Stunned Myocardium and Oxygen Free Radicals
— Sarcolemmal membrane damage due to oxygen free radicals —

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Reperfusion after reversible ischemia has been shown to result in prolonged depression of contractile function ("myocardial stunning"). Recent studies suggest that oxygen free radicals may mediate postischemic dysfunction. Since heart sarcolemmal membranes, which contain several types of enzymes, ion channels and receptors play important roles to maintain cell functions, the present study was undertaken to examine the effects of oxygen free radicals on heart sarcolemmal membrane functions in vitro. In the presence of a superoxide anion radical-generating system (2mM xanthine plus 0.03 U/ml xanthine oxidase), sarcolemmal Ca^{2+}-stimulated ATPase activity and ATP-dependent Ca^{2+} accumulation were inhibited in an incubating time-dependent manner. Both lipid peroxidation ($r=0.82$) and sulfhydryl group content ($r=0.95$) showed significant correlations with Ca^{2+}-stimulated ATPase activity. ATP-indenpendent Ca^{2+} bindings were increased upon treating the membranes with xanthine plus xanthine oxidase. Voltage-dependent Ca^{2+}-channels were also affected by oxygen free radicals. The maximal number of binding sites (Bmax) for [^{3}H]-nitrendipine binding was depressed without any changes in dissociation constant (Kd). The effects of oxygen free radicals on adrenergic receptors were more complex. Bmax for [^{3}H]-dihydroalprenolol (DHA) binding (β-receptor) was increased whereas Bmax for [^{3}H]-prazosin binding (α_1-receptor) was decreased after incubating the membrane with xanthine plus xanthine oxidase. Kd for [^{3}H]-DHA or [^{3}H]-prazosin binding was increased. Superoxide dismutase showed protective effects on the changes in these membrane functions due to xanthine plus xanthine oxidase. It is suggested that oxygen free radicals damage heart sarcolemmal mambrane functions which may lead to cardiac dysfunction in the stunned myocardium.

REPERFUSION after a brief episode of myocardial ischemia may result in prolonged contractile dysfunction without cell necrosis, a phenomenon known as "myocardial stunning"! Despite considerable investigative efforts, the mechanisms responsible for this phenomenon remain unclear.

Recently, the data in the literature support the concept that stunned myocardium is mediated, at least in part, by oxygen free radicals because 1) oxygen free radicals have been demonstrated to be increased in the myocardium[2] or in the coronary venous effluent of hearts[3] stunned by brief periods of ischemia and reperfusion; 2) exogenously administered oxygen free radical-generating

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solutions depress myocardial fuction[4,5]; and 3) the recovery of the stunned myocardium is enhanced by agents that either scavenge oxygen free radicals, such as superoxide dismutase and catalase[6], N-(2-mercaptopropiony1) glycine[7], and dimethylthiourea[8], or prevent their generation, such as allopurinol[9].

Since heart sarcolemmal membranes, which contain several types of enzymes, ion channels and receptors, play important roles to maintain the cell functions, damage of the sarcolemmal membranes may lead to dysfunction of myocardial cells. The recent study was undertaken to examine the effects of oxygen free radicals on Ca^{2+}-stimulated Mg^{2+}-dependent ATPase (Ca^{2+}-stimulated ATPase) activity, ATP-dependent Ca^{2+} accumulation, ATP-independent Ca^{2+}-binding, voltage dependent Ca^{2+} channel, and adrenergic receptors (α_1 and β receptors) in rat cardiac sarcolemmal membranes in vitro.

METHODS

Isolation of sarcolemmal membranes

Male Spague-Dawley rats weighing 200-250g were killed by decapitation. Hearts were removed, and the ventricular tissue was processed for the isolation of sarcolemmal membranes by the sucrose gradient method[10] or the hypotonic shock LiBr treatment method[11]. These purified membranes were characterized with respect to marker enzyme activities and were found to be devoid any major cross contamination[14].

Protein concentration was estimated by the method of Lowry et al[12].

