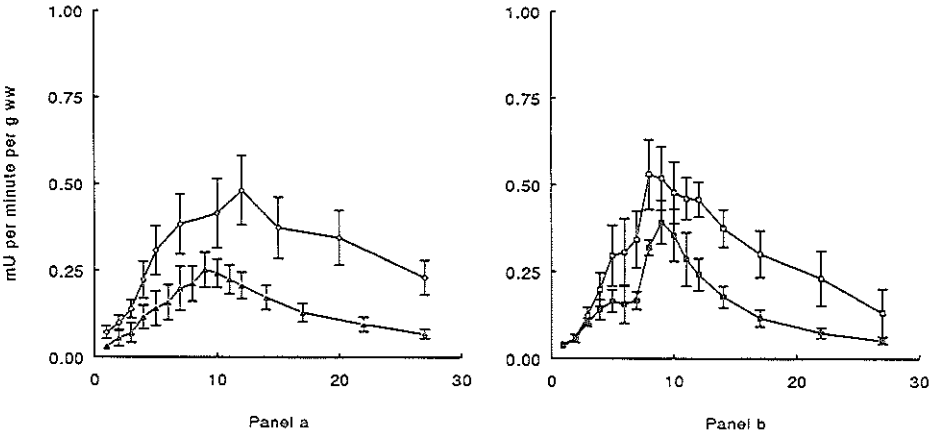

Figure 2.

Panel a. Effect of immediate reoxygenation on LDH release in the coronary effluent of hearts subjected to fifteen minutes of ischemia (open circles) and the protective effect of seven minutes of anoxic reperfusion after ischemia preceding reoxygenation (open triangles).

Panel b. Effect of 10 μM hydrogen peroxide administered during seven minutes of anoxic reperfusion after ischemia on the LDH release upon reoxygenation (open squares) and the protective effect of preloading with deferoxamine (closed squares). Reperfusion starts at time zero. (Means ± SEM, n=6 hearts in each group)

ANOXIA: To investigate whether the toxicity depends on a preceding period of ischemia hydrogen peroxide toxicity was assessed after fifteen minutes of anoxic perfusion. The results showed that hydrogen peroxide present during the last seven minutes of a period of twenty-two minutes of anoxia did not impair the contractility of the reoxygenated hearts compared to the hearts subjected to anoxic perfusion without

these hearts very little LDH was detectable (<0.05 mU/g wt weight/min). Furthermore, we have shown earlier that reoxygenation after anoxic perfusion did not lead to any loss of contractility and that LMW iron does not increase under those conditions [13].

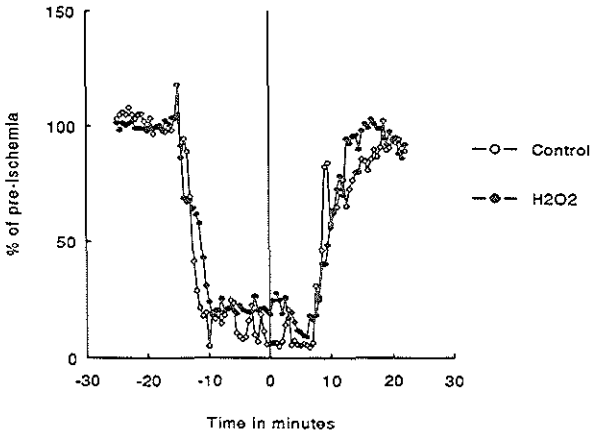

Figure 3.
Effect of reoxygenation on contractility after twenty-two minutes of anoxic perfusion of hearts perfused without (open circles) or with (closed circles) 10 μM hydrogen peroxide present during the last seven minutes of anoxia.
A period of fifteen minutes of anoxic perfusion (time = -15 to 0) preceded anoxic perfusion with or without hydrogen peroxide (time = 0 to 7) after seven minutes the hearts were reoxygenated (time = 7 and onwards). Means of five hearts per group, standard errors are omitted for clarity.

Low Molecular Weight Iron.

Because deferoxamine pretreatment abolished hydrogen peroxide toxicity after ischemia the amount of iron in the LMW pool was determined under the various conditions (Fig. 4). During ischemia the amount of iron in the LMW pool increased from 49±16 to 183±45 pmole/mg protein ($p < 0.05$) confirming our earlier findings[13]. Upon reperfusion with anoxic buffer in the presence or absence of hydrogen peroxide the LMW iron pool returned to about normal values over the next seven minutes (22±18 and 59±29 pmole/mg protein, respectively; $p < 0.05$) and remained fairly constant after reoxygenation. Hearts preloaded with deferoxamine showed a slightly higher LMW iron content than normal (85±23 and 49±45 pmole/mg protein, respectively; n.s.), but

content than normal (85±23 and 49±45 pmole/mg protein, respectively; n.s.), but ischemia did not lead to a significant increase of LMW iron in those hearts (136±42 pmole/mg protein after 15 min; n.s.). Anoxic perfusion for fifteen minutes did not cause a significant change in LMW iron and neither did the additional seven minutes of anoxic reperfusion in the presence of hydrogen peroxide. (61±29, 55±32 and 42±25 pmole/mg protein respectively, n.s.).

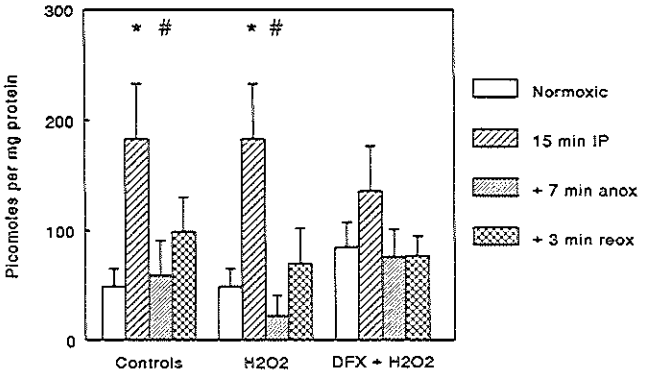

Figure 4.
The effect of anoxic reperfusion on LMW iron in hearts reperfused with anoxic Tyrode for the first seven minutes after ischemia either without hydrogen peroxide (control), with 10 μM hydrogen peroxide (H^2O^2) or with hydrogen peroxide and deferoxamine pretreatment (DFX + H^2O^2). Hearts were homogenized as described after normoxic perfusion (normoxic), fifteen minutes of ischemia (ischemic), after fifteen minutes of ischemia and seven minutes anoxic reperfusion (+ 7 min anox), or after fifteen minutes ischemia + seven minutes anoxic reperfusion + three minutes reoxygenation (+ 3 min reox).
Note that the first two groups in the control and the H2O2 group are identical. (* denoted $p < 0.05$ vs normoxic hearts, # denotes $p < 0.05$ vs ischemic hearts in that group, means ± SEM, n=5 in each group).

Antioxidant Defense System.

Besides a role of LMW iron it is possible that the increased susceptibility of the (post-)ischemic heart to hydrogen peroxide is caused by a decrease in the hydrogen peroxide metabolizing enzymes, i.e. catalase and glutathione peroxidase. A decrease of reduced glutathione, the substrate of the latter antioxidant enzyme, will also lead to impaired detoxification of hydrogen peroxide. Therefore, the activities of catalase and

glutathione peroxidase, and the total glutathione and reduced glutathione content were determined under the various conditions. Because hydrogen peroxide had no effect after a period of anoxia these parameters were not determined in these hearts. Table I shows that the antioxidant defense system did not vary significantly between all groups (one way analysis of variance, using the Bonferonni correction for multiple comparisons).

