Status of myocardial antioxidants in ischemia–reperfusion injury

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Abstract

Background: Myocardial ischemia–reperfusion represents a clinically relevant problem associated with thrombolysis, angioplasty and coronary bypass surgery. Injury of myocardium due to ischemia–reperfusion includes cardiac contractile dysfunction, arrhythmias as well as irreversible myocyte damage. These changes are considered to be the consequence of imbalance between the formation of oxidants and the availability of endogenous antioxidants in the heart. Observations: An increase in the formation of reactive oxygen species during ischemia–reperfusion and the adverse effects of oxyradicals on myocardium have now been well established by both direct and indirect measurements. Although several experimental studies as well as clinical trials have demonstrated the cardioprotective effects of antioxidants, some studies have failed to substantiate the results. Nonetheless, it is becoming evident that some of the endogenous antioxidants such as glutathione peroxidase, superoxide dismutase, and catalase act as a primary defense mechanism whereas the others including vitamin E may play a secondary role for attenuating the ischemia–reperfusion injury. The importance of various endogenous antioxidants in suppressing oxidative stress is evident from the depression in their activities and the inhibition of cardiac alterations which they produce during ischemia–reperfusion injury. The effects of an antioxidant thiol containing compound, N-acetylcysteine, and ischemic preconditioning were shown to be similar in preventing changes in the ischemic-reperfused hearts. Conclusions: The available evidence support the role of oxidative stress in ischemia–reperfusion injury and emphasize the importance of antioxidant mechanisms in cardioprotection. © 2000 Published by Elsevier Science B.V.

Keywords: Contractile function; Coronary disease; Free radicals; Ischemia

1. Introduction

Ischemia–reperfusion injury may occur as damage to the myocardium following blood restoration after a critical period of coronary occlusion [1]. In fact, ischemia–reperfusion 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 arrhythmias, (b) microvascular damage, (c) myocardial stunning ‘reversible mechanical dysfunction’ and (d) cell death, which may occur either together or separately [2–7]. There are two main hypotheses, namely oxidative stress and Ca2+-overload, which have been proposed to explain the pathogenesis of ischemia–reperfusion injury [8–11]. Both these mechanisms are most likely related to each other but it is not known whether they operate simultaneously or if one precedes the other (Fig. 1). Oxidative stress, which is usually associated with increased formation of reactive oxygen species (ROS), modifies phospholipids and proteins leading to lipid peroxidation and oxidation of thiol groups; these changes are considered to alter membrane permeability and configuration in addition to producing functional modification of various cellular proteins [12–15]. Oxidative stress may result in cellular defects including a depression in the sarcolemmal (SL) Ca2+-pump ATPase and Na+–K+ ATPase activities; these changes lead to decreased Ca2+-efflux and increased Ca2+-influx.
respectively [16,17]. 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 [18–21]. 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 [22–25]. These alterations were markedly reduced by antioxidants such as catalase and superoxide dismutase. The depression in Ca\(^{2+}\)-regulatory mechanism by ROS ultimately results in intracellular Ca\(^{2+}\) ([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 superoxide radicals [1]. 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 [26,27]. Allopurinol, an inhibitor of xanthine oxidase, has also been shown to be beneficial in protecting myocardium from ischemia–reperfusion injury [28–30]. Since Ca\(^{2+}\)-overload hypothesis has been discussed in detail elsewhere in recent reviews [9,11], this article is focussed on oxidative stress and antioxidant mechanisms in ischemia–reperfusion.

### 2. Role of oxidative stress in ischemia–reperfusion

Several studies have proposed the essential role of ROS in the pathogenesis of myocardial ischemia–reperfusion injury [31–37]. ROS including hydrogen peroxide (H\(_2\)O\(_2\)), superoxide radical, hydroxyl radical and peroxynitrite have been shown to increase upon reperfusion of the heart following ischemia [31–37]. In ischemic-reperfused hearts, many alterations such as depression in contractile function, arrhythmias, change in gene expression, and loss of adrenergic pathways have been observed [21,38]. In fact, similar changes have been reported in hearts perfused with various ROS generating systems. Furthermore, pretreatment of cardiac subcellular organelles with ROS showed similar changes [39,40]. Thus, alterations in the myocardium during ischemia–reperfusion were suggested to be in part due to oxidative stress. It should be pointed out that global ischemia (30 min) followed by reperfusion (60 min) in isolated rat hearts was associated with depressed contractile function as indicated by decreased left ventricular developed pressure (LVDP), \(+dP/dt\) (rate of pressure development), \(-dP/dt\) (rate of pressure decline) and increased left ventricular end-diastolic pressure (LVEDP).