Measurement of Mg^{2+}-ATPase and Ca^{2+}-stimulated ATPase activities

For the estimation of Mg^{2+}-ATPase, sarcolemmal vesicles (20—50 μg protein) were preincubated at 37°C for 5 min in 0.5 ml of medium containing 140 mM KCl-10 mM MOPS-Tris (pH 7.4), 2 mM $MgCl_2$, 5 mM sodium azide, and 0.2 mM ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetra-acetic acid (EGTA). The reaction was started by the addition of 4 mM Tris-ATP (pH 7.4) and was terminated 5 min later with 0.5 ml of cold 12% trichloroacetic acid; the liberated phosphate was measured by the method of Tausky and Shorr[13]. Estimation of total (Ca^{2+}-stimulated $+Mg^{2+}$) ATPase was carried out in the medium containing 140 mM KCl-10 mM MOPS-Tris (pH 7.4), 2 mM $MgCl_2$, 5 mM sodium azide, and 1×10^{-5}M free Ca^{2+}. The concentration of free Ca^{2+} in the medium was adjusted by using EGTA[14]. The Ca^{2+}-stimulated ATPase activity was the difference between the total ATPase and Mg^{2+}-ATPase activities.

ATP-dependent Ca^{2+}-accumulation assay

Sarcolemmal vesicles (100 μg protein) were preincubated in 0.5 ml of medium containing 140 mM KCl-10 mM MOPS-Tris (pH 7.4), 2 mM $MgCl_2$, and desired amout of $^{45}CaCl_2$-EGTA ($^{45}CaCl_2$ 39.56 mCi/ng; New England Nuclear, Boston, MA) to give 1x10^{-5}M free Ca^{2+}[14]. Ca^{2+} accumulation was initiated by adding 4 mM Tris-ATP (pH 7.4). After 5 min of incubation at 37°C, 250 ul aliquots were immediately filtered through Millipore filters (0.45 μm), washed with 2×3-ml ice-cold KCl-MOPS and 1 mM $LaCl_3$ (pH 7.4), dried, and the radioactivity determined for calculating the total Ca^{2+} accmulation.

Non-specific Ca^{2+} binding was measured in the absence of ATP for each set of experiments. The ATP-dependent Ca^{2+} accumulation was calculated by subtracting non-specific Ca^{2+} binding from total Ca^{2+} accumulation.

Determination of lipid peroxidation and sufhydryl group content

The lipid peroxidation was assayed by measuring the formation of malondialdehyde by the thiobarbituric acid method as described by Buege et al[15].

Sulfhydryl content of the sarcolemmal membranes was determined with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), according to the procedure described elsewhere[16]. The assay system contained 20 mM imidazole-HCl buffer (pH 7.0), 1 mM EDTA, and 0.2 mM DTNB. For the determination of free sulfhydryl groups, 200 μg membrane grotein was incubated for 3 min in the above medium, and the absorbance at 412 nm was measured. For total sulfhydryl groups determination, the membrane was incubated for 30 min in the assay medium in the presence of 0.1% sodium dodecyl sulfate

TABLE I EFFECTS OF XANTHINE+XANTHINE OXIDASE ON ATP-DEPENDENT Ca^{2+} ACCUMULATION AND ATP-ASE ACTIVITIES

Treatment	ATP-dependent Ca^{2+} accumulation (n mol Ca^{2+}/mg protein/5 min)	ATPase activity (μ mol Pi/mg protein/hr)	
		Mg^{2+}-ATPase	Ca^{2+}-stimulated ATPase
Control	27.0±1.7	195±3	13.6±0.7
X	24.7±1.4	193±3	13.4±0.4
XO	24.2±1.3	194±3	14.0±0.4
X+XO	9.5±0.8*	176±2*	2.6±0.5*
X+XO+SOD	21.8±0.8*	192±2	10.1±0.4*
X+XO+SOD+CAT	21.7±0.8*	192±2	10.2±0.5*
SOD	30.6±2.0	193±3	13.9±0.5
CAT	26.4±1.1	193±3	13.3±0.3

Prior to assay for ATP-dependent Ca^{2+} accumulation and ATPase activity, sarcolemmal membranes (200 μg protein/ml for Ca^{2+} accumulation and 300–400 μg protein/ml for ATPase activity) were incubated for 30 min at 37°C in 140 mM KCl-10mM MOPS, pH 7.4 plus the additions shown. Controls were also incubated under similar conditions without any additions. The final concentrations of xanthine (X), xanthine oxidase (XO), superoxide dismutase (SOD), and catalase (CAT) were 2 mM, 23 μg/ml (0.03 U/ml), 80 μg/ml, and 10 μg/ml, respectively. Each value is a mean±S.E. of 6 different preparations.

