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The Role of Antioxidants in Myocardial Ischemia-Reperfusion Injury

by

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Abbreviations:

AA: ascorbic acid;
$[Ca^{2+}]i$: intracellular concentration of calcium;
CuZnSOD: cupper zinc superoxide dismutase;
DHA: dehydroascorbic acid;
$+dP/dt$: rate of pressure development;
$-dP/dt$: rate of pressure decline;
ECSOD: extracellular superoxide dismutase;
GPx: glutathione peroxidase;
GR: glutathione reductase;
GSH: glutathione;
GSSG: glutathione disulfide;
LVDP: left ventricular developed pressure;
LVEDP: left ventricular end-diastolic pressure;
MDA: malondialdehyde;
MnSOD: manganese superoxide dismutase;
NAD^+: nicotinamide adenine dinucleotide;
NADPH: nicotinamide adenine dinucleotide phosphate;
NFkB: nuclear factor kappa B;
$ONOO^-$: peroxynitrite;
ROS: reactive oxygen species;
SL: sarcolemmal;
SOD: superoxide dismutase;
SR: sarcoplasmic reticulum

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Abstract

Myocardial ischemia-reperfusion causes damages to the myocardium including vascular and microvascular injury, dysfunction of endothelial cells, myocyte edema, increased myocyte apoptosis, increased myocyte necrosis and cardiac contractile dysfunction. These changes are primarily considered to be the consequence of increased reactive oxygen species (ROS) during ischemia-reperfusion. Many studies have shown the deleterious effects of ROS on myocardium by both direct and indirect measurements. Various antioxidants such as superoixde dismutase (SOD), catalase, glutathione peroxidase (GPx) have been shown to prevent these myocardial ischemia-reperfusion injury. The severity of ischemia-reperfusion injury and the subsequent level of oxidative stress, the interaction of antioxidants with ROS may determine the effectiveness of antioxidants for cardioprotection. The conversion of xanthine dehydrogenase to xanthine oxidase during ischemia-reperfusion injury is in part contributed to the increased production of superoxide ($O_2^{\bullet -}$). Xanthine oixdase inhibitors can afford a promising myocardial protection. Overexpression of various antioxidants such as manganese SOD (MnSOD) can protect against myocardial ischemia-reperfusion injury. This paper will focus on oxidative stress and the function of antioxidant enzymes in myocardial ischemia-reperfusion.

Introduction

Myocardial ischemia-reperfusion injury may occur as damage to the myocardium following blood restoration after a critical period of coronary occlusion [1]. It is a clinical problem

associated with procedures such as thrombolysis, angioplasty and coronary bypass surgery which are commonly used to establish the blood reflow and minimize the damage of the heart due to severe myocardial ischemia. The ischemia-reperfusion injury includes a series of events: (a) reperfusion arrhythemias, (b) microvascular damage, (c)myocardial stunning 'reversible mechanical dysfunction' and (d) cell death, which may occur either together or seperately [2]. There are two main hypotheses, namely oxidative stress and Ca^{2+}-overload, which have been proposed to explain the pathogenesis of ischemia-reperfusion injury [2,3]. Both mechanisms are most likely related to each other but it is not known whether they operate simutaneously or if one precedes the other (Figure1) [4].

