Mechanisms of alterations in cardiac β-adrenoceptor linked signal transduction due to ischemia-reperfusion

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MECHANISMS OF ALTERATIONS IN CARDIAC B-ADRENOCEPTOR LINKED SIGNAL TRANSDUCTION DUE TO ISCHEMIA-REPERFUSION

BY

SUJATA PERSAD

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

This thesis is dedicated to

my husband, Rabindranath

and

my children, Amit and Neesha

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ABSTRACT

While reperfusion of the ischemic heart is generally thought to be a beneficial process, some detrimental effects including contractile deterioration broadly known as reperfusion injury have been noted when the ischemic myocardium is reperfused after a certain time. Although an attenuated inotropic response to catecholamines in the ischemic heart disease is well documented, the mechanism of this abnormality in the myocardium is not well understood. Since the β-adrenoceptor-G protein-adenylyl cyclase system is known to mediate the sympathetic control of heart function, we examined the effects of ischemia-reperfusion on β-adrenoceptor linked signal transduction pathway. For this purpose rat hearts were made globally ischemic for 30 min and then reperfused for 60 min. The ischemic hearts failed to develop the left ventricular pressure (LVSP) and showed a marked increase in the left ventricular end-diastolic pressure. Not only the recovery of ischemic heart to develop LVSP was decreased upon reperfusion, the ischemic-reperfused hearts also showed attenuated inotropic responses (LVSP, rate of contraction and rate of relaxation) to isoproterenol.

Determination of specific binding of ^{125}I-cyanopindolol with cardiac membranes revealed a significant increase in the density and affinity of β_1-adrenoceptors without any changes in the characteristics of β_2-adrenoceptors in the ischemic hearts. On the other hand, the affinities and densities of β_1-adrenoceptors mainly and of β_2-adrenoceptors to a lesser extent were decreased in the ischemic-reperfused hearts. The basal and forskolin-stimulated adenylyl cyclase activities were unaltered due to ischemia but were increased upon

reperfusion. Isoproterenol-stimulated adenylyl cyclase activity was depressed in a similar manner in both the ischemic hearts and the ischemic-reperfused hearts. The NaF- and Gpp(NH)p-stimulated adenylyl cyclase activities were depressed in the ischemic hearts and increased in the ischemic-reperfused hearts. Addition of superoxide dismutase plus catalase in the perfusion medium prevented the reperfusion-induced changes in contractile function, responses of the heart to isoproterenol, responses of membrane adenylyl cyclase to isoproterenol, as well as densities and affinities of β-adrenoceptors. Cholera toxin (CT)-stimulated adenylyl cyclase, the CT-catalysed ADP-ribosylation activity and G_s-protein immunoreactivity were decreased in the ischemic hearts and increased upon reperfusion. Although pertussis toxin (PT)-stimulated adenylyl cyclase activity was unaltered in both ischemic and ischemic-reperfused hearts, the PT-catalysed ribosylation and G_i-protein immunoactivity were slightly increased in the reperfused myocardium. These results suggest that the depressed contractile activity as well as the attenuated inotropic responses of ischemic-reperfused hearts to isoproterenol may be due to the formation of oxyradicals in the myocardium. Furthermore, the inability of isoproterenol to stimulate adenylyl cyclase in the ischemic-reperfused hearts may be due to alterations mainly in the characteristics of β_1-adrenoceptors including density, affinity and coupling with the adenylyl cyclase.

Since our studies suggested the involvement of oxygen derived free radicals in attenuating the inotropic response to catecholamines as well as derangement of the β-adrenoceptor signal pathway in the ischemic-reperfused heart, in the regulation of heart function, we examined the effect of oxyradicals on the cardiac β-adrenoceptors, G-proteins

and adenylyl cyclase. Accordingly, rat heart membranes were incubated with xanthine plus xanthine oxidase (X plus XO), a well known oxyradical generating system, for 10 to 40 min. Treatment of rat heart membranes with X plus XO revealed a biphasic change in the basal adenylyl cyclase activity whereby 10 min incubation increased and 30 min incubation decreased the enzyme activity. Likewise, the forskolin-, NaF- and Gpp(NH)p-stimulated adenylyl cyclase activities showed an increase at 10 min and a decrease at 30 min incubation with X plus XO. The stimulation of adenylyl cyclase by isoproterenol was also increased and decreased at 10 and 30 min incubations with X plus XO, respectively.

Determination of specific binding of ^{125}I-cyanopindolol with cardiac membranes revealed a decrease in the density of β_1-adrenoceptors by both 10 min and 30 min treatments with X plus XO. On the other hand, the affinity of the β_1-adrenoceptors was increased after 10 min and reduced after 30 min incubation with X plus XO; the β_2-adrenoceptors at 10 min were not modified significantly. The proportion of G protein-coupled β-receptors in the high affinity state was increased at 10 min and decreased at 30 min of treatment with X plus XO. Addition of superoxide dismutase (SOD) plus catalase (CAT) in the incubation medium prevented the X plus XO induced alterations in adenylyl cyclase activities and β_1-adrenoceptor density. Furthermore, treatment of cardiac membranes with low concentrations of hydrogen peroxide (H_2O_2) (50 to 100 μM) increased the adenylyl cyclase activities in the absence or presence of different stimulants whereas higher concentrations of H_2O_2 exerted a depressant effect. Cholera toxin (CT)-stimulated adenylyl cyclase activity, CT-catalysed ADP-ribosylation activity and G_s-protein immunoreactivity unlike the pertussis (PT)-

stimulated activities were increased at 10 min incubation with X plus XO and decreased at 30 min incubation. These data suggest that alterations in the β_1-adrenoceptor-G_s protein-adenylyl cyclase pathway due to X plus XO are biphasic in nature. Furthermore, the observed changes in the β-adrenoceptor mechanisms due to X plus XO are primarily due to the formation of H_2O_2 in the myocardium.

In view of the increased formation of H_2O_2 as a dismutation product of superoxide radicals and generation from the mitochondria during ischemia-reperfusion, we investigated the effect of this activated species of oxygen on the β-adrenoceptor-G-protein-adenylyl cyclase complex. Rat heart membranes were incubated with high concentrations of H_2O_2 (0.1 to 5 mM) for 10 min and then the biochemical activities were measured. A concentration-dependent decrease in the isoproterenol-stimulated adenylyl cyclase activity was observed upon incubating membranes with H_2O_2. Although both the affinity and density of β_1-adrenoceptors were decreased by H_2O_2, the density of the β_2-adrenoceptors was decreased whereas their affinity was increased. The basal as well as forskolin-, NaF- and Gpp(NH)p-stimulated adenylyl cyclase activities were depressed by the treatment with H_2O_2. CAT alone or in combination with mannitol (MAN) was able to decrease the magnitude of the alterations due to H_2O_2-treatment significantly. However, the CT-stimulated adenylyl cyclase activity and CT-catalyzed ADP ribose labelling of G_s-protein were depressed by H_2O_2 indicating a depressed G_s-protein activity, while the G_i-protein activity as reflected by PT-stimulation of the adenylyl cyclase and ADP-ribosylation was unaltered. The G_s and G_i protein immunoreactivities estimated by labelling with antibodies

against $G_{s\alpha}$ and $G_{i\alpha}$ subunits to G_s and $G_i$proteins, respectively, indicated that while binding of antibodies to the 45kD band of G_s-protein was decreased by treatment with H_2O_2, binding of antibodies to neither the 52 kD subunit of G_s nor the 40 kD subunit of G_i was altered by H_2O_2. These results suggest that H_2O_2 may modify the β-adrenoceptor linked signal transduction in the heart by depressing the β_1-adrenoceptor, G_s-proteins and adenylyl cyclase enzyme.

In order to examine the validity and significance of the *in vitro* effects of oxyradicals on the observed cardiac β-adrenoceptor linked signal transduction mechanisms, rat hearts were perfused with X plus XO (X: 2mM and XO: 10 U/L); for 10 and 30 min as well as with H_2O_2 (1mM) for 10 min. The positive inotropic effect of 1 μM isoproterenol was augmented upon perfusion with X plus XO for 10 min but was attenuated by perfusion for 30 min. On the other hand, the positive inotropic action of isoproterenol was markedly depressed upon perfusing the hearts with 1mM H_2O_2 for 10 min. Cardiac membranes from hearts perfused for 20 min with X plus XO showed an increase in the basal as well as forskolin-, NaF-, Gpp(NH)p- and isoproterenol-stimulated adenylyl cyclase activities. On the other hand, these activities were depressed upon perfusing the hearts with X plus XO for 30 min. Saturation binding curves using ^{125}I-cyanopindolol (70 pM) as a competing ligand and isoproterenol as an agonist (10^{-10} - 10^{-4}M), indicated that while 10 min perfusion with X plus XO increased the proportion of high affinity coupled receptors in cardiac membranes, there was an increase in the proportion of low affinity coupled receptors upon perfusion with X plus XO for 30 min. The observed changes in the contractile parameters as well as β-

adrenergic receptor-adenylyl cyclase system due to X plus XO were prevented by the addition of SOD plus CAT in the perfusion medium. In another series of experiments, the basal as well as forskolin-, NaF- and isoproterenol-stimulated adenylyl cyclase activities in cardiac membranes from hearts perfused with H_2O_2 were depressed. The density and affinity of β_1-adrenoceptors were also depressed without any changes in the β_2-adrenoceptors upon perfusing the hearts with H_2O_2. The H_2O_2-induced changes were prevented by perfusing the hearts with CAT. The results obtained by perfusing the hearts with X plus XO or H_2O_2 were essentially similar to those obtained under *in vitro* conditions. Furthermore, the augmentation and depression of the positive inotropic responses of isoproterenol by oxyradicals were associated with similar changes in the β-adrenoceptor linked signal transduction mechanisms. These studies lend further support to the concept that the changes in the signal transduction system in ischemic-reperfused hearts, unlike the ischemic hearts, may involve complex alterations in the β-adrenoceptor-G-protein-adenylyl cyclase pathway due to the generation of oxyradicals.

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I. STATEMENT OF THE PROBLEM

Early restoration of blood flow during acute myocardial ischemia is effective in reducing infarct size and improving ventricular function (1,2). While reperfusion of the ischemic heart is generally thought to be a beneficial process, some detrimental effects such as deterioration of contractile function and ultrastructural damage broadly known as reperfusion injury have been noted when the ischemic myocardium is reperfused after a certain time (2-4). In view of the important role played by β-adrenoceptors, G-proteins and adenylyl cyclase system in the regulation of heart function and metabolism, several investigators have reported that the β-adrenergic receptor mechanisms are altered during myocardial ischemia (5-8) as well as during coronary arterial reperfusion (5, 9-12). However, effects of ischemia and reperfusion on various components of the β-adrenergic pathway remain controversial because increases, decreases and no changes have been observed. (6, 10-16). These conflicting results observed by different investigators appear to be due to the usage of variable periods of ischemia as well as varying degrees of reperfusion. In addition, reperfusion may be occurring simultaneously as a consequence of opening of the collaterals under *in vivo* conditions. Nonetheless, relatively little is known regarding the mechanisms that change the β-adrenoceptor signal transduction system in the ischemic as well as ischemic-reperfused hearts.

Oxygen-derived free radicals have been implicated as a major factor in the pathophysiology of ischemia-reperfusion injury in the myocardium (17-19). These partially

reduced forms of oxygen in the ischemic-reperfused myocardium may be generated within the cardiomyocyte at the mitochondrial respiratory chain (20), or by other sources such as arachidonic acid metabolism and catecholamine oxidation (18, 21). Several investigators (22-24) have reported that exogenous free radicals produce functional and structural abnormalities in the heart. Treatment of cardiac sarcoplasmic reticulum (SR) and sarcolemmal (SL) membranes with different oxyradical generating systems has been shown to depress Ca^{2+}-pump mechanisms and these defects have been suggested to induce intracellular Ca^{2+}-overload and subsequent heart dysfunction (19, 25-28). Depression in the SL Na^+-K^+ ATPase and Na^+-Ca^{2+} exchange activity upon treatment of heart membranes with oxyradical generating systems has also been suggested to contribute towards the occurrence of intracellular Ca^{2+}-overload (29, 30). Perfusion of the isolated hearts with an oxyradical generating system has also been shown to depress both the SL Na^+-Ca^{2+} exchange and Ca^{2+}-pump activities during the development of contractile failure (31, 32). Although a decrease in the density of Ca^{2+}-channels in the SL membrane (28) and SR (33) due to oxyradicals can be seen to result in a reduction of Ca^{2+} available for cardiac contraction, the contribution of depressed Ca^{2+}-stimulated ATPase activity upon exposing myofibrils (34) to oxyradicals in promoting contractile abnormalities cannot be ruled out. Accordingly, it appears that heart dysfunction due to oxygen free radicals may be due to defects in both Ca^{2+}-handling by cardiomyocytes and the interaction of Ca^{2+} with the contractile apparatus.

Since β-adrenoceptor mechanisms including β_1- and β_2-adrenoceptors, guanine nucleotide binding proteins (G_s- and G_i- proteins) and adenylyl cyclase are known to affect

the entry of Ca^{2+} in cardiomyocytes and thus play an important role in the regulation of heart function (35, 36), some investigators have examined the effects of different oxyradical generating systems on various components of this signal transduction pathway. For example, treatment of cardiac membranes with some oxyradical generating systems increased the density but decreased the affinity of β-adrenoceptors (37) whereas treatment with H_2O_2, an active species of oxygen, decreased the affinity without any changes in the density of β-adrenoceptors (37, 38). On the other hand, treatment of heart membranes with H_2O_2 was reported to increase the density of β-adrenoceptors (39) whereas a loss in the number of β-adrenoceptors was seen upon treating cortical membranes with iron and ascorbic acid, a hydroxyl radical generating system (40). An increase or no change in the density of β-adrenoceptors in ventricular membranes has also been observed upon treatment with some oxidants (41, 42). Furthermore, it may be noted that no information regarding the effect of oxyradicals and oxidants on β_1- or β_2-adrenoceptor is available in the literature. It should be pointed out that a decrease in the adenylyl cyclase activity was observed upon treating heart membranes with H_2O_2 and some oxidants by some investigators (36, 41, 42) whereas others (43) have reported an increase in the enzyme activity due to H_2O_2 in the vascular smooth muscle cells. A transient increase followed by a decrease in the adenylyl cyclase activity was reported upon treating cardiac membranes with iron-ascorbic acid system (44). Although G-protein activities in heart membranes (38) and vascular smooth muscle cells (43) were unaltered due to oxyradical exposure, no detailed information in this regard is available for making any meaningful conclusion.

Since relatively little is known about the role of oxygen-free radicals in inducing changes in β-adrenergic receptor mechanisms due to reperfusion injury and the conflicting information on this aspect in the ischemic heart, a series of experiments in this study was undertaken to examine alterations in the various components of the β-adrenoceptor-G-protein-adenylyl cyclase in the ischemic-reperfused hearts. Furthermore, experiments were carried out to investigate the effects of superoxide dismutase (SOD) plus catalase (CAT) system, an excellent scavenger for the active species of oxygen (19), on various components of the β-adrenoceptor signal transduction pathway in the ischemic-reperfused hearts. For this purpose, global ischemia-reperfusion injury was induced by occluding the coronary flow for 30 min followed by reperfusion for 60 min in isolated rat hearts perfused in the absence or presence of SOD plus CAT. Alterations in β-adrenoceptors, G-proteins and adenylyl cyclase were studied upon isolating cardiac membranes from the ischemic as well as reperfused hearts. In addition, contractile activities as well as the inotropic responses of the ischemic-reperfused hearts to a well known β-adrenoceptor agonist, isoproterenol, were monitored. Since oxygen free radicals are prominently implicated in the derangement of the ischemic reperfused hearts and in view of the relatively conflicting and scattered information regarding the effect of oxyradicals on the β-adrenoceptor signal transduction mechanism, another set of experiments in this study was undertaken to examine in detail if any component of the β-adrenoceptor pathway in the heart is affected by oxyradicals. For this purpose, a combination of xanthine (X) plus xanthine oxidase (XO) was used as an oxyradical generating system for treatment of rat cardiac membranes under *in vitro*

conditions. Membrane preparations obtained from isolated rat hearts perfused with X plus XO were also employed for studying the validity of changes in β_1- and β_2-adrenoceptors as well as adenylyl cyclase activity seen under *in vitro* conditions. Because an increase in the production of H_2O_2 has been shown to occur in the ischemic-reperfused heart due to dismutation of the superoxide radical as well as generation in the mitochondria (19, 45, 46), effect of this active species of oxygen on the β-adrenoceptor pathway was also studied under *in vitro* and *ex-vivo* conditions. In addition, the inotropic responses of the hearts perfused with X plus XO as well as H_2O_2 to isoproterenol were investigated to establish the significance of alterations in the β-adrenoceptor mechanisms in the myocardium upon exposure to oxyradicals.

II. REVIEW OF LITERATURE

1. Introduction

Salvage of the ischemic myocardium has been the goal of numerous experimental and clinical cardiologist for many years. Of the various approaches, including the array of drugs that have been used to date (47-49) early restoration of myocardial blood flow is now generally accepted as the best means to achieve this goal. While reperfusion appears to be a prerequisite for tissue salvage, this process also carries with it a component of injury which can prove deleterious to the ischemic myocardium. It is still a matter of debate whether the ischemic tissue is injured by the reperfusion itself or whether reperfusion simply causes cells already killed by ischemia to undergo sudden changes in appearance. There is also ample evidence to suggest that the cellular injury due to myocardial ischemia may occur through mechanisms which are different from those for reperfusion. Much of the current research is aimed at minimizing the risk/benefit ratio for procedures implemented to induce reperfusion and at elucidating the mechanisms responsible in promoting reperfusion injury and subsequently confirming the relevance of adjunctive therapy in potentiating salvage of the ischemic myocardium. One of the fundamental issues in this regard therefore, is that of establishing the mechanisms responsible for the contractile abnormalities associated with myocardial ischemia and ischemia-reperfusion injury. It is understood that cardiac pump failure and changes in cardiac cell ultrastructure due to ischemia-reperfusion or hypoxia-reoxygenation involve a wide variety of complex pathological abnormalities and the present

information on these aspects are largely based on the beneficial effects of several pharmacological interventions. For example the beneficial effects of Ca^{2+}-antagonists (50, 51) have supported the role of intracellular Ca^{2+}-overload whereas those of both beta- and alpha- adrenergic blockers (52-55) point to the role of increased sympathetic activity (56) in the pathophysiology of ischemic heart disease. However, the involvement of prostaglandins and several other metabolic abnormalities during several stages of ischemic heart disease have also been identified (57, 58). In addition to producing dramatic reductions in high energy stores such as adenosine triphosphate (ATP) and creatine phosphate (CP), myocardial ischemia has been shown to result in a large accumulation of free fatty acids (FFA) and their acyl derivatives (59-63) as a consequence of changes in lipid metabolism. FFA and their acyl derivatives have been shown to promote cardiac dysfunction (64, 65) by depressing contractile force development (66). The long chain acyl derivatives of FFA are also known to bind with membranes (67) and change their properties (68, 69). Lysophospholipids that have accumulated in the ischemic heart (70-73) are known to induce arrhythmias (74-76). Accumulation of other metabolites due to lack of washout also plays an enormous part in promoting the overall loss of normal functional capacity of the heart. In this regard accumulation of protons derived from anaerobic glycolysis during ischemia is considered to exert inhibitory effects on contractile proteins (77, 78). On the other hand, acidosis due to the accumulation of both CO_2 and protons (decrease in pH) in the ischemic tissue (79) has been shown to be beneficial as long as it is mild over a short period, but may be harmful when severe over a prolonged period (80). Lactate accumulation is

known to promote decreased contractility (81) and $NADH_2$ accumulation due to impaired rate of mitochondrial metabolism has been shown to promote intramitochondrial Ca^{2+}-overload (82). Over the years, however, it is becoming clear that the mechanisms of ischemic injury and reperfusion injury may be distinct because no increase in intracellular Ca^{2+} occurs during ischemia, whereas intracellular Ca^{2+} overload is the hallmark of the ischemic-reperfused heart (83).

Although varying degrees of defects in different Ca^{2+} regulating systems located in the SL, SR and mitochondrial membranes have been identified in the reperfused heart, the exact mechanisms of these membrane abnormalities are poorly understood (36, 84, 85). A wide variety of SL defects including changes in Na^+-K^+ATPase, Na^+-Ca^{2+} exchange, Ca^{2+} pump, adenylyl cyclase, beta adrenergic receptors, alpha adrenergic receptors and G-protein mediated processes, in different models of heart disease (84-100), have been defined. Specifically, the β-adrenoceptor-G-protein-adenylyl cyclase system, which is known to play a major role in the regulation of heart function and metabolism, has been reported to be altered during both myocardial ischemia (5-8) as well as reperfusion (5, 9-12). However, reports of modifications incurred in the various components of the β-adrenergic pathway due to ischemia and reperfusion remain controversial, (6, 10-16). These conflicting observations by different investigators appear to be due to the usage of variable periods of ischemia as well as varying degrees of reperfusion in a variety of *ex-vivo* and *in-vivo* models in different species of animals. Furthermore, relatively little is known regarding the mechanism of changes in the β-adrenoceptor signal transduction system in the ischemic as well as

ischemic-reperfused hearts.

It is pointed out that oxygen derived free radicals have been implicated as a major factor in the pathophysiology of ischemia-reperfusion injury in the myocardium (17-19). These partially reduced forms of oxygen in the ischemic-reperfused myocardium can be generated from a number of sources within the cardiomyocyte including mitochondrial respiratory chain (20), and by other extracellular and systemic sources such as arachidonic acid metabolism and catecholamine oxidation (18, 21). Different types of active oxygen species have been shown to produce electrical abnormalities (101, 102), ultrastructural damage (22, 103), intracellular Ca^{2+}-overload (104) and cardiac dysfunction (105). Various oxygen radical scavengers have also been shown to exert beneficial effects on ischemia-reperfusion injury (106, 107). However, very little is known about the actions of oxyradical scavengers on the status of signal transduction mechanism such as G-protein or adenylyl cyclase-cyclic AMP system associated with the β-adrenergic receptors in SL upon exposure to oxygen radicals. Thus in light of the importance of this cascade in myocardial functional regulation, it is intended to summarize the alterations effected upon the components of this pathway during both myocardial ischemia and reperfusion as well as due to interaction with free radicals.