In addition, ischemia–reperfusion was found to increase H\(_2\)O\(_2\), [Ca\(^{2+}\)]\(_i\), malondialdehyde (MDA) content and the formation of conjugated dienes in the heart. Treatment of the heart with antioxidant enzymes, superoxide dismutase (SOD) plus catalase protected against these changes [21,41]. An increase in the formation of ROS during ischemia–reperfusion was also reported by using the electron paramagnetic resonance technique [33,42,43]. ROS 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\(_2\)O\(_2\) production and other ROS during ischemia–reperfusion leads to lipid peroxidation and sulfhydryl group oxidation.

Peroxynitrite has recently been shown to cause deleterious effects in the heart following ischemia–reperfusion [44,45]. It is formed by a fast biradical reaction of nitric oxide and superoxide anion mainly in the endothelium, myocytes, and neutrophils [46–48]. Although peroxynitrite 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 [49–53]. Interestingly, a protective effect of peroxynitrite on myocardium was also observed [54,55]. Tissue concentration of peroxynitrite and the biological environment especially the presence of detoxifying agents seem to determine its deleterious or protective effects [56,57]. The exact mechanism of such effects of peroxynitrite is not clear; however, a detailed review on the potential role of peroxynitrite on the cardiovascular system has been published earlier [58].

### 3. 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 anti-oxidants are not fully understood, it is possible that one antioxidant may equilibrate with another to establish a cellular redox potential and thus all endogenous anti-oxidants may act in concert to protect against oxidative insult. Nonetheless, 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 metals ions, (d) enhancing endogenous anti-oxidant generation and (e) reducing apoptotic cell death by upregulating the anti-death gene (Bcl-2) [59]. Antioxidants are usually classified as endogenous antioxidants (produced within the body) and exogenous antioxidants (produced outside the body). Many substances have been suggested to act as endogenous antioxidants; however, we will focus on superoxide dismutase (SOD), catalase, glutathione peroxidase and vitamin E, which have been studied extensively (Table 1). Among exogenous anti-oxidants, some drugs have been reported to exert anti-oxidant action. These include thiol-containing compounds, β-adrenergic blockers, angiotensin converting enzyme inhibitors, iron chelating agents and Ca²⁺ antagonists [60,61].

SOD catalyzes the dismutation of superoxide anion (O²⁻) to H₂O₂. Subsequently H₂O₂ is reduced to H₂O and O₂ by peroxidases such as glutathione peroxidase or catalase. SOD 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) [62]. Glutathione peroxidase catalyzes the peroxidation of H₂O₂ in the presence of reduced glutathione (GSH) to form H₂O and oxidized glutathione (GSSG). The GSSG recycles back to give GSH by glutathione reductase, which requires NADPH from the hexose monophosphate shunt. Thus, glutathione peroxi- dase, plays a significant role as H₂O₂ 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 [63]. Other endoge- nous antioxidants including vitamin E (tocopherols), vita- min C (ascorbic acid), and vitamin A are also present in the myocardium [64–66]. Vitamin E is a fat soluble substance and is mainly associated with plasma lipopro- teins. It acts as a potent peroxyl radical scavenger via breaking the lipid peroxidation chain reaction [67].

4. Role of antioxidants in myocardial ischemia–reperfusion injury

Various studies have reported the beneficial effects of antioxidants as these agents render resistance to the heart against the ischemic–reperfusion injury [68–70]. However, other investigators have failed to observe such results [71–73]. Several factors can contribute to this discrepancy such as species difference, various experimental methods and techniques applied, different time periods of ischemia–reperfusion insults, as well as different types of anti-oxidants with different properties. Thus, there is a clear need for a systematic study to determine the exact role of antioxidants in protection against the ischemic–reperfusion injury. A recent investigation has reported a depletion of endogenous antioxidants in the ischemic heart upon reperfusion; this change was dependent upon the severity of ischemia–reperfusion [74]. Hydrophilic antioxidants such as ascorbate and glutathione were decreased during 40 min of reperfusion, but not after ischemia; their oxidized forms

