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Endogenous antioxidant changes in the myocardium in response to acute and chronic stress conditions

Pawan K. Singal, Arvinder K. Dhalla, Michael Hill and Thomas P. Thomas

Division of Cardiovascular Sciences, St. Boniface General Hospital Research Center and Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Canada

Abstract

Oxygen is a diradical and because of its unique electronic configuration, it has the potential to form strong oxidants (e.g. superoxide radical, hydrogen peroxide and hydroxyl radical) called oxygen free radicals or partially reduced forms of oxygen (PRFO). These highly reactive oxygen species can cause cellular injury by oxidizing lipids and proteins as well as by causing strand breaks in nucleic acids. PRFO are produced in the cell during normal redox reactions including respiration and there are various antioxidants in the cell which scavenge these radicals. Thus in order to maintain a normal cell structure and function, a proper balance between free radical production and antioxidant levels is absolutely essential. Production of PRFO in the myocardium is increased during various *in vivo* as well as *in vitro* pathological conditions and these toxic radicals are responsible for causing functional, biochemical and ultrastructural changes in cardiac myocytes. Indirect evidence of free radical involvement in myocardial injury is provided by studies in which protection against these alterations is seen in the presence of exogenous administration of antioxidants. Endogenous myocardial antioxidants have also been reported to change under various physiological as well as pathophysiological conditions. It appears that endogenous antioxidants respond and adjust to different stress conditions and failure of these compensatory changes may also contribute in cardiac dysfunction. Thus endogenous and/or exogenous increase in antioxidants might have a therapeutic potential in various pathological conditions which result from increased free radical production. (Mol Cell Biochem **129:** 179–186, 1993)

Key words: myocardial dysfunction, ischemia-reperfusion injury, hypertrophy and heart failure, free radicals in cardiac pathology

Physico-chemical aspects of oxygen radicals

Molecular oxygen is a life supporting agent which exists as a diradical wherein the two unpaired electrons in its outer orbital have parallel spins. Although a strong oxidant, its unique electronic configuration limits the reactivity of oxygen as inversion of electron spin is required for the oxidation of a two electron donor by oxygen. At the same time, this unusual configuration renders oxygen potentially toxic to all biological material as its *in vivo* univalent reduction can occur readily giving rise to highly reactive intermediates. Oxygen requires a total of

Address for offprints: P.K. Singal, St. Boniface General Hospital Research Center, 351 Tache Ave., Winnipeg, Canada, R2H 2A6

four electrons for a complete reduction to H_2O. These four electrons can be added in one step (tetravalent reduction) as in mitochondria by the catalytic enzyme cytochrome oxidase and this is the fate of > 95% of the oxygen taken up by the cell. However, 3–5% of the oxygen taken up by the cell is also reduced in univalent steps, generating different reactive intermediates [1, 2].

In the univalent reduction pathway, addition of a single electron to molecular oxygen results in the production of superoxide anion radical (O_2^-). The addition of another electron (divalent reduction) to the O_2^- results in the formation of peroxide anion, which protonates to form hydrogen peroxide (H_2O_2). H_2O_2 is not a radical because of pairing of all oxygen electrons, however, H_2O_2 is a strong oxidizing agent and capable of causing cell damage. Interaction of H_2O_2 with transition metals such as iron, via Fenton reaction, can lead to the production of very strong oxidizing species. The trivalent reduction of oxygen or subsequent addition of an electron to H_2O_2 results in the formation of hydroxyl radical ($OH^{\cdot}$) and hydroxyl anion (OH^-). Hydroxyl radical can also be formed in a metal catalyzed Haber-Weiss reaction of O_2^- and H_2O_2. These highly reactive intermediate species (O_2^-, H_2O_2, $OH^{\cdot}$) are also termed partially reduced forms of oxygen (PRFO).

Chemically, different species of PRFOs possess different reactivities and biological half-lives. Superoxide radical is highly reactive and has a relatively short half-life as it spontaneously dismutates to H_2O_2. This reaction is speeded up many fold in the presence of an enzyme known as superoxide dismutase. H_2O_2 has a relatively longer half-life and therefore can diffuse some distance before reacting with a molecule. Thus, H_2O_2 can cause damage away from the site of its production. The extremely reactive nature of hydroxyl radical limits its field of action. It has the shortest half-life and is the most reactive of these species.

