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# Oxygen free radicals and calcium homeostasis in the heart

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## Abstract

Many experiments have been done to clarify the effects of oxygen free radicals on $Ca^{2+}$ homeostasis in the hearts. A burst of oxygen free radicals occurs immediately after reperfusion, but we have to be reminded that the exact levels of oxygen free radicals in the hearts are yet unknown in both physiological and pathophysiological conditions. Therefore, we should give careful consideration to this point when we perform the experiments and analyze the results.

It is, however, evident that $Ca^{2+}$ overload occurs when the hearts are exposed to an excess amount of oxygen free radicals. Though ATP-independent $Ca^{2+}$ binding is increased, $Ca^{2+}$ influx through $Ca^{2+}$ channel does not increase in the presence of oxygen free radicals. Another possible pathway through which $Ca^{2+}$ can enter the myocytes is $Na^+$-$Ca^{2+}$ exchanger. Although, the activities of $Na^+$-$K^+$ ATPase and $Na^+$-$H^+$ exchange are inhibited by oxygen free radicals, it is not known whether intracellular $Na^+$ level increases under oxidative stress or not. The question has to be solved for the understanding of the importance of $Na^+$-$Ca^{2+}$ exchange in $Ca^{2+}$ influx process from extracellular space. Another question is 'which way does $Na^+$-$Ca^{2+}$ exchange work under oxidative stress? Net influx or efflux of $Ca^{2+}$ ?' Membrane permeability for $Ca^{2+}$ may be maintained in a relatively early phase of free radical injury. Since sarcolemmal $Ca^{2+}$-pump ATPase activity is depressed by oxygen free radicals, $Ca^{2+}$ extrusion from cytosol to extracellular space is considered to be reduced. It has also been shown that oxygen free radicals promote $Ca^{2+}$ release from sarcoplasmic reticulum and inhibit $Ca^{2+}$ sequestration to sarcoplasmic reticulum. Thus, these changes in $Ca^{2+}$ handling systems could cause the $Ca^{2+}$ overload due to oxygen free radicals. (Mol Cell Biochem **139**: 91–100, 1994)

*Key words:* sarcolemma, myofibrils, $Na^+/Ca^{2+}$ exchange, sarcoplasmic reticulum, cardiac contraction, $Ca^{2+}$ pump

## Introduction

During the last decade, the role of oxygen free radicals in myocardial ischemia-reperfusion injury has been studied extensively. Although, the most important pathways through which free radicals may be formed during ischemia-reperfusion have not been defined precisely, several lines of evidence have shown that a burst of oxygen free radicals occurs immediately after reperfusion [1–4]. It has been also reported that oxygen free radicals have the ability to cause the dysfunction of the heart, and that various types of free radical scavengers have protective effects on ischemia-reperfusion injury [5–10]. These data suggest that oxygen free radicals is one of the crucial factors in ischemia-reperfusion injury. It is also well accepted that intracellular $Ca^{2+}$ overload plays an important role in ischemia-reperfusion injury [11–16]. $Ca^{2+}$ overload could have serious consequences for the myocyte through activation of a variety of enzymes such as proteases, lipases, and phospholipases [17] or ATPase [11, 12]. In addition, $Ca^{2+}$ is actively accumulated and precipitated with phosphate in the matrix space of mitochondria [18]. This process is associ-

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ated with inhibition of ATP synthesis [18] via mitochondrial oxidative phosphorylation and the ATP synthetase. Therefore, clarification of the relationship between oxygen free radicals and $Ca^{2+}$ homeostasis is necessary to understand the detail mechanisms for cellular injury in myocardial ischemia-reperfusion. The purpose of the present treatise is to discuss on this subject. In the first section, we discuss on the effects of oxygen free radicals on $Ca^{2+}$ handling systems in the heart. In the next section, effects of oxygen free radicals on intracellular $Ca^{2+}$ concentration are discussed.

## Effects of oxygen free radicals on $Ca^{2+}$ handling systems in the heart

Cytosolic $Ca^{2+}$ overload can occur either because of increased $Ca^{2+}$ influx from extracellular space to cytosol or because of insufficient $Ca^{2+}$ extrusion from cytosol. Cytosolic $Ca^{2+}$ concentration is also affected by subcellular $Ca^{2+}$ store sites such as sarcoplasmic reticulum. Therefore, the following discussion is divided into three parts. The first part is the effects of oxygen free radicals on $Ca^{2+}$ influx from the extracellular space to intracellular space. These processes include ATP-independent $Ca^{2+}$ binding, $Ca^{2+}$ channels, adrenergic receptors, $Na^+$-$Ca^{2+}$ exchange, $Na^+$-$K^+$ ATPase, $Na^+$-$H^+$ exchange, and permeability of cardiac sarcolemmal membranes. The second part is the effects of oxygen free radicals on $Ca^{2+}$ extrusion systems, which include $Na^+$-$Ca^{2+}$ exchange and $Ca^{2+}$ pump ATPase of sarcolemmal membranes. The final part is the effects of oxygen free radicals on $Ca^{2+}$ translocating processes of sarcoplasmic reticulum.

