EFFECTS OF HYDROGEN PEROXIDE ON STIMULATORY GUANINE NUCLEOTIDE-BINDING PROTEIN IN RAT HEART

HISAMICHI MASUDA, M.D., MASANORI KANEKO, M.D., RONG BANG HONG, M.D.
TAKAYOSHI IKEGAYA, M.D., HIDEHARU HAYASHI, M.D.*, AKIRA KOBAYASHI, M.D.
AND NOBORU YAMAZAKI, M.D.

This study was undertaken to examine the effects of hydrogen peroxide on stimulatory guanine nucleotide-binding protein (Gs), and coupling in the β-aderenergic receptor - Gs - adenylate cyclase system in rat heart, in vitro. Cardiac membranes were preincubated with various concentrations (0.1, 1, and 10 mM) of hydrogen peroxide at 30 °C for 5, 10, 30 and 60 min. Although the assay of β-adrenergic receptors involving [^{3}H]-dihydroaloprenolol ([^{3}H]-DHA) binding revealed that the maximal number of binding sites (B_{max}) was not altered, the dissociation constant (K_d) for [^{3}H]-DHA was increased in the presence of 1 mM and 10 mM hydrogen peroxide (control 0.68 ± 0.16 nM, vs 1 mM H_2O_2 1.13 ± 0.16, 10 mM H_2O_2 1.01 ± 0.12). Conversely, no significant changes in Gs activities were observed in hydrogen peroxide-treated groups. Adenylate cyclase activity (stimulated by forskolin) was significantly reduced by 10 mM hydrogen peroxide after a 5 min preincubation period (control 277.1 ± 19.2 pmol cAMP/mg protein/min, H_2O_2 230.3 ± 14.9). The amounts of cyclic AMP produced by the stimulation of membranes with GTP, GTP+(l)-isoproterenol, guanylimidodiphosphate (Gpp(NH)p) or Gpp(NH)p+(l)-isoproterenol were significantly lower in 10 mM hydrogen peroxide-treated groups than those in controls (GTP: control 57.6 ± 5.6 pmol cAMP/mg protein/min vs H_2O_2 46.4 ± 6.9, GTP+(l)-isoproterenol: control 83.9 ± 10.2 vs H_2O_2 67.7 ± 10.3, Gpp(NH)p: control 77.5 ± 8.8 vs H_2O_2 61.0 ± 8.6, Gpp(NH)p+(l)-isoproterenol: control 105.0 ± 13.1 vs H_2O_2 83.9 ± 12.2, forskolin: control 223.2 ± 13.8 vs H_2O_2 182.8 ± 18.4). In the presence of hydrogen peroxide, the extents of the depression in cAMP production induced by GTP, GTP+isoproterenol, Gpp(NH)p, and Gpp(NH)p+(l)-isoproterenol were similar to those induced by forskolin stimulation. These results indicate that hydrogen peroxide does not affect Gs activity or coupling in the β-receptor - Gs - adenylate cyclase system, but does depress cAMP production by inhibiting adenylate cyclase.

(*Jpn Circ J* 1993; **57:** 1007−1015)

Key words:
β-adrenergic receptor
Guanine nucleotide-binding protein
Adenylate cyclase
Oxygen free radicals
Hydrogen peroxide

OXYGEN free radicals have been implicated as mediators of cellular injury under some pathophysiological conditions[1−3] Although exogenously-generated oxygen free radicals are known to cause con-

(Received August 21, 1992; accepted March 18, 1993)
*Third Department of Internal Medicine, and *Photon Medical Research Center, Hamamatsu University School of Medicine, Hamamatsu, Japan*
This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan
Mailing address: Masanori Kaneko, M.D., Ph.D., Third Department of Internal Medicine, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31, Japan

tractile dysfunction in the heart[4,5] the detailed mechanisms which underlie this dysfunction are still unclear. Myocardial contractility is regulated by several physiological factors[6,7] Calcium ion, inositol 1, 4, 5-triphosphate, 1, 2-diacylglycerol, and cyclic AMP (cAMP) are all thought to be potentially important second messengers in the heart[6] Cyclic AMP levels are modulated by the β-adrenergic receptor-stimulatory guanine nucleotide-binding protein (Gs) - adenylate cyclase system[8,9] The β-adrenergic receptor - Gs - adenylate cyclase system is a plasma membrane-bound protein assembly consisting of three major components. The heterotrimetric guanine nucleotide-binding regulatory proteins couple with extracellular receptors and cause the stimulation (Gs) or inhibition (Gi) of the effector enzyme adenylate cyclase, which is the primary regulator of the intracellular concentration of the second messenger cAMP[9,10] Since cAMP modulates cardiac contractility[6,7] changes in β-adrenergic receptor, Gs, and/or adenylate cyclase activities, as well as in coupling of the component, can affect cardiac contractility.