	Normoxic	15' ischemia	+ 7 min N_2	+ 3' O_2
Glutathion Peroxidase			µmoles NADPH per minute	
Controls	106.0 ± 18.0	172.0 ± 44.0	174.0 ± 34.0	168.0 ± 40.0
H_2O_2	106.0 ± 18.0	172.0 ± 44.0	186.0 ± 38.0	175.0 ± 35.0
DFX + H_2O_2	164.0 ± 45.0	136.0 ± 36.0	195.0 ± 65.0	183.0 ± 59.0
Catalase			µmoles H_2O_2 per minute	
Controls	20.9 ± 2.4	27.1 ± 5.5	24.1 ± 4.0	22.7 ± 6.1
H_2O_2	20.9 ± 2.4	27.1 ± 5.5	29.3 ± 5.5	26.2 ± 5.2
DFX + H_2O_2	16.9 ± 5.4	19.5 ± 4.7	22.7 ± 5.1	27.6 ± 4.0
Reduced Glutathione			nmoles per mg protein	
Controls	16.6 ± 3.2	20.3 ± 5.4	25.5 ± 5.2	17.8 ± 5.0
H_2O_2	16.6 ± 3.2	20.3 ± 5.4	25.4 ± 1.8	16.2 ± 3.1
DFX + H_2O_2	21.8 ± 5.4	18.5 ± 4.2	26.8 ± 5.1	18.1 ± 1.3
Total Glutathione			nmoles per mg protein	
Controls	14.4 ± 3.6	28.9 ± 5.1	24.4 ± 3.9	18.8 ± 3.9
H_2O_2	14.4 ± 3.6	28.9 ± 5.1	16.1 ± 1.9	15.8 ± 5.1
DFX + H_2O_2	19.1 ± 4.1	16.8 ± 2.4	20.3 ± 3.4	10.5 ± 1.2

Table 1.
Hydrogen peroxide specific defenses in rat hearts perfused with normoxic buffer (normoxic) for fifteen minutes, after fifteen minutes of no flow ischemia (15' ischemia), after seven minutes of anoxic reperfusion (+ 7' N2) and after the subsequent three minutes of reoxygenation (+ 3' O2).
Control hearts (control) were perfused with Tyrode only. H_2O_2: hearts that were reperfused with anoxic Tyrode containing 10 µM H_2O_2. DFX + H_2O_2: hearts that were perfused with normoxic Tyrode containing 50 µM deferoxamine during ten minutes before ischemia and reperfused with anoxic Tyrode containing 10 µM H_2O_2.

DISCUSSION

The main conclusion to be drawn from the present study is that the increased susceptibility to hydrogen peroxide of the post-ischemic heart is related to the amount of catalytic LMW iron and not due to a decrease in hydrogen peroxide metabolizing capacity.

Our data showed that a short period of anoxic reperfusion after ischemia causes a much better recovery of the contractility compared to immediate reoxygenation. Previously, the beneficial effect of hypoxic reperfusion was attributed to a decrease in the production of ROS during reoxygenation [24]. However, others have shown that after a short period of anoxic reperfusion a burst of free radicals occurs during reoxygenation, that is of similar magnitude to the burst that occurs during immediate reoxygenation. In that study no radicals were observed during the anoxic reperfusion period [25]. In the present study we have shown that during the anoxic reperfusion period the LMW iron pool, which was increased during ischemia, returned to values that did not differ from normoxic hearts. Apparently, under those conditions the amount of free iron became too low to catalyze the formation of hydroxyl radicals in sufficient amounts to cause tissue damage upon reoxygenation. This hypothesis is substantiated by our finding that hydrogen peroxide was only toxic when the LMW pool was increased. Hearts could cope with hydrogen peroxide immediately after ischemia only if the iron had been rendered non-catalytic by the iron chelator deferoxamine [16]. Therefore, our results expand the notion of a crucial role for iron in post-ischemic oxygen radical toxicity. In the present study we have shown that hydrogen peroxide is toxic due to an iron dependent mechanism. Furthermore, this was established under conditions were no superoxide is generated [25] and this indicates that the catalytic post-ischemic iron is ferrous iron.

How the drop in the LMW iron pool during anoxic reperfusion occurred is not known, but it is conceivable that the free iron was washed out. Calculation showed that approximately ten nanomoles of iron were lost. This is so little that it cannot be determined if diluted in the coronary effluent. Re-uptake of iron by ferritin is not very likely because this process is oxygen dependent[26].

Normally, hydrogen peroxide is effectively detoxified by the antioxidant enzymes catalase and glutathione peroxidase but if the oxygen supply is suddenly increased after an ischemic period the formation of ROS may overwhelm the antioxidant enzyme system. Under such a condition and if accompanied by an increased amount of ferrous iron in the LMW iron pool hydrogen peroxide is immediately converted to the highly reactive hydroxyl radical leading to loss of cardiac function and tissue damage.

From a clinical point of view our results indicate that it may be beneficial to flush a ischemic organ with anoxic buffer or venous blood before reoxygenation, because this could lead to normalized levels of LMW iron and attenuation of reperfusion injury without the need for pharmacological interventions.

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CHAPTER SIX

IMPAIRED GLYCOLYSIS ATTENUATES THE RELEASE OF FERROUS IRON AFTER ISCHEMIC PRECONDITIONING.

Adapted from: Arthur Voogd, Mustafa Catak, Wim Sluiter, Tom J.C. Ruigrok and Johan F. Koster. Impaired Glycolysis Attenuates the Release of Ferrous Iron after Ischemic Preconditioning. Circulation submitted

ABSTRACT

Background: The amount of iron in the low molecular weight pool (LMW) increases during no-flow ischemia and is thought to be essential to oxygen radical damage upon reperfusion. Iron release during ischemia may be related to accumulation of reducing agents which can be prevented by decreasing the rate of anaerobic glycolysis. The present study investigates whether pre-ischemic glycogen depletion, known to reduce glycolytic activity and improve post-ischemic function also decreases iron release.
Methods and results: Isolated rat hearts were partially depleted of glycogen by perfusion with (1) anoxic buffer, (2) buffer containing glucagon and (3) by three short periods of ischemia before being subjected to fifteen minutes of no-flow ischemia. All the glycogen depleted groups had lower glycolytic activity during ischemia measured as glycogen consumption, lactate accumulation and acidosis.
Control hearts recovered to 44 ± 3.8 % of pre-ischemic contractility after thirty minutes of reoxygenation. The three protocols used to deplete glycogen caused hearts to recover to 80 % ± 5.2 % (1), 81 ± 6.2 % (2) and 73 ± 5.6 % (3) of pre-ischemic contractility. LMW iron content at the end of ischemia in control hearts was 192 ± 45 pmoles/mg protein while pretreated hearts contained 60 ± 19 (1), 74 ± 25 (2) and 23 ± 32 (3) pmoles/mg protein ($p<0.05$ for the difference between control and each group, $n=6$).
Conclusion: The amount of iron released during ischemia into the LMW pool is related to glycolytic activity. Therefore, all pretreatments decreasing pre-ischemic glycogen, including various "pre-conditioning" protocols can be expected to decrease iron release and thus attenuate post-ischemic radical toxicity.

INTRODUCTION

The generation of reactive oxygen species upon reoxygenation of ischemic tissue contributes to the detrimental events that together constitute reperfusion injury (1,2,3,4,5). The toxicity of superoxide and hydrogen peroxide is thought to be caused by the generation of the highly oxidizing hydroxyl radical mediated through transition metal dependent reactions (6). The most abundant intracellular transition metal, iron, is safely deposited in ferritin or bound to haem proteins (7) and as such is not available for Haber-Weiss chemistry. Only a very small amount is present in what is known as the low molecular weight (LMW) pool (8,9,10,11) in which form it can participate in the Haber-Weiss reactions (12,13).