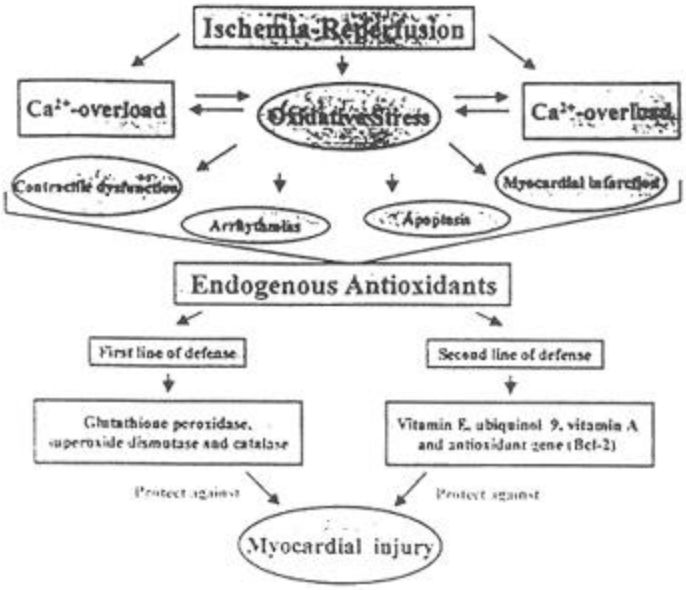

Figure 1. Schematic diagram showing pathophysiologic and therapeutic implications of oxidative stress and endogenous antioxidants in ischemia-reperfusion injury in the heart (from 4).

Pathophysiological Features of Reperfusion Injury

Vascular and microvascular injury

In the majority of experimental models of reperfusion injury release of vascular occlusion does not lead to full restoration of blood flow to the ischemic territory. Instead, flow often declines following the initial reactive hyperaemia. Possible causes for the 'no reflow' phenomenon are endothelial cell swelling, impaired nitric oxide release and capillary occlusion with platelets/neutrophils [5]. A variety of associated processes are also implicated including complement activation, increased endothelial permeability, $O_2^{\bullet -}$ generation, microemboli, cytokine release and activation of platelets with release of serotonin and ADP. Oxygenated reperfusion also paradoxically leads to further development of microvascular incompetence and in addition leads to ultrastructural damage in severely ischemic myocardium. Thus, ischemia-reperfusion can result in early and severe injury to the vasculature which may further jeopardize the survival of the myocytes. Whilst ischemia alone can cause endothelial disturbance and smooth muscle injury, reperfusion can inflict microvascular damage which may compromise the return of normal coronary perfusion. This can range from a transient increase in vascular resistance which is initiated during ischemia, to a sustained loss of competent capillaries and total 'no-reflow' tissue non-reperfusion. Although the latter is less important for infarcted myocardium, 'no-reflow' may be important in potentially salvageable (often referred to as 'stunned') myocardium, for example, after successful thrombolysis [6].

Endothelial dysfunction

Considerable experimental evidence points towards endothelial dysfunction in reperfusion injury and also indicates that endothelial cell damage (increased permeability) occurs following reperfusion due to oxygen-dependent mechanism [7]. Endothelial cells may themselves be a

source of $O_2^{\bullet-}$ during reperfusion. When endothelial cells are reoxygenated, $O_2^{\bullet-}$ is produced. Thus, endothelial cells are not only a source of oxygen free radicals but also a target.

Accelerated cell necrosis of reperfusion

Although ischemia will ultimately destroy living tissues and cause a number of recognizable ultrastructural and biochemical changes, reperfusion of doomed cells may alter the rate and manner of necrosis. The latter changes may involve cell edema, disruption of the sarcolemma, contracture and increased leakage of enzymes [8]. These changes may be produced by damage to cell membranes or disturbance of ion homeostasis, for example, too much calcium and sodium during reintroduction of oxygen.

Apoptosis in ischemia-reperfusion

Apoptosis or programmed cell death is a distinct form of destruction of the cell which is associated with synthesis of enzymes that degrade and fragment its own DNA. The signal pathway of apoptosis involves the stimulation of cell membrane death receptor which leads to the activation of caspases (aspartate-specific proteases), protein cleavage, DNA fragmentation and cell death [9]. It has been shown that myocardial ischemia-reperfusion is associated with an increase in apoptotic cells [9]. However, the exact mechanisms underlying the induction of this apoptotic process and the long term consequences of this process in myocardial ischemia-reperfusion are not completely understood. Hypoxic conditions, upregulations of death receptors, and oxidative stress have been suggested to trigger apoptosis during ischemia-reperfusion [10]. The study has shown upregulation of an antioxidant oncogene by ischemic adaptation which is associated with a reduction of cell apoptosis and DNA fragmentation [10]. Furthermore, it was demontrated that ROS generated during preconditioning can activate

antioxidant oncogene by stimulating a specific nuclear transcription factor (NFkB) which in turn reduces apoptosis. This signal pathway reducing apoptosis was blocked by free radical scavengers. This results indicate an additional antioxidant pathway for the protection of myocardium by ischemic preconditioning.