Oxygen radicals and tissue injury

Collectively PRFOs can mediate cell damage via lipid peroxidation, oxidation of structural and functional proteins as well as lesions and cross linking of nucleic acids [2, 3]. There is considerable evidence to suggest that peroxidation of membrane lipids in itself is a characteristic feature of PRFO mediated injury. Owing to the presence of polyunsaturated fatty acids (PUFA) in their phospholipids, biomembranes and subcellular organelles are particularly sensitive to oxidative attack. Lipid peroxidation chain reaction in the membrane is 'initiated' by the removal of hydrogen from the unsaturated site in a fatty acid resulting in the production of a lipid radical. The radical thus generated can interact with more PUFA and the chain is 'propagated'. Addition of oxygen to these lipid radicals results in the formation of lipid peroxides. Formation of malondialdehyde (MDA), a relatively stable intermediate product of lipid peroxidation, is considered to be a reasonable indicator of oxidative injury. Malondialdehyde itself also has cytotoxic properties and therefore can alter intrinsic membrane properties such as cell shape, ion transport and membranous enzyme activity [4]. Additionally, MDA has been shown to be mutagenic as well as carcinogenic [5, 6]. Membrane lipid peroxidation modifies membrane structure, usually resulting in a more rigid membrane which can cause changes in the activity of essential membrane transport proteins. These types of membrane alterations impair the ability of the cell membrane to maintain ionic gradients, with the potential for eventually accumulating calcium in the cell. Excess calcium can cause extensive membrane damage by activation of phospholipases and proteases as well as impair energy production due to the accumulation of calcium in the mitochondria [7].

Considerable evidence suggests that PRFO can modify various cell proteins which can adversely affect normal functioning of the cell. In this regard, proteins rich in sulphydryl-group containing amino acids are most susceptible to PRFO attack. In addition, the susceptibility of proteins also depends on their location as well as potential for repair. In addition to the ability of PRFO to directly alter protein structure and/or function, oxidative reactions can have other effects. Oxidized intracellular proteins may undergo selective and accelerated endogenous proteolysis [2]. Although oxidative attack does not always lead to a decrease in enzymatic activity, oxidation of proteins yields many possible products. Some of these are cross-linked reaction products, fragmentation products and specific lesions in protein structure that may render them inactive or alter their function. More specifically in the myocardium, oxygen radicals have been shown to influence Ca^{2+}-ATPase activity in the heart sarcolemma [8], sarcoplasmic reticulum [9] as well as Na^+/Ca^{2+} exchange [10] and Na^+-K^+ ATPase activity in the microsomal fractions [11].

Cell death and mutation from exposure to ionizing radiation is primarily due to PRFO generation and their interaction with nucleic acids. PRFO damage nucleic acids by producing base damage, single-strand breaks,

cross-linking of DNA-fragments and chromosomal aberrations. Strand breaks have important implications in terms of the development of pathological states and they must be repaired for the cell to function properly. DNA repair enzymes could also be altered by PRFO; therefore the probability of misincorporating the wrong base in the repaired DNA is higher. Such modifications can cause cellular abnormalities [5, 6].

Thus subcellular injury due to PRFO as well as lipid radicals plays an important role in the pathogenesis of a variety of disease states including pulmonary disorder [12], ischemia-reperfusion and hypoxia-reoxygenation injury of the heart [13–15], adriamycin-cardiomyopathy [16] and catecholamine cardiotoxicity [17].

Antioxidant defence system

Since all these PRFO are continually being produced in the cell under normal conditions, several biochemical defence mechanisms have developed during evolution to quench these reactive species and protect the cell from their deleterious effects. In addition, there are non-enzymatic defence systems. Collectively these enzymatic and non-enzymatic antioxidants constitute the antioxidant reserve. Notable enzymatic defences include superoxide dismutases (SOD), glutathione peroxidase (GSHPx) and catalase whereas tocopherols, carotenes and ascorbic acid are the major non-enzymatic antioxidants [18].