Neely and Grotyohan (14) have shown that the recovery after ischemia is inversely related to the glycolytic activity, measured as the amount of lactate that accumulates during ischemia, but independent of tissue ATP just before reperfusion. This was achieved by pre-ischemic glycogen depletion through anoxic perfusion immediately before ischemia. By now it is well established that short periods of ischemia protect against a longer ischemic insult and the concept is known as ischemic preconditioning (15,16). Among the explanations proposed for this phenomenon are reduced energy demand (17), the induction of stress proteins and effects mediated through oxygen radicals, adenosine receptor and arachidonic acid metabolites (15,16).

Glycogen depletion must be a common feature of all the experimental pre-conditioning protocols used and is known to attenuate acidification and metabolite accumulation during ischemia (18,19). In a recent study from our laboratory we have shown that iron is released into the low molecular weight pool during ischemia. Furthermore, it was shown that this iron reallocation does not occur during perfusion with anoxic buffer (20). It was proposed that iron reallocation is due to the reduction of ferric iron from ferritin leading to an increase of ferrous iron in the catalytic low molecular weight pool. Because the reductive iron release from ferritin can be driven by reductants such as NADPH, NADH and xanthine (21) and has been shown to proceed at a faster rate at lower pH (22) we hypothesise that metabolite accumulation and the acidosis that occur during no flow ischemia are the driving force behind the iron release. In our working hypothesis oxygen radical mediated damage during reperfusion will be

attenuated if acidification and metabolite accumulation during ischemia are reduced because less iron will be mobilized. In the present study we have addressed this hypothesis by evaluation of the effect of pre-ischemic glycogen depletion on post ischemic recovery, intracellular pH and iron release during ischemia in the isolated rat heart.

MATERIALS AND METHODS

Animals and Langendorff perfusion protocol.

Twelve to fourteen week old male Wistar rats were used. After a brief anaesthesia with diethyl ether the hearts were excised and placed in ice cold Tyrode buffer. The hearts were cannulated through the aorta and perfused retrogradely according to Langendorff (23) while beating spontanuously. Perfusions were carried out at 37 °C with Tyrode buffer containing 128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$ and 11 mM glucose, pH 7.4. The buffer was saturated with 95% O_2 and 5% CO_2. Tyrode buffer was made anoxic by saturation with 95% N_2 and 5% CO_2 for at least one hour. Perfusion pressure was kept constant at 80 cm water pressure.

In order to investigate the effects of pre-ischemic glycogen depletion hearts were subjected to three different protocols that are known to decrease cardiac glycogen content and subjected to fifteen minutes of no-flow ischemia. Pre-ischemic glycogen depletion was achieved by preperfusion before ischemia with 1) anoxic buffer for fifteen minutes (14), 2) three five-minute periods of no-flow ischemia interrupted by ten minutes reperfusion (18) and 3) perfusion with 250 μg/l glucagon for five minutes (24). These groups were compared to control hearts, which were subjected to fifteen minutes of no-flow ischemia after perfusion with only normoxic Tyrode for one hour.

Separate sets of experiments were performed in order to determine different parameters. Functional recovery, LDH and lactate release were determined in one set of four groups (n=6 in each group) of hearts. Glycogen content was determined in one group (n=5) after fifteen minutes of normoxic perfusion. A further eight groups of hearts (n=5 in each group) were used to determine cardiac glycogen immediately before ischemia but after pretreatment and at the end of ischemia in all four experiments. LMW iron was determined in each group (n=6 in each group) of hearts at the end of the ischemic period. Nuclear Magnetic Resonance (^{31}P NMR) measurements were performed using the final set of four groups (n=3 in each group).

Functional parameters.

Lactate dehydrogenase (LDH) release, as a measure of tissue damage, and coronary flow were measured as described earlier (25). Apex displacement was detected with a smooth muscle transducer. Contractility, as a measure of cardiac work, was calculated as the product of apex-amplitude and apex-frequency, which were recorded every thirty seconds. The mean value of the contractility of each heart during five minutes before ischemia was set at 100 %.

Glycogen, Lactate, LMW iron and pH determination.

Hearts were frozen in freezing isopentane and homogenized in 5 % perchloric acid. Glycogen was determined in the neutralized homogenate as described elsewhere (26). Glycogen values are expressed as a percentage of normoxic control hearts which contained 2.21 ± 0.11 mg glycogen per g wet wt.

Lactate was determined in the coronary effluent as described elsewhere (27) and cumulative release is expressed as μmoles per gram wet weight (μmole/g wet wt).

Low molecular weight iron was determined as described in detail elsewhere (20) and expressed as picomoles per milligram
protein (pmole/mg protein).

Intracellular pH values were calculated from the chemical shift of the intracellular inorganic phosphate peak in the ^{31}P NMR spectra as described in detail elsewhere (28).

Chemicals.

Glucagon was obtained from NOVO Nordisk A/S, Copenhagen, Danmark, and dissolved in Tyrode at a concentration of 250 μg/l.

Statistics.

Intergroup differences were evaluated with analysis of variance using the Bonferroni option for multiple comparisons (STATA release 2.0).

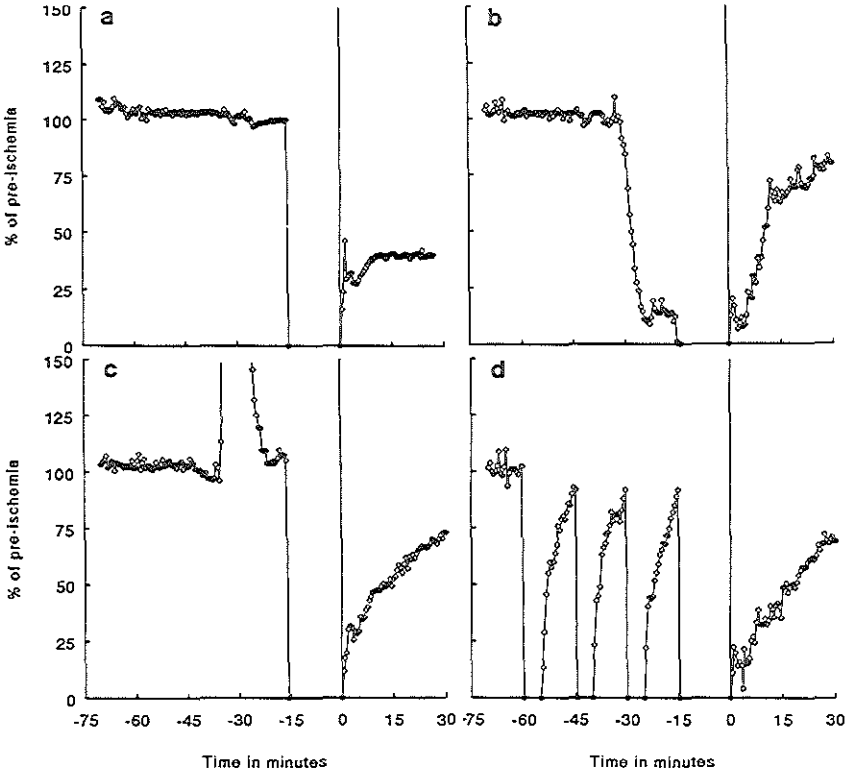

Figure 1.
The effect of pretreatment on post-ischemic recovery. Data aquisition starts at time = -70. Contractility is expressed as percentage of the contractility between time = -65 and time = -60. All hearts were perfused with normoxic Tyrode before pretreatment.
Pretreatments: none (control, panel a), anoxic Tyrode for fifteen minutes starting at time = -30 (anoxia, panel b), normoxic Tyrode containing 0.25 mg/l glucagon for five minutes starting at time = - 35 followed by fifteen minutes without glucagon (glucagon, panel c) three five minute periods of ischemia and ten minutes of reperfusion (3 x 5'IP, panel d).