2. Free radicals during ischemia-reperfusion

A free radical is any molecule that has an odd number of electrons. Because of their molecular configuration, free radicals are normally highly reactive and can cause cellular injury. Under normal physiological conditions, free radical reactions are critical for the normal operation of diverse biologic processes. Radical species are often generated *in vivo* as byproducts of normal metabolism or under conditions such as exposure of organisms to ionizing radiation, to drugs which are capable of redox cycling or to xenobiotics that can form free radical metabolites *in situ.* The occurrence of reactive oxygen species therefore is an attribute of normal aerobic life. The steady state formation of these partially reduced forms of oxygen is balanced by a similar rate of their consumption by antioxidants such as superoxide dismutases and hydroperoxidases that may be enzymatic or non-enzymatic. "Oxidative stress" results from an imbalance in this oxyradical-antioxidant equilibrium in favor of the oxyradicals either due to their overproduction or as a consequence of reduced antioxidant reserve in the body.

Highly toxic oxygen radicals have been implicated in the pathogenesis of ischemia/reperfusion injury. Two popular theories of how reperfusion injury may occur are a) the Ca^{2+}-overload hypothesis and b) the free radical hypothesis (108). The former theory suggests that defects in the cellular capacity to regulate Ca^{2+} established during ischemia, result in the accumulation of toxic levels of intracellular Ca^{2+} during reperfusion. The free radical theory is based on the understanding that supra-normal quantities of reactive oxygen

species including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) are generated during reperfusion. Much of the support for this premise is due to many investigators, but not all (109), who have found that free radical scavengers can reduce the injury in the isolated heart model. Subsequently, these reactive oxygen species are implicated in the induction of membrane defects which promote excessive Ca^{2+} entry into the cell, thus unifying both theories.

It has been proposed that large quantities of different oxygen radicals formed upon reperfusion of the ischemic myocardium promotes tissue necrosis, arrhythmias (110), myocardial stunning and cellular damage (111-113). The superoxide radicals serve a key role in this scheme. It is thought to be cytotoxic but its relatively short half life in biological systems limits its diffusion away from the site of its generation. The superoxide radicals can be reduced further by SOD-catalysis to form H_2O_2, which although not a free radical species, it is a potent oxidiser. H_2O_2 is also membrane permeable and may traverse considerable distances in the cell and thus cause damage at sites distant from its origin (19). H_2O_2 is also a precursor for the formation of the $^{\cdot}OH$ radicals which, although short lived, are extremely reactive with biological membranes to form the carbon centered alkoxy and peroxy-radicals (19). The involvement of free radicals during ischemic-reperfusion has been documented by the detection of bursts of oxygen radicals in the coronary sinus blood as well as systemic blood within minutes of reperfusion (114-117). It has also been suggested that the breakdown of homeostatic mechanisms during hypoxia/ischemia can result in increased cytosolic Ca^{2+}, activating a Ca^{2+} dependent cytosolic protease that covalently modifies

xanthine dehydrogenase, converting it to xanthine oxidase (17). The enzyme catalyses the univalent oxidation of purine substrates with the concomitant formation of $\cdot O_2^-$ radicals, H_2O_2 (118, 119) and perhaps singlet oxygen ($^1O_2$) (120). Accumulation of these substrates during ischemia has been established by Jennings and Reimer (121) who reported that conversion of xanthine dehydrogenase to xanthine oxidase increased during the course of myocardial ischemia in dogs. Other studies report damage to isolated organ systems by exogenous xanthine oxidase and purine substrate (22, 122). Allopurinol or oxypurinol, which are xanthine oxidase inhibitors, protected against oxidative damage in ischemia/reperfusion injury in cats (123), rats (124) and dogs (125). However studies done in man (126) or rabbit (127) indicate the failure of xanthine oxidase inhibitors to protect against ischemia/reperfusion injury and in fact it has not been possible to detect measurable quantities of xanthine oxidase in human myocardium (128). However Yokoyama et al (129) postulated that large amounts of xanthine oxidase may be produced in the liver following ischemia and initiate systemic production of free radicals. The superoxide radicals once formed promote the generation of an entire family of activated O_2 species formed by sequential reduction as follows:

$$O_2 + e^- \rightarrow O_2^{\cdot -}$$

$$2O_2^{\cdot -} + 2H \rightarrow H_2O_2 + O_2$$

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^- + \cdot OH$$

Other well known biological sources of free radicals constitute activated neutrophils (130), direct donation of electrons from the reduced mitochondrial electron transport chain

(NADH-dehydrogenase, ubiquinone-cytochrome b regions) to molecular oxygen (131), catecholamine oxidation (132), and cyclooxygenase and lipoxygenase enzymes (prostaglandins) (133).

Alterations of membrane lipids and proteins by free radicals is one of the critical and important factors in the evolution of ischemia/reperfusion damage. Cell membranes contain large amounts of polyunsaturated fatty acids complexed to phospholipids which when peroxidized, results in loss of cellular integrity and function (134, 135). The alkoxy- and peroxy radicals that are the intramembranal products of lipid peroxidation can further promote polymerization, peptide chain breakage and altered amino acid structure of membranal proteins and enzymes. Proteins containing the amino acids with sulfhydryl groups in their structure, such as tryptophan, tyrosine, phenylalanine, histidine, methionine and cysteine, are most sensitive to modification of their structure (131). Functional alterations due to disruption of membrane integrity leading to loss of activity of the membrane bound enzymes, receptors and ion channels are some of the free-radical related effects that promote irreversible cellular destruction. Treatment of cardiac SR and SL membranes with oxygen free radicals has been shown to depress Ca^{2+}-pump mechanisms and these defects have been suggested to induce intracellular Ca^{2+}-overload and heart dysfunction (19, 25-28). Depression in the SL Na^{+}-K^{+} ATPase and Na^{+}-Ca^{2+} exchange activity upon treatment of heart membranes with oxyradical generating systems has been suggested to contribute towards the occurrence of intracellular Ca^{2+}-overload (29, 30). Reeves et al (136) reported a 10-fold increase in Na^{+}-Ca^{2+} exchange activity by reduced oxygen species in

bovine cardiac sarcolemmal vesicles. The stimulation of activity required the presence of reducing agents ($O_2^{\cdot}$) as well as oxidizing agents (H_2O_2) and probably resulted due to their influence on conformation of the exchanger by modifying the thiol-disulphide groups in its structure. Kim and Akera (137) reported decreased Na^+-K^+ ATPase activity, depressed specific [^{3}H]-ouabain binding, and lowered sodium pump activity of SL from ventricular muscle. These studies also revealed that scavengers of all species of activated oxygen ($O_2^{\cdot-}$, H_2O_2, $^{\cdot}OH$ and $^1O_2$) had protective effects to various degrees. Perfusion of the isolated heart with an oxyradical generating system also had been shown to depress both SL Na^+-Ca^{2+} exchange and Ca^{2+}-pump activities during the development of contractile failure (31, 32). Although a decrease in the density of Ca^{2+}-channels in the SL membrane (28) and SR (33) due to oxyradicals can be seen to result in the reduction of Ca^{2+} available for cardiac contraction, the contribution of depressed Ca^{2+}-stimulated ATPase of myofibrils (34) due to oxyradicals may also promote contractile abnormalities.

In the heart, the contractile force development and/or heart rate are regulated by receptor systems acting via accumulation of intracellular cAMP (G_s-protein coupled), by receptor systems acting via inhibition of cAMP formation (G_i-protein coupled), and by receptor systems acting independently of cAMP. Amongst all these receptors, the β-adrenoceptor-G_s protein-adenylyl cyclase-cAMP system is by far the most powerful contributor to the regulation of cardiac function. Since this pathway has also been shown to control the entry of Ca^{2+} into cardiomyocytes (35, 36), alterations of this signal transduction pathway during ischemia-reperfusion and due to oxidative stress may promote

significant increase in intracellular Ca^{2+} levels and subsequent cellular destruction and dysfunction. Thus one of the aims of this review is to describe the characteristics and function of the components of this pathway and define some of the alterations in these components under conditions of ischemia-reperfusion and oxidative stress. It should be pointed out that the β-adrenergic receptor system is a multi transport system composed of five functional units: a) a stimulatory receptor (β-adrenoceptor) which binds the stimulatory hormone or the neurotransmitter. The β-adrenergic receptor, like all known receptors which interact with guanine nucleotide binding proteins (G-proteins), is a transmembrane glycoprotein with seven putative membrane-spanning sequences; b) the stimulatory G-proteins (G_s) are coupled with adenylyl cyclase and upon activation these initiate the production of cyclic AMP and thus regulate diverse metabolic functional events. G_s protein is composed of three subunits: α_s, which possesses the GTP-binding site and is the target for cholera toxin catalyzed ADP-ribosylation by NAD^+; the β and γ subunits are tightly associated with each other. The G_s proteins are located in the inner leaflet of the plasma membrane and are less hydrophobic than either the β-adrenoceptor or the catalytic unit of adenylyl cyclase; c) the catalytic unit of adenylyl cyclase has a multitude of transmembrane spanning domains carrying its catalytic function at the cytoplasmic side of the membrane; d) inhibitory receptors which bind inhibitory neurotransmitters or inhibitory hormones and suppresses the activity of adenylyl cyclase by transducing their signal via the G_i proteins; e) the inhibitory GTP-binding protein (G_i). This protein like G_s is a heterotrimer composed of three subunits: α_i, β and γ where the βγ complex is highly similar to the one found in G_s.

3. β-adrenergic receptors and their regulation

The β-adrenoceptors belong to the G-protein coupled super family of receptors that have seven hydrophobic transmembrane-spanning regions and are proteins consisting of 402-560 amino acids (138). Human β-adrenoceptors have been shown to comprise of three subtypes β_1, β_2 and β_3 which are encoded by three distinct genes (139, 140). From structural studies carried out with these receptors it is evident that the trans-membrane domain appears to be the site of agonist and antagonist binding, whereas the cytoplasmic domain is where G-protein interacts and the terminal -COOH tail appears to be where phosphorylation can take place. Comparison of the distribution of β-adrenergic receptors with marker enzymes of the SL, SR and mitochondria has shown that β-adrenoceptors exist only in the SL membrane. This location renders them accessible to circulating catecholamines as well as those released from the sympathetic nerve terminals. It is now generally accepted that the β_1- and β_2-adrenoceptors coexist in the human heart; however, at present, there is no evidence for the existence β_3-adrenoceptors in the human heart (141). Some investigators have reported that the number of β-adrenoceptors in the normal human heart is quite evenly distributed in the right and left atrial as well as ventricular tissues, and this has been demonstrated by radioligand binding studies (80-90 mol/mg protein in all four chambers of heart) (142, 143) by quantitative autoradiographic studies (144), and *in vivo* by positron

emission tomography (PET) studies (145). On the other hand, the work from other laboratories have suggested that the proportion of β_2-adrenoceptor is somewhat higher in the atria (approx. 1/3 of total β-adrenoceptor population) than in the ventricular myocardium (approx. 20% of the total β-adrenoceptor population) (142, 143) and even higher in the atrio-ventricular conducting system (up to 50%) (146). Although the β_1-adrenoceptors predominate in the human heart especially the ventricles, human cardiac adenylyl cyclase is preferentially stimulated by β_2-adrenoceptors (142, 147, 148). This is in contrast to most other mammalian species (rabbit, cat, dog, rat) where the predominant type of β-adrenoceptor is the β_1-adrenoceptor, which is also responsible for the activation from these receptors of adenylyl cyclase by catecholamines (149).

The performance of the normal myocardium is not under the influence of the adrenergic system (150), which plays a major role under conditions of stress when sympathetic outflow is increased markedly. The number of receptors per unit area of SL (the receptor density) is not fixed but can rise (upregulation) or fall (downregulation) in response to certain physiological and pathophysiological circumstances. There is a lot of controversy surrounding the exact meaning of the term downregulation of receptors and possibly the most correct explanation lies in the supposition that there occurs a decrease in receptor number. Thus downregulation of receptors would result from 1) internalization of receptors, 2) decreased rate of receptor synthesis and 3) increased rate of receptor degradation. Prolonged exposure of receptors to its agonists results in a time dependent attenuation of responsiveness or refractoriness to continued stimulation by that agent. This phenomenon termed

"homologous desensitization" exhibited by the β-adrenoceptor is thought to involve phosphorylation of the β-adrenoceptor (151) by a specific β-adrenergic receptor kinase (βARK) (152). Once phosphorylated the receptors are no longer able to couple to the G_s protein (152) and the modified receptor becomes internalized. It should be mentioned that uncoupling of the β-adrenergic receptors and G_s after βARK-mediated phosphorylation requires the presence of another protein, β-arrestin (153); the exact function of β-arrestin in this regard is however unclear. The internalized receptors are not necessarily degraded but may actually be stored in an intracellular "pool" to be eventually resensitized, by removal of the phosphate groups, and returned to the cell surface membrane. Another form of desensitization is called heterologous desensitization, which results from desensitization of the receptors by a cAMP dependent kinase, and therefore can be triggered by a continued high rate of cAMP formation due to stimulation from not only β-receptors but also other receptors linked to adenylyl cyclase (154). Not all the receptors present at the surface membrane are in use at all times, since some of these receptors termed "spare receptors", do not react to agonist stimulation. Thus an altered number of receptors is not necessarily indicative of a corresponding alteration in the activity of the system. The human heart however, contains only a few spare receptors for β-adrenoceptor-mediated positive inotropic effects, and nearly all the receptors present at the surface membrane at any one time are necessary to evoke maximal response to adrenergic stimulation (155). It should be understood that even if the receptor density remains fixed, the activity of the receptors may be altered by molecular changes that regulate the affinity of the receptors for their agonists

(156).

The "Upregulation" of β-adrenergic receptors occurs under reduced exposure of cell to β-adrenoceptor agonists as achieved by denervation and treatment with β-adrenoceptor antagonists. Although the mechanisms involved in receptor upregulation are not well established it may occur due to reversal of all the parameters discussed in association with desensitization and downregulation. Acute exercise in rats was seen to induce rapid elevation of membrane bound β-adrenoceptors accompanied by a reduction in the intracellular receptor "stores" (157) suggesting that externalization of intracellular receptors may be a mechanism. It should be mentioned that increments of β-adrenoceptor mRNA levels with 30 - 60 min incubation with adrenaline followed by an inhibition of β-adrenoceptor transcription after 24 hrs incubation has been demonstrated (158). To this end, cAMP (158, 159) as well as glucocorticoids (160) have been shown to regulate receptor gene transcription. Therefore it appears that in the case of myocardial β-adrenergic receptors, both cycling of receptors between internal and external stores (161) as well as rapid changes in receptor protein synthesis (158) may provide means whereby the sensitivity of the heart to a given amount of β-stimulation can be modified.

4. Adenylyl cyclase and its regulation

Situated on the outer surface of the SL membrane, the β-adrenergic receptors couple to their intramembranal effector enzyme, adenylyl-cyclase, when it is occupied by

catecholamines such as norepinephrine and epinephrine. It should be clarified that although the best understood pathway for the regulation of adenylyl cyclase activity involves stimulation by β-adrenergic agonists, this enzyme broadly serves as a final common effector which integrates and interprets convergent inputs from many other signal generating pathways. Binding of agonist to an appropriate receptor causes activation of a stimulatory guanine nucleotide binding protein, G_s, which in turn stimulates adenylyl cyclase; the enzyme can also be inhibited by receptor-G_i protein pathways. To date, ten species of adenylyl cyclase enzymes (isoenzymes) have been identified in different tissues. Pfeuffer and coworkers (162, 163) were the first to purify the $G_{s\alpha}$- and Ca^{2+}-calmodulin-activated enzyme (Type I adenylyl cyclase) using forskolin-affinity chromatography. Thereafter, Krupinski et al (164), were able to derive a partial amino acid sequence and thus a full length cDNA encoding of this Type I adenylyl cyclase enzyme. Low-stringency homology probing and PCR techniques have since permitted molecular cloning of seven additional adenylyl cyclase cDNAs as well as information regarding two partial sequences (165). Structurally the adenylyl cyclase enzyme is made up of a short amino terminal followed by six transmembrane spans, a large cytoplasmic domain of approximately 360-390 amino acids, then a second set of six transmembrane spans followed by another large cytoplasmic domain of 255-330 amino acids (165). Due to the low levels of their expression in all tissues (0.01-0.001% of membrane protein) and the unavailability of satisfactory antibodies, there is limited information available regarding the tissue distribution of adenylyl cyclase. It has been shown, however, that the isoforms of the enzyme present in the heart are types III to

VII - with the greatest quantity being types V and VI (166, 167). Although the proposed molecular structure of adenylyl cyclase resembles the structure of certain ion channels and pore-forming molecules, there are no sequence similarities between these two classes of molecules (164). Also, unlike the channel proteins most of this enzyme protein has been reported to be located on the cytoplasmic side of the membrane (168), the presumed site of interaction with the G-protein α subunit.

When an agonist occupies the G_s coupled receptor, the complex thus formed catalyzes an exchange of GDP for GTP on the α-subunit of the G-protein. The GTP bound $G_{s\alpha}$ separates from the βγ subunits of this heterotrimeric complex and thereafter interacts with adenylyl cyclase and activates the enzyme. Since the $G_{s\alpha}$ subunit possesses an intrinsic GTPase activity, the hydrolysis of $G_{s\alpha}$ -bound GTP to GDP terminates the activation of adenylyl cyclase by $G_{s\alpha}$-GTP within seconds. $G_{s\alpha}$-GDP then reassociates with the βγ subunit and awaits a new cycle of activation (169). All mammalian species of adenylyl cyclase are thus far known to be stimulated directly by $G_{s\alpha}$. Type I and Type VIII adenylyl cyclase are capable of integrating and interpreting signals that are received from G_s-linked receptors and from pathways that increase intracellular Ca^{2+} concentrations (170, 171). The βγ subunit complex however appears to be a potent inhibitor of the type I adenylyl cyclase and its effect seems to be exerted directly on the enzyme (172). It also seems that adenylyl cyclase type I is able to discriminate the source of the βγ complex since only high concentrations of βγ achieved by the activation of G_i or G_o are able to inhibit the enzyme significantly while lower concentrations obtained by the activation of $G_{s\alpha}$ are unable to exert this effect (172).

This discrepancy is thought to be essential if dissociation of the G_s oligomer to yield $G_{s\alpha}$ and $\beta\gamma$ is to successfully activate the enzyme. On the other hand, the $\beta\gamma$ subunit exerts striking stimulatory effects on type II and type IV adenylyl cyclase, and these effects are dependent on coincidental activation by $G_{s\alpha}$ (173) such that the system is designed to respond synergistically when two pathways are activated simultaneously (173). Direct interaction of $G_{i\alpha}$ with adenylyl cyclase is by far the most well known mechanism for inhibition of adenylyl cyclase activity. Substantial (60-80%) inhibition of cAMP synthesis has been reported for $G_{s\alpha}$- and forskolin-stimulated adenylyl cyclase activity by nanomolar to micromolar concentrations of different isoforms of $G_{i\alpha}$ ($G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$ and $G_{2\alpha}$) (174, 175). Other regulators of adenylyl cyclase include Ca^{2+} (either directly or via PKC-mediation) and Ca^{2+}-calmodulin. Both Ca^{2+} and calmodulin appears to have a stimulatory effect on adenylyl cyclase at approximately 10-100 μM concentration range (176). Finally it has been reported that while PKA is thought to be an inhibitor of adenylyl cyclase activity (177), phosphorylation of the cyclases by PKC has a stimulatory effect on their activity (177).

5. G-proteins and their regulation

The heterotrimeric guanine nucleotide-binding proteins (G-proteins) form the switchboard between a family of receptors and intracellular effector molecules (178). Two types of G-proteins namely the stimulatory G-proteins (Gs) and the inhibitory G-proteins

(Gi) are involved in the conveyance of signals from receptors to adenylyl cyclase. All G proteins share a common structure, ie they are all made up of 3 subunits, α, β and γ; the α-subunit is thought to be the subunit responsible for the specific action of directly interacting and regulating the effector. The α-subunit of the G_s proteins is of a molecular weight ranging from 39 to 52 kD while the α subunit of G_i has a molecular weight of 40-41 kD (178). It should be pointed out that although a total of eight G_s proteins have been purified, the cDNAs derived from a total of nine genes encoding the α subunit have been cloned and they can be formally divided into four major classes derived from amino acid homology- α_s, α_i, α_q and α_{12} (179). All the isoforms of $G_{s\alpha}$ are encoded by a single gene (180) and are produced as separate proteins by alternate splicing of the precursor mRNA transcript (181). Members of all four classes have been detected in the myocardium (179). Homology cloning has revealed that there are at least four G_β genes and three Gγ genes. The beta (35 kD) and gamma (8-10 kD) subunits appear to be always tightly coupled as the βγ complex. The α-subunit of G_s-protein is also a substrate for cholera toxin catalyzed ADP-ribosylation where NAD serves as the ADP-group donor (182). Cholera toxin catalyzed ADP ribosylation locks the G_s-protein in the active state by virtue of the fact that it inhibits the GTPase activity of the protein. The ADP-ribosylation modifies an argenine residue located near the postulated GTP-binding region of the α-subunit. The G_i protein is an inhibitory regulatory component of adenylyl cyclase when activated by muscarinic-cholinergic receptor, α2-adrenoceptors, adenosine-,somatostatin- and neuropeptide Y- receptors (183). Three distinct isoforms of the $G_{i\alpha}$ subunit generated from three different genes are known to exist (184). All three

isoforms have been shown to be expressed in every tissue although their specific mRNA expression varies (185). In heart, the predominant isoform is the $G_{i\alpha 2}$ although the others are also present (184). As far as its inhibitory function is concerned, the mechanism seems to involve first a dissociation of the α_i-subunit from the $\beta\gamma$-complex, following which the $\beta\gamma$ subunit associates with and inhibits the activity of $G_{s\alpha}$-proteins. $G_{i\gamma}$ has not yet been demonstrated to have any direct inhibitory effect on adenylyl cyclase (186). Pertussis toxin catalyses the ADP-ribosylation of $G_{i\alpha}$-subunit in a manner similar to the cholera-toxin catalysed ADP-ribosylation of $G_{s\alpha}$ (187). The result of ADP-ribosylation of $G_{i\alpha}$ is an increased affinity of this subunit for the $\beta\gamma$ complex, thereby promoting their reassociation. The physiological consequence of this is the abolition of the inhibitory effect of G_i-proteins on adenylyl cyclase enzyme.

