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THE EFFECT OF HYDROGEN PEROXIDE ON β-ADRENOCEPTOR FUNCTION IN THE HEART

G.R.M.M. HAENEN‡, P. VAN DANSIK, N.P.E. VERMEULEN, H. TIMMERMAN and A. BAST

Department of Pharmacochemistry, Subfaculty of Chemistry, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

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A membrane preparation of calf heart left ventricle has been used to study the effect of radical stress on the β-adrenoceptor complex. To this end the membranes were incubated for 30 minutes with several concentrations of hydrogen peroxide. This resulted in a dose dependent peroxidation of the membrane lipids. Preincubation with hydrogen peroxide in the concentration range 10^{-7}–10^{-3} M caused an increase in specific (−)-[^{125}I]-Iodocyanopindolol binding, possibly due to a decrease in membrane fluidity as a result of lipid peroxidation, thus making the receptor protein more accessible. Higher concentrations H_2O_2 reduced the specific (−)-[^{125}I]-Iodocyanopindolol binding, which is most likely the effect of deterioration of the receptor protein by the more pronounced radical stress induced by these higher concentrations. Also adenylate cyclase activity was affected by radical stress. Basal cyclic-AMP production and cyclic-AMP production induced by NaF (10^{-2} M) or guanylylimidodiphosphate (10^{-4} M), was suppressed after pretreatment with concentrations of H_2O_2 above 10^{-4} M. This indicates a higher sensitivity of the adenylate cyclase toward radical stress when compared to the receptor protein. Our results show that radical stress can perturb β-adrenoceptor function considerably in the heart.

KEY WORDS: Hydrogen peroxide, lipid peroxidation, β-adrenoceptors, adenylate cyclase, heart.

INTRODUCTION

The cardiotoxicity of several catecholamines is well known. The most obvious explanation for this toxicity is β-adrenoceptor hyperstimulation. The strong positive chronotropic and inotropic effect of catecholamines may lead to hypoxia, depletion of high-energy phosphate stores and abnormal calcium accumulation in the myocardium. Apart from that, it has been stated that radical mechanisms play a role in catecholamine induced myocardial necrosis.[1] During the (aut)oxidation of catecholamine superoxide anion radicals, hydrogen peroxide, hydroxyl radicals and semiquinone radicals are generated. This hypothesis is supported by the protection which is afforded by vitamine E and the membrane stabilizing agent zinc, against isoproterenol induced cardiomyopathy[1] and the inability of propranolol to protect against the toxic effect of isoproterenol on cultured cardiac muscle cells,[2] whereas ascorbate protects.[3]

Both mechanisms, receptor hyperstimulation and autoxidation, are interrelated. Hypoxia induced by excessive β-adrenoceptor stimulation induces an increase in the radical production. In heart tissue, low oxygen tension gives conversion of xanthine

‡To whom correspondence should be addressed.

dehydrogenase into xanthine oxidase by a Ca^{2+} dependent proteolysis.[4] Xanthine oxidase utilizes molecular oxygen as an electron acceptor to catalyze the production of superoxide anion radicals and hydrogen peroxide. Other sources of radicals induced by hypoxia originate from the accumulation of neutrophils[5] and an excessive formation of reduced electron carriers in mitochondria in the heart.[6] On the other hand radical processes influence several receptor systems.[7-10]

The aim of the present study was to evaluate the effect of radical stress on the β-adrenoceptor system in the heart. To this end we preincubated membranes of the calf heart left ventricle with several concentrations hydrogen peroxide which resulted in a dose dependent radical stress. After washing the membranes, β-adrenoceptor density was measured by means of a receptor binding assay using (−)-[^{125}I]-iodocyanopindolol (ICYP). Adenylate cyclase activity was measured by a RIA procedure.