RESULTS

Functional Recovery.

In order to determine the effect on the recovery after reperfusion rat hearts were subjected to preperfusion with anoxic Tyrode, Tyrode containing 250 μg/l glucagon and to a standard "pre-conditioning" protocol consisting of three five-minute periods of ischemia interrupted by ten minutes of reoxygenation. Control hearts, which were subjected to fifteen minutes of ischemia without pretreatment recovered to 44 ± 3.8 % of pre-ischemic contractility after thirty minutes of reoxygenation (Fig. 1a). Hearts which were subjected to anoxic perfusion during fifteen minutes immediately before ischemia recovered to 80 ± 5.2 % after thirty minutes of reperfusion (Fig. 1b). Perfusion with glucagon led to an increase in contractility of up to 285 % of control level due to the positive inotropic action of glucagon. Hearts were stabilised for fifteen minutes by changing to buffer without glucagon and then made ischemic. This treatment led to a recovery of 81 ± 6.2 % after thirty minutes (Fig. 1c). Preconditioning of hearts with three repeated periods of ischemia caused hearts to recover to 73 ± 5.6 % of pre-ischemic contractility (Fig. 1d).

LDH release.

In order to evaluate tissue damage induced by ischemia after glycogen depletion, cumulative LDH release was measured in the coronary effluent during thirty minutes of reperfusion. Control hearts released 5.23 ± 1.13 (means ± SD, n=6) units LDH during thirty minutes of reperfusion (Fig. 2). Tissue damage was markedly attenuated by pretreatment with fifteen minutes of anoxic perfusion, five minutes preperfusion with glucagon and with three short periods of ischemia (0.81 ± 0.20, 0.57 ± 0.11 and 1.52 ± 0.36 U/g wet wt, respectively. $p < 0.01$ for the difference between each group and the control group, n=6 in each group)

Glycogen Depletion.

In order to investigate the effect on glycogen content, separate groups of hearts were subjected to either of the protocols and glycogen content was determined directly before ischemia and after fifteen minutes of ischemia.

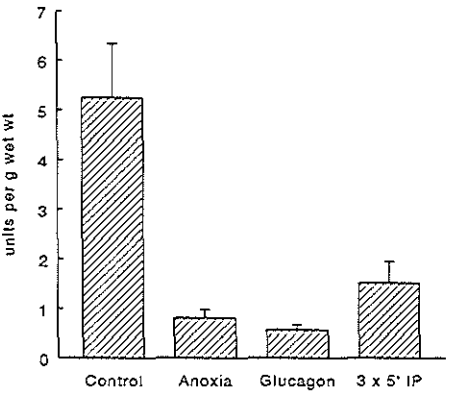

Figure 2.
The effect of pretreatment on the total amount of LDH released in the coronary effluent during thirty minutes of reperfusion after fifteen minutes ischemia of rat hearts that were not pretreated (control) or pretreated with anoxic Tyrode for fifteen minutes (anoxia), normoxic Tyrode containing 0.25 mg/l glucagon for five minutes followed by fifteen minutes without glucagon (glucagon) and three five minute periods of ischemia and ten minutes of reperfusion (3 x 5'IP).

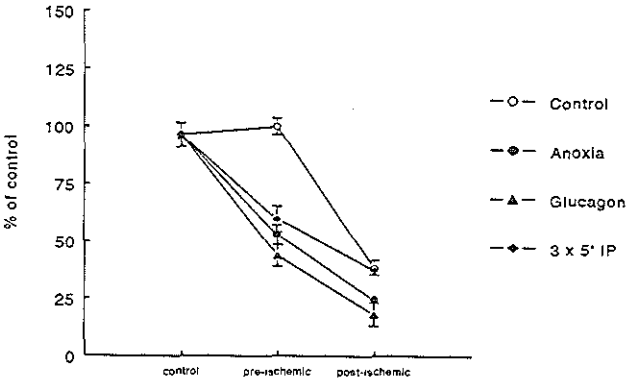

Figure 3.
The effect of pretreatments and of fifteen minutes of ischemia after pretreatment on glycogen content of rat hearts. Glycogen content is expressed as a percentage of the pre-ischemic control value. Separate groups of hearts (n=5) were sacrificed either after pretreatment of at the end of the ischemic period. Control hearts contained 2.21 ± 0.11 mg glycogen/g wet wt. For group legends please refer to figure 2.

Control hearts perfused with normoxic Tyrode for fifteen minutes contained 2.11 ± 0.10 mg/g wet wt glycogen. After a further forty-five minutes of normoxic perfusion glycogen content was 2.21 ± 0.11 mg/g wet wt (n.s., n=5, Fig. 3). After fifteen minutes of ischemia, glycogen content decreased to 38.7 ± 2.1 % of the original glycogen content. Preperfusion with anoxic Tyrode for fifteen minutes depleted the cardiac glycogen stores to 52.9 ± 4.3 % and this decreased further to 24.6 ± 2.1 % ($p<0.05$, n=5) after fifteen minutes of ischemia. Glucagon perfusion during five minutes depleted glycogen stores down to 43.8 ± 4.8 % and this decreased further to 18.1 ± 5.2 % ($p<0.05$, n=5) during ischemia. Preconditioning with three short periods of ischemia depleted glycogen stores to 59.7 ± 5.7 % of the normoxic value which decreased further to 38.4 ± 1.7 % after fifteen minutes of ischemia ($p<0.05$, n=5).

Lactate Release.

The amount of lactic acid that is released by the ischemic heart upon reperfusion is a reflection of the glycolytic activity that has taken place during ischemia. Therefore the cumulative amount of lactic acid in the coronary effluent during thirty minutes of reperfusion was determined. All three of the pretreated groups released less lactate than the control group (Fig. 4, $p< 0.05$ for the difference between the control group, n=6 in each group).

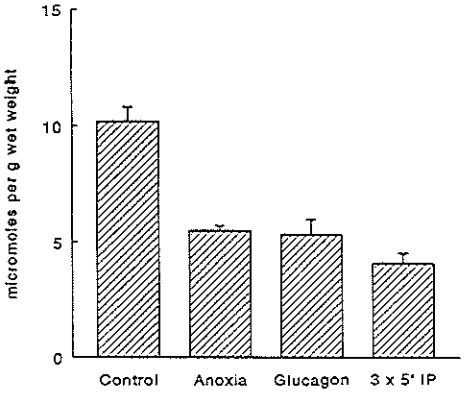

Figure 4.
The effect of pretreatments on the total amount of lactate released in the coronary effluent during thirty minutes of reperfusion after ischemia. Cumulative lactate release was lower in pretreated hearts compared to control hearts (n=6 hearts in each group, $p<0.05$ oneway analysis of variance using the Bonferroni correction for multiple comparissons). For group legends please refer to figure 2.

Intracellular pH.