It is thought that receptors bind the α-subunit of G proteins at the carboxy terminal, and this interaction can be blocked by a number of factors such as: by pertussis toxin-induced ADP ribosylation of a cysteine residue located at the C-terminal of α_i and α_0 subunits, by mutagenesis of the C-terminal residues, and by antibodies raised against the C-terminal (188). Furthermore the C-terminal designates the specificity of receptor-G-protein interactions (189). The N-terminals on the other hand is essential for binding to the $\beta\gamma$ subunit; binding of $\beta\gamma$ subunit to the α subunit is required for the receptor stimulated exchange of GTP for GDP by the α-subunit (178). The effector binding area of the α-subunit of G proteins is also located at the C-terminal (189). In the inactive state, G-proteins are bound to a GDP molecule at its nucleotide binding site. Upon interaction with an

activated or occupied receptor, a conformational change occurs at the GDP binding site such that GDP is released and GTP gets preferentially bound. This exchanges is considered to be the rate limiting step in the activation process and different isoforms of G-proteins exhibit different rates of exchange of GTP for GDP and thus get activated at different rates (190). GTP binding is a Mg^{2+}-dependent process with different G-proteins requiring different Mg^{2+} concentrations for maximal guanine nucleotide mediated activation (191). The conformationally altered GTP-bound protein now splits into two compounds: the activated α-subunit and the $\beta\gamma$ complex. The α-subunit can now stimulate various effector molecules including (adenylyl cyclase and ion channels). Eventual hydrolysis of the GTP to GDP by the α-subunit itself increases its affinity for the $\beta\gamma$ complex and the two portions reunite to produce the inactive G-protein ready for another cycle.

The role of the α_s-subunit of G-protein in β-adrenergic signalling includes activation of adenylyl cyclase and formation of cAMP and also direct activation of Ca^{2+} channels; both these effects are therefore considered to explain relaxant as well as positive inotropic actions of catecholamines on cardiac muscle. Gating of ion channels is another important function of G-proteins. G-proteins are thought to increase the opening probability of Ca^{2+} channels but the mechanism of this effect is not known; such channels are called G-protein-gated channels (192). In addition G_s-protein has also been reported to mediate a direct (ie. cAMP independent) inhibition of Na^+ channels in rat neonatal ventricular myocytes (193). Although, the role of G-proteins in cardiovascular responses and in disease states are an area of intense research and their altered status has been reported in a number of pathologies (194,

195), modifications of these heterotrimeric proteins during ischemia reperfusion are not well established.

6. Cardiac β-adrenoceptor-G protein-adenylyl cyclase in acute myocardial ischemia and reperfusion

In acute myocardial ischemia, large amounts of catecholamines are liberated and accordingly receptor desensitization/downregulation would be the expected end result. However, in reality the opposite seem to occur. Myocardial ischemia in dog, guinea pig and rat has been consistently associated with increased β-adrenoceptor number (6, 12, 13, 16, 196). However, disparity exists as to the integrated activity and coupling of the increased β-receptor density to G-protein-adenylyl cyclase complex and its physiological responsiveness. Although the mechanism for this increased receptor numbers in the face of elevated endogenous catecholamine levels is not understood, it may be associated with the depleted ATP levels in the myocardium leading to either enhanced externalization (196, 197) or impaired internalization (6, 198) of receptors. In isolated guinea pig hearts, global ischemia of 35 - 90 min increased β-adrenoceptor density and moreover stimulated adenylyl cyclase activity (196) indicating a sensitization of β-adrenoceptor-adenylyl cyclase system. Studies done with isolated rat heart model of global ischemia revealed an increase in β-receptor number immediately upon onset of ischemia, followed by an increased forskolin-stimulated adenylyl cyclase activity after 15 min of ischemia (6). This is indicative of an

independent activation of the catalytic unit of the enzyme and suggestive of a dual sensitization of the β-receptor and adenylyl cyclase systems. Using the canine *in vivo* model of acute regional myocardial ischemia, it was found that β-adrenoceptor density increased within 30 min of regional ischemia in only those dogs that developed ventricular fibrillation (199, 200), while both β-adrenoceptor density and adenylyl cyclase remained unchanged in hearts without ventricular fibrillation (12, 13, 199). In these studies, the β-adrenoceptor affinities remained unchanged; however, in other studies the high affinity receptors for isoproterenol have been reported to be either reduced (7, 15) or unaffected (201). In early ischemia ($\leq$ 30 min), an enhancement of β-adrenoceptor stimulation is associated with an increase in the adenylyl cyclase activity (6, 196, 198) as well as an impairment of the inhibitory adenylyl cyclase regulation, thereby suppressing the tonic inhibition of adenylyl cyclase (202). However, an unaltered adenylyl cyclase activity in early ischemia has also been reported (199). It should be noted that while an increase in tissue levels of cAMP occurs within 5 min by ischemia, the activity of adenylate cyclase is increased only after 15 min of ischemia. This temporal disparity between cAMP content and adenylyl cyclase activity may be due to the dual sensitization of the β-receptor-adenylyl cyclase reported by Strasser et al (6), whereby an initially increased β-receptor number may stimulate an unaltered adenylyl cyclase to produce greater quantities of cAMP, followed by an activation of adenylyl cyclase at a later phase of ischemia resulting in the same end point. Ischemia for a duration greater than 30 min but less than 60 min is reported to be associated with a persistent increased number of β-adrenoceptors (6) but reduced adenylyl cyclase activity (6,

8, 16, 198) leading to a decreased β-adrenoceptor responsiveness. In spite of the increased density of β-adrenoceptors in late ischemia, it was noted that the number of these β-adrenoceptors in the high affinity coupled state was actually reduced (15). The decreased adenylyl cyclase activity in late ischemia has been observed when the enzyme was stimulated in the presence of isoproterenol, Gpp(NH)p or forskolin as well as at the level of the catalytic subunit in the presence of Mn^{2+} (16). It has been suggested that the enhancement of adenylyl cyclase in acute ischemia is linked to a simultaneously occurring activation of the protein kinase C (PKC) (203) whereas the decrease in adenylyl cyclase during prolonged ischemia appears to be accompanied by and related to a decrease of the G_s-protein activity (8, 11, 204). While there exists a lack of consistency with regards to the observed alterations in the β-adrenoceptor pathway due to ischemia, relatively little is known about the status of this pathway in the reperfused hearts. Although reperfusion of the ischemic myocardium maintains the increased β-adrenoceptor density (12), it reverses the depressed adenylyl cyclase activity (11).

The changes in the components of the signal transduction pathway are thought to have important implications during both ischemia and reperfusion. Although discrepancies in findings exist with regards to the ischemic myocardium, it has been suggested that there is an association between increased β-adrenergic receptor stimulation, cyclic AMP elevation and the development of ventricular fibrillation in early ischemia of less than 30 min duration. It is hypothesized that since cAMP phosphorylates Ca^{2+}-channels, enhanced quantities of this nucleotide may induce intracellular Ca^{2+}-overload and precipitate the Ca^{2+}-mediated

electrophysiological alterations that underlie ventricular fibrillation. On the other hand, the recovery of signal transduction with respect to increased β-adrenergic receptors and the adenylyl cyclase enzyme during reperfusion is thought to have beneficial implications. Since adenylyl cyclase regulates Ca^{2+}-fluxes across the SL and SR membranes, the alterations in the reperfused myocardium may represent mechanisms for the recovery of the ischemic-reperfused myocardium (205). This explanation may help rationalize the ability of the "stunned" myocardium to respond to adrenergic stimulation.

It should also be pointed out that in spite of the considerable effort which has been made for the understanding of alterations in the β-adrenoceptor pathway during ischemia, an enormous amount of discrepancy exists in the reports on this subject by various investigators. Although most of these studies have employed a variety of animal models, it is not known if similar changes occur in the human. Nonetheless, it is interesting to observe that, 1 hr of cardiopulmonary bypass with cardioplegic cardiac arrest promoted marked desensitization of β-adrenoceptor mediated right atrial adenylyl cyclase activation without affecting right atrial β-adrenoceptor number in children with acyanotic congenital heart disease (206).

7. Mechanisms responsible for alterations in β-adrenoceptor pathway during ischemia-reperfusion

Different explanations have been provided for the increase in β-adrenoceptor density by ischemia. Several investigators have argued that β-adrenoceptors were redistributed from the intracellular vesicles to the membranes during ischemia (196, 197, 201, 207, 208). On the other hand, it was suggested that redistribution of β-adrenoceptors was due to impaired internalisation, an effect attributed to ATP depletion (201, 209).. However, Mukherjee et al. (13) have rejected the mechanism of receptor translocation as a possible explanation for ischemia induced increase in β-adrenoceptor density. An increase in protein synthesis, although attractive, is not a satisfactory explanation for the increase in receptor numbers because of the relatively short period of ischemia (13). Likewise, the observed increase in receptor density may not be artefactual because no difference in membrane protein content, was seen between control and ischemic hearts (13). One explanation that has been gaining considerable recognition recently is that the effect of ischemia-reperfusion on β-adrenoceptors may be mediated via oxidative stress. In this regard it should be noted that ischemia and reperfusion have been shown to be associated with oxidative stress which has been observed to affect the β-adrenoceptor density (39). On the other hand, oxygen radical generating systems were demonstrated to induce a decrease in maximal specific binding of ^{125}ICYP to membrane fractions from lungs, lymphocytes or adipocytes (210, 211). More recently it has been described that relatively mild oxidative stress by H_2O_2 may increase the density of β-adrenoceptors in cardiac membranes, whereas higher concentrations of H_2O_2 decrease the density of the receptors without any alterations in their affinity (39). However, in another study mild oxidative stress using a variety of radical-generating system decreased

the affinity of ^{3}H-DHA binding to cardiac membranes and increased the maximal binding of ^{3}H-DHA (37). Nonetheless, maximal binding of a hydrophilic ligand ^{3}HCGP-12177 was unaffected. This has been interpreted to be indicative of oxygen radical induced alteration in membranes such that there is an increase in the accessibility of β-receptors to hydrophobic but not hydrophilic ligands (37). Extensive oxidative stress, however, decreased maximal binding to β-adrenoceptor for both hydrophilic and hydrophobic ligands (37). Oxidative stress has also been shown to impair the overall β-adrenoceptor response in several organs. A reduced β-adrenergic response in airways smooth muscle has been observed due to both treatment with H_2O_2 (212) as well as oxygen free radicals produced by activated macrophages (213). The positive inotropic response in cardiac muscle to catecholamines (214) and adrenergic relaxation of the small intestines (215) have been shown to be compromised by treatment with H_2O_2.

Similar to the divergent reports with respect to the β-adrenoceptor status in ischemia and reperfusion, there is also no consensus with regards to the status of adenylyl cyclase activity and cAMP formation under the same conditions. Both reduction (7, 11, 15, 16) as well as increase (196, 201) in the activity of adenylyl cyclase due to ischemia have been reported. Will-Shahab et al (216) reported that while the reduction in adenylyl cyclase in moderate ischemia was reversible, a more pronounced ischemia caused irreversible damage. It has been suggested that while reversible damage to adenylyl cyclase may be due to increased Ca^{2+} content of the heart, the irreversible damage may be due to oxidative stress (216). However, Will-Shahab et al. (216) indicated that adenylyl cyclase is the main target

for oxidative stress as seen in conditions such as ischemia-reperfusion whereas Haenen et al. (41), suggested that the intermediary G-proteins are affected by the oxidative stress. However, not much is known about the effect of ischemia-reperfusion or oxidative stress upon the coupling G-proteins in the β-adrenoceptor signal transduction pathway.

It is tempting to speculate that the effects of ischemia-reperfusion upon β-adrenoceptors-adenylyl cyclase is mediated via oxidative stress. If the reported effects during this condition are due to oxidative stress, this might to an extent explain the conflicting results obtained by various investigators. In this regard it should be noted that peroxidation of membrane lipids due to oxidative stress has been reported to lower the receptor density and alter plasma membrane viscosity which affects receptor coupling (215). Reactive oxygen species may also interact with thiol/disulphide groups in the protein components of the signal transduction system and lead to altered receptor coupling. To this end, both β-adrenergic receptors (217) and adenylyl cyclase (218, 219) have been shown to contain sulfhydryl groups which are essential for their function and these are vulnerable targets for reactive oxygen species. G-proteins also contain essential sulfhydryl groups, the alkylation or oxidation of which can alter their ability to couple receptor signal to the effector namely adenylyl cyclase (218, 220). From the above discussion it is evident that the functional responsiveness of the β-adrenoceptor-G protein-adenylyl cyclase system is altered during ischemia and reperfusion. However upon studying the modifications of the components during these conditions, it is strikingly obvious that there exists very little consistency in the findings by the various investigators and several mechanisms have been

proposed as mechanisms for these modifications. Ischemia-reperfusion has been shown to be associated with oxidative stress which in turn is known to affect β-adrenoceptors and adenylyl cyclase in various tissues. Accordingly, oxidative stress is currently believed to promote the altered status of the β-adrenoceptor pathway during ischemia-reperfusion. Intracellular targets for these reactive oxygen species appears to be both membrane lipids and proteins especially the sulfhydryl groups and disulfide bridges of the proteins. However relatively little is known about the effect of oxidative stress on β-adrenoceptors and adenylyl cyclase in the heart and moreover the information that is available to date is divergent. In addition, almost nothing is known with regards to oxidative stress induced alterations to G-proteins and their coupling activity. Thus information with regards to the status of β-adrenoceptor pathway during oxidative stress as well as the possible involvement of such modifications in promoting the altered inotropic state of the ischemic-reperfused heart would be helpful in extending our existing knowledge. With this in mind, we investigated alterations in the component of the β-adrenoceptor-G-protein-adenylyl cyclase due to oxidative stress and attempted to extrapolate the relative importance of these changes to the modifications which may occur in this pathway during ischemia and reperfusion.

III. METHODS

1. Perfusion of isolated rat hearts: Male Sprague-Dawley rats (200-250 g) were sacrificed by decapitation, their hearts rapidly removed and perfused according to the Langendorff procedure at a constant flow of 10 ml/min using the Krebs-Henseleit buffer (K-H buffer) oxygenated with 95% O_2 and 5% CO_2, pH 7.4 (221). The composition of K-H buffer was in mM: NaCl 120.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.25, glucose 11.0. After an equilibration period of 15 min, total ischemia was induced by stopping the perfusion for 30 min while the hearts were kept at constant humidity and temperature of 37°C. The hearts were electrically stimulated (Phipps and Bird stimulator) at 300 beats/min via a square wave of 1.5 ms duration at twice the threshold voltage. The left ventricular developed pressure (LVDP), the rate of change in developed pressure (+dP/dt) and the rate of change in relaxation (-dP/dt) were measured by using a water-filled latex balloon inserted into the left ventricle. The volume of the balloon was adjusted at the left ventricular end-diastolic pressure (LVEDP) of 10 mM Hg at the beginning of experiment and the balloon was connected to the pressure transducers Model 1050BP-BYOPAC SYSTEM INC). Data were recorded online through an analogue-digital interface (MP 100, BIOPAC SYSTEM INC), stored and processed with AcqKnowledge 3.0.1 for Windows (BIOPAC SYSTEMS INC).

2. Protocol for ischemia-reperfusion: In ischemic-reperfused hearts, perfusion with normal K-H buffer was reinstated to 60 min after 30 min of global ischemia. In hearts

treated with SOD plus CAT, the enzyme mixture was infused into the perfusion medium as a stock solution in K-H buffer via a side arm close to the cannula for 10 min before inducing ischemia; this infusion was also started at the time of initiating reperfusion. Preliminary experiments revealed that SOD when used alone had no beneficial effects on ischemia-reperfusion induced changes reported in this study. Control hearts were perfused for 30 to 90 min periods with K-H buffer and because of the overlapping values, the results were grouped together. In some experiments, the effect of isoproterenol (1 μM) on the cardiac contractile activity of the control and ischemic-reperfused hearts was studied by infusing this agent through the side arm of the perfusion apparatus.

*3. **Perfusion with oxygen radicals:*** To generate the superoxide radical, X (2 mM, Sigma) and XO (10 U/l, Sigma) were dissolved in the above buffer solution and mixed for 60 min in order to ensure sufficient generation of the superoxide radical. When using H_2O_2, this activated species of oxygen (200 μM, 500 μM and 1 mM) was mixed in the buffer solution in separate experiments. All hearts were allowed to equilibrate for 20 to 30 min with normal oxygenated K-H buffer before exposure to any experimental intervention. Hearts were either perfused with X plus XO for 5, 10, 15 and 30 min or with H_2O_2 (200 μM or 500 μM) for 10 min, to examine changes in contractile parameters. In separate experiments hearts were perfused with X plus XO for 10 min or 30 min in the presence or absence of SOD (1.5×10^5 U/l) and CAT (1.0×10^5 U/l) or with 1 mM H_2O_2 for 10 min in the presence or absence of CAT (1.0×10^{-5} U/l) or D-mannitol (120 mM). Control hearts were perfused for 10 to 30 min periods with K-H buffer and because of the overlapping

values, the results were grouped together. In some experiments, the effects of 1 μM isoproterenol infusion on contractile parameters of hearts perfused in the absence or presence of X plus XO or H_2O_2 were examined.

*4. **Preparation of heart membranes:*** At the end of each perfusion period, the hearts were used to prepare crude membrane according to the method described by Dixon et al (222), which were then used for the various assay. Briefly, the ventricular tissue was minced and then homogenized in 50 mM Tris-HCl, pH 7.4 (15 ml/g tissue) with a PT-20 polytron (Brinkman Instruments, Westbury, NY, USA), twice for 20 s each at a setting of 5. The resulting homogenate was centrifuged at 1000 x g for 10 min and the pellet was discarded. The supernatant was centrifuged at 48000 x g for 25 min. The resulting pellet was suspended and centrifuged twice in the same buffer and at the same speed; the final pellet was resuspended in 50 mM Tris-HCl, pH 7.4 and used for various biochemical assays.

*5. **In vitro treatment of membranes with oxygen radicals:*** In order to examine the effects of oxyradicals under *in vitro* conditions rats were decapitated, hearts removed and ventricular tissue used for membrane preparation (222). Aliquots of membrane were either incubated with X plus XO at the concentration of 2 mM and 0.03 U/ml, respectively, or H_2O_2 at the concentrations range of 5 μM - 5 mM for different time periods at 30°C. SOD, CAT or D-mannitol (MAN), when used as scavengers, were at the concentration of 80 μg/ml, 10 μg/ml and 2 mM, respectively. Membranes incubated without any addition for the relevant time period served as controls. Membranes treated with X plus XO (in the presence or absence of SOD, CAT, MAN), or H_2O_2 (in the presence or absence of CAT and MAN), were

washed and resuspended in 50 mM Tris-HCl (pH 7.4), prior to their use for various assays.

6. β-Adrenergic receptor binding: To determine β_1- and β_2-adrenoceptors binding, aliquots (0.1 mg/ml) of membrane preparations from control or treated hearts (ischemic, ischemic-reperfused as well as X + XO- or H_2O_2-perfused) were used in this study. Experiments were also carried out by employing cardiac membranes treated in the absence (control) and the presence of X plus XO or H_2O_2 under *in vitro* conditions. Membranes were incubated for 60 min at 37°C with various concentrations (5 - 400 pM) of ^{125}I-cyanopindolol (2200 Ci/mMol) in the presence or absence of either 100 μM CGP-20712A (a selective β_1 antagonist) or 100 μM ICI-118,551 (a selective β_2 antagonist). Incubations were terminated by rapid vacuum filtration through Whatman GF/C filters. The non-specific binding was approximately 20% of the total binding values. Specific binding to β_1-receptors was calculated as the difference between ^{125}I-CYP binding values in the presence and absence of ICI-118,551, whereas β_2-receptor specific binding was the difference between ^{125}I-CYP binding values in the presence and absence of CGP-20712A. The values for maximal binding (B_{max}) and dissociation constant (K_d) were calculated from the Scatchard plot analysis of the data according to the interactive LIGAND program of Munson and Rodbard (223). In agonist competition studies, a concentration of ^{125}I-CYP (0.07 nM) was used in the presence of increasing concentrations of the β-receptor agonist, isoproterenol (10^{-10} - 10^{-4} M). In this set of experiments, assays were performed in triplicate by incubating membranes for 40 min at 37°C and terminating the reaction by rapid filtration on Whatman GF/C filters. The agonist binding curves for each preparation were plotted by subtracting the nonspecific

binding (displaced by 0.1 mM isoproterenol) from the total ^{125}I-CYP binding at each concentration of isoproterenol.

*7. **Determination of adenylyl cyclase activity:*** Adenylyl cyclase activity was determined by measuring [^{32}P] cAMP formation from [α-^{32}P] ATP as described by Sethi et al (224). Unless otherwise indicated the incubation assay medium contained 50 mM glycylglycine (pH 7.5), 0.5 mM MgATP, [^{32}P] ATP (1 - 1.5 x 10^6 cpm), 5 mM $MgCl_2$ (in excess of the ATP concentration), 100 mM NaCl, 0.5 mM cAMP, 0.1 mM EGTA and an ATP-regenerating system comprising of 2 mM creatine phosphate and 0.1 mg creatine kinase/ml in a final volume of 200 μl. Incubations were initiated by the addition of membranes (30 - 70 μg) to the reaction mixture, which had been equilibrated for 3 min at 37°C. The incubation time was 10 min at 37°C and the reaction was terminated by the addition of 0.6 ml 120 mM zinc acetate containing 0.5 mM unlabelled cAMP.

cAMP was determined by co-precipitation of other nucleotides with $ZnCO_3$ by the addition of 0.5 ml 144 mM Na_2CO_3 and subsequent chromatography by a double column system as described by Salomon et al (225). The unlabelled cAMP served to monitor the recovery of [^{32}P] cAMP by measuring absorbency at 259 nM. Under the assay conditions used, the adenylyl cyclase activity was linear with respect to protein concentration and time of incubation. For studying the effect of pertussis and cholera toxins on the adenylyl cyclase activity (determination of the functional activity of G-proteins), the membrane preparations were treated with or without toxins for 60 min at 30°C in the same reaction

mixture as that employed for ADP-ribosylation (described below) except that 10 mM NAD was used instead of [α-^{32}P] NAD. These membranes were washed 2 - 3 times with Tris-buffer and finally suspended in the same buffer for the estimation of adenylyl cyclase activity.