Among the antioxidant defence mechanisms, superoxide dismutases probably are the first line of defence against oxidative damage. SOD is part of the family of metalloenzymes that catalyze the dismutation of O_2^- radical to H_2O_2. SOD found in the cytosol of mammalian cells is a dimeric protein with a molecular weight of about 32,000 daltons containing CuZn complex at the active site [19]. Copper atom is essential for the catalytic activity of the molecule, whereas zinc atom imparts stability to the molecule [19]. Mammalian cells also contain a manganese (Mn) SOD (Mol. wt. $\sim$ 95,000 daltons), which is primarily localized in the mitochondrial matrix. Other forms of SOD which have been identified recently include an iron-containing SOD and a Cu-containing SOD [20]. The former is primarily found in prokaryotic and plant cells while the latter is found in human blood plasma [21].

Glutathione peroxidase is a member of the family of peroxidases that are the second line of antioxidant defence which catalyze the reduction of hydroperoxides using glutathione (GSH) to form oxidized glutathione (GSSG), water and/or an organic alcohol [22].

$$H_2O_2 + 2\,GSH \xrightarrow{GSHPx} GSSG + H_2O$$

$$ROOH + 2\,GSH \xrightarrow{GSHPx} GSSG + ROH + H_2O$$

Glutathione peroxidase enzyme is a selenium-dependent, tetrameric protein with a molecular weight of $\sim$ 85,000 daltons containing four selenocystine moieties which form the active site [23]. For the continuous functioning of the enzyme i.e. to reduce peroxides, oxidized glutathione (GSSG) must be reduced back to glutathione (GSH). This is accomplished by a NADPH-dependent enzyme, glutathione reductase, which catalyzes the reduction of GSSG to GSH by NADPH. Since glutathione is a rate limiting factor for GSHPx activity, maintenance of an adequate cellular glutathione concentration is important. GSHPx can reduce a variety of lipid peroxides and hydroperoxides with little degree of specificity.

Catalase, a tetrameric hemeprotein, also serves to protect the cells from excessive accumulation of H_2O_2 by converting H_2O_2 to water and oxygen. Molecular weight of catalase is approximately 240,000 daltons and it is primarily located in peroxisomes. Catalase can use H_2O_2 in peroxidative reactions with ethanol or methanol as substrates but only at low H_2O_2 concentrations. At higher H_2O_2 concentrations the catalytic mode of the enzyme predominates with the production of water and oxygen [22]. The concentration of catalase in the heart is reported to be relatively low [24]. Glutathione peroxidase has a lower Km for H_2O_2 and is more effective at low H_2O_2 concentrations whereas at higher concentrations catalase is more important. Therefore, both glutathione peroxidase and catalase are important in regulating the H_2O_2 concentration within the cardiac cell. A variety of other peroxidases having different affinities for H_2O_2 have also been found in biological systems. These may play a protective role, especially in tissues otherwise lacking catalase and/or GSHPx. These include ascorbate, cytochrome C-oxidase, NADPH-oxidase and Chloro-peroxidase.

Non enzymatic defences also include small molecules such as glutathione, uric acid and taurine in addition to tocopherols, ascorbate and carotenes. However, tocopherols are considered to be the most powerful of all these. Tocopherols are naturally occurring chroman derivatives found within the biological membranes. Among tocopherols, alpha tocopherol (Vitamin E) has