### *I: $Ca^{2+}$ influx from extracellular space to intracellular space*

It is well accepted that excitation-contraction coupling in mammalian heart include two critical $Ca^{2+}$ components. One component of $Ca^{2+}$ comes directly from influx across the sarcolemmal membrane, and another component of $Ca^{2+}$ is derived from the sarcoplasmic reticulum via the process of $Ca^{2+}$-induced $Ca^{2+}$-release [19, 20]. The $Ca^{2+}$-channel and the $Na^+$-$Ca^{2+}$ exchanger are recognized as responsible for this transsarcolemmal influx of $Ca^{2+}$ [21], though $Na^+$-$Ca^{2+}$ exchanger also operates to produce a net efflux of $Ca^{2+}$ (22, 23].

#### *1. ATP-independent $Ca^{2+}$-binding*

The sources of $Ca^{2+}$ that enters the cell across the sarcolemma are considered to be the extracellular space and $Ca^{2+}$-binding sites within the sarcolemmal membranes [24–26]. Langer *et al.* [24–26] reported that membrane bound $Ca^{2+}$ in cardiac cell is a superficial store of $Ca^{2+}$, which becomes available for entry upon excitation of the myocardium. Kinetic studies of $Ca^{2+}$ binding with sarcolemmal membrane in the absence of ATP have revealed the presence of both low-affinity, high-capacity $Ca^{2+}$-binding site and high-affinity, low-capacity $Ca^{2+}$-binding site [26]. The amount of $Ca^{2+}$ that enters the cell is a major determinant of the concentration of $Ca^{2+}$ at the myofilaments during systole, and is a major determinant of the level of contactile force as a result [26]. In fact, ATP-independent $Ca^{2+}$-binding in heart sarcolemma has been shown to exhibit linear relationship with the contactile force development in the normal myocardium [24–28].

We have examined the effects of oxygen free radicals on this ATP-independent $Ca^{2+}$-binding activity in rat heart sarcolemmal membrane [29]. In the presence of xanthine plus xanthine oxidase or hydrogen peroxide, both low- and high-affinity $Ca^{2+}$-binding activities were increased after 5 min incubation. Philipson *et al.* [30] showed that more than 80% of $Ca^{2+}$ was bound to membrane phospholipids at physiological level of extracellular $Ca^{2+}$. Since oxygen free radicals are known to promote the peroxidation of membrane phospholipids [31], it is likely that the changes in ATP-independent $Ca^{2+}$-binding by oxygen free radicals are due to alterations in the phospholipid composition of the membrane.

#### *2. $Ca^{2+}$ channels*

$Ca^{2+}$ channels are intrinsic membrane glycoproteins that participate in the regulation of transmembrane ion flow and cellular function in the heart [32–34]. $Ca^{2+}$ channels open in response to changes in membrane potential and allow $Ca^{2+}$ to enter cells. This inward movement of $Ca^{2+}$ depolarizes the heart cell membrane and contributes to the plateau phase of the action potential, pacemaker activity, and impulse conduction [35–37]. In addition, the influx of $Ca^{2+}$ causes a transient rise in intracellular $Ca^{2+}$ concentration, which in turn can initiate the cardiac contraction [38–41].

We have examined the effects of oxygen free radicals on the binding of $Ca^{2+}$ channel antagonists by employing [$^3$H]-nitrendipine as a ligand. Isolated rat heart membranes were incubated with various types of oxygen free radicals-generating system, and the assay of the [$^3$H]-nitrendipine binding activity revealed that the maximal number of binding sites (Bmax) was reduced in a time-dependent manner without any significant changes in the binding constant (Kd); a significant reduction of Bmax was seen after incubating the membranes with free radical-generating systems for a 10-min-period [42]. These results indicate that oxygen free radicals may reduce the number of voltage-dependent $Ca^{2+}$ channels and this change may contribute towards decreasing the voltage-dependent $Ca^{2+}$ influx of the cardiac cells.