*8. **Toxin-catalyzed ADP-ribosylation***: ADP-ribosylation of G_i-proteins and G_s-proteins was studied by treating the membranes with pertussis toxin and cholera toxin according to the methods described by Sethi et al (224). For the determination ADP-ribosylation of G_i-proteins, 50 μg of the control, treated experimental membranes were incubated for 60 min at 30°C in 100 μl of 100 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM GTP, 10 mM thymidine, 2 μM [α-^{32}P] NAD (2 Ci/mmol) and activated pertussis toxin (5 μg/ml). G-protein substrates of cholera toxin (G_s-proteins) were assayed in an analogous fashion; the membrane protein was incubated for 90 min at 30°C in 100 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM ATP, 10 mM thymidine, 0.1 mM GTP, 10 mM arginine, 1 mM $NADP^+$, 2 μM [α-^{32}P] NAD (20 Ci/mmol) and activated cholera toxin (20 μg/ml). The reactions were stopped by addition of cold 20% trichloroacetic acid (TCA). Cholera toxin and pertussis toxin were activated by incubating in 50 mM DTT for 30 min at 37°C. The samples were applied to a 12% SDS polyacrylamide gel according to the method of Laemmli. (226). The gels were dried and subjected to autoradiography using Kodak X-AR5 film at -70°C for 24 - 72 hrs. An LKB Ultroscan XL laser sensitometer

was used to quantitate the G_s and G_i proteins under control and experimental conditions.

9. Electrophoresis and immunoblot assay: The G_s and G_i proteins were quantified by an immunoblotting method modified from that described by Hathaway and Haeberle (227). Control experimental membranes were suspended in 50 μl H_2O and 50 μl sample buffer (6.4 M urea, 17 mM Tris-HCl, 19.5 mM glycine (pH 8.6), 10 mM DTT, 10 mM EGTA, 1 mM EDTA, 5 mM NaF, 1 mM phenylsulphonyl fluoride and 0.4% bromophenol blue) and then denatured by boiling for 3 min. The proteins were resolved in 12% sodium dodecyl sulphate (SDS) polyacrylamide gel and then electroblotted to nitrocellulose sheets by employing a 25 mM Na_2HPO_4 transfer buffer. After transfer, the nitrocellulose sheets were shaken for approximately 2 hr in blocking buffer which contained 10 mM Tris-buffered saline (TBS), 5% fat-free powdered milk and 1% Tween-20. The blots were then incubated for 14 hr with specific antisera [AS/7 specific for $G_{i\alpha}$ and RM/1 specific for $G_{s\alpha}$] (1:3,000) in TBS and then washed twice for 10 min each with 1% Tween-20 and TBS alternately. The antigen-antibody complexes were detected by chemiluminescence whereby the nitrocellulose sheets were dipped in luminol substrate solution (Amersham). To visualize the bands, chemilumigrams were developed on Hyperfilm-ECl; normal exposure times ranged from 30 sec - 1 min. An LKB ultrascan XL laser densitometer was used to scan the developed films and quantitate the specific bands for $G_{i\alpha}$ and $G_{s\alpha}$ under control and experimental conditions.

10. Statistical analysis of the data: All results were expressed as mean ± SE and analysed statistically by using the Student's "t" test. The P value < 0.05 was taken to reflect a significant difference between the control and experimental preparations.

IV. RESULTS

1. Ischemia-Reperfusion

a. ***Contractile Parameters:*** Alterations in LVDP, LVEDP, +dP/dt and -dP/dt were studied in the isolated rat hearts upon inducing global ischemia for 30 min as well as upon reperfusion for 60 min following 30 min of ischemia and the results are shown in Figs. 1 and 2. Global ischemia resulted in a rapid decline of LVDP; complete inability of the heart to generate developed pressure was seen within 2 to 3 min of inducing ischemia. The LVEDP increased to its peak (90%) at approximately 10 min of ischemia and remained persistently increased during the further period of ischemia. Reperfusion induced a slow recovery of LVDP, +dP/dt and -dP/dt; in fact these parameters showed about 40% recovery over a 60 min reperfusion period whereas LVEDP remained elevated (about 90% of the control value) (Figs. 1 and 2). On the other hand, a 70 to 80% recovery of LVDP, +dP/dt and -dP/dt was associated with a marked reduction in the elevated LVEDP upon reperfusion of the ischemic hearts with a medium containing SOD plus CAT (Figs. 1 and 2). A time-course effect of isoproterenol (1 μM) infusion revealed a smaller ($P < 0.05$) increase in LVDP, +dP/dt and -dP/dt in the ischemic-reperfused hearts compared to the control hearts (Fig. 3). These attenuated inotropic responses to isoproterenol stimulation in the ischemic-reperfused hearts were normalized to a large extent (81 to 94%) in the presence of SOD plus CAT (data from two hearts not shown).

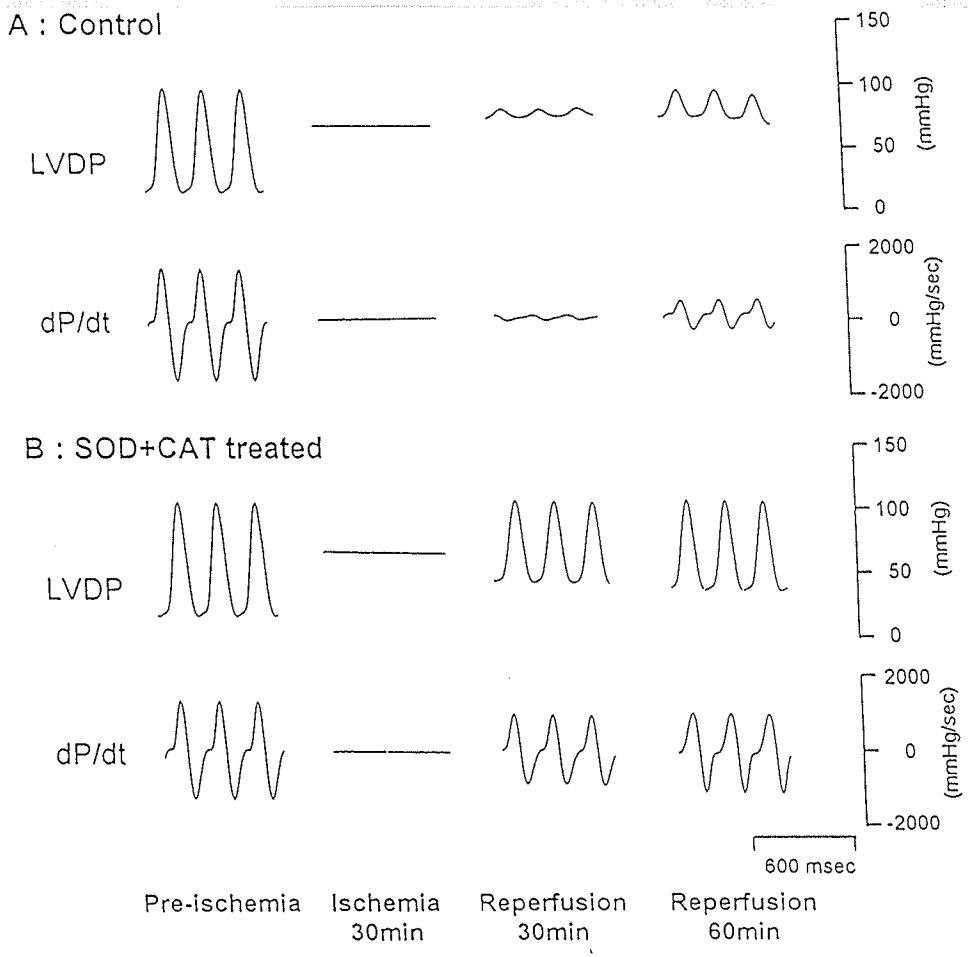

Fig. 1. A typical recording showing the effects of 30 min global ischemia and 60 min reperfusion on the left ventricular developed pressure (LVDP) and the rate of change in the left ventricular pressure development (dP/dt) in the isolated perfused rat heart in the absence (A) or presence (B) of SOD (80 μg/ml) plus CAT (10 μg/ml) in the perfusion medium. SOD - superoxide dismutase; CAT - catalase.

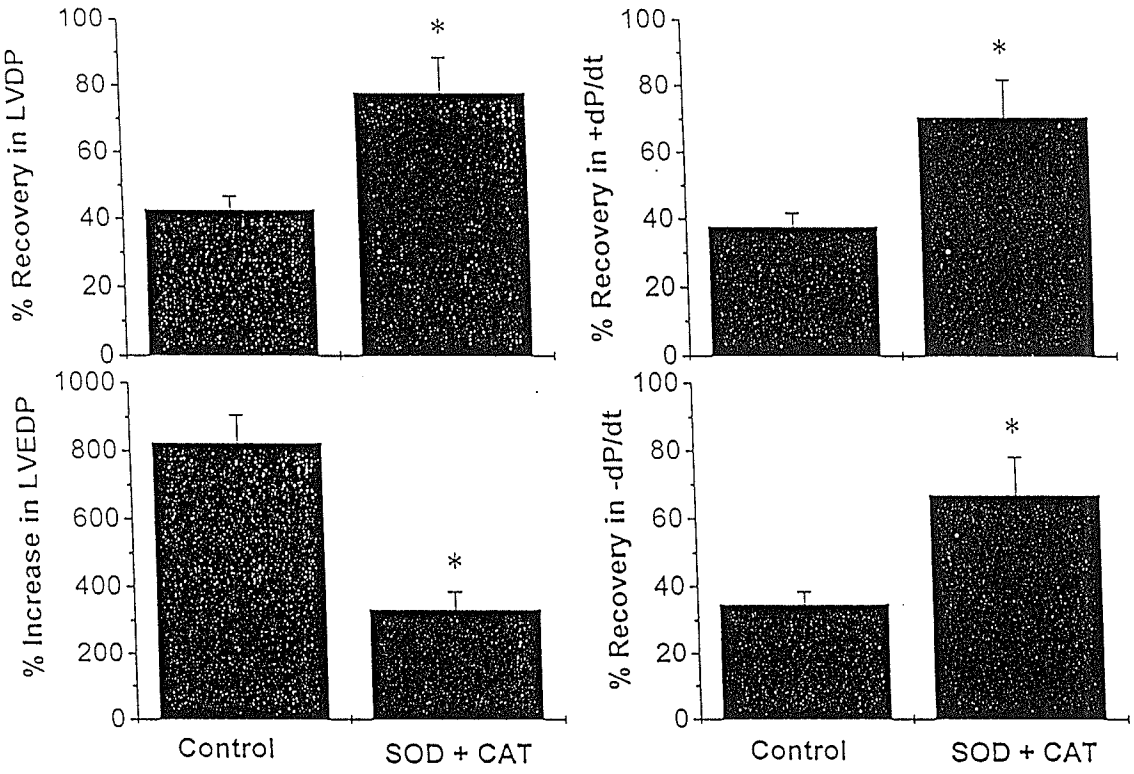

Fig. 2. Effect of perfusion with superoxide dismutase (SOD) plus catalase (CAT) on the recovery of contractile parameters in 30 min ischemic and 60 min reperfused rat hearts. Both SOD and CAT were present during the preperfusion period (10 min) and reperfusion period. Each value is the mean ± SE of 4 - 6 experiments in each group. LVDP: left ventricular developed pressure; LVEDP: left ventricular end diastolic pressure; +dP/dt: rate of contraction; -dP/dt: rate of relaxation. * - Significantly different from the respective control value.

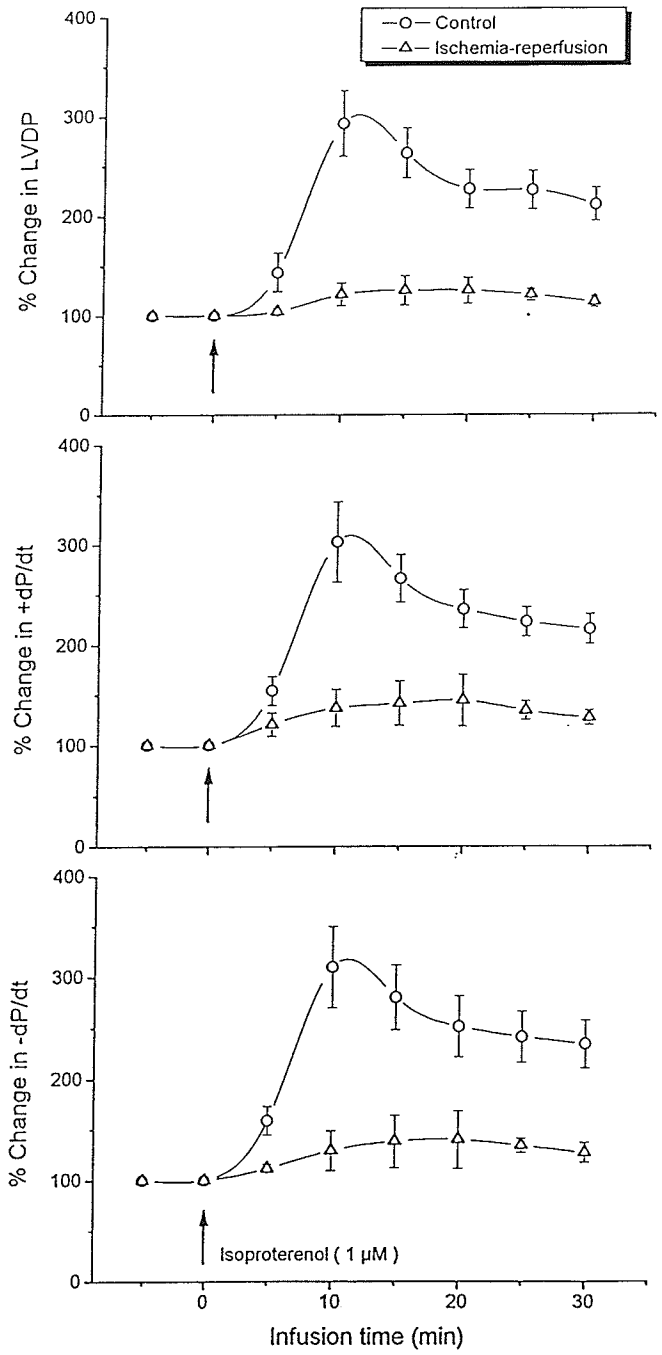

Fig. 3. Effect of infusion of isoproterenol (1 μM) on contractile parameters in control hearts and hearts made ischemic for 30 min and then reperfused for 60 min. Each value is a mean ± SE of 4 - 6 experiments in each group. LVDP: left ventricular developed pressure; +dP/dt: rate of contraction; -dP/dt: rate of relaxation. * - Significantly different from the respective control value.

b. ***Beta-adrenergic receptors*:** In order to show if β-adrenergic receptors were altered in hearts upon inducing ischemia for 30 min as well as upon reperfusion for a 60 min period, the specific binding of ^{125}I-CYP to both β_1-adrenoceptors and β_2-adrenoceptors was studied in cardiac membranes. Fig. 4 shows the specific binding data for β_1-adrenoceptors as well as the Scatchard plot analysis of ^{125}I-CYP binding to β_1-adrenergic receptors in control and ischemic-reperfused hearts. Both the affinity (1/Kd) and the density of the β_1-adrenoceptors were severely reduced in the ischemic-reperfused hearts compared to controls (Fig. 4 and Table 1). Although the Scatchard plot analysis of data on ^{125}I-CYP with β_2-adrenoceptors also revealed a depression in both the affinity ($1/K_d$) and the density of β_2-adrenoceptors in ischemic-reperfused hearts (Table 1), these changes were much smaller in comparison to those seen in β_1-adrenoceptors. Nonetheless, the presence of both SOD and CAT in the perfusion medium prevented these reperfusion-induced alterations in both β_1- and β_2-adrenoceptors almost completely (Table 1). It should be noted that both the density and affinity ($1/K_d$) of β_1-adrenoceptors were slightly but significantly increased whereas no such changes were evident in the β_2-adrenoceptors in the 30 min ischemic hearts (Table 1). Agonist competition curves using isoproterenol (Fig. 5), demonstrated a shift in the curve towards the left in ischemic myocardium compared to control, indicating an increase in the number of coupled β-adrenoceptors in the high affinity state. On the other hand, the curve for ischemic-reperfused myocardium moved to the right with respect to the control curve indicating an increase in the low affinity state of the coupled β-adrenoceptors.

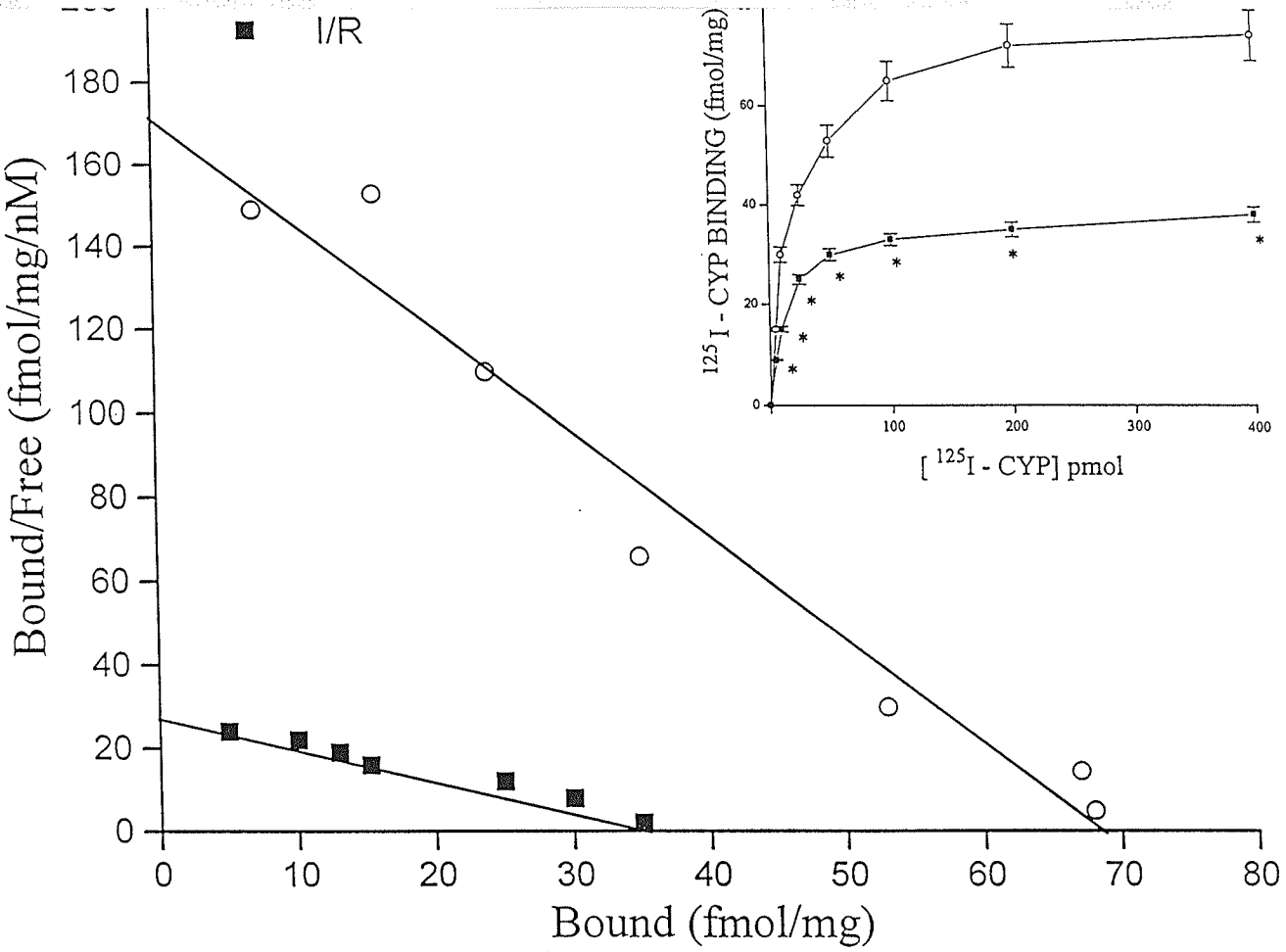

Fig. 4. Scatchard plot analysis of [^{125}I]-iodocyanopindolol (CYP) binding in membranes isolated from control (○) and ischemic (30 min)/reperfused (60 min) rat hearts (■). Data represents a typical experiment performed in triplicate. Inset: Equilibrium specific binding of [^{125}I] CYP with cardiac membranes by using ICl 118,551 in 5 - 6 experiments in each group. * Significantly different from control ($P < 0.05$). I/R: ischemia/reperfusion

Table 1. Binding characteristics of [^{125}I]-iodocyanopindolol to crude membrane preparations from control, ischemic and ischemic-reperfused rat hearts

	β_1-adrenergic receptors		β_2-adrenergic receptors	
	K_d (pmol)	B_{max} (fmol/mg)	K_d (pmol)	B_{max} (fmol/mg)
Control	39 ± 2	70 ± 4	15 ± 1.0	22 ± 1.5
Ischemia	30 ± 2*	84 ± 3*	14 ± 1.0	21 ± 1.5
Ischemia/ Reperfusion	64 ± 6*	39 ± 3*	20 ± 1.2*	17 ± 1.1*
I/R + SOD + CAT	40 ± 2$^{\#}$	65 ± 4$^{\#}$	16 ± 1.1$^{\#}$	21 ± 1.2$^{\#}$

Each value is the mean ± SE of 6 separate preparations for each group. Crude membranes were isolated from hearts subjected to global ischemia for 30 min and reperfused for 60 min. Control hearts were perfused with normal medium for 30 to 90 min. Since the values for 30 to 90 min perfused hearts were overlapping, the results were grouped together. Specific binding of [^{125}I]-cyanopindolol at different concentrations with B_1- and β_2-adrenergic receptors were determined in the presence of ICI 118,551 and CGP-20712A compounds, respectively. K_d and B_{max} values were determined from the Scatchard plot analysis of the data. *Significantly different from control ($p < 0.05$). $^{\#}$ Significantly different from I/R ($p < 0.05$). I/R = Ischemia/reperfusion; SOD = Superoxide dismutase (80 μg/ml); CAT = Catalase (10 μg/ml).