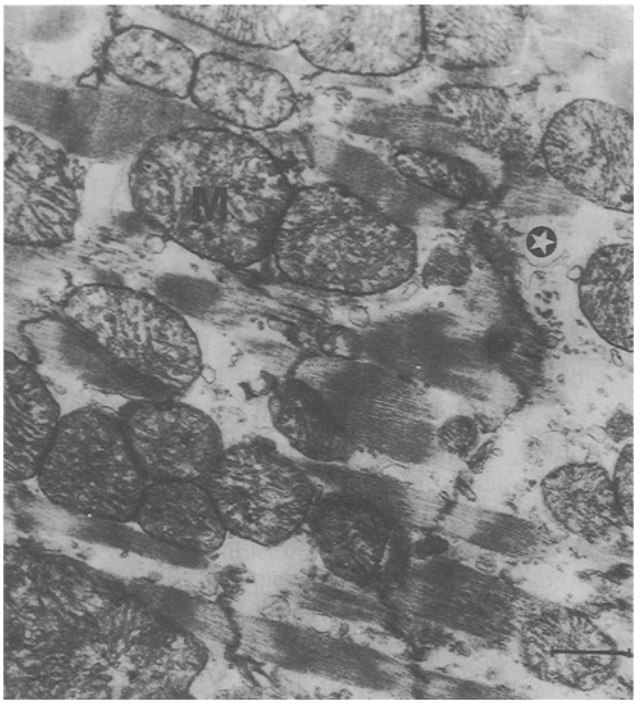

Fig. 1. Ultrastructural damage in isolated rat hearts perfused *ex vivo* with the buffer containing xanthine-xanthine oxidase for 20 min. Intracellular edema (*), swelling of mitochondria (M), disrupted sarcotubular system and generalized cell damage due to oxygen radicals are apparent. Magnification line represents one micron.

been the most widely studied. Its lipophilic nature is responsible for its excellent antioxidant properties. It has access into the hydrophobic regions of the membranes thereby positioning for maximum effectiveness. Although chemistry of its antioxidant properties is not fully understood, vitamin E is considered to directly react with PRFO, yielding lipid hydroperoxides which can be removed by the activity of phospholipase-GSHPx systems. This is thought to interrupt the radical chain-reaction processes that propagate the peroxidation of membranes. Thus vitamin E is often termed as a chain-breaking antioxidant. Being intercalated into the membranes, vitamin E can scavenge PRFO generated from outside as well as from inside the membrane, thus limiting the extent of phospholipid peroxidation. In this functional aspect, vitamin E may be aided by many biological systems to suppress lipid peroxidation [25].

Ascorbate/Vit C is a water soluble molecule found both intra- and extracellulary in most biological systems. Ascorbate may directly reduce free radicals with the concomitant formation of dehydroascorbate via the semidehydroascorbate free radical which is then reduced by other enzymes. Ascorbate also plays an important role in regenerating α-tocopherol. β-carotene is known both to quench excited species and also to react directly with PRFO. Thus, it is apparent that aerobic cells contain extensive antioxidant defence system to protect against PRFO generation and injury. For aerobic cells to maintain normal function and prevent the occurrence of oxidative injury, a balance between PRFO production and antioxidant defences is required. For the most part, this balance is well maintained under normal conditions; however, when the balance shifts in the favor of free radical production, e.g. during pathological conditions, cell injury occurs.

Oxygen radicals and myocardial cell injury

There is ample evidence of PRFO being cytotoxic to the heart. Using *in vivo* as well as *ex vivo* myocardial preparations exposed to O_2 free radicals generated by different sources such as electrolysis of the perfusion medium [26], xanthine-xanthine oxidase (x-xo) system [27, 28], activated neutrophils [29], it has been demonstrated that PRFOs result in depressed cardiac function, depletion of high energy phosphates, cell edema and structural damage [26–29]. Since most of these deleterious effects were reversed or reduced by the exogenous administration of antioxidants e.g. superoxide dismutase and catalase, oxygen free radicals were held responsible for these

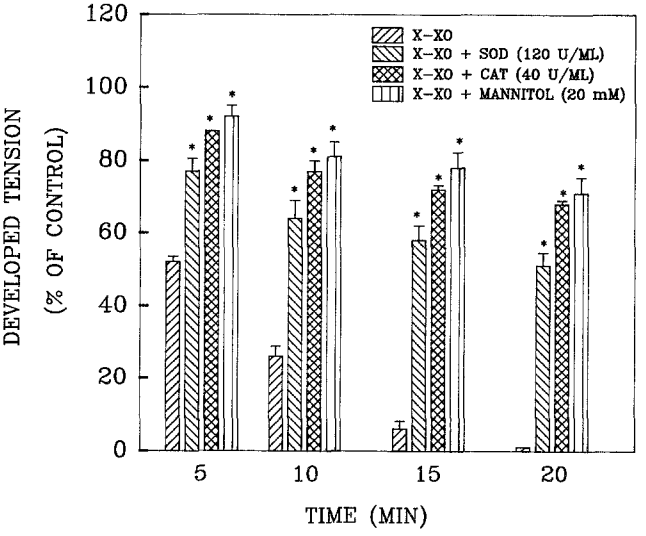

Fig. 2. Effects of xanthine-xanthine oxidase (x-xo) with and without antioxidants (superoxide dismutase, catalase, mannitol) on developed tension in isolated perfused rat heart at different durations of exposure. Values are percent of control data and are expressed as mean ± SEM of 7–8 experiments. *) $p < 0.05$ significantly different from control. Data reexpressed from Gupta and Singal [28].