In electrophysiological studies, Burrington *et al.* [43],

Hayashi *et al.* [44], Beresewicz *et al.* [45], and Jabr and Cole [46] have reported that oxygen free radicals produced an initial prolongation of action potential duration of rat and guinea pig ventricular myocytes, and that the prolongation of action potential duration was followed by a shortening of duration. Pallandi *et al.* [47] reported that xanthine oxidase-generated free radicals reduced the amplitude after 20 to 30 min exposure but did not affect the duration of the action potential in guinea pig ventricular strips. Cerbai *et al.* [48] reported a prolongation of action potential duration by dihydroxyfumarate in guinea pig ventricular myocytes. On the other hand, Nakaya *et al.* [49] reported that organic hydroperoxides produced decreases in both the amplitude and the duration of action potential of guinea pig papillary muscles and canine Purkinje fibers, and that it took 10 min of superfusion with hydrogen peroxides for the changes in resting potential and action potential to occur. It was, therefore, shown that the effects of free radicals on action potential duration were variable according to duration of the perfusion and to the species of free radicals. On the other hand, action potential duration can be influenced not only by $Ca^{2+}$ current but by $K^+$ current.

Goldhaber *et al.* [50] observed that 1 mM $H_2O_2$ or 1 mM xanthine plus 0.01 U/ml xanthine oxidase caused a rapid decrease in the amplitude of the $Ca^{2+}$ current in patch-clamped guinea pig single ventricular myocytes. Cerbai *et al.* [48] also reported that $Ca^{2+}$ current was rapidly reduced by the superfusion of guinea pig ventricular myocytes with 5 mM dihydroxyfumarate (DHF). They observed that after 2 min of exposure to DHF, the peak $Ca^{2+}$ current was decreased about 70% of its initial value, without any appreciable modification of its kinetics. Tarr *et al.* [51] showed that rose bengal-generated oxygen free radicals suppressed $Ca^{2+}$ currents in single frog atrial cells. Nakaya *et al.* [52] also reported that 10 to 30 μM cumene hydroperoxide decreased the $Ca^{2+}$ currents of guinea pig ventricular cells.

These results indicate that $Ca^{2+}$ influx through the voltage-dependent $Ca^{2+}$ channels could not be the cause of $Ca^{2+}$ overload during the exposure to oxygen free radical-generating systems.

*3. Adrenergic receptors*

The β-adrenergic receptor – stimulatory guanine nucleotide-binding protein (Gs) – adenylate cyclase system is a plasma membrane-bound protein assembly consisting of three major components [53, 54]. The heterotrimetric guanine nucleotide-binding regulatory proteins couple with extracellular receptors and cause stimulation (Gs) or inhibition (Gi) of the effector enzyme adenylate cyclase, which is the primary regulator of the intracellular concentration of the second messenger cyclic AMP (cAMP) [53–55]. In the heart, α subunit of the Gs(Gsα) plays a central role in the regulation of cardiac function [56] as in addition to transducing β-adrenergic receptor-mediated activation of adenylate cyclase. The Gsα regulates the phosphorylation of $Ca^{2+}$ channels indirectly by cAMP-dependent protein kinase [57], and it activates voltage-dependent $Ca^{2+}$ channels by a more direct interaction [58]. Cyclic AMP-dependent protein kinases can also promote the phosphorylation of other regulatory proteins such as phospholamban in the sarcoplasmic reticulum and troponin I in myofibrils [59]. Therefore, the changes in β-receptor – Gs – adenylate cyclase system can affect $Ca^{2+}$ homeostasis in the heart.

Haenen *et al.* [60] reported that although a low concentration ($1 \times 10^{-7} - 1 \times 10^{-3}$ M) of hydrogen peroxide increased Bmax for [$^{125}$I]-iodocyanopindrol binding, while a high concentration ($1 \times 10^{-1}$ M) of hydrogen peroxide decreased Bmax. We observed that both Bmax and Kd for [$^3$H]-dihydroalprenol (DHA) binding were increased by xanthine plus xanthine oxidase after 10 min of incubation in rat heart. One mM of hydrogen peroxide increased the Kd value whereas Bmax was unaffected [61]. When a hydrophilic ligand, [$^3$H]-CGP-12177, was used for the β-adrenergic receptor assay, an increase in Kd value without any significant changes in Bmax was seen on treating the membrane with xanthine plus xanthine oxidase [61]. We also examined the direct effects of hydrogen peroxide on Gs activity, and observed that hydrogen peroxide (0.1 to 10 mM) did not cause any significant effects on both Gs activity and the coupling in the β-adrenergic receptor – Gs – adenylate cyclase system in rat cardiac membranes [62]. Will-Shahab *et al.* [63], Shimke *et al.* [64], and we [62] showed that adenylate cyclase activity was depressed by oxygen free radicals. Furthermore, in the presence of oxygen free radical-generating systems, cAMP productions after stimulation with GTP, GTP + (1)-isoproterenol, Gpp(NH)p, and Gpp(NH)p + (1)-isoproterenol were reduced [62]. Therefore, it seems that the changes in β-adrenergic receptor systems under oxidative stress may not lead to $Ca^{2+}$ overload in the heart.