To evaluate the influence of glycogen depletion on the development of the ischemic acidosis, pH was measured by ^{31}P NMR in the four groups of hearts. In control hearts the intracellular pH decreased from 7.04 ± 0.02 immediately before ischemia to 5.95 ± 0.05 after fifteen minutes of ischemia. In the three glycogen depleted groups there was a significant attenuation of the acidosis ($p < 0.05$ for the pH after fifteen minutes ischemia in control versus ischemic the pH in each group, n=3). In these groups of hearts pH decreased during ischemia from 6.97 ± 0.02 to 6.24 ± 0.05 (anoxic perperfusion), 7.05 ± 0.01 to 6.12 ± 0.01 (glucagon) and from 7.07 ± 0.03 to 6.18 ± 0.05 (3 x 5 min ischemia).

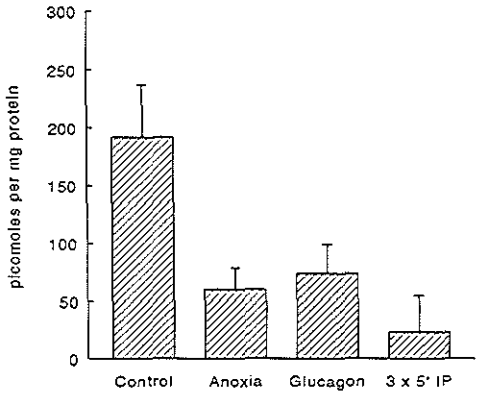

Figure 5.
The effect of pretreatment on LMW iron in rat hearts at the end of fifteen minutes of ischemia. LMW iron was lower in pretreated groups than in control hearts. (n=6 hearts in each group, $p<0.05$ oneway analysis of variance using the Bonferroni correction for multiple comparissons). For group legends please refer to figure 2.

LMW iron.

The amount of iron in the LMW pool upon reoxygenation is thought to be related to the functional recovery. Therefore, the effect of the pretreatment on the amount of LMW iron after fifteen minutes of ischemia was determined in separate groups of hearts. Control hearts contained 192 ± 45 pmoles of LMW iron per mg protein (Fig. 5).

Pretreatment with anoxia, glucagon and three ischemic periods all led to a reduction in the amount of iron that was released into the LMW pool during ischemia (60 ± 19, 74 ± 25 and 23 ± 32 pmole/mg protein respectively, $p<0.05$ for the difference between control and each group, $n=6$).

DISCUSSION

The results of the present study show that partial glycogen depletion before ischemia caused an improvement of post-ischemic contractility. During fifteen minutes of ischemia control hearts degraded 1.35 mg/g wet wt glycogen. This corresponds to 7.5 μmole/g wet wt glucose and could lead to a maximal lactate accumulation of 15.0 μmole/g wet wt. The cumulative lactate release during reoxygenation in our control hearts was 10.2 ± 1.2 μmole/g wet wt. All pretreated hearts in our study degraded approximately half of the amount of glycogen and accumulate half the amount of lactate.

The relation between reduction in glycolytic activity during ischemia and improved post-ischemic recovery is a matter of debate. Anoxic preperfusion has been used to deplete glycogen before ischemia and a negative correlation existed between tissue lactate content and post-ischemic recovery (14). Volovsek et al (18) have shown that preconditioning of rat hearts lowers glycogen content before ischemia and causes a reduction of glycogen utilisation during ischemia. Here the protective effect was attributed to a reduction in lactate accumulation and acidosis. The relation between ATP content and recovery was unclear. For instance no relation was found (14,18,29,30) which indicates that energy production during ischemia, which would sustain cellular ATP, is not essential for post-ischemic recovery. On the other hand Murry et al (17) have proposed that ischemic preconditioning lowers energy demand, preserves ATP and thereby causes a decreased rate of anaerobic glycolysis during ischemia. However, the lower glycolytic activity can also be due to a reduction of the amount of glycogen. Because oxygen deprivation during pretreatment can be argued to interfere with energy demand during subsequent ischemia we have also used glucagon to cause glycogen depletion. Our results show that in these hearts the extent of glycogen depletion and the rate of glycolysis during ischemia is similar to both the anoxic and preconditioned group, suggesting that the decrease of available substrate is the main reason for the reduction in glycolysis and the attenuation of metabolite accumulation and acidosis. Recently it was shown that when glycogen content was allowed to recover to original levels the effect of preconditioning disapears (31) explaining earlier findings that the effect of ischemic preconditioning is diminished by increasing the intervening period of reperfusion before the real insult (32).

From our present results we conclude that pre-ischemic glycogen depletion causes an attenuation of acidosis and metabolite accumulation during ischemia which is in agreement with other studies (30,31,33). It also caused a decrease of the amount of iron in the LMW pool at the end of ischemia compared to control hearts and lead to a better recovery. Based on the finding that hydrogen peroxide toxicity is increased after ischemia by an iron dependent mechanism (Voogd A, Sluiter W, Koster JF: submitted) it is likely that the iron is in the ferrous state. In that case the increased toxicity would be explained by the direct reaction between ferrous iron and hydrogen peroxide (Fenton reaction).

The mechanism of the iron release during ischemia is not known but a reductive release seems most likely. Direct mobilisation of ferritin iron by biological chelators has been shown to occur (34,35,36) but it is a slow process compared to reductive release (22,37,38). During ischemia reducing equivalents accumulate and therefore could cause the reductive release of iron from ferritin. This could explain that any pre-ischemic intervention that leads to a reduction of glycogen causing a lower rate of anaerobic glycolysis decreases ferrous iron accumulation and hence attenuates post-ischemic oxygen radical toxicity.

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CHAPTER SIX

IRON AND THE OXYGEN PARADOX: GENERAL DISCUSSION

Overview of the study.

In the present thesis the role of superoxide, hydrogen peroxide and iron was investigated after oxygen deprivation in the isolated rat heart. Until now the essential role of iron in post-ischemic oxygen toxicity was only shown indirectly with the use of chelators (88) or iron overloading (93). In chapter four it was shown that during no flow ischemia iron is released into a low molecular weight pool. Furthermore, the released amount is directly related to the duration of ischemia. The released iron is in the ferrous state allowing the Fenton reaction immediately upon reoxygenation. This is based on the increased susceptibility for hydrogen peroxide after an ischemic period which is prevented by iron chelation. Hydrogen peroxide toxicity after ischemia was assessed during anoxic reperfusion so that no superoxide was generated (22) which points to the presence of ferrous iron and hydrogen peroxide specific defences were not affected by the preceding ischemia. The role of iron was further substantiated by the fact that anoxia does not increase the susceptibility of for hydrogen peroxide which is explained by the fact that there is no iron in the LMW pool. The finding that LMW iron is increased in

ischemic but not in anoxic hearts indicates that the release is related to metabolite accumulation. Therefore, the effect of ischemic preconditioning (106,107) can ultimately be related to the LMW iron pool. The depletion of glycogen that occurs during preconditioning attenuates the accumulation of reduced metabolites and the release of iron.

In addition evidence is presented that superoxide dismutase is not always beneficial in post-ischemic rat hearts. After global ischemia ventricle fibrillation (VF) is induced while the enzyme prevents VF after LAD occlusion.

When all the data of the LMW iron content at the moment of reoxygenation and post-ischemic recovery are combined an inverse relation appears (figure 1). This relation is reminiscent of the reported inverse relation between lactate and recovery (108). It is proposed that metabolite accumulation, reflected in this lactate increase, causes the increase of iron in the LMW pool.