MATERIALS AND METHODS

Heart membranes were prepared from calf heart left ventricles according to the method of Ijzerman.[11] The membranes were taken up in buffer A (50 mM Tris-HCl, 140 mM NaCl and 5 mM $MgCl_2$, pH 7.4 at 37° C) and stored in liquid nitrogen until use. Lipid peroxidation was measured with the thiobarbituric acid method, as previously described.[12] Lipid peroxidation was expressed as nmoles malondialdehyde equivalents, using an extinction coefficient of $1.56 \cdot 10^5\,M^{-1}\,cm^{-1}$. Protein determinations were made by the method of Bradford[13] using bovine serum albumin as standard.

Heart membranes (final concentration 1.5 mg protein/ml) were incubated at 37° C, with shaking air being freely admitted in buffer A (pH 7.4 at 37° C) which contains sodium azide (final concentration 1 mM) in order to inhibit endogenous catalase, and several concentrations hydrogen peroxide (0–0.1 M) for 30 min. The incubation was terminated by the addition of one volume of ice-cold buffer A (pH 7.4 at 37° C) and centrifugation for 3 min in an eppendorf centrifuge (15,000 × g) at 4° C. The precipitated membranes were resuspendend in 1 ml fresh buffer and centrifugation again for 3 min (15,000 × g) at 4°C. This washing procedure was repeated twice. The receptor binding and adenylate cyclase activity assay were performed with membranes suspended in buffer A (pH 7.4 at 37° C).

All receptor binding assays were performed in triplicate in a final volume of 350 μl using the ^{125}I labeled β-adrenoceptor antagonist (−)-Iodocyanopindolol (ICYP). Due to the lipophilic character of ICYP all β-adrenoceptors, e.g. also those in inside-out vesicles, are determined. For the saturation experiments membranes were incubated for 60 min with several ICYP concentrations (0–700 pM). In the other experiments β-adrenoceptor density B_{max} (fmol/mg protein) was determined with a single ICYP concentration (150–250 pM). The binding reaction was terminated by the addition of 3 ml of ice-cold buffer A (pH 7.4 at 0° C), followed by rapid filtration through Whatman G/FC filters. Each filter was washed with an additional 2 × 3 ml buffer. The radioactivity of the filter was assessed in an Auto-Gamma scintillation spectrometer (Packard, USA). In all experiments non-specific binding was determined with 10^{-6} M (−)-timolol and filter binding was determined by omission of the membranes. Binding data from the saturation curves were evaluated using the computer program LIGAND on a Zenith Z-110 microcomputer.

TABLE I
Lipid peroxidation in membranes of the left ventricle of the calf heart induced by incubation with hydrogen peroxide during 30 min

concentration hydrogen peroxide	nmol malondialdehyde/mg protein
$1 \cdot 10^{-5}$ M	< 0.02
$1 \cdot 10^{-4}$ M	0.29 ± 0.05
$1 \cdot 10^{-3}$ M	0.64 ± 0.06
$1 \cdot 10^{-2}$ M	1.10 ± 0.10
$5 \cdot 10^{-2}$ M	2.55 ± 0.15

The results are expressed as mean ± S.E. of four separate experiments.

Cyclic-AMP production was performed in buffer A (pH 7.4 at 37° C) using several stimulators, indicated in Figure 4. Phosphodiesterase was inhibited by the addition of methylisobutylxanthine (final concentration $5 \cdot 10^{-5}$ M) in the incubation medium. All reactions were carried out at 37° C for 30 min, and terminated by heating at 95°C for 3 min. The cyclic-AMP content was determined by a RIA.

The chemicals used were (-)-timolol maleate and guanylylimidodiphosphate (GppNHp) (Sigma); hydrogen peroxide (perhydrol) and sodium azide (Merck). (−)-[^{125}I]ICYP was obtained from New England Nuclear and the cyclic-AMP assay kit (code TRK. 432) was obtained from Amersham. All other chemicals were of reagent grade.