We and other investigators have reported that oxygen free radicals can affect β-adrenergic receptor density[11,12] and adenylate cyclase activity[4,13,14] Furthermore, it has been reported that the efficacy of β-adrenoceptor agents is reduced in oxygen free radical-treated hearts[4] However, little is presently known about the effect of oxidative stress on Gs activity. Therefore, this study was undertaken to examine the effects of hydrogen peroxide on Gs activity and coupling in the β-adrenergic receptor - Gs - adenylate cyclase system in rat heart, in vitro.

METHODS

Cardiac Membrane Preparation

Male Sprague-Dawley rats weighing 200—300 g were sacrificed by cervical dislocation and their hearts were removed. The hearts were trimmed free of atria and large vessels using scissors. The ventricular tissue was homogenized in 50 ml/g wet tissue weight of cold homogenizing buffer (50 mM Tris-HCl, pH 7.4), using a Polytron PT-20 (setting 6.5) for 30 sec. The homogenate was filtered through four layers of gauze, and centrifuged at 1,000×g for 10 min. KCl was added to the supernatant (final KCl concentration 0.65 M), and the resulting mixture was stirred at 4 °C for 15 min. The supernatant was then centrifuged at 40,000×g for 20 min. The resulting pellet was washed twice in cold incubation buffer (50 mM Tris-HCl, pH 7.4) by resuspension and centrifugation, and was finally resuspended at 2 mg of protein/ml in incubation buffer[15] Protein concentration was estimated by the method of Lowry et al[16]

[^{3}H]-Dihydroaloprenolol ([^{3}H]-DHA) Binding Assay

Membranes were preincubated with various concentrations (0.1, 1, and 10 mM) of hydrogen peroxide at 30 °C for 10 and 60 min. To determine β-adrenergic receptor binding, the membranes were incubated with six different concentrations (0.16—5.0 nM) of [^{3}H]-DHA, in a total volume of 0.5 ml either with or without 20 μM of (l)-propranolol at 30 °C for 30 min. All incubations were terminated by rapid vacuum filtration through Whatman GF/B filters. Specific binding for [^{3}H]-DHA was calculated by subtracting nonspecific binding in the presence of (l)-propranolol from the total binding value obtained in the absence of (l)-propranolol[17,18]

Membrane Preparation of Mouse S49 Lymphoma Cells (cyc$^-$)

Plasma membranes of S49 lymphoma cells (cyc$^-$) were prepared according to the method of Ross et al[19] Cells were harvested at a density of 2.0 to 3.5×10^6/ml by low speed centrifugation, and washed twice at room temperature in 137 mM NaCl, 5.36 mM KCl, 1.1 mM KH_2PO_4, and 1.08 mM Na_2HPO_4 (pH 7.2). Subsequent steps were performed at 0—4 °C. Cells were suspended at 3×10^7/ml in 20 mM Na-HEPES, 2 mM $MgCl_2$, and 1 mM EDTA (pH 8.0)(HME buffer), and homogenized using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 43,000×g at 4 °C for 20 min, and the resulting pellet was suspended in HME buffer to a protein concentration of 3.5 mg/ml.

Assay of Stimulatory Guanine Nucleotide-

binding Protein (Gs) Activity

Gs activity was assessed using a reconstitution assay originally described by Sternweis et al[20] Briefly, the technique makes use of the capacity of Gs to be solubilized from cardiac membranes and then functionally coupled to S49 mouse lymphoma cell (cyc$^-$) membranes. These membranes are functionally deficient in Gs, but possess the adenylate cyclase catalytic unit. Because cardiac membranes also contain the catalytic unit of adenylate cyclase, we inactivated the membrane adenylate cyclase by heating the membrane preparations to 37 °C for 5 min[10] Adenylate cyclase activity was rapidly lost when the cardiac membranes were heated to 37 °C. The activity of Gs protein in cardiac membranes was more heat-stable than that of adenylate cyclase.

Cardiac membranes (0.8 mg/ml) were preincubated either with various concentrations (0.1, 1, and 10 mM) of hydrogen peroxide or without hydrogen peroxide, and heated at 37 °C for 5 min. Heat-inactivated cardiac membranes were suspended in 20 mM Tris-HCl (pH 8.0) containing 0.1% lubrol-PX, 1 mM EDTA, 100 mM NaCl, and 1 mM DTT. An aliquot of this cardiac membrane suspension (10 μl) was then mixed with 30 μl of cyc$^-$ membranes and allowed to sit on ice for 30 min. Preactivation was then performed by adding 20 μl of a buffer containing 150 mM HEPES-NaOH (pH 8.0), 2 mM ATP, 0.3 mg/ml bovine serum albumin (BSA), 9 mM phosphoenolpyruvate-KCl (pH 7.0), 15 mM $MgCl_2$, 30 μg/ml pyruvic acid, 60 μM $AlCl_3$ and 30 mM NaF. This was followed by incubation at 30 °C for 10 min. Adenylate cyclase activity was then assayed by adding 10^6 cpm [α-^{32}P]-ATP (in a total volume of 40 μl with 125 mM HEPES-NaOH (pH 8.0), 2.5 mM EDTA-Na (pH 7.0), 0.25 mM ATP, 0.25 mg/ml BSA, 7.5 mM phosphenolpyruvate-KCl (pH 7.0), 20 mM $MgCl_2$, 25 μg/ml pyruvic acid, 0.25 mM 3-isobutyl-1-methylxanthine) and incubating the mixture for an additional 30 min at 30 °C. The reaction was terminated by adding 900 μl of a stop solution containing 2.5% dodesylthionatrium (SDS), 40 mM ATP, 1.75 mM cAMP (pH 7.5), and about 10,000 cpm [^{3}H]-cAMP.