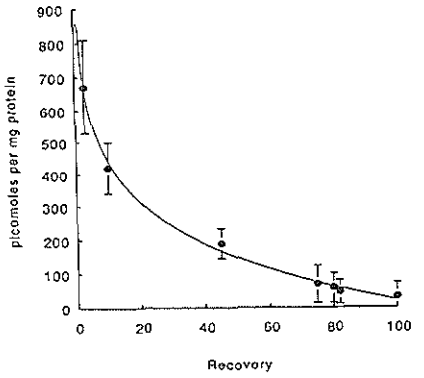

Figure 1. Relation between the amount of iron in the LMW pool and post-ischemic recovery. This data set contains all groups of hearts that were used in this thesis of which both LMW iron and recovery were measured.

Based on these findings a working hypothesis can be proposed that explains radical toxicity upon reoxygenation and is compatible with most of the available experimental evidence. Central point in this hypothesis is that it is the increase in ferrous iron in the low molecular weight iron pool during ischemia that makes the post-ischemic

tissue susceptible to the oxygen radicals. Reoxygenation injury is caused by the direct reaction between ferrous iron and hydrogen peroxide generated either directly or by the dismutation of superoxide that is formed upon reperfusion. This situation allows the Fenton reaction immediately upon reoxygenation and obliviates the need for a superoxide driven reductive release of iron in the early stages of reperfusion as proposed earlier (14,110). The disadvantage of the latter proposal being that it consumes superoxide and it is not very efficient (111) which seems to contradict the immediate burst of radical products observed upon reoxygenation (21,22). Results from chapter four and five showed that iron is released to some extent upon reoxygenation and it may contribute to the total catalytic iron pool but it is not essential.

The Fenton reaction in the reperfused tissue oxidises Fe^{2+} to Fe^{3+} and this must be reduced to participate in a following cycle. There is ample reducing power in the early stages of reoxygenation, including superoxide, to provide a reductor and cause the redox cycling of the iron. Although it seems likely, no solid experimental evidence is available to substantiate that this actually happens and that it is important.

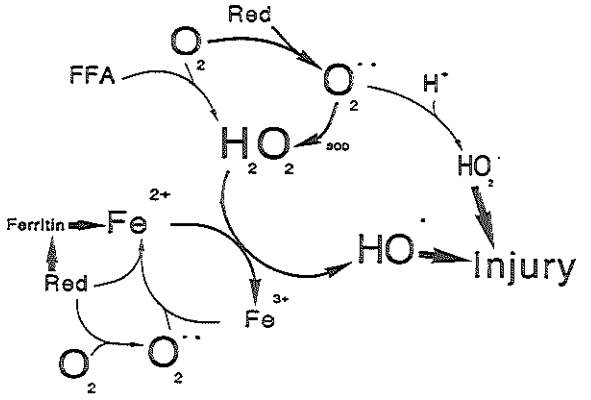

Figure 2. Reperfusion injury hypothesis. Ischemia causes an increase in protons, free fatty acids, reducing equivalents and ferrous iron. The catalytic iron reacts with hydrogen peroxide, generated by the dismutation of superoxide or by peroxisomal beta-oxidation to form the hydroxyl radical. Many reducing agents are available to regenerate the ferric iron, superoxide is only one of those. The lower pH in the early minutes of reperfusion may enhance hydroperoxyl mediated injury.

It cannot be ruled out that an iron independent component of oxygen toxicity exits. The protonated form of superoxide, the hydroperoxyl radical, may initiate lipid peroxidation independent of iron (32). Since this radical is uncharged it has better access to membranes and the pKa of the acid lies around 4.7 (30). so a decrease in intracellular pH due to ischemia, that recovers only slowly upon reoxygenation, will force more superoxide in the protonated form. This hypothesis (figure 2) will be examined in the light of other experimental evidence.

Mechanism of iron release during ischemia.

Central point in this hypothesis is the presence of reduced iron at the moment of reoxygenation. This is based on the presence of iron that is rapidly chelatable by DFX in supernatant of ischemic rat hearts (chapter 4). DFX chelates both Fe^{3+} and Fe^{2+} although with a different stability constant (112). Therefore, in these experiments we cannot make a distinction between Fe^{3+} and Fe^{2+}. The presence of Fe^{2+} is proposed on the basis of the hydrogen peroxide toxicity and on the mechanism that seems most plausible to explain the release.

Oxygen deprivation leads to complete cessation of mitochondrial ATP production and anaerobic glycolysis takes over using the endogenous glycogen as substrate. This causes the generation of lactate and protons which will accumulate in the cell during no flow ischemia (figure 3). After about fifteen minutes glycogen consumption stops and after about twenty minutes all glycolytic activity has stopped as shown by the cessation of glycogen consumption and lactate and proton accumulation. This is a compilation of the results of a number of studies that have measured these metabolites during cardiac ischemia. A number of studies have substantiated the inverse relation between lactate accumulation and post ischemic recovery. Conversely, there is no relation between ATP content and post-ischemic recovery (108,132)

From our finding that during anoxic perfusion no iron accumulates and that pre-ischemic glycogen depletion lowers iron release it is clear that iron release is related to accumulation of metabolites. Ferric iron can be extracted directly from ferritin by chelators (114,115) but this is a slow process. Reductive release from ferritin occurs in vitro with reducing agents such as $FMNH_2$, ascorbate, uric acid, GSH and cysteine

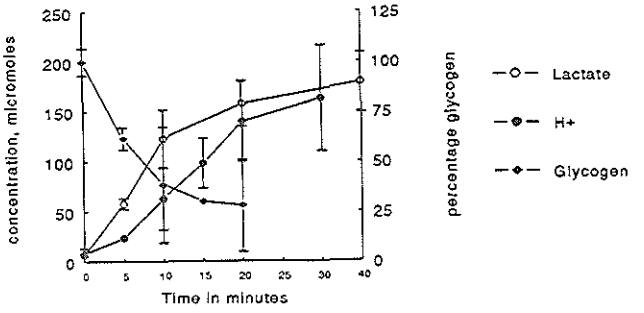

Figure 3.
Accumulation of substrate and tissue acidosis with the concomittant decline of glycogen during no flow ischemia. Data were compiled from references 123,132,134,135,136,161 and 163. Glycogen is expressed as % of control (right y-axis).

(116,117,118,119,120) and this is a much faster process. Especially the reductive release by $FMNH_2$ is very rapid and is enhanced by a lower pH (117). In view of the ischemic acidosis such a mechanism could account for the LMW iron. Biemond et al have demonstrated that iron is released from ferritin by xanthine and xanthine oxidase in the presence of oxygen (121). The larger part of this activity could be inhibited by SOD showing that it was driven by superoxide. However, thirty percent could not be inhibited by SOD and was attributed to a direct transfer of electrons from xanthine/xanthine oxidase to ferritin. Topham (122) has shown that rat liver homogenate contains ferrireductase activity which drives the release of ferrous iron from ferritin using NADH, NADPH or xanthine as reducing agents in the absence of oxygen. The NADH dependent activity from rat liver had a Km for NADH of 2.79 mM. Cytosolic NADH concentration in rat myocytes has been estimated to be 0.89 mM (148). The lactate to pyruvate ratio increases during ischemia (123) this will increase cytosolic NADH. If an NADH dependent ferrireductase activity is present in rat hearts with a similar Km, the accumulation of this reductor during ischemia could well account for the iron release. The xanthine dependent activity, which was only two percent of the NADH dependent activity, could

be inhibited by allopurinol suggesting that xanthine dehydrogenase is involved. Both substrates hypoxanthine and xanthine accumulate during ischemia and could be oxidised by the enzyme and electrons transferred to ferritin iron. The same reaction, using molecular oxygen as electron acceptor, has been proposed to be a source of superoxide upon reoxygenation. The protective effect of allopurinol assigned to inhibition of this reaction. It is unlikely however, that the xanthine oxidase reaction is the only source of superoxide during reperfusion and therefore the extent of the protection of allopurinol is curious. It is very tempting to propose that the protective effect of allopurinol may in part be due to the inhibition of iron release during ischemia.