Metabolic changes in myocardial ischemia-reperfusion

A severe and sustained reduction in blood flow to the myocardium reduces oxidative phosphorylation leading to failure to resynthesize energy-rich phosphates including ATP and creatine phosphate (Table 1) [11]. Eventually, the purine precursors necessary for resynthesis of ATP are degraded to hypoxanthine and xanthine, both substrates for the enzyme xanthine dehydrogenase/oxidase. At the same time the enzyme changes conformation from its dehydrogenase (D form) to become an oxidase (O form) (Figure 2) [11].

Table 1. Metabolic and ultrastructural changes during myocardial ischemia-reperfusion injury (from 11).

ATP loss
Creatine phosphate loss
Potassium ions into the extracellular environment
Active tension generating capacity lost
Myocytes swell
Acidosis
Ion homeostasis lost
Defective ATP resynthesis
Structural disorganization
Electrically unstable and are unable to relax
Depletion of glutathione

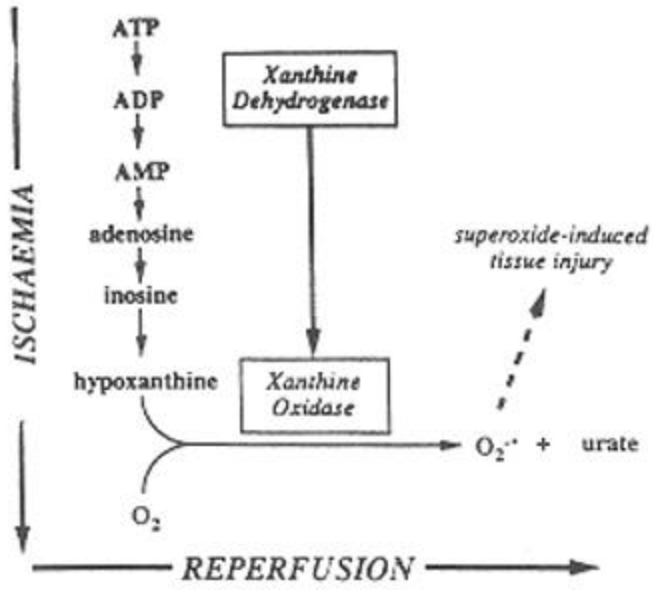

Figure 2. The simultaneous conversion of the enzyme xanthine dehydrogenase to its oxidase form and the breakdown of purine nucleotides to hypoxanthine create an ideal environment for the production of $O_2^{\bullet-}$ when O_2 is readmitted at reperfusion (from 11).

With the profound reduction in energy stores active tension generation is reduced, ion homeostasis is lost with leakage of potassium ions into the extracellular environment and calcium ions into the cytoplasm. Ischemia proceeds until the myocytes are swollen, acidotic, and show signs of structural disorganization.

Cellular ischemia also leads to the depletion of glutathione. Glutathione (GSH) is the major intracellular nonprotein sulphydryl and plays an important role in the maintenance of cellular proteins and lipid in their functional state. Glutathione acts primarily to protect these important structures against the threat of oxidation. The reducing equivalents of glutathione are used at the expense of its own oxidation to its conjugated form (GSSG) (Figure 3) [11]. Glutathione has a number of important roles including, (I) detoxification of intracellular oxidants as diverse as hydrogen peroxide, toxic intermediates of drug metabolism and lipid peroxides, and (II) the regeneration of ascorbate (AA) from dehydroascorbate (DHA), its oxidized form. When GSH

is experimentally reduced (e.g. by protein deficiency or with diethylmaleate) the toxic effects of oxidant stress are exacerated.