The reaction mixture was then passed through a column containing 1 ml of Dowex 50AG WX8 resin. The eluate from this passage and two successive 1 ml H_2O washes were discarded. Three ml of H_2O was then added to the column, and the eluate was collected and passes through a column containing 1 ml of neutral alumina type WN-3[21] The eluate from this passage and from successive washes with 1 ml of 0.1 M imidazol-HCl (pH 7.5) was discarded. An aliquot of 0.1 M imidazol-HCl (pH 7.5, 2 ml) was added to each column, and the eluate was then counted in scintillation vials containing 15 ml of a toluene-based scintillation cocktail. This cocktail contained 30% (v/v) Triton-X-100, 0.4% (w/v) 2, 5-diphenyloxazole, and 0.01% (w/v) 1, 4-bis (2-(4-methyl-5-phenyloxazolyl))-benzene. Recovery of added [^{3}H]-cAMP was about 50%[10,21]

Assay of Adenylate Cyclase Activity

The adenylate cyclase activity of cardiac membrane was assayed according to the method of Salomon et al[21] Cardiac membranes (0.8 mg protein/ml, 20 mM Tris) were preincubated with various concentrations (0.1, 1, and 10 mM) of hydrogen peroxide at 30 °C for 5, 10, 30, and 60 min. The assay mixture contained the following in a final volume of 100 μl: 25 mM Tris HCl (pH 8.0), 1 mM EDTA-NaOH (pH 7.0), 1 mM ATP, 0.16 mg/ml BSA, 20 mM creatinephosphate, 10 U/ml creatinekinase, 5 mM $MgCl_2$, 1 mM cAMP, 0.1 mM 3 isobutyl-1-methylxanthine, 40 μg protein of cardiac membranes, 10^6 cpm [α-^{32}P]-ATP, and 100 μM forskolin. Incubation was carried out at 30 °C for 10 min. The reaction was stopped by adding 900 μl of a stop solution containing 2.5% SDS, 40 mM ATP, 1.75 mM cAMP, and 10,000 cpm of [^{3}H]cAMP.

The reaction mixture was then passed through a Dowex 50AG WX8 resin column and a neutral alumina type WN-3 column using the method of Salomon[21]

Coupling in the β-receptor - Gs - Adenylate cyclase System

Cardiac membranes were preincubated with various concentrations (0.1, 1, and 10 mM) of hydrogen peroxide at 30 °C for 10 and 60 min. The treated membranes were stimulated with either 100 μM guanosine triphosphate (GTP), 100 μM GTP+(l)-isoproterenol, 100 μM 5-guanylimidodiphos-

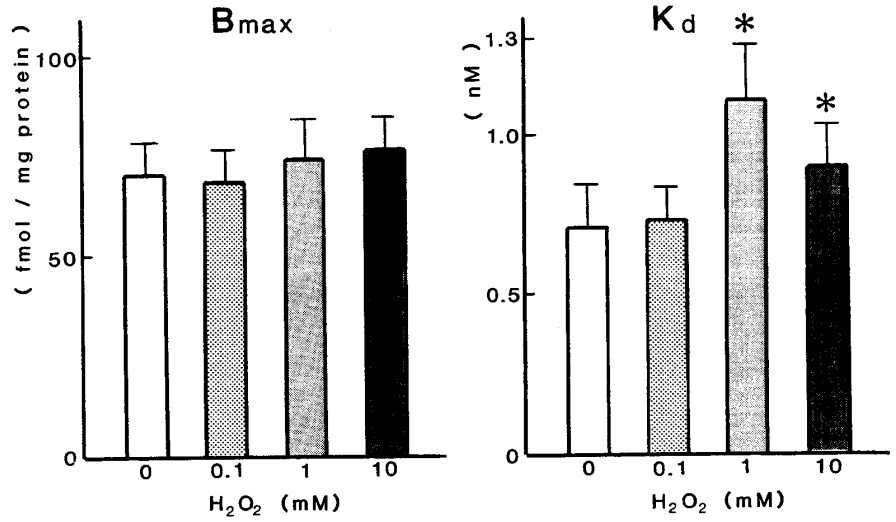

Fig.1. Effects of hydrogen peroxide on B_{max} and K_d for [^{3}H]-DHA binding. Cardiac membranes were preincubated either without (control) or with hydrogen peroxide at 30 °C for 10 min. The left panel shows the B_{max} of [^{3}H]-DHA binding, and the right panel shows the K_d for [^{3}H]-DHA binding. Values are means±S.D. of 7 different preparations. * indicates significant difference from control value ($p<0.05$)