<table>
<thead>
<tr>
<th>Name</th>
<th>Site</th>
<th>Action</th>
</tr>
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<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>–</td>
<td>Catalyzes O²⁻ dismutation to H₂O₂</td>
</tr>
<tr>
<td>Cu,Zn SOD</td>
<td>Cytoplasm, cell surface and mitochondrial membrane</td>
<td>2O²⁻ + 2H⁺ → H₂O₂ + O₂</td>
</tr>
<tr>
<td>Mn SOD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>Peroxisomes and mitochondrial membrane</td>
<td>H₂O₂ → 2H₂O + O₂</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>Cytoplasm</td>
<td>H₂O₂ + 2GSH → 2H₂O + GSSG</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Intracellular</td>
<td>Cellular reductant</td>
</tr>
<tr>
<td>Coenzyme Q10 (ubiquinone)</td>
<td>Cell membrane</td>
<td>Redox active electron carrier</td>
</tr>
<tr>
<td>Vitamin E (α-tocopherol)</td>
<td>Cytoplasm and plasma</td>
<td>Break lipid peroxidation chain and LDL reaction</td>
</tr>
<tr>
<td>β-Carotene (pro-vitamin A)</td>
<td>Plasma</td>
<td>Inhibits oxidation of LDL</td>
</tr>
<tr>
<td>Vitamin C (ascorbic acid)</td>
<td>Cytoplasm and plasma</td>
<td>Directly as an antioxidant or as a cofactor for vitamin E</td>
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(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$_2$O$_2$ resulted in tissue lipophilic antioxidant depletion. Addition of H$_2$O$_2$ (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 (Fig. 1). These differences in the lines of defense for the endogenous antioxidants may explain in part the conflicting results of vitamin E in various animal models and clinical studies [71–73].

4.1. Catalase and superoxide dismutase in 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 [75,76]. Although the activity of catalase in the myocardium has been reported to be low, many earlier studies have revealed its important role of protecting the heart from ischemia–reperfusion [77–79]. Using 3-amino triazole, an inhibitor for catalase, it was shown that 57% of the mitochondrial H$_2$O$_2$ was inhibited by catalase in isolated rat hearts [80]. In another study, 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 [81]. 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 rat hearts in vivo [63,82]. 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$_2$O$_2$ [83–85]. Thus, the demonstration regarding the protective effects of endogenous catalase in ischemia–reperfusion is controversial in the literature and further extensive studies are needed to resolve these controversial results.

Conflicting results regarding the protective effects of SOD against ischemia–reperfusion injury have also been reported. An earlier study has shown that myocardial mitochondrial Mn-SOD activity was decreased only after a long period of ischemia (60 min) [86]. Another study, however, has reported that even with 60 min of ischemia, Cu- and Zn-SOD did not change in the rat heart while myocardial glutathione peroxidase and reductase activities were increased [87]. To add more to the controversy, glutathione peroxidase and reductase activities were observed to be unaltered after a longer period of ischemia (90 min) in rabbit hearts [31]. On the other hand, alteration in Ca$^{2+}$ homeostasis in cardiomyocytes due to an increase in ROS has been suggested to explain the adverse effects of ischemia–reperfusion. It has 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 [21]. The contractile function represented by LVDP, +dP/dt and −dP/dt 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 to 22.4 nmol/mg protein/min which is almost the same value as in the control group [19]. SOD 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 ischemic-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 endogeneous antioxidant enzymes such as SOD and catalase in ameliorating the oxidative stress injury in animals; however, many questions regarding the status of both SOD and catalase activities as well as their interaction in the ischemic-reperfused hearts need to be answered by future investigations.

4.2. Glutathione peroxidase in ischemia–reperfusion

An increasing number of investigators have demonstrated the importance of glutathione peroxidase in protecting myocardium from ischemia–reperfusion. Earlier studies have used inhibitors for glutathione peroxidase such as maleic acid diethyl ester or buthionine sulfoximine which reduce the recovery of contractile function in isolated rat and cat hearts following ischemia or perfusion with peroxide-derived free radicals [88–90]. 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 [91]. Recently, a study has confirmed the essential role of glutathione peroxidase in cardioprotection against ischemia–reperfusion injury by using knockout mice; these transgenic mice exhibited markedly depressed contractile function, as seen by decreased developed force of the heart as well as the first derivative of developed force (dF/dt), following 30 min of ischemia and 120 min of reperfusion. Furthermore, the damage to cardiomyocytes, as indicated by creatine kinase release and size of infarction, was significantly higher in transgenic mice compared to non-transgenic mice [92]. Thus, enhancing the activity of endogenous glutathione peroxide is still a promising
avenue for protecting the heart against ischemia–reperfusion injury.