RESULTS

Incubation of heart membranes with hydrogen peroxide resulted in a dose dependent increase in lipid peroxidation (Table 1). Preincubation of heart membranes with 10^{-7}–10^{-3} M hydrogen peroxide increased the amount of specific ICYP binding (Figure 1). Incubation with higher concentrations hydrogen peroxide decreased β-

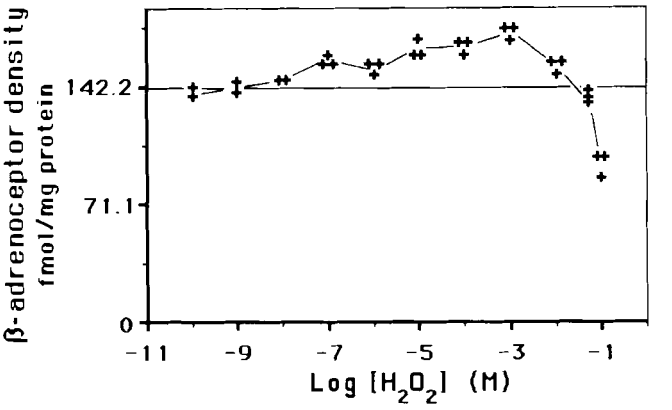

FIGURE 1 Concentration dependent effect of hydrogen peroxide pretreatment on β-adrenoceptor density. β-adrenoceptor density was calculated from specific ICYP binding, using a K_d of 55 pM. The concentration ICYP used was 150–250 pM. The contole value was (± S.E.; n = 3) 142.2 ± 2.5 fmol/mg protein. Each data point is the mean value of a separate experiment performed in triplicate.

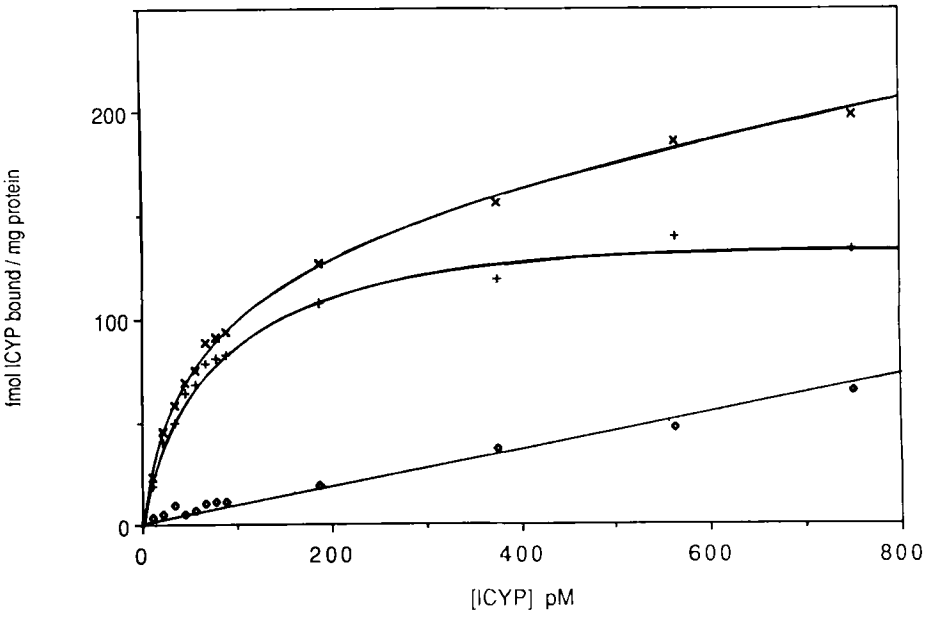

FIGURE 2 Total (×), non-specific (◇) and specific (+) binding of an increasing concentration of ICYP on control heart membranes. Non-specific ICYP binding was determined with the addition of 10^{-6} M (−)-timolol. Specific ICYP binding was calculated from the difference between total and specific binding. K_d = 54.4 ± 2.3 pM and B_{max} = 141.4 ± 2.7 fmol/mg protein.

adrenoceptor density. The same phenomenon was observed in the saturation curves shown in Figures 2 and 3. Preincubation with 10^{-5} M hydrogen peroxide for 30 min resulted in an increase in β-adrenoceptor density of approximately 15%, whereas preincubation with 10^{-1} M H_2O_2 decreased the amount of β-adrenoceptors approximately 40%. The pretreatment had no effect on the non-specific binding of ICYP, determined with 10^{-6} M (−)-timolol. Further analysis of the data revealed that pretreatment had no effect on the affinity of ICYP for the receptor since no significant change of the K_d was observed. Analysis of the data using more than one binding site were not significantly better.