TABLE I EFFECTS OF HYDROGEN PEROXIDE ON GUANINE NUCLEOTIDE-BINDING PROTEIN ACTIVITY

Concentration of H_2O_2	*Adenylate cyclase activity (pmol cAMP/mg protein/min)*	
	10 min	*60 min*
Control	*556.4±32.8*	*480.2±33.0*
0.1 mM	*563.5±28.7*	*487.8±18.0*
1.0 mM	*540.5±26.2*	*477.9±22.9*
10 mM	*541.6±23.8*	*467.1±19.5*

Cardiac membranes were preincubated with 0.1, 1, and 10 mM hydrogen peroxide at 30 °C for 10 and 60 min. Adenylate cyclase activity was assayed in the presence of 30 mM NaF. Each value is a mean±S.D. of 6 different preparations.

phate (Gpp(NH)p), or 100 μM Gpp(NH)p +100 μM (l)-isoproterenol before cAMP contents were measured.

Statistic Analysis

Results were calculated as means±SD. 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 a population. P level<0.05 was taken to reflect a significant difference.

RESULTS

Effects of Hydrogen Peroxide on [^{3}H]-Dihydroaloprenolol ([^{3}H]-DHA) Binding

Fig. 1 shows the effects of various concentrations of hydrogen peroxide on [^{3}H]-DHA binding in rat heart membranes. Prior to the assay for [^{3}H]-DHA binding, the membrane preparation was preincubated either without (control) or with various concentrations (0.1, 1, and 10 mM) of hydrogen peroxide at 30 °C for 10 min in a medium containing 20 mM Tris-HCl, pH 7.4. The Scatchard plot of the data revealed that although 0.1 mM hydrogen peroxide did not show any significant effect on [^{3}H]-DHA binding, the dissociation constant (K_d) was increased by both 1 and 10 mM hydrogen peroxide. No significant changes in the maximal number of binding sites (B_{max}) were observed in the hydrogen peroxide-treated groups.

In another set of experiments, cardiac membranes were preincubated either without (control) or with hydrogen peroxide at 30 °C for 60 min. Even after the 60 min preincubation period, B_{max} for [^{3}H]-DHA binding was not increased in the hydrogen peroxide-treated groups (control 74.3±9.4 fmol/mg protein, 0.1 mM H_2O_2 76.0±8.8, 1

TABLE II EFFECTS OF HYDROGEN PEROXIDE ON CARDIAC MEMBRANE ADENYLATE CYCLASE ACTIVITY

Concentration of H_2O_2	*Adenylate cyclase activity (pmol cAMP/mg protein/min)*			
	5 min	*10 min*	*30 min*	*60 min*
Control	*277.1±19.2*	*223.2±13.8*	*150.5±12.0*	*114.5±21.2*
0.1 mM	*268.2±15.6*	*212.6±19.5*	*153.9±7.2*	*107.0±17.6*
1.0 mM	*275.3±15.1*	*226.8±21.0*	*144.4±11.0*	*104.3±16.5*
10 mM	*230.3±14.9**	*182.8±18.4**	*123.1±17.1**	*84.1±13.3**

Cardiac membranes were preincubated with 0.1, 1 and 10 mM hydrogen peroxide at 30°C for 5, 10, 30 and 60 min. Adenylate cyclase activity was assayed in the presence of 100 μM forskolin. Each value is a mean±S.D. of 7 different preparations.
** significant difference from control value ($p<0.05$)*

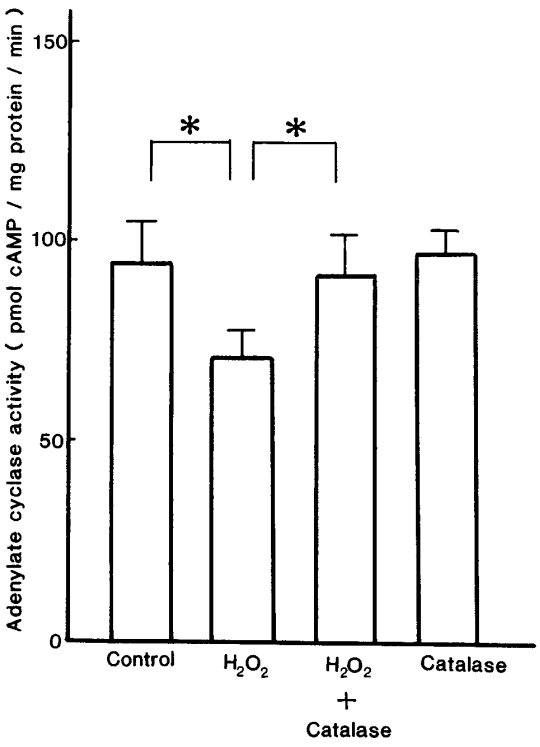

Fig.2. Protective effects of catalase on adenylate cyclase activity inhibited by hydrogen peroxide. Cardiac membranes were incubated with 10 mM hydrogen peroxide in either the absence or presence of 10 μg/ml catalase at 30°C for 60 min. Adenylate cyclase activity was assayed in the presence of 100 μM forskolin. Values are means±S.D. of 7 different preparations. * indicates a significant difference ($p<0.05$)