4.3. Vitamin E in ischemia–reperfusion

Vitamin E (α-tocopherol) is a fat soluble antioxidant, which is mainly present in the plasma, low density lipoprotein particles and the cell membrane. Some studies have demonstrated that vitamin E inhibited the oxidation of low density lipoprotein and subsequently protected against various deleterious effects on vascular wall such as deposition of atherogenic oxidized low density lipoprotein and proliferation of smooth muscle cells [68,93]. Vitamin E-treated rats showed a decrease in mortality and infarct size due to coronary occlusion by approximately 50% compared to the control group; the arrhythmic changes were also attenuated significantly [94]. In addition, vitamin E supplement prevented the depression of left ventricular function, as well as the elevation of malondialdehyde content and conjugated diene formation in the infarcted rat heart [94]. These results indicate that the beneficial effects of vitamin E in the acute ischemic syndromes may be occurring through the reduction of oxidative stress. Despite the evidence regarding the protective effects of vitamin E in animal models against ischemia–reperfusion, observational and epidemiological data on patients are contradictory [64,67,68,70]. The hypothesis which suggests that vitamin E may act as a second line of defense (Fig. 1), may explain in part these conflicting results. The beneficial effects of vitamin E may be related to the degree of the reactive oxygen production and subsequently to the severity of coronary artery disease. For example, vitamin E may be more beneficial in patients with massive myocardial infarction rather than in patients with typical angina pectoris. Obviously further studies are needed to establish this hypothesis which would clarify the exact mechanism of protection by vitamin E in ischemic heart disease.

4.4. Antioxidants protect the alteration of β-adrenoreceptor signal pathway in ischemia–reperfusion

β-Adrenergic receptors, G-protein and adenylyl cyclase system play an important role in regulating the heart function [39,69,95–98]. Conflicting results have been reported by many investigators regarding changes in the β-adrenoreceptor linked signal transduction which occurs during ischemia–reperfusion [7,99–101]. It has been shown that isolated rat hearts when subjected to ischemia (30 min)–reperfusion (60 min) show a depression in the contractile activity and a decrease in the inotropic responses to isoproterenol [69]. Furthermore, the densities of β₁ and β₂-adrenoreceptors were decreased in the ischemic-reperfused hearts and the isoproterenol-stimulated adenylyl cyclase activity was depressed. However, the basal and forskolin-stimulated adenylyl cyclase activities as well as stimulatory G-protein and inhibitory G-protein levels were increased following reperfusion. Since these changes were prevented by adding SOD plus catalase in the perfusion medium, it was evident that antioxidants protected against alterations in the β-adrenoreceptor linked signal transduction mechanism following ischemia–reperfusion [69]. It should be mentioned that SOD in absence of catalase did not prevent these changes, which indicated that H₂O₂, but not superoxide, was more likely to be involved in the β-adrenoreceptor linked signal transduction alterations due to ischemia–reperfusion. These results were further substantiated by treating the rat heart membranes with H₂O₂ which showed changes in the β-adrenoreceptor linked signal transduction pathway similar to those seen with ischemia–reperfusion [38,69]. These reports are also consistent with those of other investigators showing a depression in β-adrenergic receptor density and attenuated in adenylyl cyclase response to isoproterenol due to ischemia–reperfusion [95].

The pattern of changes in β-adrenoreceptor linked signal transduction following ischemia was different from that observed due to ischemia–reperfusion and the changes due to ischemia were also not prevented by oxyradical scavengers [69]. In the ischemic heart, the density and affinity of β₁-adrenergic receptors were increased while β₂-adrenergic receptors did not change. The increase in β-adrenergic receptors due to ischemia has been observed by some investigators [100,102]; however, others have failed to confirm these findings [99,103]. The increase in β₁-adrenergic receptors, but not β₂-adrenergic receptors, appears to be due to an increase in mRNA expression in the ischemic heart [104]. A decrease in the stimulation of adenylyl cyclase activity by isoproterenol was observed in the ischemic heart and this suggests an uncoupling of β-adrenoceptors from adenylyl cyclase or a derangement at the post receptor level of the signal transduction pathway. The basal and forskolin-stimulated adenylyl cyclase were also unaffected in the ischemic heart which indicate that the catalytic site of the effector enzyme was not altered. Other investigators showed either a decrease or an increase in the enzyme activity using hearts from different species and by employing different durations of ischemia [7,105,106]. Nonetheless, the stimulatory G-protein seems to play a major role in adenylyl cyclase derangement in the ischemic heart since a depression of NaF–, Gpp(NH)p–, and cholera toxin-stimulated adenylyl cyclase activities were observed. Attenuated cholera toxin stimulated GDP-ribosylation of stimulatory G-proteins as well as reduced binding of antibodies against stimulatory alpha G-proteins were also observed. However, there was no alteration in the inhibitory G-protein linked adenylyl cyclase activity, GDP-ribosylation or antibody binding. Similar results were reported by different groups using different animal models [107–109]. In view of the role of β-adrenoreceptor pathway in promoting Ca²⁺-influx, it appears that attenuation of the β-adrenoreceptor mechanisms by ischemia–reperfusion injury may decrease the entry of Ca²⁺ in cardiomyocytes.
and thus may serve as an adaptive change for cardioprotection. Interestingly, several laboratories have shown that different β-blocker agents such as propanolol, metoprolol and carvedilol can protect against the damage due to ischemia–reperfusion not only due to their negative inotropic and chronotropic effects but also via their antioxidant properties [60,61,110].