cytotoxic effects. Retrograde perfusion of isolated rat hearts with a solution containing x-xo results in significant structural damage including swelling of mitochondria, dissolution of mitochondrial cristae and formation of contraction bands (Fig. 1). The sarcoplasmic membrane system is swollen and disrupted. The number of lysosomal granules is increased significantly. Exposure of isolated perfused rabbit ventricular septa to superoxide radicals caused vacuolization, dilation of T-tubules, blebbing in the endothelium and accumulation of membrane debris in the vascular and extracellular spaces [27]. Ultrastructural damage was exacerbated when ventricular septa were exposed to hydroxyl radicals [27]. Pretreatment with the oxygen radical scavengers showed a protective effect, suggesting that the oxygen free radicals may have been responsible for these ultrastructural alterations.

Exposure of sarcolemma to PRFO generated by x-xo resulted in a significant decrease in the binding capacity for muscarinic receptors which was reversed by the addition of SOD and catalase [30]. Exposure to H_2O_2 and x-xo oxidase system also decreased Ca^{2+} pump activity in heart sarcolemmal preparations which was prevented by the addition of SOD, catalase as well as mannitol [8]. Exogenously generated free radicals increased vascular permeability to macromolecules leading to edema formation and subsequent tissue damage [26, 31]. These investigators also showed that a variety of antioxidants were effective in preventing the increase in vascular permeability.

Perfusing the hearts with SOD, catalase as well as mannitol significantly modulates the depressant effect of x-xo on the contractile force in *ex vivo* system (Fig. 2). Mannitol was found to be the most effective in offering the protection at all time points suggesting a major role for hydroxyl radical. *In vivo* evidence for the involvement of free radicals was also provided by the administration of xanthine oxidase with purine plus iron-loaded transferrin through a coronary vein in an anaesthetized canine preparation in which left ventricular wall motion abnormalities were noted [32].

Thus there is little doubt that PRFO can cause subcellular alterations leading to myocardial cell injury and contractile dysfunction. What remains to be established is the *in vivo* source of the free radicals as well as the precise mechanism of their action in various pathological conditions where PRFO are implicated.

Antioxidant changes during chronic conditions

Under normal conditions, antioxidants are sufficient to quench the PRFO generated. However, enhanced production of free radicals and/or reduced antioxidant levels during various pathological conditions can result in cell damage via the above listed processes including lipid peroxidation, oxidation of proteins and nucleic acids. It is now clear that certain oxidative stress conditions are also associated with changes in the antioxidant defence mechanisms. Antioxidant status of the heart is shown to be a dynamic function adjusting to various physiological as well as pathophysiological conditions imposed [18]. In the past few years, there have been numerous reports on changes in myocardial antioxidant reserve during chronic conditions. For example, with aging, different organs show different changes in their antioxidant enzyme activities. More specifically, cardiac catalase activity was increased whereas lipid peroxidation was significantly decreased at 24 months of age in rats [33]. An age-dependent increase in SOD activity has been reported in case of rats up to about 6 months of age [34]. In another study, SOD and catalase activities were demonstrated to decline in rat cardiac tissue in 72 weeks old rats [35]. Interestingly, $O_2^{\cdot-}$ and H_2O_2 generation in the myocardium of these rats was found to be increased with age [35]. These data clearly show an increase as well as a decrease in SOD and catalase activities depending on the age of the animal [33–35].