*4. $Na^+$-$Ca^{2+}$ exchange, $Na^+$-$K^+$ ATPase, and $Na^+$-$H^+$ exchange*

The $Na^+$-$Ca^{2+}$ exchange system can move $Ca^{2+}$ either into or out of the cytosol, across the plasma membrane, in exchange for $Na^+$ [65]. The net direction of $Ca^{2+}$ movement mediated by the exchanger depends on the $Na^+$ and/or the $Ca^{2+}$ electrochemical gradient, the stoichiometry, and the membrane potentials during the action potential [65]. Since the reversal potential for the exchange ($E_{Na/Ca}$) is slightly positive to the resting membrane potential during diastole, there is a small net outward movement of $Ca^{2+}$, mediated by the exchange that operates in parallel with the $Ca^{2+}$-pump ATPase to maintain the low resting $Ca^{2+}$ level in the cytosol. During the upstroke of the action potential, the driving force becomes positive, and this tends to drive $Ca^{2+}$ into the cells in exchange for $Na^+$. The net $Ca^{2+}$ entry mediated by the

exchange may continue through the plateau phase of the action potential. Then as the cell repolarizes because of the opening of $K^+$-selective channels, the driving force becomes negative; this results in net efflux of $Ca^{2+}$ mediated by the exchange operating in the $Ca^{2+}$ efflux mode [66].

$Na^+$-$Ca^{2+}$ exchange is also affected by the changes in the activities of $Na^+$-$K^+$ ATPase and $Na^+$-$H^+$ exchange. Inhibition of the $Na^+$-$K^+$ ATPase produces a rise in intracellular level of $Na^+$ [67, 68]. An elevation of the intracellular $Na^+$ will lead to the rise in intracellular $Ca^{2+}$ either by a decreased $Ca^{2+}$ efflux or by an increased $Ca^{2+}$ influx via $Na^+$-$Ca^{2+}$ exchange [23]. The inhibition of the $Na^+$-$K^+$ ATPase also results in an intracellular acidification that is thought to be a consequence of a rise in intracellular $Ca^{2+}$ produced via $Na^+$-$Ca^{2+}$ exchange [69–71]. Furthermore, measurements of intracellular $Na^+$ have shown that inhibitors of $Na^+$-$H^+$ exchange decrease the rise in intracellular $Na^+$ which was produced by ouabain [70, 72–74]. These results suggest that even in the presence of ouabain, $Na^+$-$H^+$ exchange is producing a net $Na^+$ influx which increases intracellular $Ca^{2+}$ via $Na^+$-$Ca^{2+}$ exchange [23, 70]. Thus, these three ion transporting systems can affect each other, and play important roles in $Ca^{2+}$ handling in cardiac cells.

Kramer *et al.* [75] demonstrated that $Na^+$-$K^+$ ATPase activity was reduced by oxygen free radical-generating system (dihydroxyfumarate and $Fe^{3+}$-ADP) in canine cardiac sarcolemmal membranes. Kukreja *et al.* [78] also showed that the inhibition of $Na^+$-$K^+$ ATPase activity was seen in the presence of $H_2O_2$, $H_2O_2$ plus $Fe^{2+}$, HOCl, $NH_2Cl$, or PMA-stimulated human neutrophils whereas the enzyme activity was not changed by xanthine plus xanthine oxidase in dog heart sarcolemmal vesicles. Bhatnagar *et al.* [77] observed that superfusion of frog ventricular single cells with *tert*-butyl hydroperoxide(t-BHP) increased intracellular $Na^+$ which could result from a decrease in $Na^+$ efflux via $Na^+$-$K^+$ pump. On the other hand, Xie *et al.* [102] reported that both $Na^+$-$K^+$ ATPase and $Na^+$-$H^+$ exchange activities were reduced by xanthine plus xanthine oxidase and that $Na^+$-$H^+$ exchange was more sensitive to oxidative stress than that of $Na^+$-$K^+$ ATPase. Furthermore, they observed that $^{45}Ca^{2+}$ uptake by myocytes was increased in the presence of ouabain, however, $^{45}Ca^{2+}$ uptake was decreased by xanthine plus xanthine oxidase. Their data suggest that intracellular $Na^+$ may not increase to the level which can promote $Ca^{2+}$ influx via $Na^+$-$Ca^{2+}$ exchange under oxidative stress, since $Na^+$-$H^+$ exchange is inhibited by free radicals as well as $Na^+$-$K^+$ ATPase.