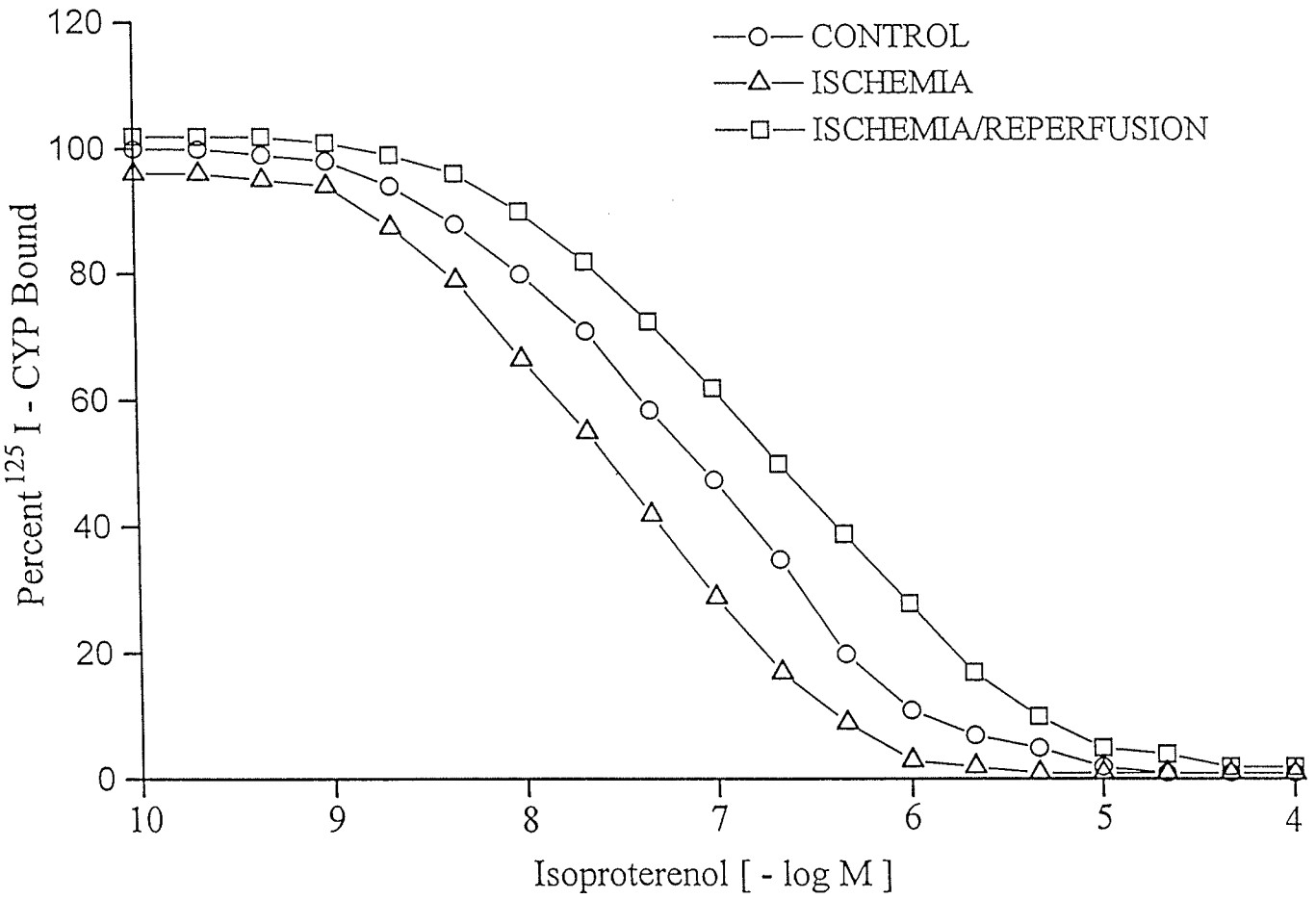

Fig. 5. Agonist competition curves for membranes in control, (○) ischemic (△), and ischemic-reperfused (□) hearts. Hearts were made ischemic 30 min and then reperfused for 60 min; control hearts were perfused for 30 to 90 min. Data represent a typical of 3 experiments using 0.07 nM ^{125}I-CYP as a ligand and different concentrations of isoproterenol as a competing agonist. ^{125}I-CYP: ^{125}I-cyanopindolol.

c. ***Adenylyl cyclase activity*:** The status of adenylyl cyclase in the ischemic and ischemic-reperfused hearts was monitored by measuring the enzyme activity in the absence or presence of different interventions. While the basal and forskolin-stimulated activities of the enzyme were unaltered by 30 min ischemia, both NaF- and Gpp(NH)p-stimulated activities of adenylyl cyclase were significantly depressed in the ischemic heart (Table 2). On the other hand, the adenylyl cyclase activity in membrane preparations was increased significantly in the ischemic-reperfused myocardium compared to control (Table 2); this was the case whether the activity was measured in the absence (basal), or presence of different stimulants such as forskolin, NaF or Gpp(NH)p. The presence of SOD plus CAT in the perfusion medium completely prevented these alterations in enzyme activity associated with ischemia-reperfusion. In another set of experiments, the effect of ischemia-reperfusion on cardiac adenylyl cyclase activity in the presence of different concentrations of isoproterenol was examined. The results in Fig. 6 indicate a marked reduction in the isoproterenol-stimulated adenylyl cyclase activity in the ischemic-reperfused hearts; this attenuation of the isoproterenol-induced stimulation of the enzyme was prevented upon reperfusing the ischemic hearts in the presence of SOD and CAT. It should also be mentioned that the adenylyl cyclase enzyme was markedly unresponsive to stimulation by increasing concentration of isoproterenol after a 30 min period of global ischemia; the activation curve of the ischemic hearts was similar to that shown for the ischemic-reperfused preparations in Fig. 6.

Table 2. Effect of forskolin, NaF and Gpp(NH)p on adenylyl cyclase activity in crude membrane preparations from control, ischemic and ischemic-reperfused rat hearts.

	Adenylyl cyclase activity (pmol/mg protein/min)			
	Control	Ischemia	Ischemia|Reperfusion	I/R + SOD + CAT
Basal	36 ± 3.0	30 ± 2.5	58 ± 4.5*	41 ± 3#
+ Forskolin (100 μM)	352 ± 29	358 ± 20	476 ± 34*	365 ± 28#
+ NaF (5 mM)	160 ± 13	120 ± 9*	227 ± 15*	178 ± 13#
+ Gpp(NH)p (30 μM)	180 ± 13	131 ± 10*	252 ± 16*	195 ± 14#

Values are mean ± SE of 4-6 experiments in each group. Adenylyl cyclase assays were done on membranes isolated from rat ventricles subjected to global ischemia for 30 min and reperfused for 60 min. Control hearts were perfused for 30 to 90 min with normal perfusion medium. Since the values for 30 to 90 min perfusion with control medium were overlapping, the results were grouped together. The concentrations of superoxide dismutase (SOD) and catalase (CAT) were 80 μg/ml and 10 μg/ml, respectively. * Significantly different from its respective control ($p < 0.05$). # Significantly different from its respective I/R group. I/R = ischemia/reperfusion.

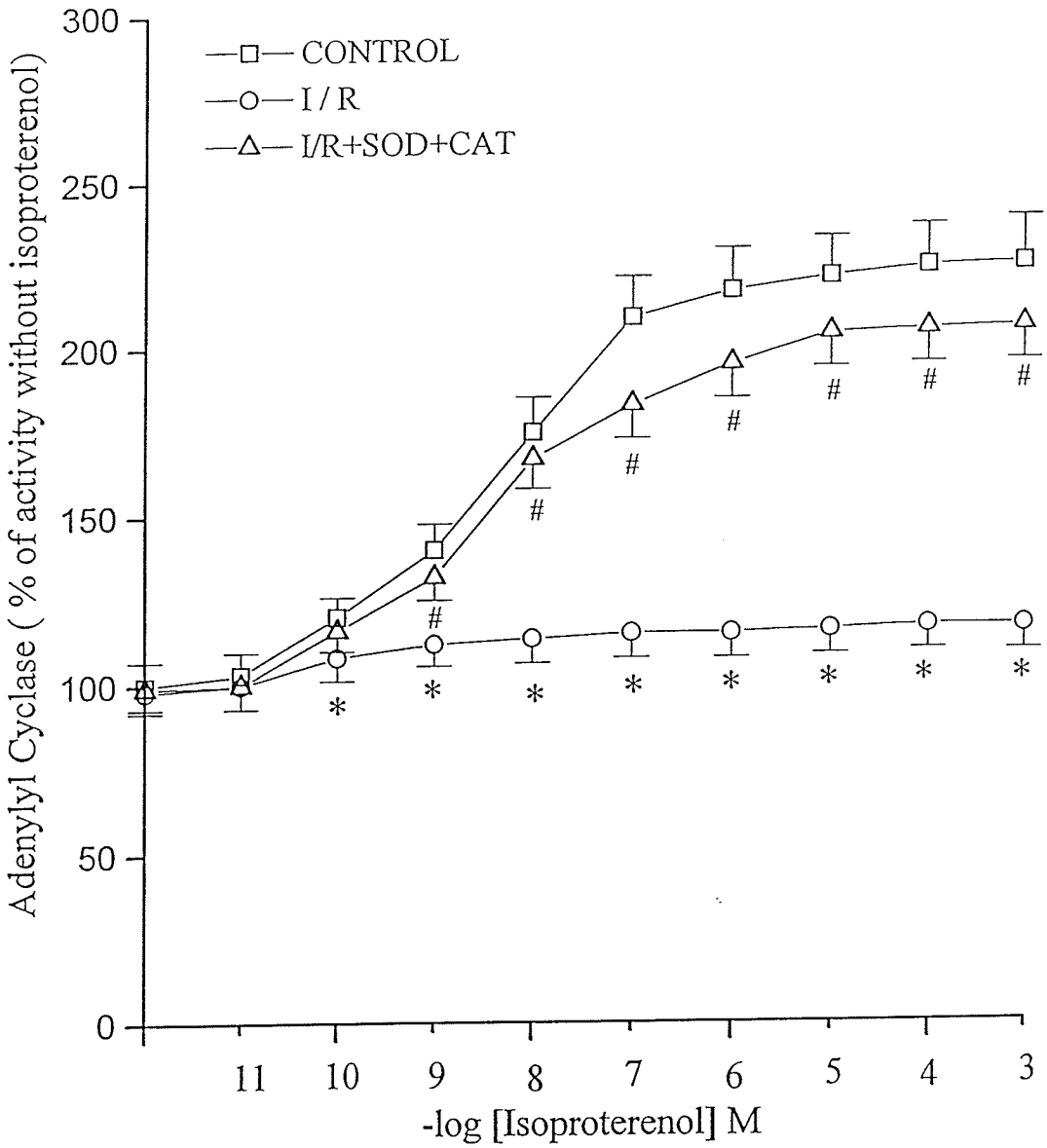

Fig. 6. Adenylyl cyclase activity in the presence of different concentrations of isoproterenol in membrane preparations from control hearts and hearts subjected to 30 min ischemia and 60 min reperfusion in the presence and absence of superoxide dismutase (SOD) plus catalase (CAT). The assay mixture contained 10 μM Gpp(NH)p. Each value is the mean ± SE of 5 - 6 membrane preparations for each group. The concentrations of SOD and CAT were 80 μg/ml and 10 μg/ml, respectively. * Significantly different from control ($P < 0.05$); # Significantly different from I/R ($P < 0.05$); IR: ischemia-reperfusion.

d. ***G-Protein activities and contents:*** For gaining information regarding changes in G-proteins in the ischemic and ischemic-reperfused hearts, the adenylyl cyclase activities were determined in the absence or presence of cholera toxin (CT), an activator of G_s-proteins, and pertussis toxin (PT), an inhibitor of G_i-proteins, and the results are shown in Fig. 7. The CT-induced increase in adenylyl cyclase was depressed in ischemic hearts but was augmented upon reperfusing the ischemic hearts (Fig. 7). On the other hand, the PT-induced increase in adenylyl cyclase was unaltered in the ischemic as well as the ischemic-reperfused hearts (Fig. 7). Alterations in G-proteins in the ischemic and ischemic-reperfused hearts were further monitored by measuring ADP-ribosylations as well as G-protein content. It should be mentioned that the CT-stimulated ADP ribosylation of G_s-proteins as well as the anti G_s-protein antibody binding detected bands at 45 kD and 52 kD bands (Fig. 8) whereas, the PT-stimulated ADP-ribosylation of G_i-proteins as well as the anti G_i-protein antibody binding produced a band at 40 kD band (Fig. 9). The CT-stimulated ADP-ribosylation activities as well as G_s-protein content (as determined by anti G_s-protein antibody binding) at both 45 kD and 52 kD bands were depressed in the ischemic myocardium and increased in the ischemic-reperfused hearts (Fig. 8). No change in the PT-stimulated ADP ribosylation of G_i-proteins at 40 kD or G_i-protein content (as measured by anti G_i-protein antibody binding at 40 kD) was seen in the ischemic hearts (Fig. 9). However, both PT-stimulated ADP ribosylation activity and G_i-protein content were slightly but significantly ($P < 0.05$) increased in the ischemic-reperfused hearts (Fig. 9).

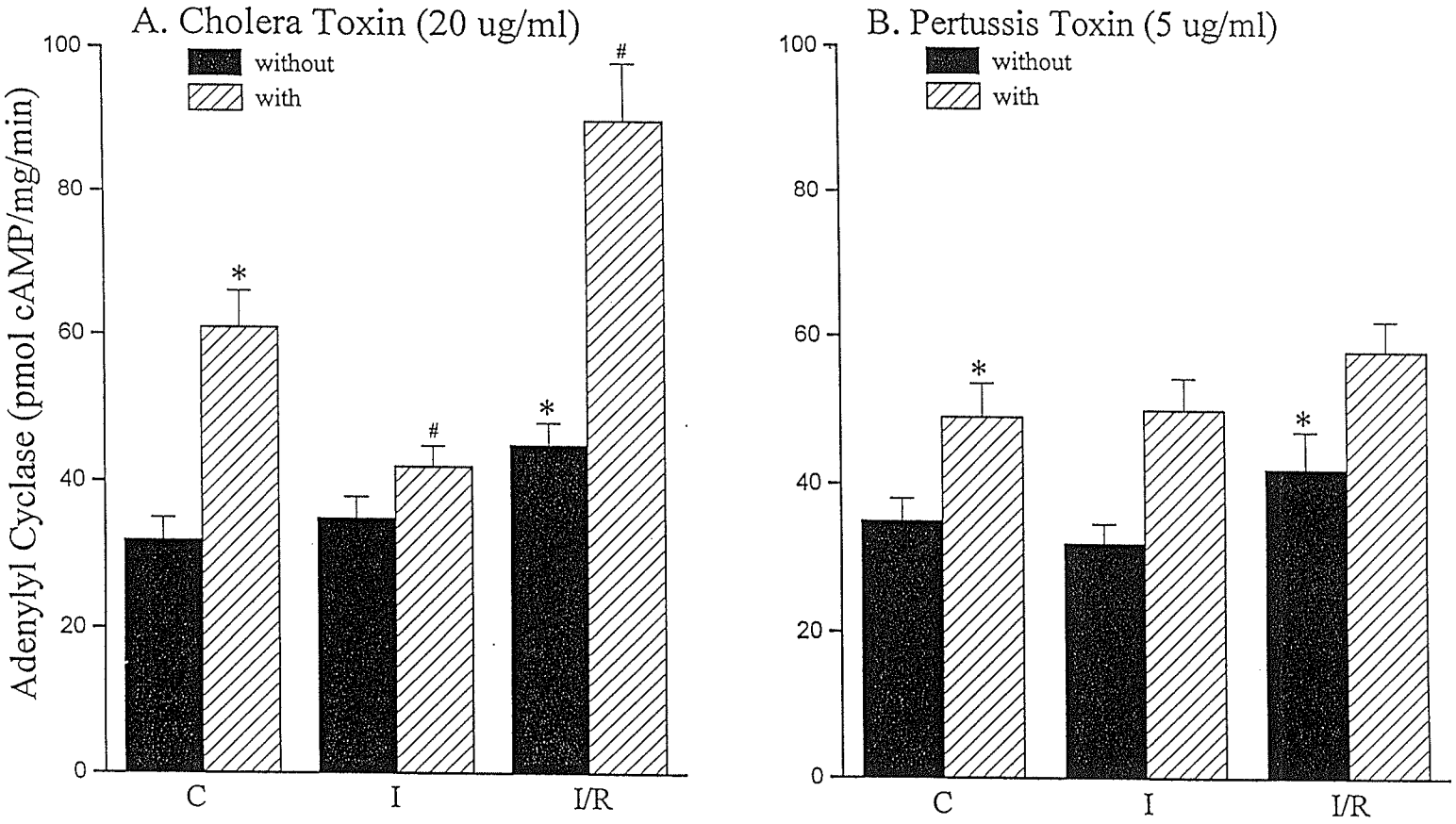

Fig. 7. Effect of cholera toxin and pertussis toxin on the adenylyl cyclase activity in membranes from control hearts, ischemic hearts (30 min) and 30 min ischemic/60 min reperfused hearts. Values are mean ± SE of 6 preparations in each group. * Significantly different from basal control ($p < 0.05$); # Significantly different from its respective value in the presence of toxin ($P < 0.05$). C: control; I: ischemia; I/R: ischemia/reperfusion

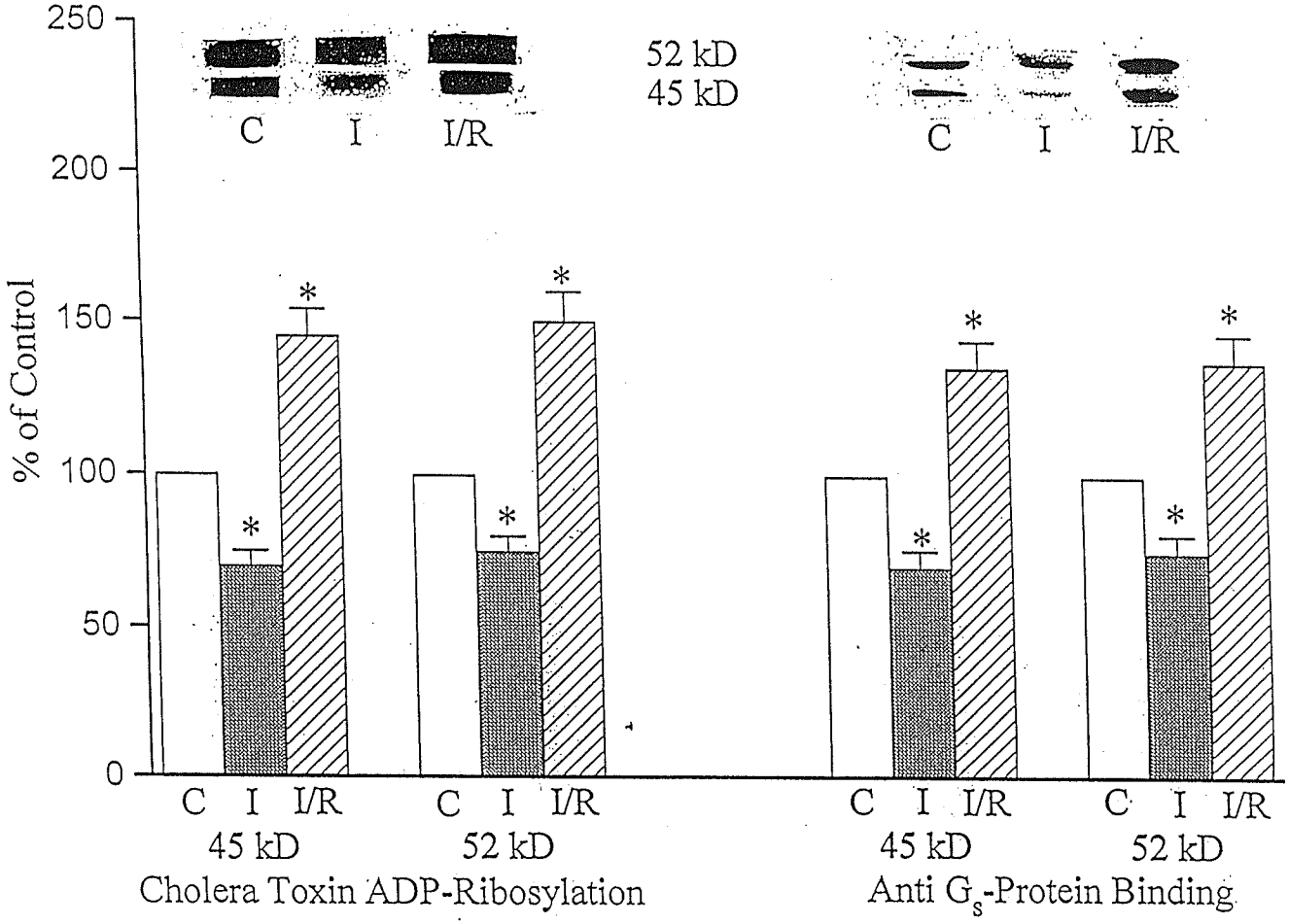

Fig. 8. Cholera-toxin stimulated ADP-ribosylation and G_s-protein content in membranes from control, ischemic and ischemia/reperfused rat hearts. Lower panel shows barographs for the densitometric analysis of cholera toxin-stimulated ADP-ribosylation and G_s-protein immunoblots in membranes from control hearts, ischemic hearts and ischemic/reperfused rat hearts. Upper panel shows the cholera toxin-stimulated ADP-ribosylation and G_s-protein immunoblots from control, ischemic and ischemia/reperfusion-treated membranes. Each value is a mean ± SE of 5 preparations in each group. * Significantly different from control ($P < 0.05$). C: control; I: ischemic (30 min); I/R: ischemic (30 min)/reperfused (60 min). The concentration of cholera toxin was 20 μg/ml.