The β-adrenoceptor complex consists of at least 3 proteins; receptor, N_s-protein and adenylate cyclase. The ICYP binding data only provide information about the receptor protein. In order to get more insight about the effect of oxidative stress on the complete β-adrenoceptor system, we also determined the effect of hydrogen peroxide preincubation on cyclic-AMP production. As shown in Figure 4 incubation of untreated membranes with GppNHp or NaF resulted, as expected, in a 2–3 fold increase in adenylate cyclase activity. Pretreatment with hydrogen peroxide resulted in a dose dependent reduction of adenylate cyclase activity independent of the stimulator (Figure 4).

DISCUSSION

Both β-adrenoceptor hyperstimulation and excessive radical formation are thought to

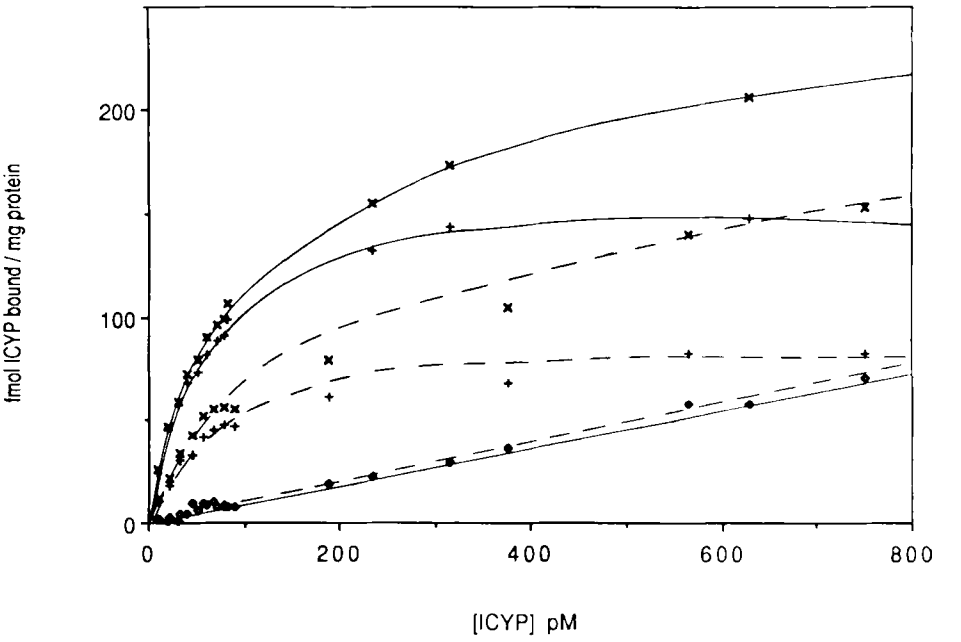

FIGURE 3 Total (×), non-specific (◇) and specific (+) binding of an increasing concentration of ICYP on heart membranes pretreated with 10 $^{-}$M H_2O_2 (——), K_d = 61.5 ± 5.4 pM and B_{max} = 162.9 ± 5.5 fmol/mg protein, or on heart membranes pretreated with 10^{-1} M H_2O_2 (- - -) K_d = 62.5 ± 5.8 pM and B_{max} = 86.9 ± 3.7 fmol/mg protein.