Superoxide Dismutase Protection

In an aqueous environment and in the absence of a chelator the Fenton reaction has a second order constant of only 76 (124). By chelation of the iron to the appropriate chelator this can be raised to 10^5 (100) which could cause a rapid burst of hydroxyl radical generation upon reperfusion in the present hypothesis. This may suffice to start the radical chain reactions needed to induce tissue injury and the contribution of the redox cycling of the iron might be negligible.

Superoxide dismutase has been tested in numerous studies of ischemia and reperfusion, both in vivo and in vitro, with the misconception that superoxide is toxic and hydrogen peroxide is not. In fact both are more or less non-toxic in the absence of a catalytic transition metal (29). However, the result were ambiguous with as many studies reporting positive as negative results (6,23). The SOD proponents explained the negative findings by, dose dependence, short plasma half life, failure of the enzyme to reach the site of superoxide generation and peculiarities of the experimental model. In addition assessment of infarct size by tetrazolium staining can be confounded by SOD, giving false positive results in in vivo studies. The hypothesis presented in this thesis yields a very straightforward explanation for the "no-effect" studies: Superoxide is not involved in the Fenton reaction and SOD will only speed up hydrogen peroxide formation. Ferrous iron is already present so it need not be liberated by superoxide. Therefore, SOD could only aggravate the situation. Indeed, it was shown that SOD induces ventricle fibrillation after

global ischemia in chapter two of this thesis and not to protect buffer perfused hearts after ischemia (125). This is compatible with our hypothesis. However, protection after global ischemia has been demonstrated in isolated rabbit (126) and rat hearts (127). Moreover, the protection of SOD against reperfusion arrhythmias after LAD occlusion in the isolated rat and guinea-pig heart has been extensively demonstrated elsewhere (128,129,130,131) and again in our study. In view of these studies and all other positive studies a superoxide specific contribution to reperfusion injury cannot be discounted. The presented hypothesis allows for several possibilities.

The protonated form of superoxide, the perhydroxyl radical, may initiate lipid peroxidation (32) and SOD will prevent its formation. There is no evidence that substantiates the role of the hydroperoxyl radical but in view of the acidosis and the pKa it should not be disregarded.

Secondly, the recycling of iron during reperfusion appears to be important and this task could be performed by superoxide. If additional SOD is present the enhanced dismutation could prevent reduction of the ferric iron generated in the Haber-Weiss cycle which would attenuate continuity of hydroxyl radical formation.

Thirdly, in the present study additional iron was released upon reoxygenation. This was inhibited by (+)-cyanidanol-3 (chapter four) and reperfusion with anoxic buffer, either with or without hydrogen peroxide (chapter five), did not cause a further increase in LMW iron, suggesting it is superoxide dependent.

Fourthly, in the cycle proposed by Winterbourne superoxide and SOD form a mechanism to detoxify radicals in general (83). In this scheme SOD takes two superoxide anions out of the cycle, producing one hydrogen peroxide, while recycling in the absence of SOD will produce one hydrogen peroxide (and one GSSG) for each superoxide. In this way SOD decreases the total production of hydrogen peroxide. If, as Winterbourne proposes, SOD has a regulatory role the amount of active enzyme may be just enough for the usual activity and insufficient for the attack during reoxygenation. Finally, superoxide is known to react with nitric oxide, the endothelium derived relaxing factor causing the generation of the hydroxyl radical through peroxynitrite (65,63).

Ischemic preconditioning.

Short periods of ischemia and reperfusion induce protection against a subsequent period of ischemia. This effect is known as ischemic preconditioning (106,107,131) and the mechanism is not understood. Our present hypothesis explains many of the effects of ischemic preconditioning.

It has been proposed that a short period of ischemia causes myocardial stunning. This is a state of decreased metabolic activity, which would lead to a better ATP status and thus afford protection (133,134). However, in subsequent studies stunning and preconditioning have been shown to occur separately (135) and no difference was found between ATP depletion during ischemia with or without preconditioning (132). More important is that ATP levels and recovery are not even correlated (108) but that accumulation of lactate is inversely related to recovery. The reduction in metabolite accumulation was achieved by pre-ischemic glycogen depletion. This is consistent with the findings that preconditioning and glycogen depletion attenuate ischemic acidosis (136,137) and improve post ischemic recovery. Preconditioning is a transient effect because it is lost when the intervening period is prolonged (146). This is explained by the recovery of glycogen levels that occurs in this period as was recently confirmed by Wolfe et al. (147). These findings do not adress the question why substrate accumulation and acidosis may be harmful. This issue was addressed in chapter six and it was shown that glycogen depletion before ischemia decreases the amount of LMW iron during ischemia. This is consistent with the proposal that release of iron is made possible either by accumulation reduced metabolites or acidosis, or both.

Several recent observations on "preconditioning" merit further discussion because these are not simply explained by our acidification hypothesis. Omar et al.(138) have shown that the protective effect of preconditioning disappears when glucose is replaced by pyruvate as post-ischemic substrate although normoxic hearts can utilise both substrates. The issue was addressed in preliminary experiments and largely confirmed. Rat hearts, subjected to anoxic perfusion to deplete glycogen before ischemia, do not regain contractile activity when reperfused either without glucose or with pyruvate. As shown in chapter five reperfusion with glucose leads to 80 % recovery. Without glycogen depletion there is no difference in recovery either with or without glucose, but

reperfusion with pyruvate leads to a marked increase of recovery. The latter finding could be due to the antioxidant effect of pyruvate (139) while the lack of recovery after glycogen depletion could be explained by the inability of post-ischemic hearts to utilise pyruvate. LDH in the coronary effluent was lower the glycogen depleted hearts showing that the lack of functional recovery was not due to tissue injury but to the lack of substrate.

Ischemia has been shown to enhance the expression of a group of proteins known as the heat shock proteins (140) and rat hearts that had been exposed to in vivo heat shock were less sensitive to ischemia and reperfusion (141). However, it has been shown that although HSP 70 was overexpressed in hearts from heat shocked rabbits, the hearts were not protected from ischemia any better than the controls (142). In addition, studies with blockers of both transcription and translation have been shown to be without effect on preconditioning making it unlikely that newly expressed enzymes are important. Although the issue is debated, the protective effect of heat shock on subsequent ischemia and reperfusion could lie in the fact that the amount of cardiac catalase was doubled (141) again putting hydrogen peroxide in the spotlight.

The source of the hydrogen peroxide and the effect of catalase.

Hydrogen peroxide is generated continuously during aerobic metabolism either directly or by the dismutation of superoxide. The peroxysomal beta oxidation of fatty acids accounts for 10 to 30 % of the total cardiac capacity for oxidation of fatty acid (143). During ischemia fatty acids accumulate (144) and are oxidized upon ischemia (145) and so this may be a direct source of hydrogen peroxide in reperfused myocardium. However, the peroxisomes contain abundant catalase, so it is not clear whether the hydrogen peroxide produced in these organelles during reperfusion has a chance to participate in reperfusion injury.