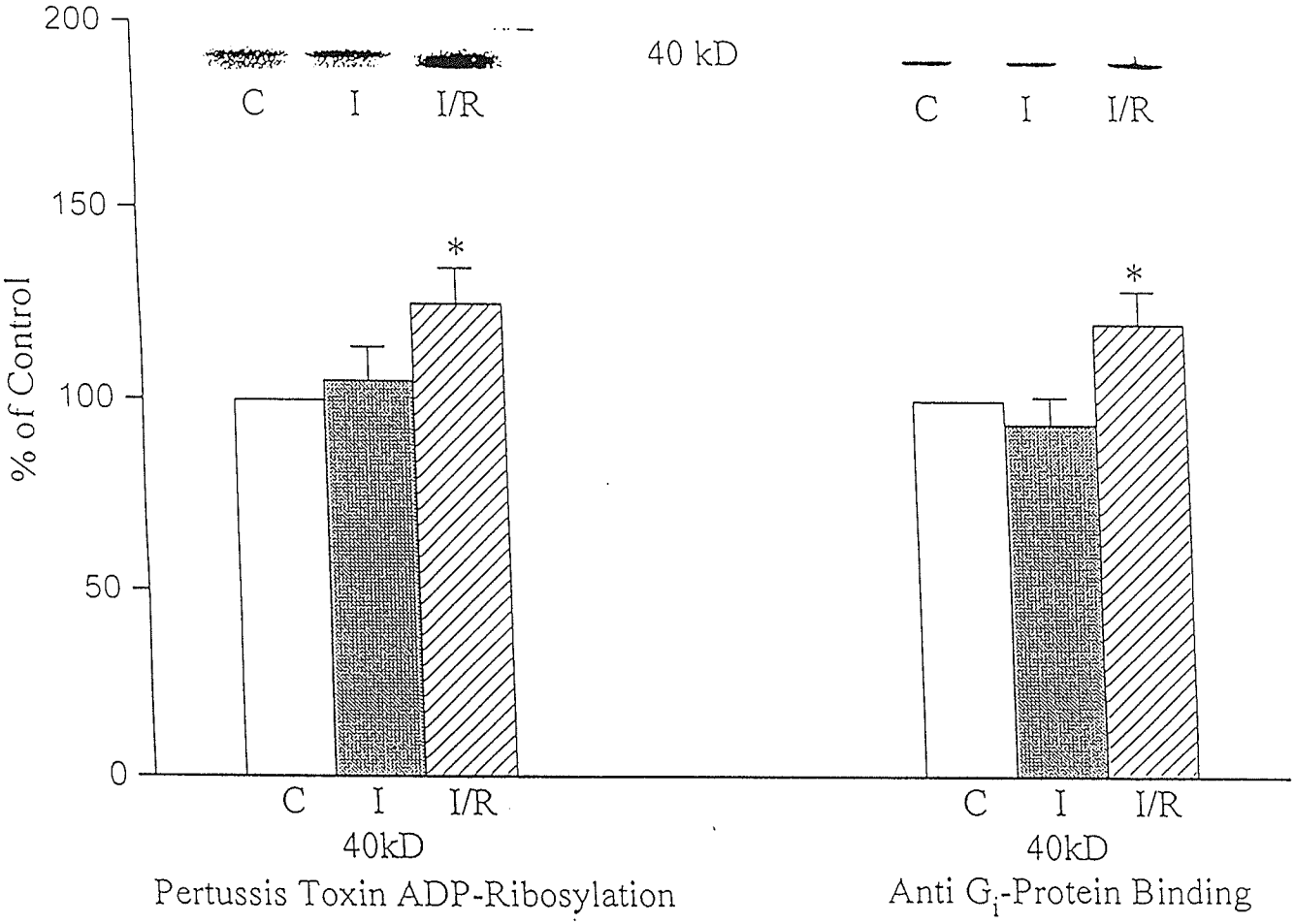

Fig. 9. Pertussis toxin stimulated ADP-ribosylation and G_i-protein content in membranes of control, ischemic and ischemia/reperfused rat hearts. Lower panel shows barographs for the densitometric analysis of pertussis toxin-stimulated ADP-ribosylation and G_i-protein immunoblots in membrane from control hearts, ischemic hearts and ischemia/reperfused hearts. Upper panel shows pertussis toxin ribosylation and G_i-protein immunoblots in membranes from control, I and I/R ventricles. Each value is the mean ± SE of 5 preparations in each group. * Significantly different from control ($P < 0.05$). C: control; I: ischemic (30 min); I/R: ischemic (30 min)/reperfused (60 min). The concentration of pertussis toxin was 5 μg/ml.

In vitro and *ex-vivo* treatment of membranes with X plus XO

a. Adenylyl cyclase activity: The basal adenylyl cyclase activity in membranes treated *in vitro* with X plus XO for different periods exhibited a biphasic pattern of changes whereby 10 min incubation increased and 30 min incubation decreased the activity of the enzyme compared to control (Fig. 10). This was also the case when the enzyme activity was measured in the presence of various stimulants such as forskolin, NaF or Gpp(NH)p (Table 3). The presence of SOD plus CAT was able to prevent these biphasic alterations associated with 10 min and 30 min incubation with X plus XO by 85 to 90%. The inclusion of MAN did not increase the magnitude of the protection to the enzyme activity afforded by SOD plus CAT. In another set of experiments, the effect of X plus XO on cardiac adenylyl cyclase activity in the presence of different concentrations of isoproterenol was examined. The results in Fig. 11 indicate that while there is a marked reduction of the isoproterenol-stimulated adenylyl cyclase activity upon treating the membranes for 30 min with X plus XO, there was an enhancement of the isoproterenol-stimulated enzyme activity upon 10 min incubation. Fig. 12 shows that while SOD in combination with CAT was able to significantly prevent the biphasic alteration in the isoproterenol-stimulated activity at both 10 min and 30 min incubation periods, SOD on its own was ineffective. The presence of MAN did not provide any further benefit in modifying the X plus XO induced alterations above those produced by SOD plus CAT (Fig. 12). Since CAT was necessary to prevent the X plus XO mediated changes, a set of experiments was carried out to study the direct involvement of H_2O_2 in inducing these biphasic alterations by treating membrane

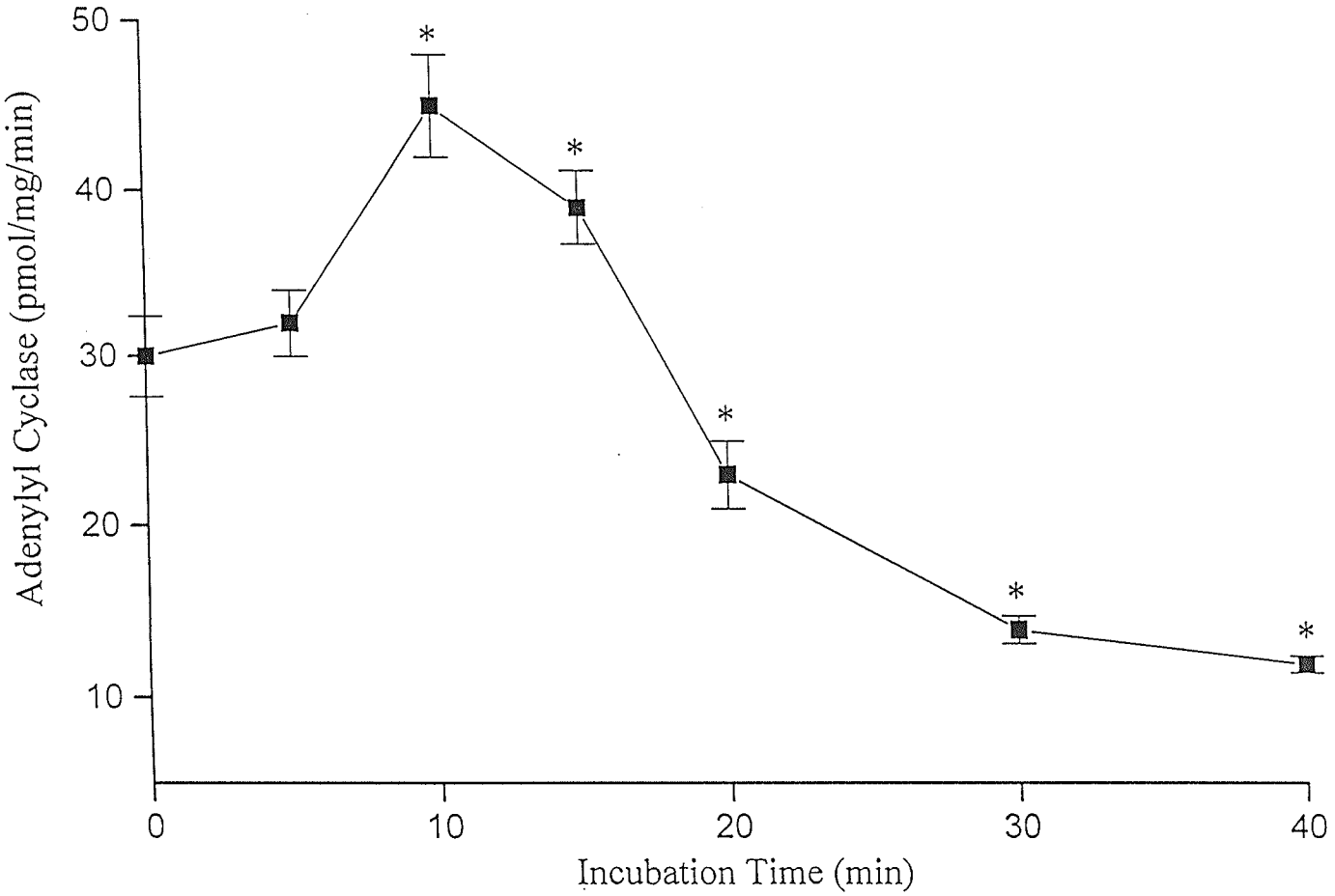

Fig. 10. Effect of different times of incubation with xanthine (2 mM) plus xanthine oxidase (0.03 U/ml) on the adenylyl cyclase activity in cardiac membranes. Incubations for different intervals were carried out at 30°C prior to the adenylyl cyclase assay. Control preparations were incubated in the absence of xanthine plus xanthine oxidase for different times. Although 10 to 15% depression in the enzyme activity was seen upon incubating the control membranes for 40 min, the changes were not significant ($P > 0.05$) and thus the results were grouped together with 0 time incubation. Each value is a mean ± S.E. of 6 separate membrane preparations. * Significantly different from control ($P < 0.05$).

Table 3. Effect of forskolin, NaF, and GppNHp on the adenylyl cyclase activity in rat cardiac membranes treated without (control) or with xanthine plus xanthine oxidase.

	AdenylylCyclase Activity (pmol/mg protein/min)		
	Control	X + XO (10 min)	X + XO (30 min)
Basal	30.5 ± 4	44.9 ± 4*	16.3 ± 1*
+ Forskolin (100 μM)	321 ± 30	600 ± 50*	222 ± 16*
+ NaF (5 mM)	140 ± 10	255 ± 19*	92 ± 7*
+ GppNHp (30 μM)	153 ± 11	265 ± 19*	97 ± 8*

Values are mean ± SE of 4-6 separate preparations for each group. Before assay for adenylyl cyclase activity, membrane preparations were incubated at 30°C for 10 or 30 min with xanthine plus xanthine oxidase (X plus XO). Controls were incubated under similar conditions without X plus XO and due to overlap, the values were grouped together. Final concentrations of X and XO were 2 mM and 0.03 U/ml, respectively. * Significantly different from its respective control ($P < 0.05$).

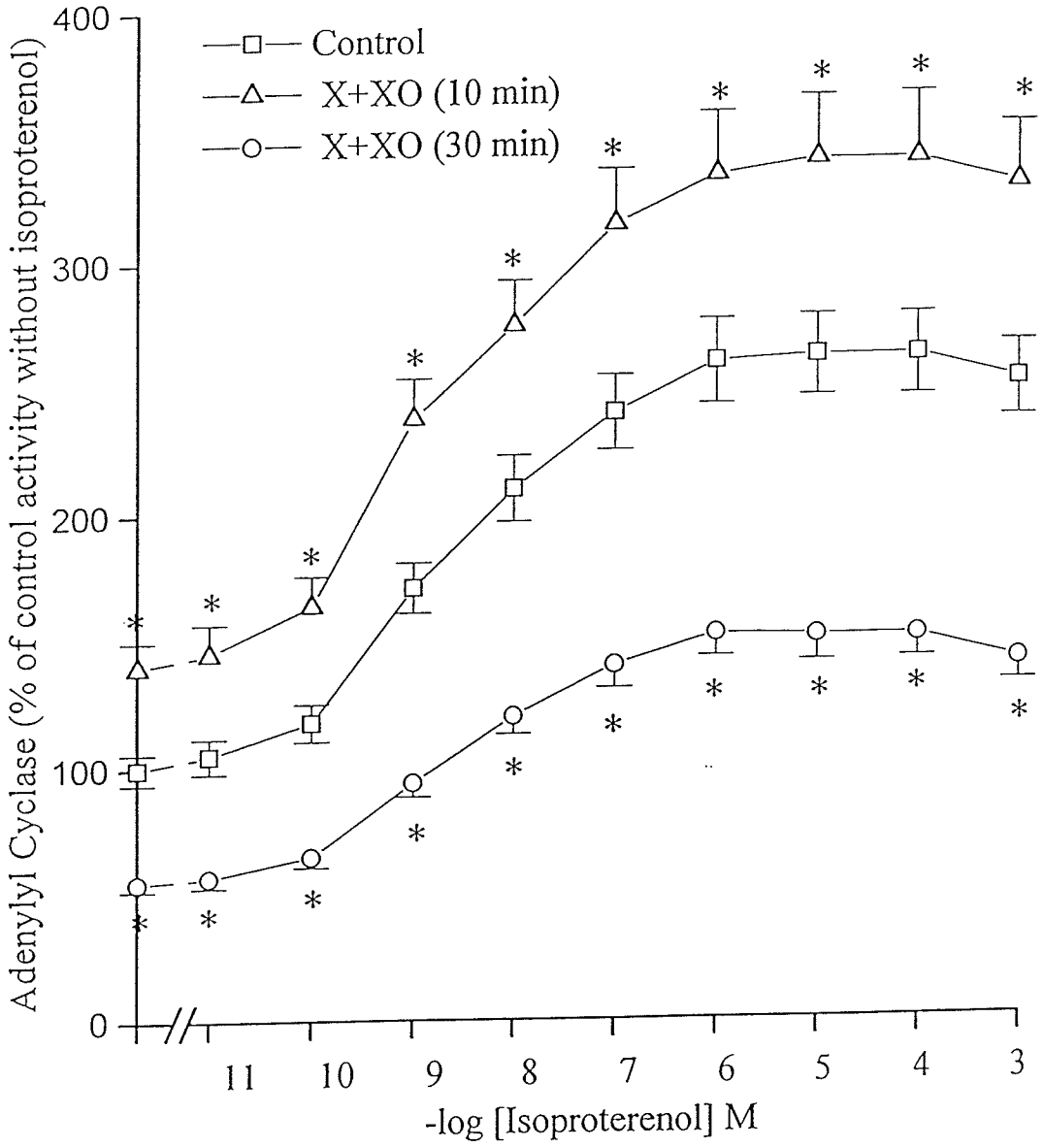

Fig. 11. Effect of different concentrations of isoproterenol on the adenylyl cyclase activity in cardiac membranes treated with xanthine (X, 2 mM) and xanthine oxidase (XO, 0.03 U/ml) for either a 10 min or a 30 min period at 30°C. The assay medium in this set of experiments contained 10 μM Gpp(NH)p. Each value is the mean ± S.E. of 6 different membrane preparations. * Significantly different from control ($P < 0.05$).

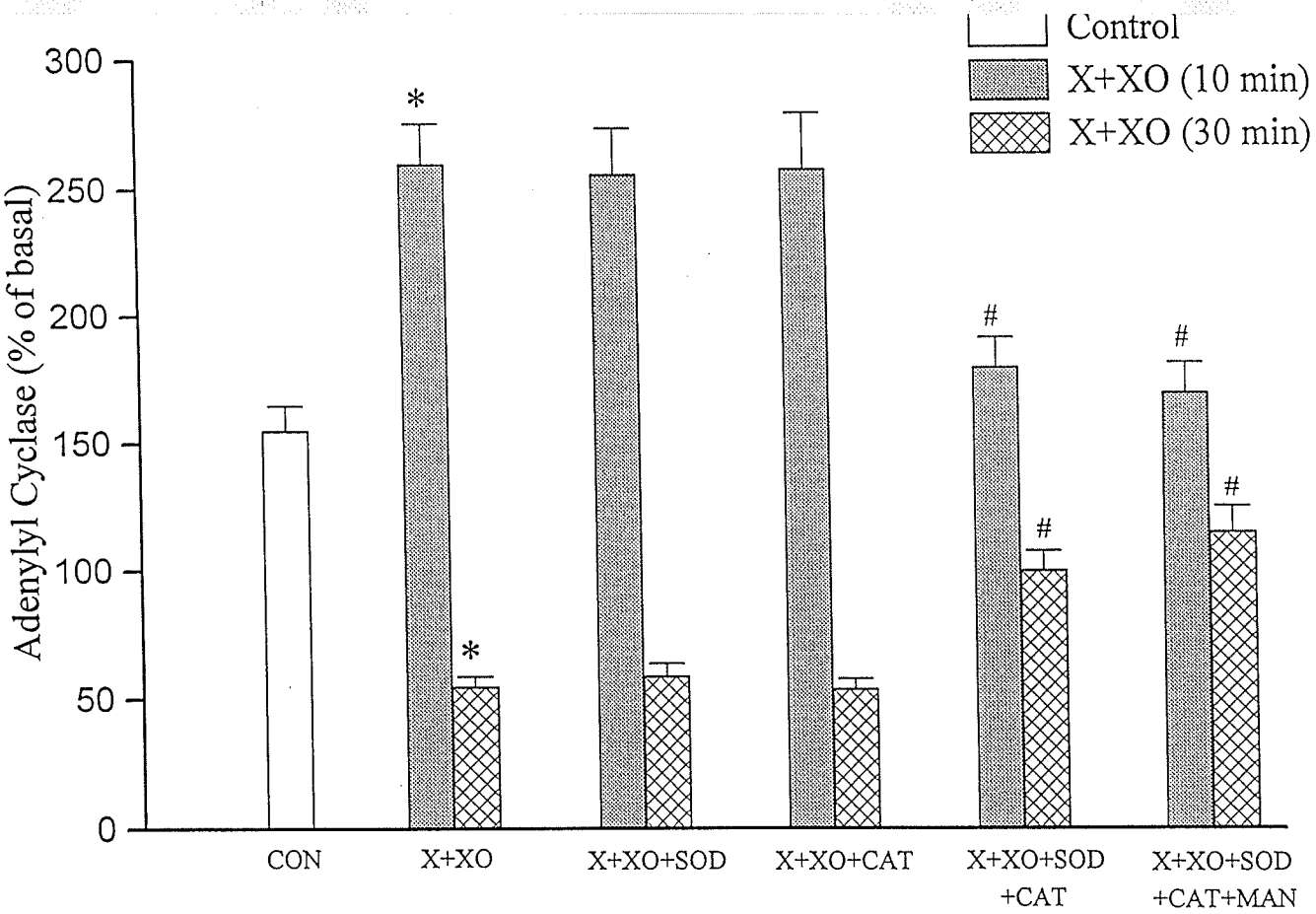

Fig. 12. Effect of xanthine (X, 2 mM) plus xanthine oxidase (XO, 0.03 U/ml) in the absence and presence of the scavengers such as superoxide dismutase (SOD), catalase (CAT), mannitol (MAN) on isoproterenol stimulated adenylyl cyclase activity. Incubations of the rat heart membranes with xanthine (X) plus xanthine oxidase (XO) with or without scavengers were done for 10 min and 30 min periods at 30°C. The assay medium contained 10 μM Gpp(NH)p; the concentration of isoproterenol was 100 μM. The results are expressed as % of the basal activity which was determined in the control preparation in the absence of both Gpp(NH)p and isoproterenol. The concentrations of SOD, CAT and MAN were 80 μg/ml, 10 μg/ml and 20 mM respectively. Each value is the mean ± S.E. of 4-6 preparations. * Significantly different from control ($P < 0.05$); # Significantly different from X + XO for its respective time of incubation. ($P < 0.05$).

Table 4. Effect of various concentrations of H_2O_2 on adenylyl cyclase activity in membranes isolated from the rat heart.

	Adenylyl Cyclase Activity (pmol/mg protein/min)				
	Concentrations of H_2O_2 (μM)				
	Control	25	50	100	200
Basal	34 ± 2	35 ± 2	50 ± 3*	43 ± 3*	25 ± 2#
+ Forskolin (100 μM)	325 ± 21	320 ± 4	485 ± 30*	424 ± 26*	241 ± 16*
+ NaF (5 mM)	142 ± 10	150 ± 11	207 ± 14*	181 ± 12*	101 ± 7*
+ Gpp(NH)p (30 μM)	155 ± 10	155 ± 11	223 ± 15*	200 ± 14*	109 ± 7*
+ Isoproterenol (100μM)	185 ± 12	183 ± 12	261 ± 18*	240 ± 17*	122 ± 9*

Values are the mean ± SE of 4 separate preparations. Before assay for adenylyl cyclase activity, membrane preparations were incubated with various concentrations of H_2O_2 (5-200μM) for 10 min at 30°C. The effect of isoproterenol was studied in the presence of 10 μM Gpp(NH)p. Controls were incubated under similar conditions without H_2O_2. * Significantly different from its respective control ($P<0.05$).

preparations for 10 min with various concentrations of H_2O_2 (5-500 μM) prior to assaying the adenylyl cyclase activity. The results in Table 4 indicate that while concentrations of H_2O_2 between 50-100 μM enhanced the enzyme activity, higher concentrations gradually depressed the basal as well as the forskolin-, NaF-, Gpp(NH)p- and isoproterenol-stimulated activities of the enzyme.