These data support that heart sarcolemmal activities of $Na^+$-$K^+$ ATPase and $Na^+$-$H^+$ exchange are reduced by oxygen free radicals. Kim *et al.* [76] reported that ischemia-reperfusion of isolated guinea pig heart reduced $Na^+$-$K^+$ ATPase activity and specific [$^3$H]-ouabain binding to the enzyme; these effects of ischemia-reperfusion were prevented to various degrees by oxygen free radical scavengers, such as superoxide dismutase, catalase, dimethylsulfoxide, histidine, or vitamin E, or by the xanthine oxidase inhibitor, allopurinol. It was, therefore, suggested that an increase in free radicals during ischemia-reperfusion was implicated in reduced $Na^+$-$K^+$ ATPase activity. The direct effects of oxygen free radicals on $Na^+$-$Ca^{2+}$ exchange is discussed in the next part of this chapter.

*5. Membrane permeability, membrane fluidity, and phosphatidylethanolamine N-methylation*

One of the main targets of oxygen free radicals appears to be the polyunsaturated fatty acids of the membrane phospholipids [31]. Lipid peroxidation can be initiated by the interaction of a reactive oxidant with a fatty acid to form a lipid radical, which then can react rapidly with $O_2$ to form the corresponding peroxy radical. This peroxy radical can then attack a neighboring fatty acid to form the hydroperoxide and a new alkyl radical. This reaction will propagate until two radical species unite in a chain-terminating reaction to form a non-radical product, or until a radical reacts with an agent, such as α-tocopherol, that forms stable radicalls [31]. Such chain reactions can result in substantial membrane damage and can change ion permeability and fluidity of membranes [78–81].

Ytrehus *et al.* [82] observed the fragmentation of sarcolemma with subsequent leakage of cell organelles into the interstitium after 10 min Langendorff perfusion of rat heart with 0.96 mM hypoxanthine plus 0.025 U/ml xanthine oxidase. Levedev *et al.* [83] reported that $Ca^{2+}$ permeability of bilayer lipid membranes consisting of phosphatidylcholines in a mixture with cholesterol was increased when the membranes were formed from oxidizing lipids or when initiators of lipid peroxidation, ascorbate and iron ions, were added to both sides of the membrane. On the other hand, Bhatnagar *et al.* [77] reported that plasma membrane permeability for $Ca^{2+}$ remained intact even after 30 min exposure to t-BHP in single cells isolated from frog ventricle. Hata *et al.* [84] also showed that rat heart sarcolemmal permeability for $Ca^{2+}$ and $Na^+$ was not changed upon treatment with 2 mM xanthine plus 0.03 U/ml xanthine oxidase, 0.5 mM $H_2O_2$, or 0.01 mM $H_2O_2$ plus 0.01 mM $Fe^{2+}$ for 30 min. Thus, it seems likely that sarcolemmal membrane permeability for $Ca^{2+}$ may be maintained in a early phase of he injury, while it is increased in a final stage of membrane damage caused by oxygen free radicals. It may be difficult for the cells to maintain the cell functions in the situation in which cell membrane permeability for $Ca^{2+}$ is increased.

Lipid peroxidation of membrane phospholipids also results in the changes in membrane fluidity. Many researchers reported that membrane fluidities were decreased by the peroxidation of phospholipid vesicles, erythrocytes, microsomes, and mitochondrial membranes [85–91], whereas Grzelinska *et al.* [92] showed increased membrane

fluidity following peroxidation of erythrocytes membrane. We [93] observed that heart sarcolemmal, sarcoplasic reticular, and mitochondrial membrane fluidities were decreased by 2 mM xanthine plus 0.03 U/ml xanthine oxidase during 5 to 30 min of incubation. However, these membrane fluidities were increased after 60 min of incubation. The decrease in the membrane fluidity can be due to lipid peroxidation of membrane phospholipids by oxygen free radicals. The later increase in the fluidity is considered to reflect the disruption of the membranes, membrane permeability for $Ca^{2+}$ may increase in such condition.

The intramembranal rearrangement of the two major membrane phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), can be regulated by phospholipid N-methylation [94]. Phospholipid N-methylation has been considered to have important implication for cell membrane properties such as membrane fluidity [95], membrane-bound enzyme activities [96–99], and β-adrenergic receptors [100]. The PE N-methylation in rat heart sarcolemmal and sarcoplasmic reticular membranes was reduced by oxygen free radicals [101]. Thus, oxygen free radicals can modify the membrane integrity by altering the PE N-methylation process as well as lipid peroxidation.