* *significantly different from control values ($P<0.05$).*

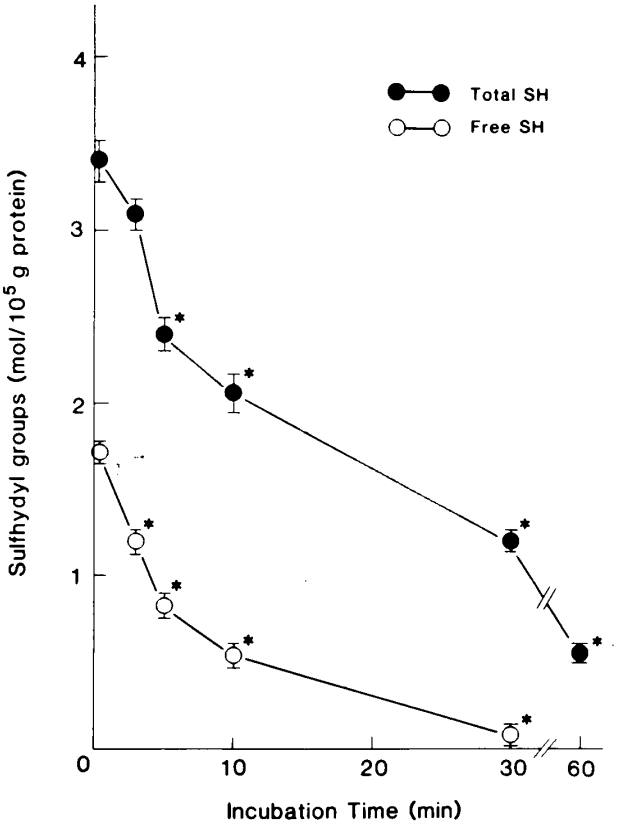

Fig.1. Time-dependent effects of xanthine+xanthine oxidase on sulfhydryl groups. At indicated times of incubation, samples were assayed for sulfhydryl content. Each value is a mean±S.E.of 6 different preparations. *significantly different from control values ($p<0.05$).

(SDS). It should be mentioned that SDS dissociates the membrane and allows the measurement of both extra- and intramembranal sulfhydryl groups. Calculation of the sulfhydryl groups was based on a molar extinction coefficient of 13,600 M/cm

TABLE II EFFECTS OF EXYGEN FREE RADICALS ON ATP-INDEPENDENT Ca^{2+} BINDING IN HEART SARCOLEMMAL MEMBRANES

Treatment	*ATP-independent Ca^{2+} binding (n mol/mg/5 min)*	
	Low affinity (1.25 mM Ca^{2+})	*High affinity (50 μM Ca^{2+})*
Control	*97.8±4.3*	*7.95±0.32*
X (2 mM)	*92.9±5.7*	*8.14±0.29*
XO (0.03 U/ml)	*101.6±6.5*	*7.88±0.40*
X (2 mM)+XO (0.03 U/ml)	*147.2±6.1**	*12.08±0.68**

*Each value is a mean±S.E. of 8 different preparations. The concentrations of Ca^{2+} were 1.25 mM and 0.05 mM for experiments on low-affinity binding and high-affinity binding, respectively. X: xanthine, XO: xanthine oxidase; the incubation period was 5 min. * Significantly different from control values ($P<0.05$).*