The adenylyl cyclase activity (basal and forskolin-, NaF-, Gpp(NH)p-stimulated) in membranes prepared from hearts perfused for 10 min or 30 min with X plus XO, were altered in a fashion similar to the *in vitro* preparations (Table 5). Essentially, the activity of the enzyme was increased and decreased from control values by 10 min and 30 min perfusion of the heart with X plus XO, respectively, (Table 5). Similarly, the isoproterenol-stimulated activity of the enzyme (Table 6) was enhanced and depressed upon 10 min and 30 min perfusion with X plus XO, respectively. The presence of SOD plus CAT in the perfusion medium was able to completely prevent the biphasic alterations in the basal and stimulated activities of the enzyme at both 10 min and 30 min perfusion periods (Table 5 and 6).

b. β-adrenergic receptors: In order to demonstrate that β-adrenergic receptors were altered in hearts upon treating cardiac membranes with X plus XO for 10 min or 30 min periods, the specific binding of ^{125}I-CYP to both β_1-adrenoceptors and β_2-adrenoceptors were measured. Fig. 13 shows the specific binding data for β_1-adrenoceptors as well as Scatchard plot analysis of ^{125}I-CYP binding to β_1-adrenergic receptors in control and X plus XO (10 min and 30 min) treated hearts. While the affinity ($1/K_d$) of the β_1-adrenoceptors

Table 5. Effect of forskolin, NaF and Gpp(NHp) on adenylyl cyclase activity in crude membrane preparation from control hearts and hearts perfused with X plus XO for 10 min and 30 min periods.

	Adenylyl Cyclase Activity (pmol/mg protein/min)				
	Control	X_{10}	X_{10} + SOD + CAT	X_{30}	X_{30} + SOD + CAT
Basal	40 ± 3.0	58 ± 4.5*	42 ± 2.5#	25 ± 1.9*	35 ± 2.0#
+ Forskolin (100 μM)	330 ± 24.0	496 ± 39.0*	387 ± 26#	225 ± 16*	278 ± 19#
+ NaF (5 mM)	145 ± 12.0	224 ± 20.5*	165 ± 11#	106 ± 7.2*	124 ± 9#
+ Gpp(NH)p (30 μM)	170 ± 12.0	270 ± 18.6*	176 ± 12#	100 ± 7.5*	145 ± 11#

Values are the mean ± SE of 5 separate experiments. Adenylyl cyclase assays were performed with membrane isolated from rat ventricles perfused with xanthine plus xanthine oxidase in the presence and absence of superoxide dismutase (SOD) and catalase (CAT) for 10 min (X_{10}) or 30 min (X_{30}). Control hearts were perfused for 10 min or 30 min with normal perfusion medium and since the values for 10 to 30 min perfusion with control medium were overlapping, the results were grouped together. Concentrations of xanthine, xanthine oxidase, SOD and CAT used were 2 mM, 10 U/L, 1.5×10^5 U/L and 1.0×10^5 U/L, respectively. * Significantly different from its respective control ($p < 0.05$); # Significantly different from respective X_{10} or X_{30} groups ($p < 0.05$).

Table 6. Adenylyl cyclase activity in the presence of different concentrations of isoproterenol in membrane preparations from control hearts and hearts perfused with X plus XO for 10 min and 30 min period.

	Adenylyl Cyclase Activity (pmol/mg protein/min)				
[Isoproterenol]	Control	X_{10}	X_{10} + SOD + CAT	X_{30}	X_{30} + SOD+CAT
None	107 ± 71	52 ± 8*	98 ± 7#	70 ± 5	92 ± 6#
0.1 μM	145 ± 10	198 ± 12*	135 ± 10#	93 ± 7*	125 ± 9#
1 μM	171 ± 11	238 ± 16*	165 ± 11#	112 ± 7*	148 ± 11#
10 μM	179 ± 11	245 ± 15*	171 ± 12#	113 ± 8*	151 ± 10#
100 μM	183 ± 12	256 ± 19*	180 ± 14#	115 ± 8*	156 ± 9#

Values are mean ± S.E. of 6 separate experiments. Assay medium in this set of experiments contained 10 μM Gpp(NH)p. Adenylyl cyclase assays were performed on membranes isolated from rat ventricles perfused with xanthine and xanthine oxidase for 10 min (X_{10}) and 30 min (X_{30}) in the presence or absence of superoxide dismutase (SOD) and catalase (CAT). Control hearts were perfused for 10 to 30 min with normal perfusion medium, and since the values for 10 to 30 min perfusion with control medium were overlapping, the results were grouped together. Concentrations of xanthine, xanthine oxidase, SOD and CAT were 2 mM, 10 U/L, 1.5×10^5 U/L and 1.0×10^5 U/L, respectively. * Significantly different from control at the respective concentration ($p < 0.05$); # Significantly different from the respective X_{10} group or X_{30} group. ($p < 0.05$) The assay medium contained 10 μM Gpp(NH)p and 0.3% ascorbic acid.

was measured after 10 min incubation with X plus XO, the density of these receptors after 30 min incubation was reduced significantly (Fig. 13 and Table 7). Although the Scatchard plot analysis of data for ^{125}I-CYP binding with β_2-adrenoceptors revealed an increase in the affinity ($1/K_d$) and a depression in density upon 30 min treatment with X plus XO, the magnitude of the alterations was significantly smaller in comparison to that seen with β_1-adrenoceptors. A 10 min treatment with X plus XO resulted in no change in either the affinity or density of the β_2-adrenoceptors (Table 7). The presence of both SOD and CAT in the incubation medium, prevented the X plus XO-induced alterations in the density of the β_1-adrenoceptors at 10 and 30 min incubations, as well as changes in both affinity and density of the β_2-adrenoceptors, but was unable to affect the increase in the affinity of the β_1-adrenoceptors at 10 min incubations (Table 7).

Table 8 shows the specific binding data of ^{125}I-CYP binding (B_{max} and K_d) to the β_1- and β_2-adrenoceptors in membrane preparations obtained from control, and X plus XO (10 min and 30 min) perfused hearts. While the density of the β_1-adrenoceptors decreased and their affinity increased dramatically upon 10 min perfusion with X plus XO, both the density and affinity of the β_1-adrenoceptor decreased upon 30 min perfusion with the same reagents. Although ^{125}I-CYP binding to the β_2-adrenoceptors revealed an increased affinity and depressed density of these receptors upon 30 min perfusion with X plus XO (Table 8), the changes were less in magnitude compared to that seen for β_1-adrenoceptors. Perfusion for 10 min with X plus XO resulted in no change to either the affinity or density of the β_2-adrenoceptor. The presence of SOD plus CAT in the perfusion medium prevented the X plus

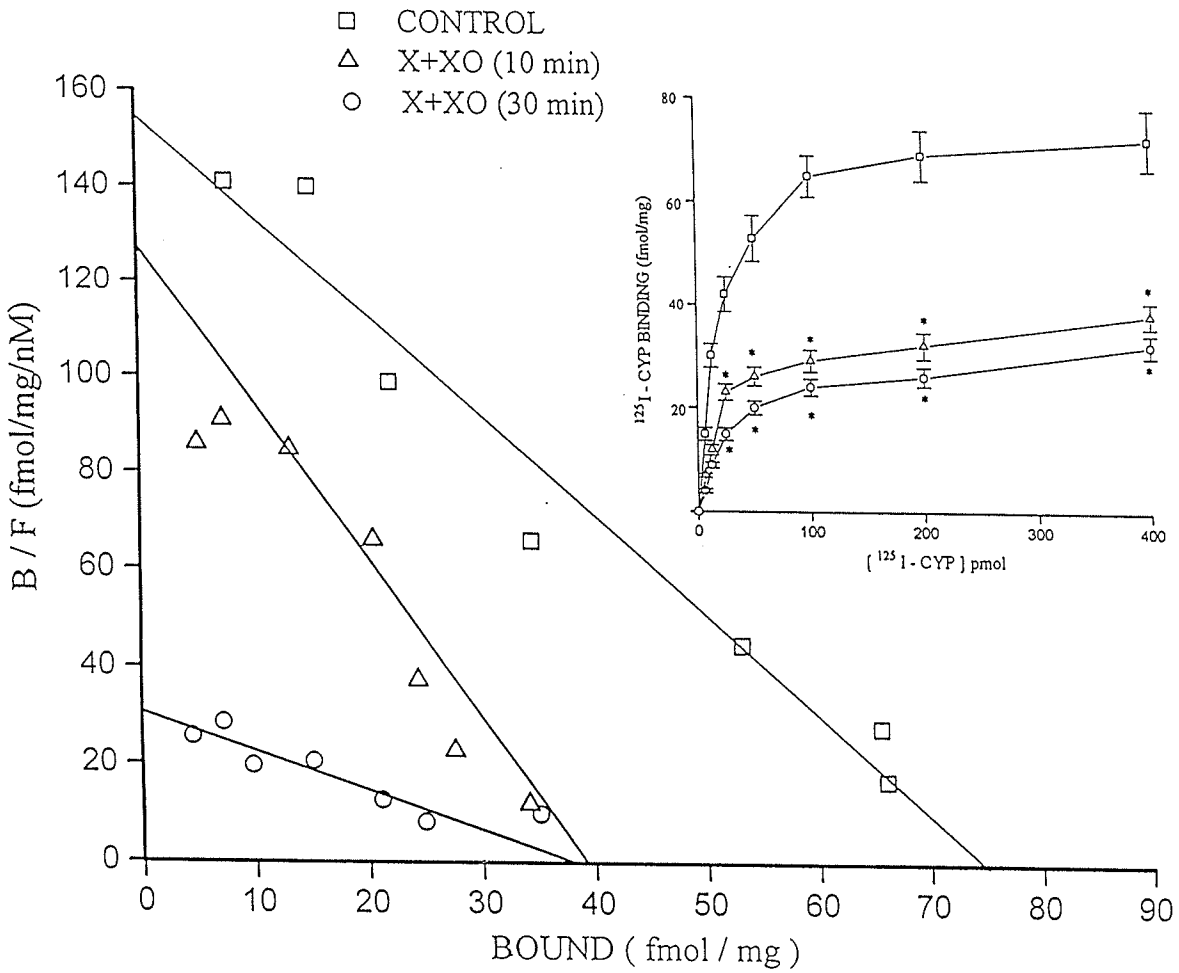

Fig. 13. Scatchard plot analysis of [^{125}I]-CYP binding in control rat cardiac membranes (□) and membranes treated with xanthine (X) plus xanthine oxidase (XO) for 10 min (△) and 30 min (○) periods. Data represents a typical experiment performed in triplicate. Inset: Equilibrium specific binding of [^{125}I]-CYP with membranes by using IC1-118,551 (100 μM) from 5-6 preparations. * Significantly different from control ($P < 0.05$). B/F: Bound/free [^{125}I]-CYP (iodocyanopindolol).

Table 7. Binding characteristics of [^{125}I]-iodocyanopindolol to rat cardiac membranes treated without (control) or with xanthine (X) plus xanthine oxidase (XO).

	β_1-adrenergic Receptors		β_2-adrenergic Receptors	
	K_d (pmol)	B_{max} (fmol/m g)	K_d (pmol)	B_{max} (fmol/mg)
A: 10 min incubation				
Control	36 ± 2.9	70 ± 6.2	14 ± 1.0	20 ± 1.7
X + XO	24 ± 1.8*	38 ± 3.0*	13 ± 1.1	19 ± 1.8
X + XO + SOD + CAT	20 ± 1.6	51 ± 3.0#	13 ± 1.0	21 ± 1.6
X + XO + SOD + CAT + Man	22 ± 1.5	52 ± 3.0#	14 ± 1.2	19 ± 1.7
B: 30 min incubation				
Control	38 ± 2.7	68 ± 4.6	14 ± 0.9	22 ± 1.8
X + XO	67 ± 2.0*	32 ± 2.5*	11 ± 0.9*	16 ± 1.5*
X + XO + SOD + CAT	54 ± 3.0#	52 ± 3.0#	14 ± 1.1#	21 ± 1.8#
X + XO + SOD + CAT + Man	55 ± 3.0#	53 ± 3.5#	14 ± 1.2#	22 ± 1.9#

Each value is the mean ± SE of 6 separate preparations in each group. Cardiac membranes were incubated for 10 min or 30 min at 30°C with the additions shown prior to the assay for β_1- and β_2-adrenergic receptors; controls were incubated under similar conditions without any additions. Specific binding at different concentrations of [^{125}I]-cyanopindolol for β_1- and β_2-adrenergic receptors were determined in the presence of ICI 118,551 and CGP 20712A compounds, respectively. The concentrations of X and XO were 2 mM and 0.03 U mM whereas those for superoxide dismutase (SOD), catalase (CAT) and mannitol (MAN) were 80 µg/ml, 10 µg/ml and 20 mM, respectively. * Significantly different from the respective control group ($P < 0.05$); # Significantly different from the respective X plus XO group ($P < 0.05$).

Table 8. Binding characteristics of [^{125}I]-cyanopindolol to crude membranes preparations from rat hearts perfused with xanthine plus xanthine oxidase for 10 min and 30 min periods.

	Control	X_{10}	X_{10} + SOD + CAT	X_{30}	X_{30} + SOD + CAT
β_1-adrenergic receptors					
K_d (pmol)	35 ± 2.7	21 ± 1.7*	20 ± 1.3	58 ± 4.6*	45 ± 3.4
B_{max} (fmol/mg)	72 ± 5.5	47 ± 4.0*	62 ± 4.0#	36 ± 2.5*	58 ± 4.3#
β_2-adrenergic receptors					
K_d (pmol)	13 ±1.0	14 ±1.1	13 ± 0.9	10 ± 0.8*	14 ± 1.0#
B_{max} (fmol/mg)	21 ± 1.4	19 ±1.4	19 ±1.3	15 ± 1.2*	22 ± 1.4#

Each value is the mean ± SE of 6 separate experiments. Crude membranes were isolated from rat ventricles perfused with X plusfor 10 min to 30 min periods. Control hearts were perfused with normal medium for 10 to 30 min, and since the values for 10 to 30 min perfused hearts with normal medium were overlapping, the results were grouped together. Specific binding ^{125}I-cyanopindolol at different concentrations with β_1- and β_2-adrenergic receptors were determined in the presence of ICI-118,551 and CGP-20712A compounds, respectively. K_d and B_{max} values wasetermined from Scatchard plot analysis of the data. * Significantly different from the respective X_{10} or X_{30} group ($P<0.05$).

XO induced alteration to both β_1- and β_2-adrenoceptor except that changes in the affinity of β_1-adrenoceptors upon perfusing the heart for 10 min were not modified (Table 8). Agonist competition curves using isoproterenol (Fig.14), demonstrated a shift in the curve towards the left upon 10 min treatment with X plus XO compared to the control curve, indicating an increase in the number of coupled β-adrenoceptors in the high affinity state. On the other hand treatment of membranes with X plus XO for 30 min moved the curve to the right of the control, indicating an increase in the low affinity state of the coupled receptors.Perfusion of the heart with X plus XO for 10 min and 30 min periods produced similar shifts in the agonist competition curves (data not shown).

c. G-protein activities and contents: The G-protein mediated activation of adenylyl cyclase were determined in the absence or presence of CT, an activator of G_s-proteins, and PT, an inhibitor of G_i-proteins, and the results are shown in Fig 15. While the CT-induced increase in adenylyl cyclase activity was enhanced in membranes incubated with X plus XO for 10 min, it was depressed upon incubation of membranes with the same agents for 30 min. It should be mentioned that CT-stimulated ADP-ribosylation of the G_s-proteins as well as anti-G_s protein antibody binding were seen at 45 kD and 52 kD bands (Fig. 16). While the CT-stimulated ADP-ribosylation activity at both 45 kD and 52 kD bands as well as G_s-protein content (as determined anti G_s-protein antibody binding) at 52 kD were increased in membranes incubated for 10 min with X plus XO, the anti-G_s protein antibody binding at the 45 kD band was markedly depressed. On the other hand, the CT-stimulated ADP-ribosylation activities as well as G_s-protein content at both 45 kD and 52 kD bands were depressed in the membranes

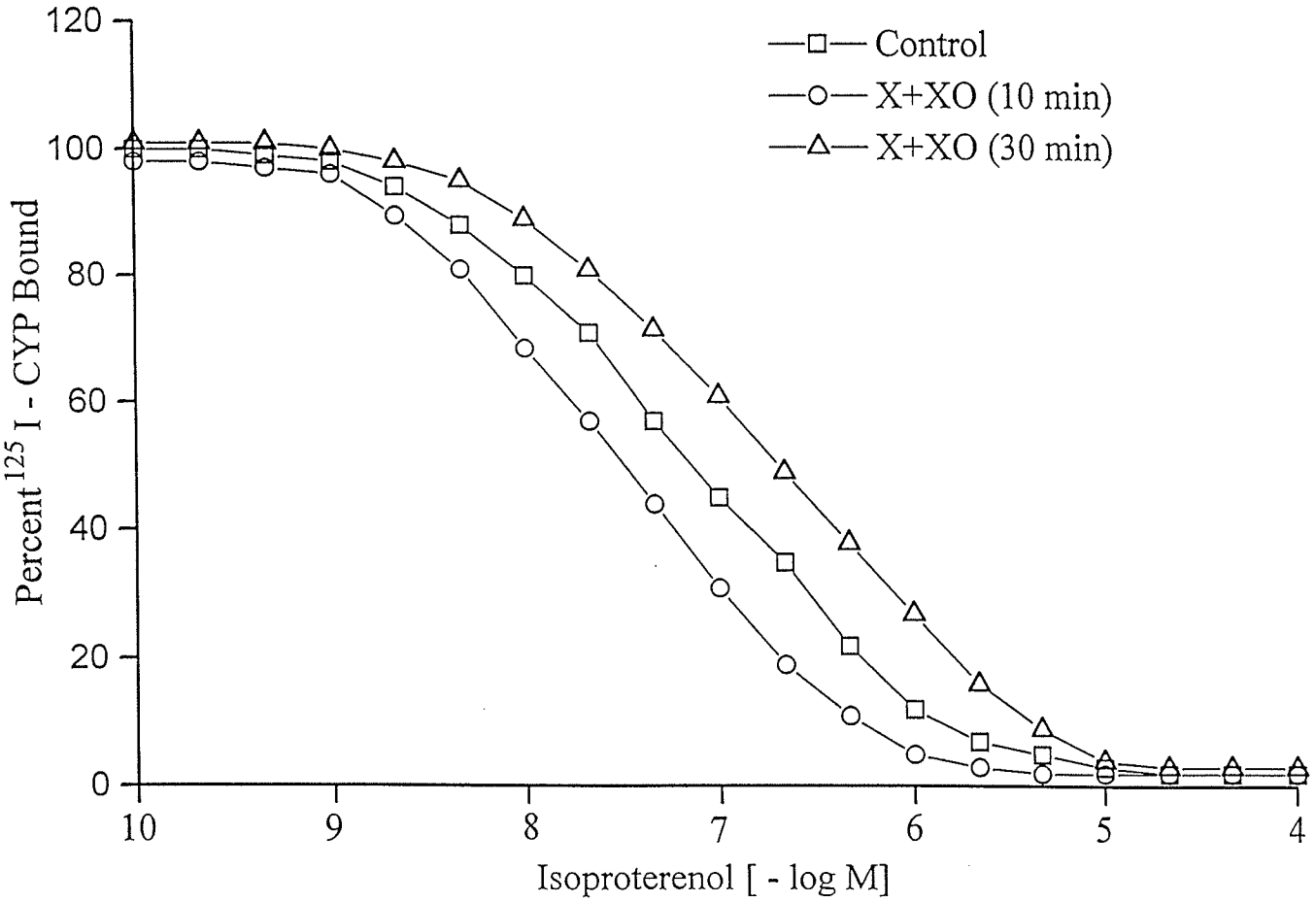

Fig. 14. Agonist competition curves for control hearts membranes (□) and membranes treated for 10 min (●) or 30 min (▲) with xanthine (2 mM) plus xanthine oxidase (0.03 U/ml). Data represents a typical experiment from 3 experiments using 0.07 nM ^{125}I-CYP as a ligand and different concentrations of isoproterenol as a competing agonist. ^{125}I-CYP: ^{125}I-cyanopindolol.

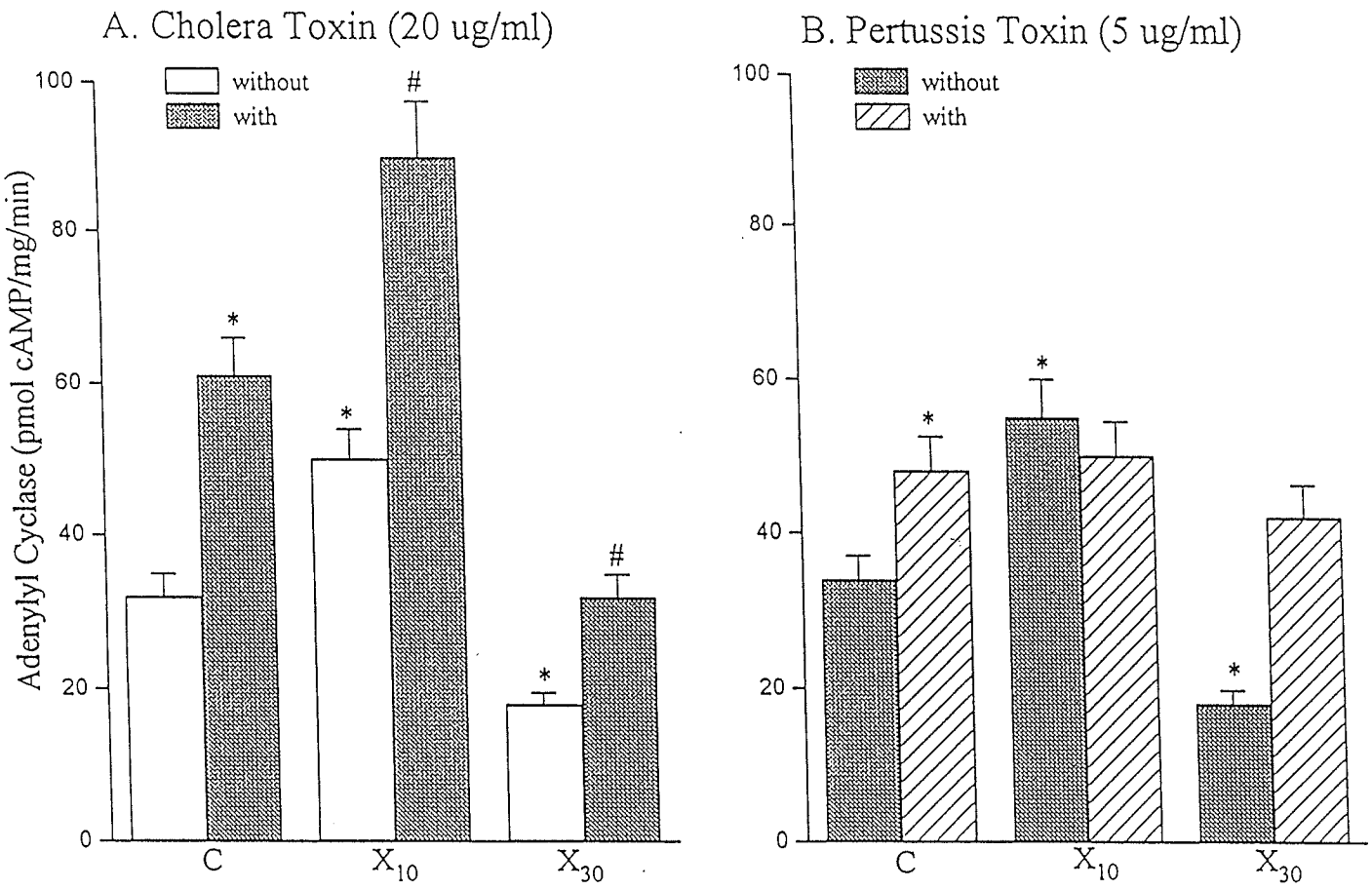

Fig. 15. Effect of cholera toxin and pertussis toxin on the adenylyl cyclase activity in control (C) and xanthine (2 mM) plus xanthine oxidase (0.03 U/ml)-treated rat cardiac membranes. Membranes were treated with xanthine (X) plus xanthine oxidase (XO) for either 10 min (X_{10}) or 30 min (X_{30}) periods at 30°C. Each value is a mean ± S.E. of 4 preparations in each group. * Significantly different from controls ($P < 0.05$); # Significantly different from its respective value in the presence of toxin ($P < 0.05$).