Another stress condition that leads to the increased production of PRFO is exercise. Strenuous physical exercise increases oxidative metabolism which increases free radical production. In this regard, free radical production was found to be increased in the skeletal muscle and the liver of exercising rats [36]. Exercise trained mice showed elevated levels of SOD, GSHPx and catalase in liver and blood [37]. These animals showed reduced mortality due to doxorubicin, a drug known to involve free radicals in causing cardiotoxicity [16]. Higuchi *et al.* [38] demonstrated a 37% and 14% increase in SOD levels in fast and slow twitch muscle fibers, respectively, in response to exercise and thyroid hormones. In another study, SOD and glutathione S-transferase activities were found to be elevated whereas catalase and GSHPx activities were decreased following strenuous exercise in female albino rats. A significant increase in free radical production was also seen in the myocardium of these animals following exhaustive endurance exer-

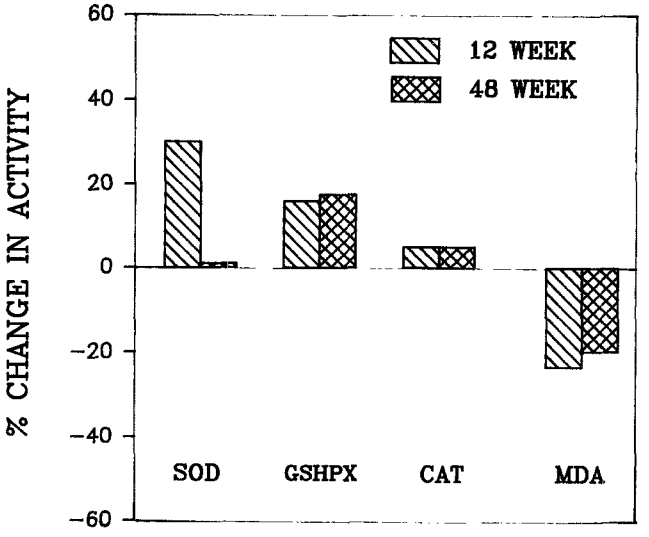

Fig. 3. Antioxidant enzyme (GSHPx, SOD, catalase) activities as well as lipid peroxidation (MDA) in hypertrophied rat hearts at 12 and 48 weeks. Hypertrophy was induced by banding of the abdominal aorta. At both stages animals had hyperfunctional hypertrophy without any signs of heart failure. Data reexpressed from Gupta and Singal [34].

cise [39]. Although significance of these changes is not clear, it is possible that strenuous physical exercise may result in a decrease in antioxidant activity due to increased oxidative stress whereas prolonged exercise, probably allowing for an adaptive response, results in increased antioxidants.

Increased PRFO production was also demonstrated in mitochondria isolated from a hypertrophied heart [40]. An increase in myocardial antioxidant capacity was reported in hypertrophic rat hearts subsequent to a gradual induction of chronic pressure overload [34]. As shown in Fig. 3, there was a 30% increase in SOD activity and 16% increase in GSHPx activity at 12 weeks of hypertrophy due to banding of the abdominal aorta in rats. At 48 weeks, SOD activity in these banded animals was not different from control hearts but GSHPx activity was still elevated. Lipid peroxidation was significantly less in the hypertrophic heart as compared to the control heart (Fig. 3). These hypertrophic hearts with increased endogenous antioxidant reserve were found to be less susceptible to *ex vivo* oxidative stress challenges than non-hypertrophied control hearts [15, 34, 41]. It was demonstrated that the hypertrophied hearts were able to maintain better contractile function on exposure to xanthine-xanthine oxidase [34, 41] and recovered better subsequent to hypoxia-reoxygenation injury [15]. Some very interesting results were obtained in the case of Guinea pigs subjected to chronic pressure overload by aortic banding for 10 and 20 weeks. Well compensated heart hypertrophy in Guinea pigs at 10 weeks was accompanied by significant increase in myocardial SOD as well as GSHPx activity and 25% decrease in lipid peroxidation. However, these antioxidant changes were more or less reversed in animals with congestive heart failure at 20 weeks (Fig. 4). In this regard, PRFO production as well as lipid peroxidation were found to be increased in mitochondria isolated from failing hearts of cardiomyopathic hamsters [42].