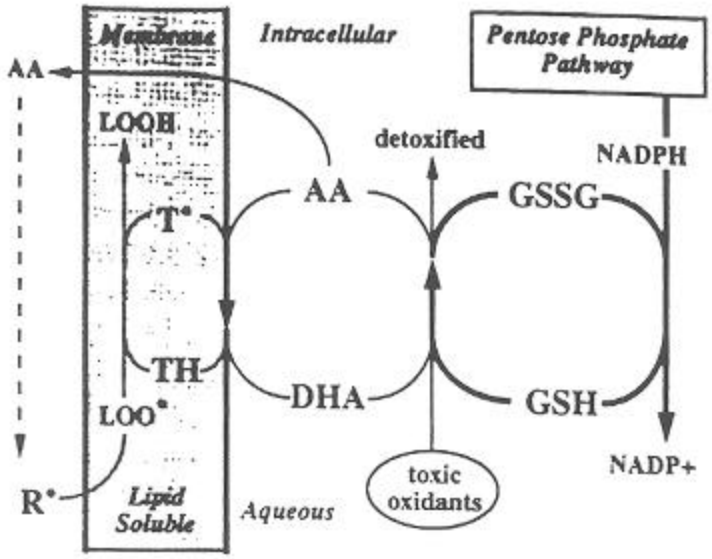

Figure 3. Glutathione acts as an intracellular antioxidant and is regenerated from its oxidized form (GSSG) using reducing equivalents from pentose phosphate pathway (from 11).

Oxidative stress species in myocardial ischemia-reperfusion

Oxidative stress is usually associated with increased formation of reactive oxygen species (ROS). Several studies have proposed the essential role of ROS in the pathogenesis of myocardial ischemia-reperfusion injury. Reactive oxygen species including hydrogen peroxide (H_2O_2), $O_2^{\bullet -}$, hydroxyl radical ($HO^{\bullet}$) and peroxynitrite ($ONOO^-$) have been shown to increase upon reperfusion of the heart following ischemia [12]. In ischemia-reperfused hearts, many alterations such as depression in contractile function, arrhythmias, change in gene expression, and loss of adrenergic pathways have been observed. Similar changes have been observed in hearts perfused with various ROS generating systems. Furthermore, pretreatment of cardiac subcellular organelles with ROS also showed similar changes [13]. Thus, alterations in the myocardium during ischemia-reperfusion were suggested to be in part duo to oxidative stress.

It was observed that global ischemia (30min) followed by reperfusion (60min) in isolated rat hearts was associated with depressed contractile function as indicated by decreased left ventricular developed pressure (LVDP), +dP/d*t* (rate of pressure development), −dP/d*t* (rate of pressure decline) and increased left ventricular end-diastolic pressure (LVEDP). In addition, ischemia-reperfusion was found to increase H_2O_2, intracellular calcium ($[Ca^{2+}]i$), malondialdehyde (MDA) content and the formation of conjugated dienes in the heart. Treatment of the heart with antioxidant enzymes such as SOD plus catalase protected against these changes [14]. An increase in the formation of ROS during ischemia-reperfusion was also reported by using the electron paramagnetic resonance technique [15]. Reactive oxygen species seem to increase significantly after a few minutes of reperfusion but its increase during ischemia alone is still controversial. On the basis of these changes it has been suggested that the increase of H_2O_2 production and other ROS during ischemia-reperfusion leads to lipid peroxidation and sulfhydryl group oxidation. Peroxynitrite ($ONOO^-$) has been shown to cause deleterious effects in the heart following ischemia-reperfusion [16]. Peroxynitrite is formed by a fast biradical reaction of nitric oxide and superoxide anion mainly in the endothelium, myocytes, and neutrophils. Although $ONOO^-$ is not a free radical , its intermediate can nitrate and hydroxylate phenolic compounds especially at tyrosine residues which in turn alter the activities of essential proteins and enzymes. Peroxynitrite has been reported to produce cellular damage by lipid peroxidation and DNA fragmentation in the heart in addition to inducing depletion of antioxidants.