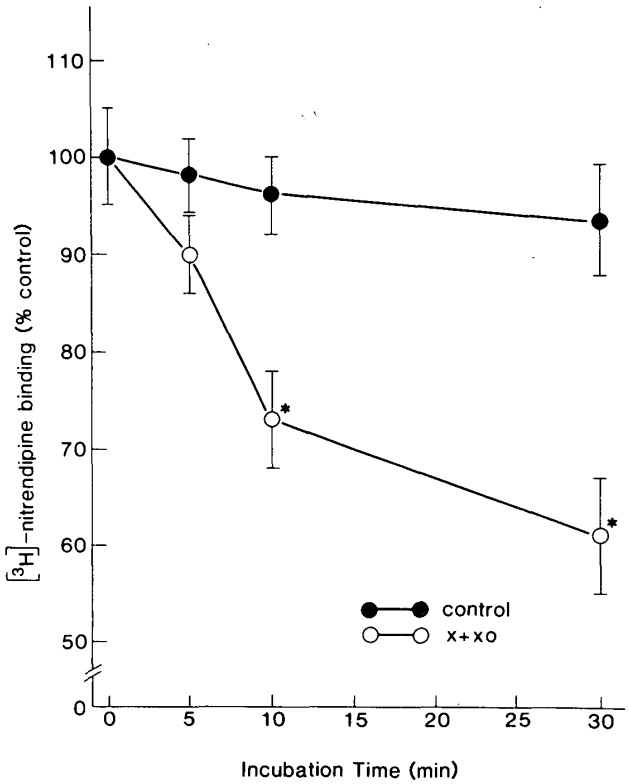

Fig.2. Incubation time-dependent effects of xanthine+xanthine oxidase on the [^{3}H]-nitredipine binding with rat heart crude membranes. Membrane preparations (1-1.5 mg/ml) were preincubated at 37°C without (control ●—●) or with (○—○) 2 mM xanthine plus 0.03 U/ml xanthine oxidase in a medium contaning 10 mM Tris-HCl, pH 7.4. At the indicated times of incubation, samples were diluted 20 times with 50 mM Tris-HCl, pH 7.4 and centrifuged at 48,000 g for 25 min and washed twice. Final pellet was suspended in 50 mM Tris buffer for [^{3}H]-nitrendipine binding assay. Each valve is a mean±S.E.of 5 different preparations. *: significantly different from control values ($p<0.05$).

at 412 nm for the thiophenol reaction product. This was verified with cysteine as a standard.

Determination of ATP-independent Ca^{2+} binding

ATP-independent Ca^{2+} binding was determined by employing 1.25 mM (low-affinity, high-capacity Ca^{2+} binding) or 0.05 mM Ca^{2+} (high-affinity, low-capacity Ca^{2+} binding) according to the procedure reported before![17]

[^{3}H]-nitrendipine binding assay

[^{3}H]-nitrendipine binding was measured according to a method reported earlier[18] Crude cardiac membrane preparations (0.08-0.1 mg protein/tube) were incubated with 0.05 to 5 nM [^{3}H]-nitrendipine in the absence or presence of 2 μM unlabelled nifedipine. The non-specific [^{3}H]-nitrendipine binding (in the presence of nifedipine) was subtracted from the total binding (in the absence of nifedipine) to obtain specific binding. The non-specific [^{3}H]-nitrendipine binding varied between 25 and 30% of the total binding values and none of the interventions employed in this study had any effect on the non-specific binding. [^{3}H]-nitrendipine was obtained from New England Nuclear Medicine (Boston, USA) as 5-methyl-[^{3}H]-nitredipine, with a specific activity of 80.9 Ci/mmol.

[^{3}H]-dihydroalprenolol (DHA) and [^{3}H]-prazosin binding assay

To determine β-adrenergic receptor binding, crude cardiac membranes were incubated with different concentrations of [^{3}H]-DHA in the presence and absence of 10 μM l-propranolol. The α_1-adrenergic receptor binding was analyzed by using an α_1-adrenergic antagonist [^{3}H]-prazosin in the presence and absence of 10 μM phentolamine hydrochloride.

Free radical-generating systems

Superoxide anion radicals were generated by the xanthine oxidase (Terochem Laboratories) reaction in which xanthine was used as a substrate[14,16] Superoxide dismutase (SOD; Sigma), was used as a scavenger for superoxide anion radicals.

Statistical analysis

Results are presented as mean$\pm$SE. For statistical evaluation, multiple analysis of variance was carried out, and Duncan's multiple-range test was used to determine differences between the means within the population. $p<0.05$ was taken to reflect a significant difference.