mM H_2O_2 73.2±9.2 10 mM H_2O_2 77.2 ±9.0), whereas K_d for [^{3}H]-DHA binding was increased in the 1 and 10 mM hydrogen peroxide-treated groups (control 0.84±0.13 nM, 0.1 mM H_2O_2 0.80±0.11, 1 mM H_2O_2 1.02±0.13, 10 mM H_2O_2 1.12±0.16). These data indicate that hydrogen peroxide reduced the affinity of [^{3}H]-DHA for β-adrenergic receptors.

Effects of Hydrogen Peroxide on Stimulatory Guanine Nucleotide Binding Protein (Gs) Activity

Table I shows the effects of hydrogen peroxide on Gs activity. Prior to the assay for Gs activity, the membrane preparations were preincubated either without (control) or with various concentrations (0.1, 1, and 10 mM) of hydrogen peroxide at 30°C for 10 and 60 min in a medium containing 20 mM Tris-HCl, pH 7.4. Gs activities were not altered by the 10 min preincubation of the membranes with 0.1, 1, or 10 mM hydrogen peroxide. Even after the 60 min preincubation with 10 mM hydrogen peroxide, no significant changes in Gs activities were observed. These data indicate that hydrogen peroxide does not affect Gs activities in our system.

Effects of Hydrogen Peroxide on Adenylate Cyclase Activity

To investigate the effects of hydrogen peroxide on adenylate cyclase activities, cardiac membranes were preincubated with 0.1, 1, and 10 mM hydrogen peroxide at 30° for 5, 10, 30, and 60 min. Adenylate cyclase activities were assayed in the presence of 100 μM forskolin. Although adenylate cyclase activities were not altered by 0.1 or 1

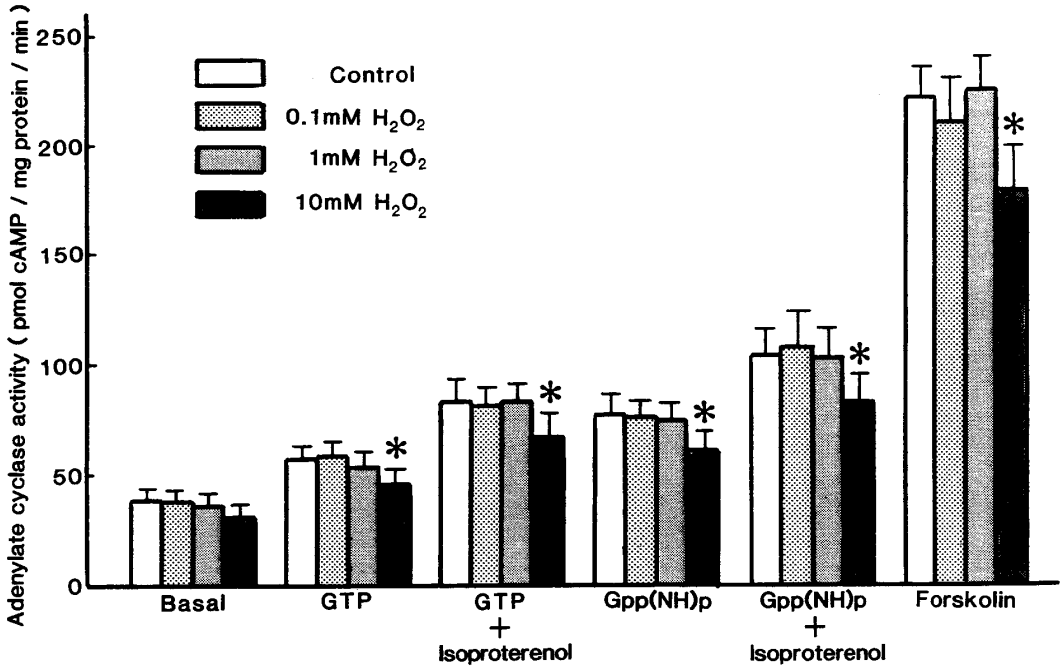

Fig.3. Effects of hydrogen peroxide on cAMP production. Cardiac membranes were preincubated with hydrogen peroxide at 30 °C for 10 min, and then stimulated with GTP, GTP and isoproterenol, Gpp(NH)p, Gpp(NH)p and isoproterenol, or forskolin. The concentrations of GTP, isoproterenol, Gpp(NH)p, and forskolin were 100 μM. Values are means $\pm$ S.D. of 7 different preparations. * indicates a significant difference from each control value, ($p<0.05$)

mM hydrogen peroxide, the enzyme activities were reduced by 10 mM hydrogen peroxide even after the 5 min preincubation (Table II). This depression of adenylate cyclase activities due to hydrogen peroxide was prevented by the addition of 10 μg/ml catalase (Fig. 2).