### *II. $Ca^{2+}$ extrusion from intracellular space to extracellular space*

To maintain the levels of $Ca^{2+}$ ion in cytosol and subcellular $Ca^{2+}$ store sites, $Ca^{2+}$ which entered the cells from the extracellular space during the action potential, has to be pumped out from the cytosol to extracellular space. It is believed that heart sarcolemma contains two important mechanisms for extruding $Ca^{2+}$; $Na^{+}$-$Ca^{2+}$ exchange and $Ca^{2+}$-pump ATPase [103–105].

#### *1. $Na^{+}$-$Ca^{2+}$ exchange*

In the heart, $Na^{+}$-$Ca^{2+}$ exchange is thought to function primarily as a mechanism for pumping $Ca^{2+}$ out of the cell, it is, however, possible that $Na^{+}$-$Ca^{2+}$ exchanger can also promote the net entry of $Ca^{2+}$ into the cell under certain circumstances such as membrane depolarization as described in the previous part. The effects of oxygen free radicals on $Na^{+}$-$Ca^{2+}$ exchange is controversial. Reeves *et al.* [106] reported that $Na^{+}$-$Ca^{2+}$ exchange system in sarcolemmal vesicles of bovine ventricular tissue was stimulated by both oxidizing and reducing agents. $Na^{+}$-$Ca^{2+}$ exchange activity was stimulated by preincubating the vesicles with 1 μM $FeSo_4$ plus 1 mM dithiothreitol (DTT) which is known to generate superoxide anion radical, $H_2O_2$, and hydroxyl radical, xanthine plus xanthine oxidase, $Fe^{2+}$ plus $H_2O_2$, or GSH plus GSSG [106]. However, the exchange activity was not affected by $H_2O_2$, GSH, or GSSG, alone [106]. Based on these data, they considered that the redox agents may activate exchange activity by promoting thiol-disulfide interchange in the carrier protein [106]. Shi *et al.* [107] reported that exposure of bovine heart sarcolemmal vesicles to 50 μM $FeSo_4$ plus 1 mM DTT for 1 to 40 min stimulated $Na^{+}$-$Ca^{2+}$ exchange and decreased the apparent Km for $Ca^{2+}$ of $Na^{+}_{i}$-dependent $Ca^{2+}$ uptake. Conversley, others [102, 108–110] reported that $Na^{+}$-$Ca^{2+}$ exchange activity was reduced by oxygen free radicals. Kutryk and Pierce [108] showed that $Na^{+}$-$Ca^{2+}$ exchange activity in canine heart sarcolemmal vesicles was depressed by 50 μmol/mg of protein $H_2O_2$ or by cholesterol oxidase. Xie *et al.* [102] also observed that $Na^{+}$-$Ca^{2+}$ exchange activity in rat myocytes was decreased by 1 mM xanthine plus 0.08 U/mol xanthine oxidase after 30 min of incubation. Furthermore, Hata *et al.* [84] showed that various types of oxygen free radical-generating systems inhibited the $Na^{+}$-$Ca^{2+}$ exchange activity in sarcolemmal membrane vesicles isolated from rat, bovine, canine, and porcine hearts. Bersohn *et al.* [112], Daly *et al.* [109], Meon *et al.* [110], and Dixon *et al.* [111, 113] reported that heart sarcolemmal $Na^{+}$-$Ca^{2+}$ exchange activity was depressed in hearts subjected to ischemia-reperfusion or hypoxia-reoxygenation, and that the reduced activity of $Na^{+}$-$Ca^{2+}$ exchange in ischemia-reperfused hearts was prevented by the addition of free radical scavengers to the perfusate [113].

#### *2. $Ca^{2+}$-pump ATPase of sarcolemmal membrane*

We [114, 115] observed the effects of oxygen free radicals on $Ca^{2+}$-pump ATPase activity and the mechanism for the effects. The $Ca^{2+}$ ATPase activity and ATP-dependent $Ca^{2+}$ accumulation in rat heart sarcolemmal inside-out vesicles were reduced by xanthine plus xanthine oxidase, $H_2O_2$, or $H_2O_2$ plus $Fe^{2+}$ both in a dose- and a time-dependent manner; a significant inhibition of the activity was seen after 1 min of incubation. Since $Ca^{2+}$-pump ATPase in cardiac sarcolemma is intimately involved in the extrusion of $Ca^{2+}$ across the cell membrane [114], the inhibition of sarcolemmal $Ca^{2+}$-pump ATPase by oxygen free radicals could lead to decrease $Ca^{2+}$ extrusion from the cytosol, resulting in an increase in cytosolic $Ca^{2+}$ concentration.