Antioxidant activities have also been shown to be changed under various other pathological conditions. Antioxidants SOD, GSHPx and catalase have been reported to be higher than normal in red blood cells from patients with β-thalassaemia minor [43]. Superoxide dismutase, catalase and GSHPx were found to be significantly increased in alcohol induced cardiomyopathy in turkeys [44]. Reduction in myocardial glutathione levels, increase in lipid peroxidation and ultrastructural changes due to chronic intake of alcohol in rats were modulated by exogenous administration of vitamin E and cyanidanol-3 [45]. GSHPx activity was increased in intestinal mucosa and liver after oral administration of peroxidized lipids [46]. A reduction of myocardial GSHPx levels in mice pretreated with doxorubicin, was proposed to account for the increased susceptibility of these hearts to anthracycline antibiotics [24]. Reduction in antioxidants and increased oxidative stress injury

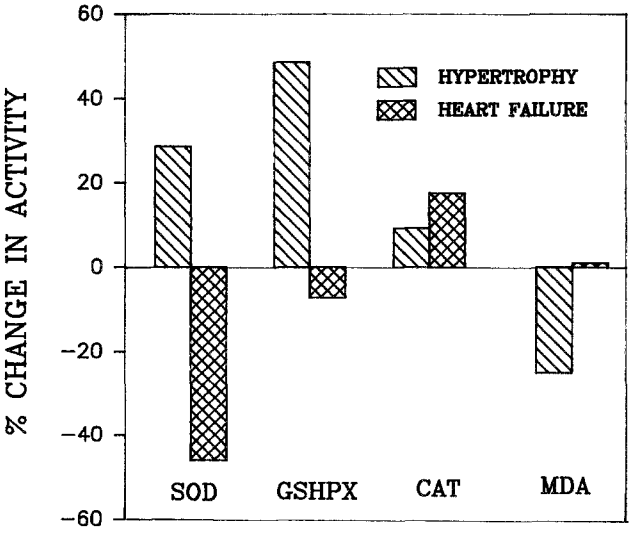

Fig. 4. Myocardial antioxidant enzyme (GSHPx, SOD, catalase) activities as well as lipid peroxidation in hypertrophied and heart failure stages in Guinea pigs. Hypertrophy was induced by banding of the ascending aorta in young Guinea pigs. Animals were in stable hypertrophy stage at 10 weeks and in heart failure stage at 20 weeks after the surgery. Data expressed as percent change in activity as compared to sham controls (N = 5).

were also seen in lung parenchymal cells following cigarette smoking [47].

Antioxidant changes during acute conditions

In contrast, during short term acute conditions of increased oxidative stress, various antioxidants in the heart have been shown to be depressed thereby predisposing the cell to oxidative damage. One of the most clearly documented conditions of increased oxidative stress involves ischemia-reperfusion injury. Antioxidant enzymes were depressed during ischemia [13, 48] as well as hypoxia conditions [14] and correlated with poor recovery of function upon reperfusion and/or reoxygenation. This point was further emphasized by another study which shows that the antioxidants in rat hearts can be upregulated by pretreatment with bacterial endotoxin [49]. In this study catalase levels were significantly higher in endotoxin-exposed animals and these hearts were more resistant to ischemia/reperfusion injury. Taking a step further, Fuji *et al.* [50] monitored antioxidant changes at the mRNA level under oxidative stress conditions. In their study, mRNA message for MnSOD was decreased during hypoxia and was found to be increased upon subsequent reoxygenation.

The exact stimulus for the altered activity of these enzymes is not known; however, increased radical formation itself during stress conditions may act as the signal. It is also clear that different antioxidants respond to various stimuli in a specific manner. At any rate, the pattern that seems to emerge is that antioxidant enzyme levels appear to adapt according to the requirements and/or conditions imposed. Failure of this adaptation may contribute to the cardiac pathology and dysfunction.

Conclusions

Oxygen free radicals are capable of causing significant myocardial injury by modification of membrane lipids, proteins and nucleic acids. Over the past two decades PRFO have been shown to be involved in a variety of cardiac pathologies. Under normal conditions, antioxidants in the cells are sufficient to protect the cells from the oxidative damage. However, it is absolutely important to maintain the balance between PRFO production and antioxidant status. The latter in the heart is dynamic in nature, responding to various stress conditions. The delicate balance between free radicals and antioxidant defences might be violated during various pathological conditions by the overproduction of PRFO and/or reduction in the defence mechanisms. Exogenous administration of antioxidants and/or approaches to increase endogenous antioxidants may prove useful in cardiac therapies.

Acknowledgements

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