4.5. 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. Briefly, the signal pathway of apoptosis involves the stimulation of cell membrane death receptors (Fas) which leads to the activation of caspases (aspartate-specific proteases), protein cleavage, DNA fragmentation and cell death. Several studies have shown that myocardial ischemia–reperfusion is associated with an increase in apoptotic cells [59,111]. 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 (Fas), and oxidative stress have been suggested to trigger apoptosis during ischemia–reperfusion [112–114]. Interestingly, a recent study has shown upregulation of an antioxidant oncogene (Bcl-2) by ischemic adaptation which was associated with a reduction of cell apoptosis and DNA fragmentation [114]. Furthermore, it was demonstrated that ROS generated during preconditioning can activate Bcl-2 gene by stimulating a specific nuclear transcription factor (NFκB) which in turn reduces apoptosis. This signal pathway reducing apoptosis was blocked by free radical scavengers [59]. These results indicate an additional antioxidant pathway for the protection of myocardium by ischemic preconditioning.

5. Role of antioxidants in myocardial ischemic preconditioning

Although the exact mechanism of ischemic preconditioning in protecting the myocardium is not fully understood, it has been proposed that an increase in the activity of endogenous antioxidants may be responsible for both the immediate and delayed preconditioning [115,116]. When the isolated rat hearts were subjected to four cycles of ischemia and reperfusion, the activities and gene expression of Mn-SOD, catalase, and glutathione peroxidase were significantly increased compared to hearts subjected to one cycle of ischemia and reperfusion [117]. In an open chest dog model, when the hearts were subjected to four cycles of ischemia (5 min) and reperfusion (10 min), the activity of mitochondrial Mn-SOD was increased immediately as well as 24 h after when compared to the non-ischemic hearts [115]. Both the activity and protein content of Mn-SOD in subendocardium and subepicardium regions were increased following ischemic preconditioning. In addition, glutathione peroxidase was increased and glutathione reductase was decreased rapidly in the ischemic myocardium; however, Cu-Zn–SOD, glutathione peroxidase and glutathione reductase did not change at 24 h. In another study, Mn-SOD activity was shown to be higher in late preconditioning than immediate preconditioning in the isolated adult rat myocyte [118]. There is evidence to suggest that protein kinase C and adenosine receptors, other major pathways for explaining the mechanism of preconditioning, may be involved in enhancing the activity of cellular antioxidants [115,116]. Surprisingly, in conscious pigs subjected to ten cycles of 2-min ischemia and reperfusion, no change was observed in Mn-SOD, catalase, glutathione peroxidase, glutathione reductase, α-tocopherol and ascorbate levels at 24 h when a marked protection against myocardial stunning was apparent [119]. However, the antioxidant hypothesis for mediating the immediate and delayed protection by ischemic preconditioning cannot be ruled out in view of a large and convincing evidence regarding the role of ROS in causing ischemia–reperfusion injury and upregulating the antioxidant enzymes. Other pathways involving ATP-sensitive K⁺ channels, protein kinase C and heat shock proteins may also contribute towards the effects of preconditioning depending upon species, ischemic conditions and other biological environments.