Effects of Hydrogen Peroxide on β-receptor - Gs - Adenylate Cyclase Coupling

Cardiac membranes were preincubated with 0.1, 1, and 10 mM hydrogen peroxide at 30 °C for 10 min, and each component of the β-receptor - Gs - adenylate cyclase system was stimulated by various interventions (Fig. 3). Although cAMP production was not altered by 0.1 or 1 mM hydrogen peroxide, cAMP production was reduced by 10 mM hydrogen peroxide in the presence of (l)-isoproterenol, GTP, Gpp(NH)p, and forskolin. In another set of experiments, cardiac membranes were preincubated with hydrogen peroxide at 30 °C for 60 min. Although there were no significant changes in cAMP production in the presence of 0.1 or 1 mM hydrogen peroxide (data not shown), cAMP production was reduced by 10 mM hydrogen peroxide (GTP: control 39.5 $\pm$ 5.2 pmol cAMP/mg protein/min vs H_2O_2 31.5 $\pm$ 4.5, GTP + isoproterenol: 53.1 $\pm$ 7.8 vs 42.5 $\pm$ 6.4, Gpp(NH)p: 46.3 $\pm$ 5.9 vs 38.5 $\pm$ 7.3, Gpp(NH)p + isoproterenol: 63.0 $\pm$ 9.2 vs 51.3 $\pm$ 7.2, forskolin: 114.5 $\pm$ 21.2 vs 84.1 $\pm$ 13.3). Adenylate cyclase activity (due to forskolin stimulation) was reduced by 18.1% of control values after a 10 min incubation with 10 mM hydrogen peroxide. Cyclic AMP production was also depressed by 19.4, 19.3, 21.3, and 20.1% of control values after stimulation with GTP, GTP + (l)-isoproterenol, Gpp(NH)p, and Gpp(NH)p + (l)-isoproterenol, respectively.

DISCUSSION

Oxygen free radicals are known to affect heart sarcolemmal membrane functions such as Na^+-K^+ ATPase;[22-24] Ca^{2+}-pump;[25,26] Na^+-Ca^+ exchanger;[27] and voltage-dependent Ca^{2+} channel.[28] Therefore, it is possible that the cardiac β-adrenergic receptor system is altered by oxygen free radicals. Indeed, some investigators have reported that oxidative stress affects the β-adrenergic

receptor system[11,12] We have previously shown that both B_{max} and K_d for [^{3}H]-DHA binding were increased by xanthine plus xanthine oxidase (superoxide anion radical - generating system), whereas only K_d was increased in the presence of 1 mM hydrogen peroxide[11] Haenen et al[12] reported that although a low concentration (1×10^{-7}-1×10^{-3} M) of hydrogen peroxide increased B_{max} for [^{125}I]-iodocyanopindolol binding, B_{max} was depressed by a high concentration (1×10^{-1} M) of hydrogen peroxide. In the present study, K_d for [^{3}H]-DHA binding was significantly increased by both 1 and 10 mM hydrogen peroxide, whereas B_{max} was unchanged. These differences in the effects of oxidative stress on β-adrenergic receptors in cardiac membranes may depend on the differences in the type of activated oxygen, ligands and/or animals used.

GTP binding proteins are the key component of the myocardial β-adrenergic signaling system[3,29-31] Alterations in the β-adrenergic receptor - Gs - adenylate cyclase system during myocardial ischemia have been described[17,32,33] A decreased Gs activity in dog hearts has been observed after 1 h of ischemia. However, the mechanism for the depression of Gs activity is still unclear, and the direct effects of oxidative stress on Gs activity have not been investigated to date. Haenen et al[4] reported that cAMP production following NaF stimulation was decreased in oxygen free radical-treated cardiac membranes, and speculated that Gs activity was depressed by oxygen free radicals. However, this interpretation may be incorrect for two reasons. First, Gs activity was not directly assessed, and, second, since oxygen free radicals inhibit adenylate cyclase activity directly[13,14] the altered NaF stimulation may simply be a result of this direct effect on the catalytic unit of adenylate cyclase. To dertermine the effects of hydrogen peroxide on Gs activity, we incubated cardiac membranes with hydrogen peroxide, and then reconstituted Gs with S49 lymphoma cell membranes which contain β-receptors and adenylate cyclase. This reconstituted S49 lymphoma cell membrane was stimulated by NaF. Therefore, we were able to observe the direct effects of hydrogen peroxide on Gs activity. Hydrogen peroxide did not show any significant effects on Gs activity in rat cardiac membranes under our experimental conditions. However, other activated oxygen species, such as superoxide anion radicals or hydroxyl radicals, may affect Gs activity, since the toxicity of each activated oxygen to cardiac membrane is different[11,25,26]