The mechanism that oxidative stress causes various damages to myocardium is not very clear. Oxidative stress may modify phospholipids and proteins leading to lipid peroxidation and

oxidation of thiol groups [4]. These changes are considered to alter membrane permeability and configuration in addition to producing functional modification of various cellular proteins. Oxidative stress may result in cellular defects including a depression in the sarcolemmal (SL) Ca^{2+}-pump ATPase and Na^{+}-K^{+} ATPase activities. These changes lead to decreased Ca^{2+}-efflux and increased Ca^{2+}-influx, respectively. Oxidative stress has also been reported to depress the sarcoplasmic reticulum (SR) Ca^{2+}-pump ATPase and thus inhibit Ca^{2+} sequestration from the cytoplasm in cardiomyocytes [4]. The oxidative stress -induced changes in the SR Ca^{2+}-pump as well as SL Na^{+}-K^{+} pump are not limited to cardiomyocytes but have also been observed in the coronary artery smooth muscle cells. These alterations were markedly reduced by antioxidants such as catalase and SOD. The depression in Ca^{2+}-regulatory mechanism by ROS ultimately results in $[Ca^{2+}]i$ overload and cell death. On the other hand, an increase in $[Ca^{2+}]i$ during ischemia induces the conversion of xanthine dehydrogenase to xanthine oxidase and subsequently results in generating $O_2^{\bullet -}$ [Figure 2].

Types of antioxidants

Myocardial antioxidants are defined as substances which inhibit or delay the oxidative damage to subcellular proteins, carbohydrates, lipids and DNA. Although the exact mechanisms and interactions among various antioxidants are not fully understood, it is possible that one antioxidant may equilibrate with another to establish a cellular redox potential and thus all endogenous antioxidants may act in concert to protect against oxidative insult [17]. It has been suggested that antioxidants can act through several mechanisms such as: (a) scavenging ROS or their precursors, (b) inhibiting the formation of ROS, (c) attenuating the catalysis of

ROS generation via binding to metal ions, (d) enhancing endogenous antioxidant generation and (e) reducing apoptotic cell death by upregulating the anti-death gene [17]. Antioxidants are classified as endogenous antioxidants and exogenous antioxidants. The most important endogenous antioxidants are SOD, catalase, GPx. Superoxide dismutase catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 . Subsequently H_2O_2 is reduced to H_2O and O_2 by peroxidases such as GPx or catalase. Superoxide dismutase is present in the cytoplasm as well as on the endothelial cell surface with either copper or zinc (CuSOD, ZnSOD) and in the mitochondria with manganese (MnSOD) [18]. Glutathione peroxidase catalyzes the peroxidation of H_2O_2 in the presence of reduced glutathione (GSH) to form H_2O and oxidized glutathione. The GSSG recycles back to give GSH by glutathione reductase (GR), which requires NADPH from the hexose monophosphate shunt. Thus, GPx plays a significant role as H_2O_2 scavenger in the heart since its activity is much higher than catalase. On the other hand, catalase is a membrane bound enzyme which is present in peroxisomes but its activity has also been observed in the mitochondrial matrix [19]. Other endogenous antioxidants including vitamin E (tocopherols), vitamin C (ascorbic acid), and vitamin A are also present in the myocardium [20].

Role of antioxidants in myocardial ischemia-reperfusion injury

Various studies have been reported the beneficial effects of antioxidants as these agents render resistance to the heart against the ischemia-reperfusion injury [21]. It has been reported that a depletion of endogenous antioxidants in the ischemic heart upon the severity of ischemia-reperfusion [22]. Hydrophilic antioxidants such as ascorbate and glutathione were decreased

during 40 min of reperfusion, but not after ischemia; their oxidized forms (dehydroascorbate and glutathione disulfide) were markedly increased during reperfusion in the isolated rat hearts. On the other hand, lipophilic antioxidants, such as ubiquinol 9 and vitamin E, did not change during ischemia-reperfusion. However, increasing the severity of ischemia-reperfusion by adding H_2O_2 resulted in tissue lipophilic antioxidant depletion. Addition of H_2O_2 (500 μM) during ischemia-reperfusion resulted in a decrease in tissue vitamin E and ubiquinol 9 by 65% and 95%, respectively. These results suggest that ascorbate and reduced form of glutathione may act as a first line of defense against oxidative stress during ischemia-reperfusion while vitamin E may act later on during severe oxidative stress (Figure 1).