#### *Mechanisms for the changes in heart sarcolemmal ion-transporting systems by oxygen free radicals*

To investigate the role of sulfhydryl groups in causing depression of the sarcolemmal $Ca^{2+}$-pump activities, we [115] examine the following aspects in heart sarcolemmal membranes:

1. the effects of sulfhydryl-reducing agents, such as DDT or cysteine on the depression of $Ca^{2+}$-pump activities due to oxygen free radicals,
2. the effects of sulfhydryl groups reagents, such as N-ethylmaleimide (NEM), on $Ca^{2+}$-pumpATPase activity, and

3. the effects of oxygen free radicals on sulfhydryl groups.

The inhibition of sarcolemmal $Ca^{2+}$-pump activities by oxygen free radicals was prevented by the addition of DTT or cysteine in a dose-dependent manner [115]. NEM inhibited $Ca^{2+}$-pump activity both in a dose- and a time-dependent manner [115]; DTT and cysteine prevented the changes in $Ca^{2+}$-pump activity because of NEM [115]. Hearts sarcolemmal sulfhydryl groups were depressed by various types of oxygen free radical-generating systems both in a dose- and a time-dependent manner; free radical scavengers showed protective effects on the sulfhydryl groups depression by oxygen free radicals [115]. Furthermore, there was a significant correlation between changes in sarcolemmal $Ca^{2+}$-pump ATPase activity and sarcolemmal sulfhydryl groups [115]. These results indicate that oxygen free radicals depress the heart sarcolemmal $Ca^{2+}$-pump activity by modifying the sulfhydryl groups in the sarcolemmal membrane. In addition, because sulfhydryl groups are known to regulated other membrane-bound ion-transporting systems such as $Na^+$-$K^+$ ATPase, $Na^+$-$Ca^{2+}$ exchange, and voltage-dependent $Ca^{2+}$ channel of sarcolemma, $Ca^{2+}$ release protein and $Ca^{2+}$-pump ATPase of sarcoplasmic reticulum [84, 106, 116–128], it is likely that the oxidation of sulfhydryl groups in the membrane-bound ion-transporting systems may lead to depression of the activities due to oxygen free radicals.

Not only the direct reactions of oxygen free radicals with membrane-bound enzyme proteins but lipid peroxidation of membrane phospholipids by free radicals can affect the enzyme activities. Accumulation of hydroperoxides resulted from peroxidation of membrane phopholipids can inactivate the enzymes activities by modifying the lipid microenvironment, by oxidizing amino acid residues, or by mediating polypeptide chain polymerization reactions [31].

*III. $Ca^{2+}$translocating processes of sarcoplasmic reticulum*

Hess *et al.* [129–135] have studied the effects of various types of oxygen free radical-generating systems on $Ca^{2+}$ transporting systems of cardiac sarcoplasmic reticulum. They observed that $Ca^{2+}$-pump ATPase activity and steady-state $Ca^{2+}$ uptake were depressed by free radicals [129–135]. Direct measurement of the number and turnover of the pump units indicated that the number of the units was unchanged but turnover rate was decreased by oxygen free radicals [131]. Furthermore, exposure to oxygen free radicals increased the passive permeability of the sarcoplasmic reticular vesicles to $Ca^{2+}$, but the increased permeability *per se* was insufficient to explain the effects of oxygen free radicals on $Ca^{2+}$-pump ATPase activity [131]. Cumming and Holmberg *et al.* [136, 137] have studied the effects of free radicals, which was produced by the illumination of rose bengal, on the sheep cardiac sarcoplasmic reticulum $Ca^{2+}$ release channel which was inserted into synthetic lipid bilayers. They found that $Ca^{2+}$ release channel open probability was increased initially, and that continued illumination resulted in an irreversible loss of channel function and subsequent bilayer disruption [136–138]. They also showed that ryanodine binding in isolated cardiac membranes was reduced by oxygen free radicals, with associated degradation of a 340 kD protein, which is thought to be the ryanodine receptor and sarcoplasmic reticular $Ca^{2+}$ release channel complex [136–138]. Abramson *et al.* [118, 119] and Zaidi *et al.* [126] reported that oxidizing agents were found to induce rapid $Ca^{2+}$ efflux from actively loaded sarcoplasmic reticular vesicles isolated from rabbit skeletal muscle. These data indicate that when sarcoplasmic reticulum is exposed to oxygen free radicals, $Ca^{2+}$ release from sarcoplasmic reticulum to cytosol is promoted and $Ca^{2+}$ sequestration from cytosol into sarcoplasmic reticulum lumen is inhibited.