Some investigators have reported that adenylate cyclase activities are decreased by oxygen free radicals[13,14] We also found that adenylate cyclase activity was reduced by hydrogen peroxide. Although the mitochondrial production of hydrogen peroxide under pathophysiological conditions[1-3] may be greater than that under physiological conditions, the exact level of hydrogen peroxide in this small subcellular region of the myocyte is unknown. The mechanisms by which oxygen free radicals affect cardiac β-receptors and adenylate cyclase activity are not clear at present. Lipid peroxidation of membrane phospholipids and/or direct modification of proteins may be responsible for the changes in [^{3}H]-DHA binding and adenylate cyclase activity in the presence of hydrogen peroxide. In this regard, it should be pointed out that there was a good correlation between the depression of adenylate cyclase activity and both oxidation of sulfhydryl groups of enzyme proteins and lipid peroxidation of membrane phospholipids due to oxygen free radicals[14]

In the presence of hydrogen peroxide, cAMP production after stimulation with GTP, GTP+(l)-isoproterenol, Gpp(NH)p, and Gpp(NH)p+(l)-isoproterenol was depressed by 19.4, 19.3, 21.3, and 20.1% of control values, respectively. These depressions in cAMP production were almost identical to those induced by by forskolin stimulation. Therefore, it seems likely that coupling in the β-adrenergic receptor - Gs - adenylate cyclase system is not affected by hydrogen peroxide. In the presence of 1 mM hydrogen peroxide, cAMP production in the presence of GTP+(l)-isoproterenol or Gpp(NH)p+(l)-isoproterenol was unchanged, whereas K_d for [^{3}H]-DHA binding increased. This discrepancy may have been caused by the dosage of (l)-isoproterenol (100 μM) we used in this experiment.

It is well known that cAMP is an important second messenger for the regulation of cardiac contraction[7-9] An increase in cyto-

solic cAMP through the stimulation of β-adrenergic receptors results in an activation of cAMP-dependent protein kinases, leading to an increase in phosphorylation of several regulatory proteins such as phospholamban in the sarcoplasmic reticulum, troponin I in myofibrils, and the 15-kDa protein in the sarcolemma[7] The phosphorylation of phospholamban and troponin I results in an enhanced rate of cardiac relaxation, whereas the phosphorylation of sarcolemmal 15-kDa protein has been shown to parallel the increase in the inotropic state[7] In the present study, we determined that hydrogen peroxide increased K_d for [^{3}H]-DHA binding and decreased adenylate cyclase activity, while Gs activity and coupling in the β-receptor - Gs - adenylate cyclase system were not changed. These changes in the β-receptor - Gs - adenylate cyclase system may lead to the depression of cAMP production, and thus result in the cardiac dysfunction associated with hydrogen peroxide.