Catalase and SOD in myocardial ischemia-reperfusion

Recently, direct evidence, using a genetically engineered animal model, has been presented to show the importance of catalase and SOD in protecting the myocardium against ischemia-reperfusion injury [23]. Although the activity of catalase in the myocardium has been reported to be low, many studies have revealed its important role of protecting the heart from ischemia-reperfusion [24]. Using 3-amino triazole, an inhibitor for catalase, it was shown that 57% of the mitochondrial H_2O_2 was inhibited by catalase in isolated rat hearts [25]. 3-amino triazole was found to decrease the recovery of changes in contractile function and SR Ca^{2+}-pump ATPase activity following 10 min of global ischemia. It was also shown that 3-amino triazole resulted in a decrease in lipid radical release due to 10 min of global ischemia in the isolated rat hearts as well as a reduction of the reperfusion arrhythmias after 5 min of regional ischemia in the isolated rat hearts *in vivo*. On the other hand, several studies did not observe such effects of 3-amino triazole on the recovery of contractile function or lipid peroxidation during reperfusion with

H_2O_2 [26]. Thus, the demonstration regarding the protective effects of endogenous catalase in ischemia-reperfusion is controvarsial and further studies are needed to resolve these controversial results.

Conflicting results regarding the protective effects of SOD against ischemia-reperfusion injury have been also reported. A study has shown that myocardial mitochondrial MnSOD activity was decreased only after a long period of ischemia (60 min) [27]. However, another study has reported that even with 60 min of ischemia, CuZnSOD did not change in the rat heart while myocardial GPx and GR activities were increased [28]. It has also been shown that SOD plus catalase prevented the changes in myocardial function and Ca^{2+}-handling in isolated rat hearts subjected to 30 min of global ischemia followed by 60 min of reperfusion [29]. The contractile function represented by LVPD, +dP/d*t and* −dP/d*t* was improved by 69%, 72% and 83%, respectively, whereas the increased LVEDP was markedly reduced in the SOD plus catalase treated hearts in comparison to the untreated ischemia-reperfusion group. SR Ca^{2+}-uptake in the SOD plus catalase treated ischemia-reperfusion hearts was recovered from 12.5 nmol/mg protein /min to 22.4 nmol/mg protein /min which is almost the same value as in the control group [30]. Superoxide dismutase plus catalase also protected the decrease in ryanodine and EGTA-sensitive SR Ca^{2+}-release as well as the density of ryanodine binding sites in the ischemia-reperfused hearts. These results suggest that the protective effect of SOD and catalase may be due to improving the SR Ca^{2+}-regulating mechanisms. Such studies provide evidence regarding the importance of endogenous antioxidant enzymes such as SOD and catalase in ameliorating the oxidative stress injury in animals.

Glutathione and Glutathione peroxidase in myocardial ischemia-reperfusion

An increasing number of investigators have demonstrated the importance of GPx in protecting myocardium from ischemia-reperfusion. The inhibitors of GPX such as maleic acid diethyl ester or buthionine sulfoxamine can reduce the recovery of contractile function in isolated rat and cat hearts following ischemia or perfusion with peroxide-derived free radicals [31]. The deficiency of a co-enzyme such as selenium, which is required in maintaining the glutathione redox cycle, also renders the isolated rat heart more susceptible to oxidative injury. Thus, enhancing the activity of endogenous GPx is still a promising avenue for protecting the heart against ischemia-reperfusion injury.

Hypothesis

Hypothesis 1: Xanthine oxidase inhibitors can protect against myocardial ischemia-reperfusion injury.

Rationale:

From figure 2, we can know that a potential source of reactive oxygen-derived free radicals in ischaemic and reperfused tissues is the xanthine dehydrogenase/oxidase. The enzyme xanthine dehydrogenase/oxidase is synthesized as xanthine dehydrogenase (type D) which accounts for 90% of the enzyme in healthy tissues [32]. This type D form uses nicotinamide adenine dinucleotide (NAD) as an electron acceptor during oxidation of xanthine:

$$\text{Xanthine} + H_2O + NAD^+ \rightarrow \text{Uric Acid} + NADH + H^+$$

Alternatively, the enzyme can exist as the oxidase (type O) using molecular oxygen as an electron acceptor to produce $O_2^{\bullet-}$:

$$\text{Xanthine} + H_2O + 2O_2 \rightarrow \text{Uric Acid} + 2O_2^{\bullet-} + 2H^+$$