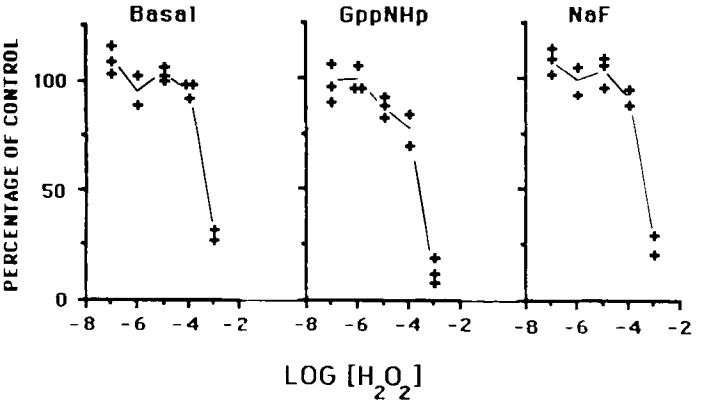

FIGURE 4 Concentration dependent effect of hydrogen peroxide pretreatment on basal cyclic-AMP production and on cyclic-AMP production stimulated by NaF (10^{-2} M) or GppNHp (10^{-4} M). The control values are (± S.E.; n = 3) basal 21.0 ± 1.2; NaF 60.3 ± 2.0 and GppNHp 41.1 ± 1.2 pmol cyclic-AMP/mg protein/min. Each data point represents a separate experiment.

be involved in the cardiotoxicity induced by several catecholamines. The aim of the present study is to evaluate the effect of radical stress on the β-adrenoceptor system in the heart. Incubation of heart membranes with hydrogen peroxide was shown to result in a dose dependent lipid peroxidation. A direct reaction of hydrogen peroxide with unsaturated fatty acids occurs only very slow. Traces of transition metals (present in the buffer or bound to the membrane) rapidly react with hydrogen peroxide to form more reactive oxygen species which can induce lipid peroxidation.

The oxidative stress induced by hydrogen peroxide markedly affects the β-adrenergic receptor binding protein (Figure 1). Neurotransmitter receptors are thought to be buried in or to float on the lipid bilayer membrane. The position of a diffusible protein in the membrane is determined by the interactions between the amino acid residues with the external aqueous and lipid domains. The two main forces controlling this are an electrostatic and a hydrophobic interaction. The lipid fluidity determines the magnitude of the hydrophobic interaction. An increase in rigidity of the phospholipid bilayer by lipid peroxidation[14] can result in a vertical displacement of the equilibrium position of the receptor protein toward the aqueous domain.[15] Thus the receptor becomes more accessible and this can explain the increase in ICYP binding we observed at low hydrogen peroxide concentrations.

Increasing the hydrogen peroxide concentration resulted in a more pronounced radical stress. Primary oxygen radicals, their secondary lipid radical intermediates and aldehydes produced during lipid peroxidation can deteriorate membrane bound proteins[16] including the receptor protein. Receptor proteins of other systems are also affected by free radicals. Ascorbic acid induced lipid peroxidation, inhibits dopamine antagonist binding in neostriatal membrane preparations[7] and in rat cortical membranes.[8] Oxygen radicals generated by the xanthine-xanthine oxidase system and hydrogen peroxide reduce muscarinic antagonist binding at sarcolemmal receptors from the canine heart.[9] Furthermore hydrogen peroxide reduces synaptic GABA receptor binding of [^{3}H]-Muscimol, whereas xanthine-xanthine oxidase treatment results in a profound stimulation of the binding of [^{3}H]-Muscimol.[10]

The adenylate cyclase data (Figure 4) show a 2–3 fold stimulation by NaF and GppNHp. Preincubation with relative low concentrations of hydrogen peroxide resulted in a reduced adenylate cyclase activity, independent of the stimulator. These data suggest that oxidative stress impairs the adenylate cyclase unit of the β-adrenoceptor complex. The reduction at a relative low concentration hydrogen peroxide is possibly due to a higher sensitivity of the adenylate cyclase toward radical stress in comparison to the β-receptor protein.

High concentrations of several catecholamines produce myocardial cell necrosis. β-adrenoceptor hyperstimulation and excessive radical formation are thought to be involved in this necrosis. Our results demonstrate that radical stress can perturb β-adrenoceptor functioning in the heart. After the onset of the catecholamine induced cardiotoxicity, the radicals formed can reduce the toxic effects mediated by β-adrenoceptor hyperstimulation. On the other hand there is evidence that, once the process of lipid peroxidation has started, the protection against radical stress is reduced.[12] So in treating cardiotoxicity, reduction of the radical stress might be more profitable then β-adrenoceptor blockade.

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