In general the studies in which catalase was added during experimental ischemia and reperfusion have shown a beneficial effect (6,23) underlining the role of hydrogen peroxide. In view of our finding that hydrogen peroxide specific defences were not affected it must be concluded that the reaction with iron is preferred. All this suggests that anti-oxidant defences are organised such that both superoxide and hydrogen

peroxide are allowed to exist in low concentrations, in order to be used for oxidative purposes. Danger is prevented by the sequestration of iron because this effectively stops unwanted oxidations. This fails during ischemia and thus added catalase may tip the balance.

Relation to in vivo studies and clinical relevance.

The presented hypothesis assigns a central role to metabolite accumulation and acidosis in the release of iron during ischemia and hence to radical toxicity early during reperfusion. This is consistent with many findings derived from in vitro studies.

In vivo the issue is much more complicated due to the complex and delicate balance that governs the interaction between granulocytes, platelets, endothelial cells, vascular smooth muscle and other elements of the vasculature to ensure homeostasis. Ample evidence exists that oxygen radicals are a part of that balance and are instrumental in the necrosis seen in infarcted areas. The majority of studies with iron chelation by deferoxamine have all shown a protective effect (6,100,149). This confidence has led to successful clinical trials using deferoxamine during cardioplegia in patients under going myocardial revascularisation (151). The studies that failed to document a protective effect all added deferoxamine after ischemia (150,152), too late to chelate the intracellular catalytic iron. The present hypothesis also stresses the possible benefits of anoxic reperfusion. This allows the washout of catalytic iron before oxygen is re-introduced, causing a better recovery. The gradual reperfusion that occurs during thrombolytic therapy may therefore be an inherent part of its success. It should be considered to perfuse transplanted with anoxic fluid before reoxygenation. The simplest form of organ preservation, hypothermic storage, may be effective not just because metabolism is slowed down, but because that prevents reductive iron release.

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SUMMARY.

Reintroduction of oxygen into ischemic tissue causes the formation of reactive oxygen species among which the oxygen radicals. This contributes to the tissue injury that becomes apparent upon reperfusion. The phenomenon is known as the oxygen paradox. It is known that iron enhances the toxicity of oxygen radicals and that iron is important in the pathology of reperfusion after ischemia. Although this had been shown extensively, it was left unexplained when and how the catalytic iron becomes available. The research described in this thesis addresses the interplay between reduced metabolites of oxygen, reactive oxygen species, and iron in the isolated rat heart.

In chapter two it is shown that superoxide dismutase, the enzyme that speeds up the conversion of superoxide to hydrogen peroxide, is not always beneficial in ischemia/reperfusion experiments showing that this is not the primary toxic species. In chapter three it was shown in hearts from iron loaded rats the iron is localised mainly in the endothelial cells and pericytes. However, no relation was found between functional deterioration and morphological abberations. Chapter four describes a new method to determine the amount of catalytic iron in ischemic hearts. It was shown that ischemia causes a dramatic rise in the amount of catalytic iron and hence predisposes the hearts to oxygen damage. Indeed, in chapter five it was shown that after ischemia rat hearts are more susceptible to hydrogen peroxide through an iron dependent mechanism. This enhanced toxicity could not be induced by anoxic perfusion, in which the hearts were perfused with nitrogen saturated buffer. During anoxia the amount of catalytic iron did not increase suggesting that the accumulation of glycolytic metabolites is essential for ischemic iron release. Subsequently, in chapter six it was shown that interventions that limit the accumulation of reducing equivalents during ischemia, did indeed attenuate the release of iron during ischemia and caused an improved post ischemic recovery.

These results of these studies lead to the conclusion that it is the reductive release of iron during ischemia that causes the toxicity of oxygen radicals upon reperfusion. The ferrous iron reacts with hydrogen peroxide to form the more toxic oxygen radicals that induce tissue injury.

SAMENVATTING.

Herintroductie van zuurstof in ischemisch weefsel leidt tot de vorming van reactieve zuurstof metabolieten waaronder de zuurstof radicalen. Dit draagt bij aan het functie verlies dat zichtbaar wordt tijdens reperfusie. Dit fenomeen staat bekend als de zuurstof paradox. Het is daarnaast bekend dat ijzer de giftigheid van zuurstof radicalen kan verhogen en dat ijzer betrokken is bij de pathologie die optreedt tijdens reperfusie. Hoewel dit in een aantal studies uitdrukkelijk was vastgesteld bleef het onduidelijk wanneer en hoe het ijzer in een katalytische vorm vrijkomt. Het onderzoek, beschreven in dit proefschrift, was gericht op de interactie tussen reactieve zuurstof metabolieten en ijzer tijdens ischemie en reperfusie in het geïsoleerde rattehart.

In hoofdstuk twee werd vastgesteld dat het enzym superoxide dismutase, dat de omzetting van superoxide naar waterstof peroxyde versnelt, niet altijd beschermt tegen zuurstof schade. Dit toont aan dat superoxide niet de primaire toxische metaboliet is. In hoofdstuk drie werd aangetoond dat in harten van met ijzer beladen ratten het ijzer ophoopt in de endotheelcellen van de coronairvaten. Er bleek echter geen verband tussen het eerder vastgestelde functie verlies en morfologische schade. Hoofdstuk vier beschrijft een nieuwe methode om de hoeveelheid katalytisch ijzer te meten. Hiermee werd vastgesteld dat deze hoeveelheid zeer sterk toeneemt tijdens ischemie hetgeen voorspelt dat ratteharten na ischemie veel gevoeliger zijn voor zuurstof radicalen. Inderdaad werd in hoofdstuk vijf vastgesteld dat waterstof peroxyde, via een ijzer afhankelijk mechanisme, veel giftiger is voor ratteharten na ischemie dan daarvoor. Dit geldt niet voor anoxie, waarbij het hart wordt geperfundeerd met een buffer die verzadigd is met stikstof. Hierbij nam ook de hoeveelheid katalytisch ijzer niet toe en dat wijst erop dat de accumulatie van metabolieten van de anaërobe glycolyse essentieel is voor het vrijkomen van ijzer. Dit werd in hoofdstuk zes onderzocht. Uit deze resultaten bleek dat wanneer de accumulatie van reductie-equivalenten tijdens ischemie wordt verhinderd, de hoeveel vrijgekomen ijzer ook daalt en het post-ischemisch herstel sterk verbeterde.

Deze resultaten ondersteunen de conclusie dat de zuurstof paradox optreedt omdat ijzer via een reductief mechanisme vrijkomt tijdens de ischemie. Het katalytische Fe^{2+} reageert direct met het gevormde waterstof peroxyde tot de veel giftiger zuurstof radicalen die de oorzaak zijn van de weefselschade tijdens reperfusie.

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Curriculum Vitae.

Arthur Voogd werd 10 juli 1959 geboren te Oude - Tonge. Na het behalen van het Atheneum-B diploma aan de Rijkscholengemeenschap Goeree - Overflakkee in 1978 begon hij de studie Scheikunde aan de Universiteit van Amsterdam. Hij behaalde in 1983 het kandidaats examen en na enige commerciële omzwervingen het doctoraal in 1987. Tijdens het hoofdvak Moleculaire Biochemie, onder leiding van Dr. P. Sloof en Prof. Dr. L.A. Grivell, bepaalde hij de sequentie van 5.2 kb maxicirkel DNA van C. fasciculata. Tijdens het bijvak Endocrinologie, onder leiding van Prof. Dr. J.J.M. de Vijlder, heeft hij gewerkt aan de zuivering van humaan schildklierperoxidase. In 1987 begon hij als AIO bij afdeling Farmacologie en in 1989 bij de Afdeling Biochemie onder leiding van Prof. Dr. J.F. Koster alwaar dit proefschrift werd bewerkt.

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