It has been established that conversion of the enzyme from the D-form to the O-form occurs as a result of tissue ischemia [33]. This may be precipitated by depletion of ATP and subsequent loss of control over membrane calcium gradients. Increased cytosolic calcium concentration activates a calcium-dependent proteases which in turn converts the dehydrogenase D-form to an oxidase O-form [34]. The failure to resynthesize ATP due to interruption of oxidative phosphorylation promotes the breakdown of adenine nucleotides such as AMP, and subsequently to adenosine, inosine, hypoxanthine and xanthine. The latter two substances are oxidizable substrates for xanthine oxidase and build up in ischaemic tissues [35]. Only when the molecular oxygen is readmitted at the time of reperfusion will rapid production of $O_2^{\bullet-}$ occur. Although xanthine dehydrogenase was not found in human and rabbit hearts, a considerable amount of the enzyme was detected in vascular endothelial cells by histochemical methods.

So, if we can use some substances that can inhibit the conversion of dehydrogenase D-form to oxidase O-form during myocardial ischemia, it will block the production of $O_2^{\bullet-}$.

Experimental design and methods:

In the experiments we need to determine the xanthine oxidase/xanthine dehydrogenase contents of myocardium in normal myocardium and ischemia-reperfusion region. Briefly, Dog is prepared with open chest. The left anterior descending branch of the dog heart is ligated for about 30 min and then biopsies are removed from both the normal and the ischemic regions. The samples are homogenized and assayed for oxidase and dehydrogenase activity. Urate production is monitored at 295 nm in the absence of NAD^+ to determine the oxidase activity

and in the presence of NAD^+ to determine the dehydrogenase plus oxidase activity. The expected result is that the content of xanthine oxidase increases with ischemia.

Further experiment we can do is that dogs can be divided into two groups: control and xanthine oxidase inhibitor. Then, The infarct size, the risk zone size and the infarct as a percentage of the risk zone are measured and compared between these two groups.

Hypothesis 2: Overexpression of MnSOD protects against myocardial ischemia-reperfusion injury.

Rationale:

From figure 2, we can know that $O_2^{\bullet-}$ has been considered one of the important contributing factors to myocardial ischemia-reperfusion injury. Antioxidant enzyme SOD are a class of enzymes that catalyze dismutation of $O_2^{\bullet-}$ to form H_2O_2 and O_2. Three distinct types of SOD have been found in mammalian tissues: manganese-containing superoxide dismutase (MnSOD), copper/zinc-containing superoxide dismutase (CuZnSOD) and extracellular superoxide dismutase containing Cu and Zn atoms at the active sites(ECSOD)[36]. The conclusion that $O_2^{\bullet-}$ causes ischemia-reperfusion injury suggests that increasing activity of cellular antioxidant enzymes should protect tissues from reperfusion damage.

Experimental design and methods:

The experiment is carried out to introduce MnSOD intracellularly by transgenic technology. The impact of altered MnSOD activity on the functional recovery of the heart can be assessed in Langendorff perfused hearts. The effect of overexpression of MnSOD on myocardial function can be evaluated in an *in vivo* regional ischemia model. All of following experiments can be used to test this hypothesis.

Generation of MnSOD transgenic mice: In order to achieve a high level of transcription of the MnSOD gene in various tissues, human MnSOD transgene is constructed by placing the corresponding cDNA downstream to a human β-actin promoter and a 5' flanking sequence. This vector contains intron 1 sequence of the human β-actin gene to allow RNA splicing to occur.

Northern blot analysis: Northern blot analysis is performed on RNA isolated from the hearts of control and transgenic dogs. The blot is pre-hybridized and hybridized with a ^{32}P-labeled human MnSOD cDNA probe and washed under stringent conditions.

Western blot analysis: Western blot can be used to measure the MnSOD proteins from the hearts of control and transgenic dogs. Specimens are electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel and then semidry transferred to membrane. Membranes are blocked with 5% nonfat milk and sequentially immunoprobed with primary antibody followed by secondary antibody. Western blots are developed with ECL and imaged on hyperfilm.