REFERENCES

1. AMBROSIO G, BECKER LC, HUTCHINS GM, WEISMAN HF, WEISFELDT ML: Reduction in experimental infarct size by recombinant human superoxide dismutase: insights into the pathophysiology of reperfusion injury. *Circulation* 1986; **74:** 1424–1433
2. JOLLY SR, KANE WJ, BAILIE MB, ABRAMS GD, LUCCHESI BR: Canine myocardial reperfusion injury: its reduction by the combined administration of superoxide dismutase and catalase. *Circ Res* 1984; **54:** 277–285
3. FU LX, ILEBEKK A, KIRKEBEN KA, AKSNES G, WAAGSTEIN F, BERGH CH, HOEBEKE J, LIANG QM, HJALMARSON A: Oxygen free radical injury and Gs medical signal transduction in the stunned porcine myocardium. *Cardiovasc Res* 1992; **26:** 449–455
4. HAENEN GRMM, VEERMAN M, BAST A: Reduction of β-adrenoceptor function by oxidative stress in the heart. *Free Radic Biol Med* 1990; **9:** 279–288
5. ELEY DW, KORECKY B, FLISS H, DESILETS M: Calcium homeostasis in rabbit ventricular myocytes. *Circ Res* 1991; **69:** 1132–1138
6. EDES I, KRANIAS EG: Phospholamban and troponin I are substrates for protein kinase C in vitro but not in intact beating guinea pig hearts. *Circ Res* 1990; **67:** 394–400
7. KAROZEWSKI P, BARTEL S, KRAUSE EG: Differential sensitivity to isoprenaline of troponin I and phospholamban phosphorylation in isolated rat hearts. *Biochem J* 1990; **266:** 115–122
8. LEVITZKI A: β-Adrenergic receptors and their mode of coupling to adenylate cyclase. *Physiol Rev* 1986; **66:** 819–854
9. GILMAN AG: G protein transducer of receptor generated signals. *Annu Rev Biochem* 1987; **56:** 615–649
10. IKEGAYA T, KOBAYASHI A, HONG RB, MASUDA H, KANEKO M, YAMAZAKI N: Stimulatory guanine nucleotide-binding protein and adenylate cyclase activities in Bio 14.6 cardiomyopathic hamsters at the hypertrophic stage. *Mol Cell Biochem* 1992; **110:** 83–90
11. KANEKO M, CHAPMAN DC, GANGULY PK, BEAMISH RE, DHALLA NS: Modification of cardiac adrenergic receptors by oxygen free radicals. *Am J Physiol* 1991; H821–H826
12. HAENEN GRMM, DANSIK PV, VERMEULEN NPE, TIMMERMAN H, BAST A: The effect of hydrogen peroxide on β-adrenoceptor function in the heart. *Free Radic Res Commun* 1988; **4:** 243–249
13. WILL-SHAHAB L, SCHIMKE I, HABERLAND A, KOTTNER I: Responsiveness of cardiac adenylate cyclase in the normal and ischemic myocardium. Role of oxygen free radicals. *Biomed Biochim Acta* 1987; **46:** 427–432
14. SHIMKE I, HABERLAND A, WILL-SHAHAB L, KUTTNER I, PAPIES B: Free radical-induced damage of cardiac sarcolemma (SL) and activity loss of β-receptor adenylate cyclase system (β-RAS). A comparison of the time course. *Biomed Biochim Acta* 1989; **48:** 69–72
15. WILLIAMS LT, LEFKOWITZ RJ: Thyroid hormone regulation of β-adrenergic receptor number. *J Biol Chem* 1977; **256:** 2787–2789
16. LOWRY OH, ROREBROUGH J, FARR AL, RANDALL RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; **193:** 265–275
17. MUKHERJEE A, BUSH LR, McCOY KE, DUKE RJ, HAGLER H, BUJA LM, WILLERSON JT: Relationship between β-adrenergic receptor numbers and physiological responses during experimental canine myocardial ischemia. *Circ Res* 1982; **50:** 735–741
18. MUKHERJEE A, WONG TM, BUJA LM, LEFKOWITZ RJ, WILLERSON JT: Beta adrenergic and muscarinic cholinergic receptors in canine myocardium. *J Clin Invest* 1979; **64:** 1423–1428
19. ROSS EM, MAGUIRE ME, STURGILL TW, BILTONEN RL, GILMAN AG: Relationship between the β-adrenergic receptor and adenylate cyclase. *J Biol Chem* 1977; **61:** 5761–5775
20. STERNWEIS PC, NORTHUP JK, SMIGEL MD, GILMAN AG: The regulatory component of adenylate cyclase. *J Biol Chem* 1981; **25:** 11517–11526
21. SALOMON Y, LONDOS C, RODBOLL M: A highly sensitive adenylate cyclase assay. *Anal Biochem* 1974; **58:** 541–548
22. KIM MS, AKERA T: O_2 free radicals: cause of ischemia-reperfusion injury to cardiac Na^+-K^+-ATPase. *Am J Physiol* 1987; H252–H257
23. KRAMER JH, MAK IT, WEGLICKI WB: Differential sensitivity of canine cardiac sarcolemmal and microsomal enzymes to inhibition by free radical-induced lipid peroxidation. *Circ Res* 1984;

55: 120–124
24. ROWE GT, MANSON NH, CAPLAN M, HESS ML: Hydrogen peroxide and hydroxyl radical mediation of activated leukocyte depression of cardiac sarcoplasmic reticulum. *Circ Res* 1983; **53:** 584–591
25. KANEKO M, BEAMISH RE, DHALLA NS: Depression of heart sarcolemmal Ca^{2+}-pump activity by oxygen free radicals. *Am J Physiol* 1989; H368–H374
26. KANEKO M, ELIMBAN V, DHALLA NS: Mechanism for depression of heart sarcolemmal Ca^{2+} pump by oxygen free radicals. *Am J Physiol* 1989; H804–H811
27. REEVES JP, BAILEY CA, HALE CC: Redox modification of sodium-calcium exchange activity in cardiac sarcolemmal vesicles. *J Biol Chem* 1986; **261:** 4948–4955
28. KANEKO M, LEE SL, WOLF CM, DHALLA NS: Reduction of calcium channel antagonist binding sites by oxygen free radicals in rat heart. *J Mol Cell Cardiol* 1989; **21:** 935–943
29. FU LX, WAAGSTEIN F, HJALMARSON A: Beta-adrenoceptor-G protein-adenylate cyclase system in cardiac disease: a new insight into desensitization. *Clin Physiol* 1991; **11:** 1–7
30. FU LX, WAAGSTEIN F, HJALMARSON A: Increased transcript of Gi-alpha 3 in human failing heart. *Eur Heart J* 1990; **11:** 1013
31. FU LX, SJOGREN KG, LIANG QM, WAAGSTEIN F, HJARMARSON A: Distribution and function of beta-adrenoceptor-adenylate cyclase in human failing heart. *Eur Heart J* 1990; **11:** 1477
32. SUSANNI EE, MANDERS WT, KNIGHT DR, VATNER DE, VATNER SF, HOMCY CJ: One hour of myocardial ischemia decreases the activity of the stimulatory guanine nucleotide regulatory protein Gs. *Circ Res* 1989; **65:** 1145–1150
33. VATNER DE, KNIGHT DR, SHEN YT, THOMAS JX, HOMCY CJ, VATNER SF: One hour of myocardial ischemia in conscious dogs increases beta-adrenergic receptors, but decreases adenylate cyclase activity. *J Mol Cell Cardiol* 1988; **20:** 75–82