RESULTS AND DISCUSSION

Effects of xanthine plus xanthine oxidase on ATP-dependent Ca^{2+} accumulation and ATPase activities

The effects of superoxide radicals on ATP-dependent Ca^{2+} accumulation and ATPase activities were examined by incubating the sarcolemmal membranes in the presence of 2 mM xanthine plus 0.03 U/ml xanthine oxidase. Table I shows that the incubation of membranes with either 2 mM xanthine, 0.03 U/ml xanthine oxidase, 80 μg/ml SOD, or 10 μug/ml catalase for 30 min did not affect ATP-dependent Ca^{2+} accumulation and ATPase activities. However, a combination of xanthine plus xanthine oxidase depressed ATP-dependent Ca^{2+} accumulation and Ca^{2+}-stimulated ATPase activities by 65% and 81% of the control values, respectively. SOD showed a protective effect on the depression in Ca^{2+} accumulation and ATPase activities due to incubation with xanthine plus xanthine oxidase. To study the time-course effects of xanthine plus xanthine oxidase on Ca^{2+}-stimulated ATPase activity, the enzyme activity was measured after 1, 5, 10, 30, and 60 min preincubation at 37°C. Ca^{2+}-stimulated ATPase activity was inhibited in a time-dependent manner.

The interaction of superoxide radicals with sarcolemmal membrane vesicles was rapid, because significant inhibition of Ca^{2+}-stimulated ATPase activity was seen after 1 min of incubation.

Lipid peroxidation and sulfhydryl group contents

Oxygen free radicals are known to attack molecules of major biological significance and these include phospholipids, proteins and nucleic acids[19] Since changes in lipid environment of membrane-bound enzyme protein can affect the enzyme activity, and since sulfhydryl groups are known to regulate the membrane-boud enzyme activities in the cell, it is possible that the lipid peroxidation or the oxidation of sulfhydryl groups in the membrane may lead to the depression of enzyme activities because of oxygen free radicals.

Lipid peroxidation as reflected by MDA formation was measured after 30 min incubation at 37°C. The incubation of sarcolemmal vesicles in the presence of xanthine plus xanthine oxidase increased the level of MDA formation by 133% (8.3 ± 0.03 nmol of MDA/mg protein) in comparison with the control samples (3.56 ± 0.18 nmol MDA/mg

TABLE III TIME-DEPENDENT EFFECTS OF XANTHINE+XANTHINE OXIDASE ON [^{3}H]-DHA AND [^{3}H]-PRAZOSIN BINDING IN RAT HEART MEMBRANE

Incubation Time	*[^{3}H]-DHA binding*		*[^{3}H]-prazosin binding*	
	Kd (nM)	*Bmax (fmol. mg^{-1})*	*Kd (nM)*	*Bmax (fmol. mg^{-1})*
10 min				
Control	*0.649±0.055*	*70.5±6.2*	*0.069±0.006*	*70.6±6.4*
X+XO	*0.884±0.072**	*107.3±8.5**	*0.077±0.007*	*67.5±5.3*
30 min				
Control	*0.652±0.070*	*68.9±5.3*	*0.070±0.007*	*69.1±5.8*
X+XO	*1.231±0.143**	*96.2±6.5**	*0.088±0.009*	*60.3±4.2*
60 min				
Control	*0.668±0.071*	*68.6±4.7*	*0.071±0.007*	*67.8±5.5*
X+XO	*1.127±0.121**	*83.5±6.9*	*0.145±0.008**	*40.2±4.0**

Each value is mean±S.E. of 5 different preparations. Prior to assay for both [^{3}H]-DHA and [^{3}H]-prazosin binding, crude membranes (1 mg/ml) were incubated without (control) or with 2 mM xanthine (X) plus 0.03 U/ml xanthine oxidase (XO) for different time periods at 37°C.
** Significantly different from control values ($P<0.05$).*

pretein). The addition of SOD to the incubation medium resulted in a significant decrease of MDA formation when compared with values obtained with xanthine plus xanthine oxidase.