Scherer *et al.* [123] and other researchers [121, 122] suggested that decline in $Ca^{2+}$-pump ATPase activity was due to oxidation of sulfhydryl groups as evidenced by the ability of sulhydryl groups reducing agents to prevent inhibition of the ATPase activity, the decline in sulfhydryl content of oxidized sarcoplasmic reticulum, and the ability of sulfhydryl groups binding agents to inhibit the $Ca^{2+}$-pump ATPase activity. Salama's group showed that reactive disulfide compounds, which are known to specifically oxidize free SH sites via a thiol-disulfide exchange reaction, trigger $Ca^{2+}$ release from sarcoplasmic reticular vesicles [116–119, 126]. Furthermore, they reported that sulfhydryl groups reducing agents can reverse the effects of reactive disulfide compounds [126]. Thus, the modification of sulfhydryl groups is important mechanism by which oxygen free radicals affect on $Ca^{2+}$-transporting systems in sarcoplasmic reticulum, as well as in sarcolemmal membrane.

## Effects of oxygen free radicals on intracellular $Ca^{2+}$ concentration

Electrophysiological studies have shown that the application of oxygen free radicals caused delayed after depolarization and aftercontraction, which indicate $Ca^{2+}$ overload of the cell [44, 49]. It is, however, important to measure intracellular $Ca^{2+}$ concentration during the application of oxygen free radicals. The development of calcium-sensitive fluorescent dyes has provided a new technique to monitor the changes in $Ca^{2+}$ during the contraction cycle and to measure intracellular $Ca^{2+}$ concentration. Using this technique, the effects of oxygen free radicals on intracellular $Ca^{2+}$ concentration have been studied [44, 139–142]. Hayashi *et al.* [44] reported that rod-shaped myocytes became shortened or rounded (contracture) after the application of 0.1 and 1 mM $H_2O_2$ in isolated guinea pig ventricular

myocytes, and that intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), measured by the fura-2 340/380 ratio, increased from the control values of 53 and 68 nmol/l to 110 and 105 nmol/l when cells were shortened during the perfusion of 0.1 and 1 mM $H_2O_2$, respectively, Burton *et al.* [139] observed that exposure of cultured rat ventricular myocytes to free radical-generating solution (2.3 mM purine, 0.01 U/ml xanthine oxidase, and 2.4 μM iron-loaded transferrin) altered $[Ca^{2+}]_i$: fura-2 fluorescence ratio which indicate $[Ca^{2+}]_i$ was 639% of the control value after approximately 30–70 min with cessation of normal $Ca^{2+}$ transients. They also reported that there was no increase in $[Ca^{2+}]_i$ when myocytes pretreated with 10 μM α-tochopherol for 18–24 hr were exposed to free radicals [139]. Josephson *et al.* [140] reported that measurements in isolated rat ventricular myocytes loaded with indo-1 demonstrated rises in both systolic and diastolic fluorescence ratio following exposure to the free radical-generating system (1–10 mM $H_2O_2$ plus $Fe^{3+}$-nitrilotriacetate): the $Ca^{2+}$ overload was prevented by the $Ca^{2+}$ channel antagonist, nitrendipine, suggesting that the $Ca^{2+}$ overload occured largely due to $Ca^{2+}$ influx through voltage gated $Ca^{2+}$ channels. Liu *et al.* [141] reported that the effect of oxidized low density lipoprotein (LDL) on the $Ca^{2+}$ transients of isolated rabbit cardiomyocytes using fura-2 technique. They observed that the systolic $Ca^{2+}$ concentration in transients was significantly increased after treatment with 100 μg of oxidized LDL cholesterol/ml for 16 min without any effect on the diastolic $Ca^{2+}$ concentration [141]. Eley *et al.* [142] showed exciting data concerning the effect of oxygen free radicals on $Ca^{2+}$ homeostasis in isolated rabbit ventricular myocytes which were loaded with fura-2 and superfused with 100 μm HOCl under voltage-clamped conditions. In these experiments, they observed that the amplitude of the $Ca^{2+}$ transients was reduced from 402 nM to 82 nM while $[Ca^{2+}]_i$ increased from 78 nM to 265 nM within 200 seconds after HOCl addition: during this time, the amplitude of the slow inward currents increased by 10%. This sustained steady-state rise in $[Ca^{2+}]_i$ occured even in the absence of extracellular $Ca^{2+}$ but was virtually abolished by preexposure to 10 mM caffeine [142]. Furthermore, although washout of HOCl failed to induce recovery, subsequent exposure to the dithiol reducing agent dithiothreitol caused a rapid restoration of both the steady-state $[Ca^{2+}]_i$ and $Ca^{2+}$ transient amplitude. Therefore, they concluded that HOCl caused a rise of $[Ca^{2+}]_i$ by inducing the release of $Ca^{2+}$ from internal stores and by impairing cellular extrusion mechanisms, and that these effects occur through alteration of protein thiol redox status [142].

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