Immunocytochemistry: Electron immunogold analysis of MnSOD in the hearts of non-transgenic mice is performed using a rabbit anti-rat SOD. MnSOD will be localized by protein A-gold immunocytochemistry. The antibody reacts with MnSOD with high specificity.

Spectrophotometric assay of antioxidant enzyme activity: MnSOD activity can be measured by the cytochrome C reduction inhibition assay. Briefly, MnSOD activity is measured by incubating amount of total specimen protein with cytochrome C, xanthine and xanthine oxidase as well as NaCN to inhibit endogenous CuZnSOD. The subsequent rate of cytochrome C reduction is determined spectrophotometrically at 550 nm.

Assessment of myocardial function after ischemic reperfusion: The ventricular functions of the mouse hearts can be measured by inserting a tiny plastic-wrapped balloon connected to a pressure transducer into the left ventricle via the mitral valves. Cardiac functions that are recorded before ischemia and after reperfusion, such as left ventricular developed pressures (LVDP), $\pm dP/dt$, heart rates and coronary flow rates, are used for comparison. The percentages of functional recovery are calculated by dividing each of the functional recovery values at the end of reperfusion by their corresponding pre-ischemic values. End-diastolic pressures prior to ischemia and after reperfusion are calculated from the functional curves. The perfusate flowing out of the heart is collected and measured. The coronary flow rate is determined by the amount of perfusate measured in a specific time period.

Regional ischemia *in vivo*: Mice will be anesthetized with chloral hydrate. An endotracheal tube can be inserted 5-8 mm from the larynx and the mice are ventilated with room air. The heart is exposed via a left lateral thoracotomy in the third intercostal space. After pericardiectomy, the left descending coronary artery is ligated near the middle of the heart using a curved needle with an nylon suture. The thorax is closed immediately, leaving one end of the coronary sutures exposed. Electrocardiography is recorded on the lead II electrode of a Microcomputer Augmented Cardiograph II, indicating a significant elevation of the ST segment. This signals successful ligation of the coronary artery. After artery occlusion, the thread is gently removed so that the heart can undergo reperfusion. The hearts are perfused as a Langendorff preparation for several minutes. The left coronary artery is occluded, and 1% Evans blue is infused into the aorta and coronary arteries to determine the area at risk. The heart is sectioned transversely into five sections, with one section at the site of the ligature. Macroscopic staining

with triphenyltetrazolium chloride is used to quantitate the infarct sizes. Both sides of each slice are imaged, and the area of infarction in each slice is determined by computerized planimetry using a digital imaging system, corrected for heart weight of each slice, and summed for each left ventricle. The area at risk is expressed as the percentage of the left ventricle, and the area of infarct is expressed as a percentage of the area at risk.

Summary

The hypothesis involving the role of ROS in myocardial ischemia-reperfusion injury has been supported by a wide range of investigation. Myocardial ischemia-reperfusion has been shown to result in vascular and microvascular injury, endothelial dysfunction, apoptosis, increased necrotic cell death, myocardial contractile dysfunction, arrhythmias and changes in gene expression. All of these damages to the myocardium are primarily contributed to the increased production of ROS. Various antioxidants such as SOD, catalase, GPx have been shown to prevent these myocardial ischemia-reperfusion injury. The conversion of xanthine dehydrogenase to xanthine oxidase in ischemia-reperfusion injury is in part contributed to the increased production of $O_2^{\bullet -}$. The blockage of xanthine oixdase will afford a profound degree of protection in ischemia-reperfusion injury. The severity of ischemia-reperfusion injury and the subsequent level of oxidative stress, the interaction of antioxidants with ROS may determine the effectiveness of antioxidants for cardioprotection. The development of high-efficiency large animal models of cardiac gene transfer will facilitate the evaluation of potential clinical applicability of antioxidant cardiac gene therapy and may have a particular role in high-risk

revascularization procedures such as angioplasty of multiple sequential lesions or surgical bypass involving reoperation, concomitant valvular procedures, or poor ventricular function.

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