Fig. 1 shows the time-dependent effects of superoxide radicals on sulfhydryl groups. Free sulfhydryl groups were significantly depressed after 3 min of incubation, whereas total sulfhydryl groups were significantly depressed after 5 min of incubation. To find a relationship between Ca^{2+}-stimulated ATP ase activity and lipid peroxidation or sulfhydryl group contents, the data from experiments concerning the effects of oxygen free radicals on heart sarcolemma were plotted. Both lipid peroxidation ($r=0.82$) and sulfhydryl groups ($r=0.95$) showed significant correlations with Ca^{2+}-stimulated ATPase activity.

ATP-independent Ca^{2+} binding

Membrane-bound Ca^{2+} in cardiac cell is believed to serve as a superficial store of Ca^{2+}, which becomes available for entry upon excitation of the myocardium[20] In fact, ATP-independent Ca^{2+} binding in heart sarcolemma has been shown to exhibit a linear relationship with the contractile force development in the normal myocardium[21,22] Table II shows the effects of oxygen free radicals on ATP-independent Ca^{2+} binding. In the presence of xanthine plus xanthine oxidase, both low and high affinity Ca^{2+} binding were increased by 51% and 52% of control values, respectively.

Voltage-dependent Ca^{2+}-channel

Figure 2 shows incubation time-dependent effects of xanthine plus xanthine oxidase on [^{3}H]-nitrendipine binding with rat heart crude membranes. Specific binding of [^{3}H]-nitrendipine was significantly reduced after incubating the preparation for 10 and 30 min with oxygen free radical-generating system; 5 min incubation did not show significant changes. Since the activities of membrane-bound enzymes, such as Na^+-K^+ ATPase[23] and Ca^{2+}-stimulated ATPase were significantly depressed within 5 min of incubation under similar experimental conditions, it appears that [^{3}H]-nitrendipine binding proteins are more resistant to the action of oxygen free radicals. The Scatchard plot analysis of the data revealed that the maximal number of binding sites (Bmax) was depressed by the oxygen free radical -generating system (control 199±12 fmol/mg protein, xanthine plus xanthine oxidase 139±7 fmol/mg protein) without any changes in the dissociation constant (Kd) (conrol 0.231±0.011 nM, xanthine plus xanthine oxidase 0.252±0.011 nM).

The presence of SOD in the xanthine plus xanthine oxidase mixture was capable of preventing the effects of this radical-generating system (Bmax 171±10 fmol/mg protein). Xanthine, xanthine oxidase or SOD did not

exert any effect on the Bmax or Kd values for [^{3}H]-nitrendipine binding. It should be pointed out that non-specific binding values for xanthine plus xanthine oxidase-treated group were not significantly different from those for the control group.

Adrenergic receprors

Table III shows the incubation time-dependent effects of xanthine plus xanthine oxidase on [^{3}H]-DHA and [^{3}H]-prazosin binding. The Scatchard plot analysis of the data revealed that Bmax for [^{3}H]-DHA binding was increased after incubating the preparations for 10 min or 30 min with superoxide radical generating system; 60 min incubation did not show any significant changes. On the other hand, Bmax for [^{3}H]-prazosin was unchanged at 10 min and 30 min incubation but was decreased following 60 min incubation with xanthine plus xanthine oxidase. The Kd for [^{3}H]-DHA or [^{3}H]-prazosin binding was increased upon treating the membranes with xanthine plus xanthine oxidase. SOD showed the protective effects on the changes in Bmax and Kd for both [^{3}H]-DHA and [^{3}H]-prazosin binding due to xanthine plus xanthine oxidase. Xanthine, xanthine oxidase, and SOD did not show any significant effects on Bmax and Kd values for the adrenergic receptors.

The data presented in this study indicate that when cardiac sarcolemmal membranes were exposed to oxygen free radicals, Ca^{2+}-pump activity, ATP-independent Ca^{2+} binding, voltage-dependent Ca^{2+}-channels, and adrenergic receptors functions were affected. Also associated with free radical damage to sarcolemmal membranes in the inhibition of Na^+-K^+-ATPase activity[23] Levedev et al[24] demonstrated that permeability of membranes to Ca^{2+} was increased by the lipid peroxidation of phospholipids. Furthermore, Ca^{2+} uptake of sarcoplamic reticulam was reduced in the presence of free radicals[25] All these mechanisms may lead to the changes in Ca^{2+} homeostasis in the cell which may result in dysfunction of the myocardium.

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