In order to test if the effects of ischemic preconditioning are simulated by treatment of hearts with antioxidants, we have compared the effects of these interventions by employing the isolated, perfused rat heart preparations according to the methods described elsewhere [18,19,21]. N-acetylcysteine (NAC), a thiol-containing compound, was used as an antioxidant since it is known to interact with oxygen radical species and result in NAC disulfide as a main end product [120–123]. NAC has also been shown to interact with superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radical (·OH), and hypochlorous acid [124]. Global ischemia for 20 min followed by reperfusion for 30 min resulted in a decrease in LVEDP and increase in LVEDP as well as increased malondialdehyde (MDA) content and decreased creatine kinase (CK) content in the rat heart (Table 2). Furthermore, cardiac free sulfhydryl (SH) content and glutathione redox potential expressed as GSH/GSSG ratio were also depressed in the ischemic-reperfused hearts (Fig. 2). Adding NAC (100 µM) into the perfusion buffer attenuated these changes due to ischemia–reperfusion. These results suggest that thiol-compounds may be one of the potent defense systems against ischemia–reperfusion and indicate that free SH content and the GSH/GSSG ratio can be used as sensitive markers for oxidative stress along with MDA formation in the heart. Preconditioning of the heart with three cycles of repetitive brief ischemia (2 min) and reperfusion (5 min) before inducing global ischemia (20 min) and reperfusion (30
Table 2
Effect of preconditioning and N-acetylcysteine (100 μM) on cardiac performance as well as MDA and CK contents in ischemic and reperfused hearts

<table>
<thead>
<tr>
<th></th>
<th>LVDP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>MDA (nmol/mg protein)</th>
<th>CK (U/g of wet weight heart)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80±3.2</td>
<td>9.8±0.4</td>
<td>0.16±0.04</td>
<td>786±24</td>
</tr>
<tr>
<td>IR</td>
<td>19±0.7*</td>
<td>71.5±2.8*</td>
<td>0.56±0.10*</td>
<td>590±18*</td>
</tr>
<tr>
<td>PC+IR</td>
<td>38±2.1*</td>
<td>39.6±2.4*</td>
<td>0.32±0.10*</td>
<td>678±16*</td>
</tr>
<tr>
<td>NAC+IR</td>
<td>64±3.5*</td>
<td>27.6±1.9*</td>
<td>0.31±0.09*</td>
<td>794±21*</td>
</tr>
</tbody>
</table>

* Values are mean±S.E. of six to eight hearts in each group. Hearts were preconditioned by three cycles of repetitive brief global ischemia for 2 min and reperfusion for 5 min in a manner similar to that described earlier [19] before subjecting them to 20 min of global ischemia followed by 30 min of reperfusion. Changes in the above mentioned parameters were measured according to the methods described elsewhere [21,130]. Treatment of the heart with NAC was carried out 10 min before inducing ischemia and this agent was also present in the perfusion medium during the reperfusion period. Statistical differences among multiple groups were analyzed using one-way analysis of variance (ANOVA) whereas differences between two groups were analyzed by the unpaired Student’s t-test. LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; MDA, malondialdehyde; IR, ischemia–reperfusion; PC, preconditioning; CK, creatine kinase; NAC, N-acetylcysteine. * P<0.05 in comparison to control; ** P<0.05 in comparison to IR group.

min) was also found to produce beneficial effects on ischemia–reperfusion induced changes in cardiac performance in addition to preserving cardiac SH groups and CK contents as well as decreasing the formation of MDA after prolonged ischemia and reperfusion (Table 2 and Fig. 2). Similar results for preconditioning have been reported in rabbit and rat hearts [86,125,126]. Such similarities between the beneficial effects of ischemic preconditioning and NAC indicate that preservation of SH groups may be one of the mechanisms for the cardioprotective action of ischemic preconditioning. However, it remains to be established whether preservation of SH groups and increased antioxidant activity in the myocardium is of general significance for cardioprotection against ischemia–reperfusion.

6. Concluding remarks

The hypothesis involving the role of ROS in myocardial ischemia–reperfusion injury has been supported by a wide range of investigations. Myocardial ischemia–reperfusion has been shown to result in contractile dysfunction, arrhythmias, loss of adrenergic pathways, changes in gene expression, apoptosis as well as necrotic cell death. Various antioxidants such as catalase, SOD, glutathione peroxidase and vitamin E have been shown to prevent these alterations in both experimental and clinical studies. The severity of ischemia–reperfusion injury and the subsequent level of oxidative stress, the interaction of antioxidants with ROS as well as among various antioxidants seem to determine the effectiveness of antioxidants for cardioprotection. The
ischemic preconditioning is also known to exert beneficial effects in suppressing the ischemia–reperfusion induced changes in the hearts; however, extensive studies are needed to determine the status of endogenous antioxidants as well as the exact signal transduction pathway in this condition.

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