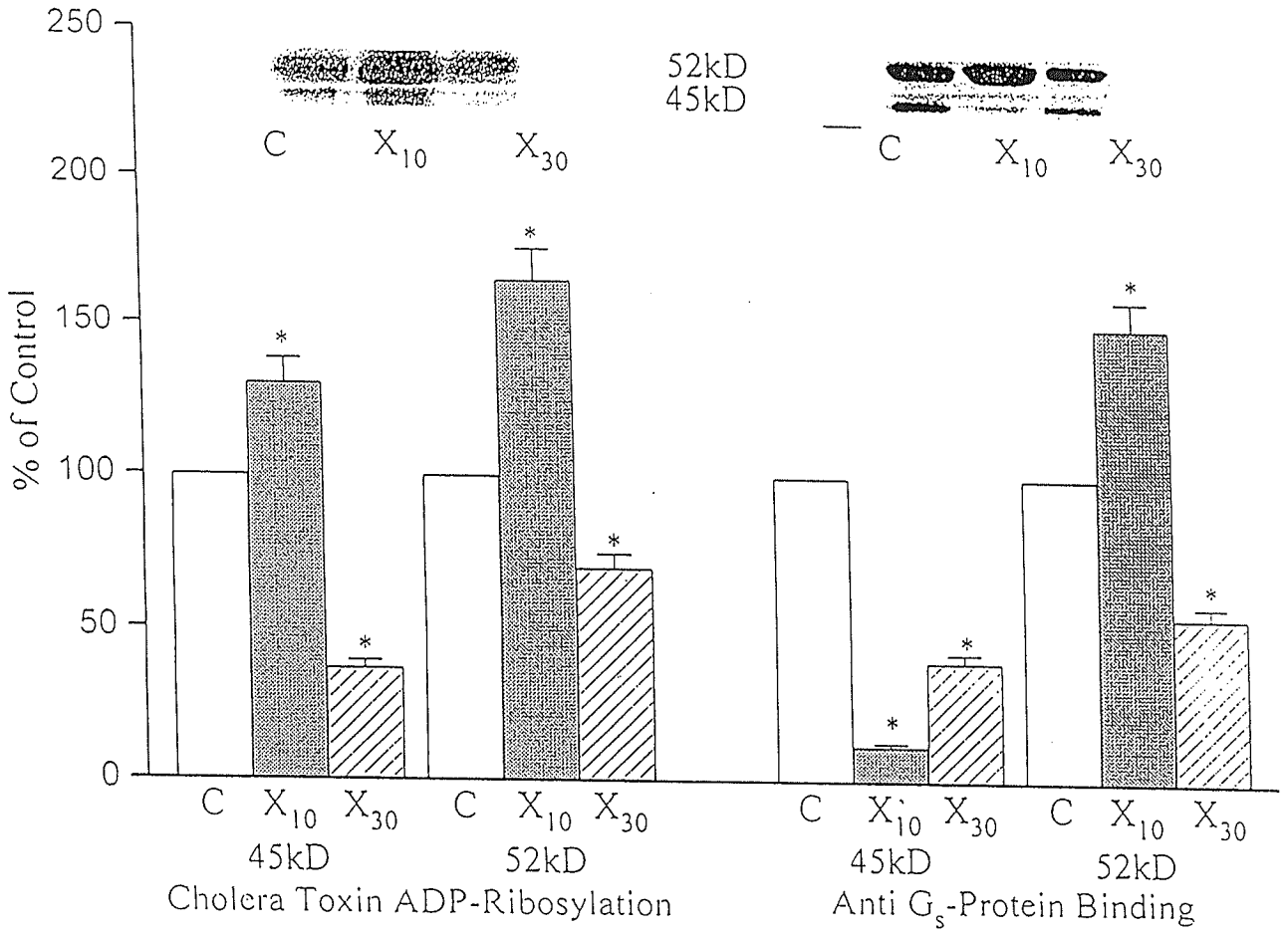

Fig. 16. Cholera toxin catalysed ADP-ribosylation and G_s-protein immunoblots in control (C) and xanthine (2 mM) plus xanthine oxidase (0.03 U/ml)-treated rat cardiac membranes. Lower panel shows barographs for the densitometric analysis of cholera toxin catalysed ADP-ribosylation and G_s-protein immunoblots at 45 kD and 52 kD bands in control membranes and membranes treated with X + XO for 10 min (X_{10}) and 30 min (X_{30}) periods. Upper panel shows immunoblots for the cholera toxin catalysed ADP-ribosylation and G_s-protein from control and X + XO treated membranes. Each value is the mean ± S.E. of 4 preparations. * Significantly different from control ($P < 0.05$). The concentration of cholera toxin was 20 µg/ml.

treated for 30 min with X plus XO (Fig. 16). The PT-catalysed ADP-ribosylation and anti G_i-protein antibody binding was seen at 40 kD, however, no modification in the PT-catalyzed ADP-ribosylation of the G_i-proteins or G_i-protein content (as measured by anti G_i-protein antibody binding) was seen upon both 10 min and 30 min incubation of membranes with X plus XO (Fig. 17).

d. Contractile parameters: A time course effect of isoproterenol (1 μM) infusion upon LVDP, +dP/dt, and -dP/dt were studied in isolated rat hearts perfused for 5, 10 and 15 min with X plus XO and the results are shown in Fig. 18. While 5 min perfusion with X plus XO significantly increased LVDP, +dP/dt and -dP/dt, 10 and 15 min perfusion with X plus XO resulted in a time dependent decline in these parameters. The enhanced and attenuated responses to isoproterenol-stimulation in the X plus XO perfused hearts were normalized to a large extent in the presence of SOD plus CAT in the perfusion medium (data from 3 experiments not shown).

In vitro and *ex-vivo* treatment of membranes with H_2O_2

a. ***Adenylyl cyclase activity*:** The adenylyl cyclase activity in membrane preparations treated *in vitro* with 1 mM H_2O_2 was decreased significantly compared to control (Table 9); this was the case whether the activity was measured in the absence (basal) or presence of various stimulants such as forskolin, NaF or Gpp(NH)p. These alterations were markedly attenuated in the presence of CAT plus MAN (Table 9). In another set of experiments the effect of H_2O_2 on the response of cardiac adenylyl cyclase to various concentrations of

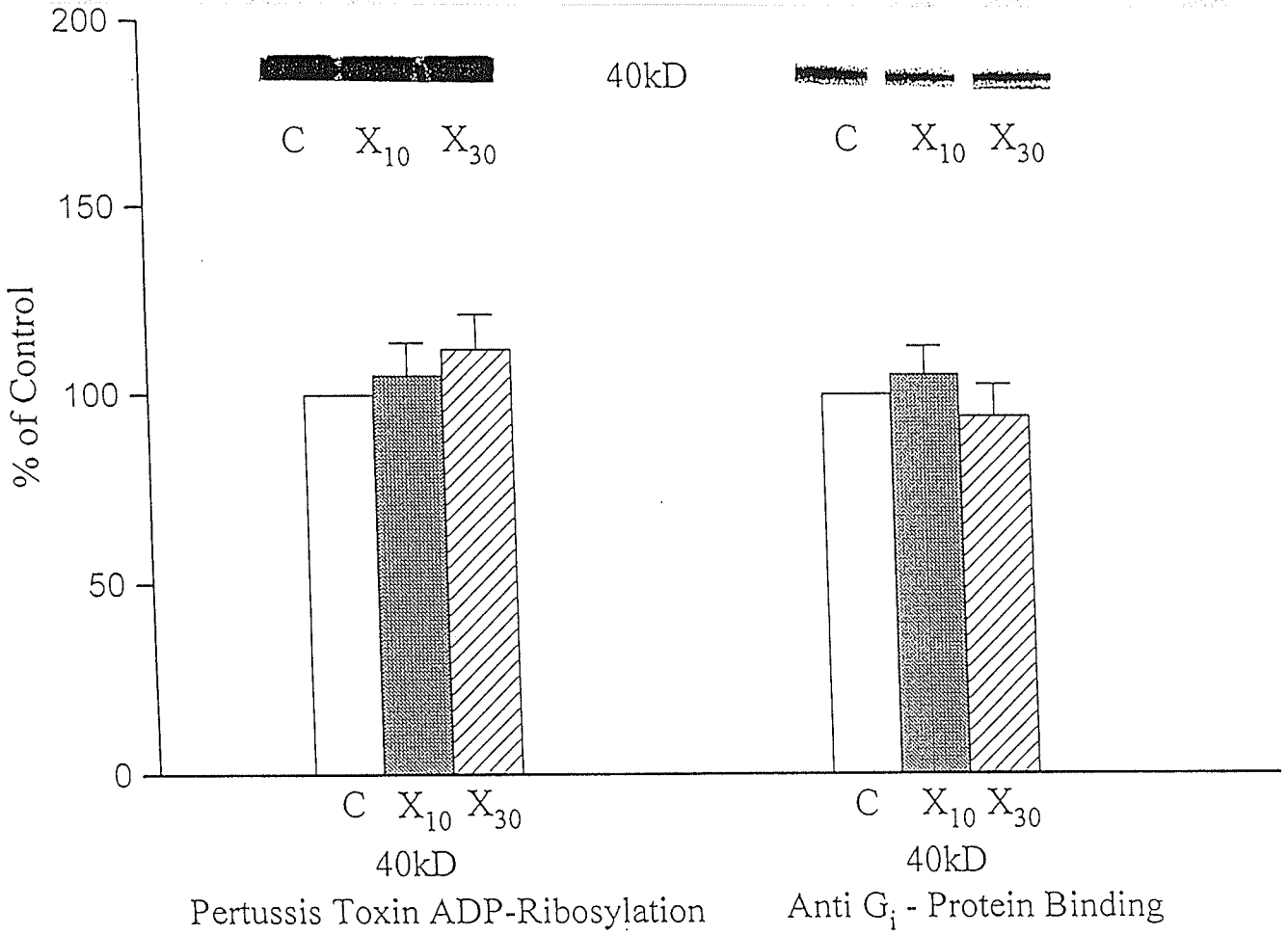

Fig. 17. Pertussis toxin catalysed ADP-ribosylation and G_i protein immunoblots at 40 kD band in control and xanthine (2 mM) plus xanthine oxidase (0.03 U/ml)-treated rat cardiac membranes. Lower panel shows barographs for the densitometric analysis of the pertussis toxin catalysed ADP-ribosylation and G_i-protein immunoblots in control membranes and membranes treated with xanthine plus xanthine oxidase for 10 min and 30 min periods. Upper panel shows immunoblots for the pertussis toxin catalysed ADP-ribosylation and G_i-protein from control and X + XO-treated membranes. Each value is the mean ± S.E. of 4 preparations. C: control; X_{10}: X + XO (10 min); X_{30}: X + XO (30 min). The concentration of pertussis toxin was 5 μg/ml.

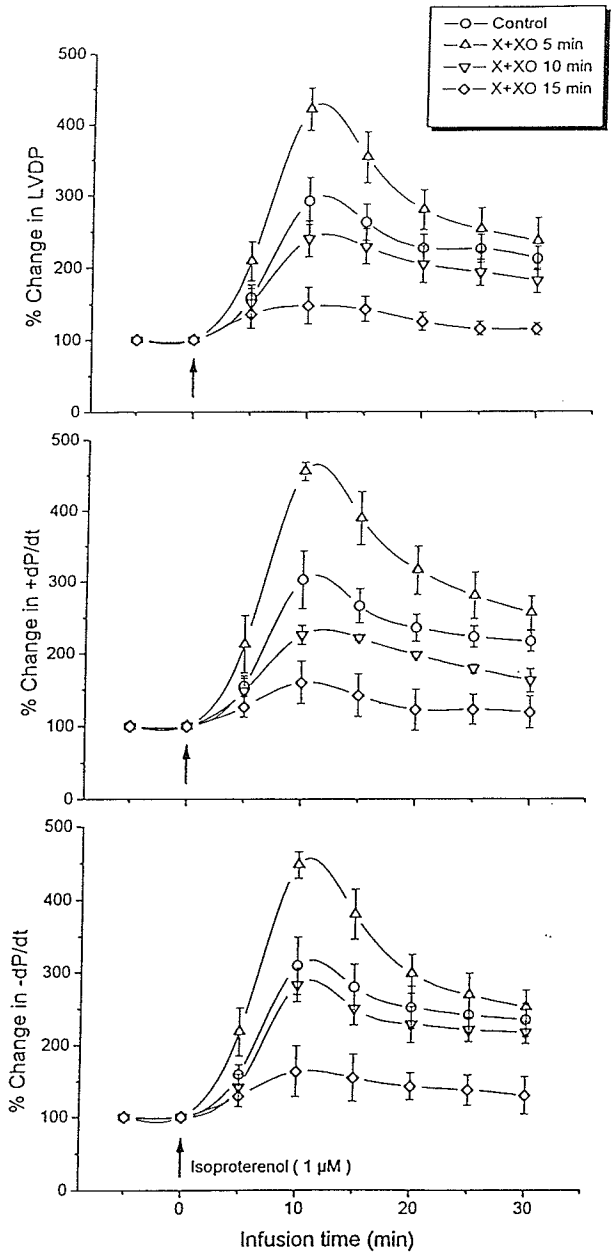

Fig. 18. Effect of infusion of isoproterenol (1 μM) on contractile parameters in control hearts and hearts perfused with xanthine (2 mM) plus xanthine oxidase (10 U/L) for 5 min, 10 min and 15 min. Each value is a mean ± SE of 5-6 experiments. LVDP - left ventricular developed pressure; +dp/dt - rate of change of developed pressure; -dp/dt - rate of change of relaxation. * Significantly different from the respective control value.

Table 9. Effect of forskolin, NaF and GppNHp on adenylyl cyclase activity in rat cardiac membranes treated without (control) or with H_2O_2.

	Adenylyl cyclase activity (pmol/mg protein/min)		
	Control	H_2O_2	H_2O_2 + CAT + MAN
Basal	28.2 ± 2.0	17.8 ± 0.8*	30 ± 2.0#
+ Forskolin (100 μM)	173 ± 14.0	130 ± 8.0*	165 ± 12.2#
+ NaF (5 μM)	259 ± 25.0	188 ± 10*	250 ± 17.0#
+ GppNHp (30 μM)	92.5 ± 7.0	67.9 ± 5.0*	85 ± 6.0#

Values are mean ± SE of 4-6 separate preparations for each group. Before assay for adenylyl cyclase activity, membrane preparations were incubated at 30°C for 10 min with H_2O_2. Controls were incubated under similar conditions without H_2O_2. Final concentrations of H_2O_2, catalase and D-mannitol were 1 mM, 10 μg/ml and 20 mM, respectively. *Significantly different from its respective control ($P < 0.05$); # Significantly different from its respective H_2O_2 group ($P < 0.05$).

isoproterenol was examined. The results in Fig. 19 indicates a marked reduction in isoproterenol-stimulated adenylyl cyclase activity upon treatment with 1mM H_2O_2. From the data regarding the effect of different concentrations of H_2O_2 on the isoproterenol-stimulated adenylyl cyclase activity in Fig. 20, it can be seen that a significant attenuation of isoproterenol responsiveness of adenylyl cyclase was evident at 0.5 to 5mM H_2O_2 whereas an augmentation of the isoproterenol response was seen at 0.1 mM H_2O_2. The decreased isoproterenol-stimulation of adenylyl cyclase activity was largely prevented in the presence of CAT or CAT plus MAN (Fig. 21), but not by MAN alone. The basal as well as forskolin-, NaF- and Gpp(NH)p-stimulated adenylyl cyclase activity, in membranes prepared from hearts perfused for 10 min with 1 mM H_2O_2, were altered in a similar fashion to that seen in the *in vitro* treatment of membrane preparations. Essentially the activity of the enzyme was decreased from control values by 10 min perfusion with H_2O_2 (Table 10). Similarly, the isoproterenol-stimulated activity of the enzyme when measured in the presence of different concentrations of isoproterenol was significantly depressed upon 10 min perfusion with H_2O_2 (Table 11). The presence of CAT and MAN in the perfusion medium was able to prevent the H_2O_2-induced alterations in the basal and stimulated activities of the enzyme (Tables 10 and 11).

b. ***β-adrenergic receptors*:** In order to show if β-adrenergic receptors were altered upon treatment with H_2O_2, the specific binding of ^{125}I-CYP to both β_1-and β_2-adrenoceptors was studied in cardiac membranes. Fig. 22 shows the specific binding data for β_1-adrenoceptors as well as Scatchard plot analysis of ^{125}I-CYP binding to β_1-receptors in control and H_2O_2-

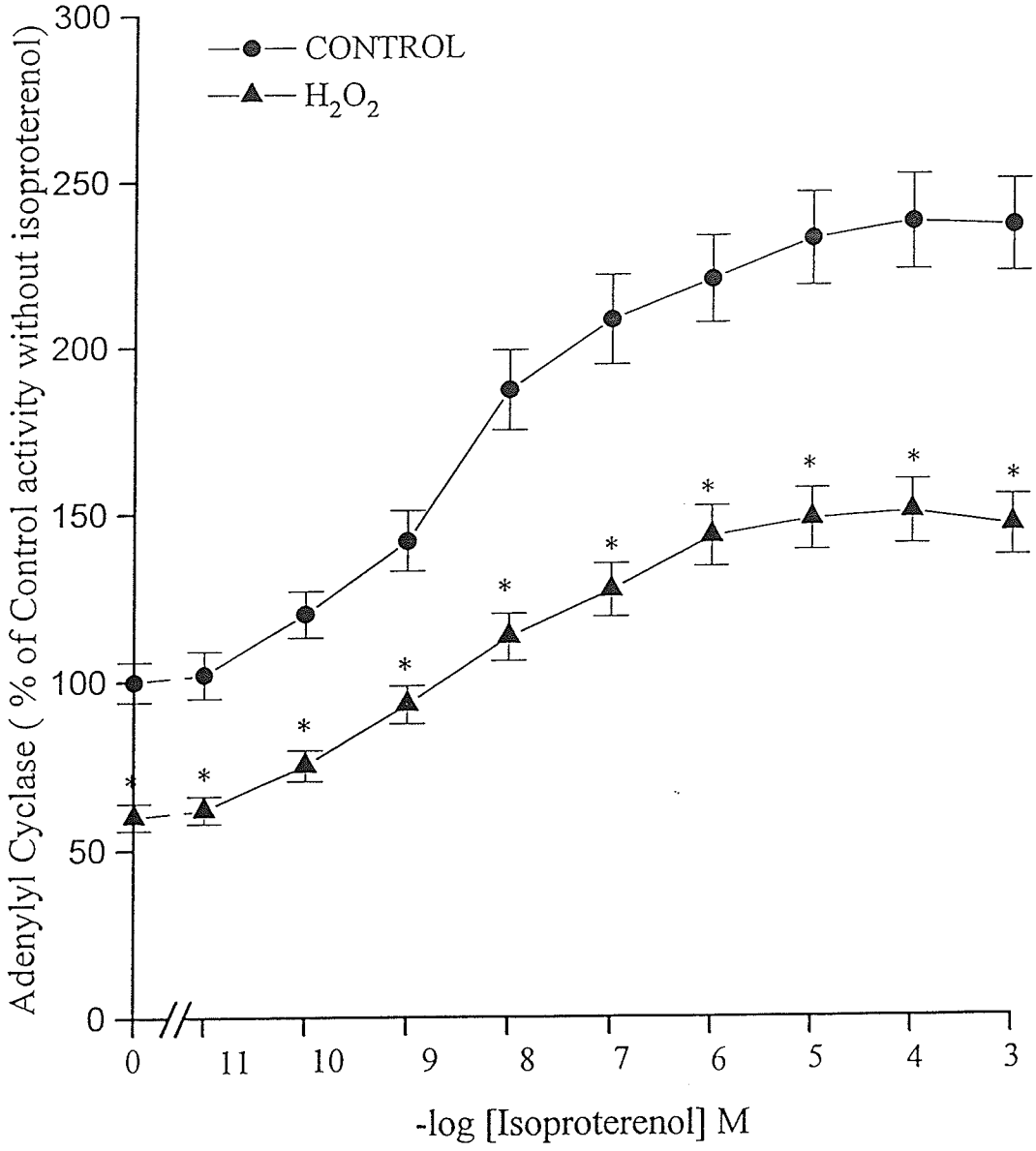

Fig. 19. Effect of different concentrations of isoproterenol (10^{-11} - 10^{-3} mM) on adenylyl cyclase activity in the rat heart membranes treated with H_2O_2 (1 mM) or without (control) for 10 min at 30°C. The assay medium in this set of experiments contained 10 μM Gpp(NH)p. Each value is the mean ± SE of 6 different membrane preparation. * Significantly different from control. ($P < 0.05$).

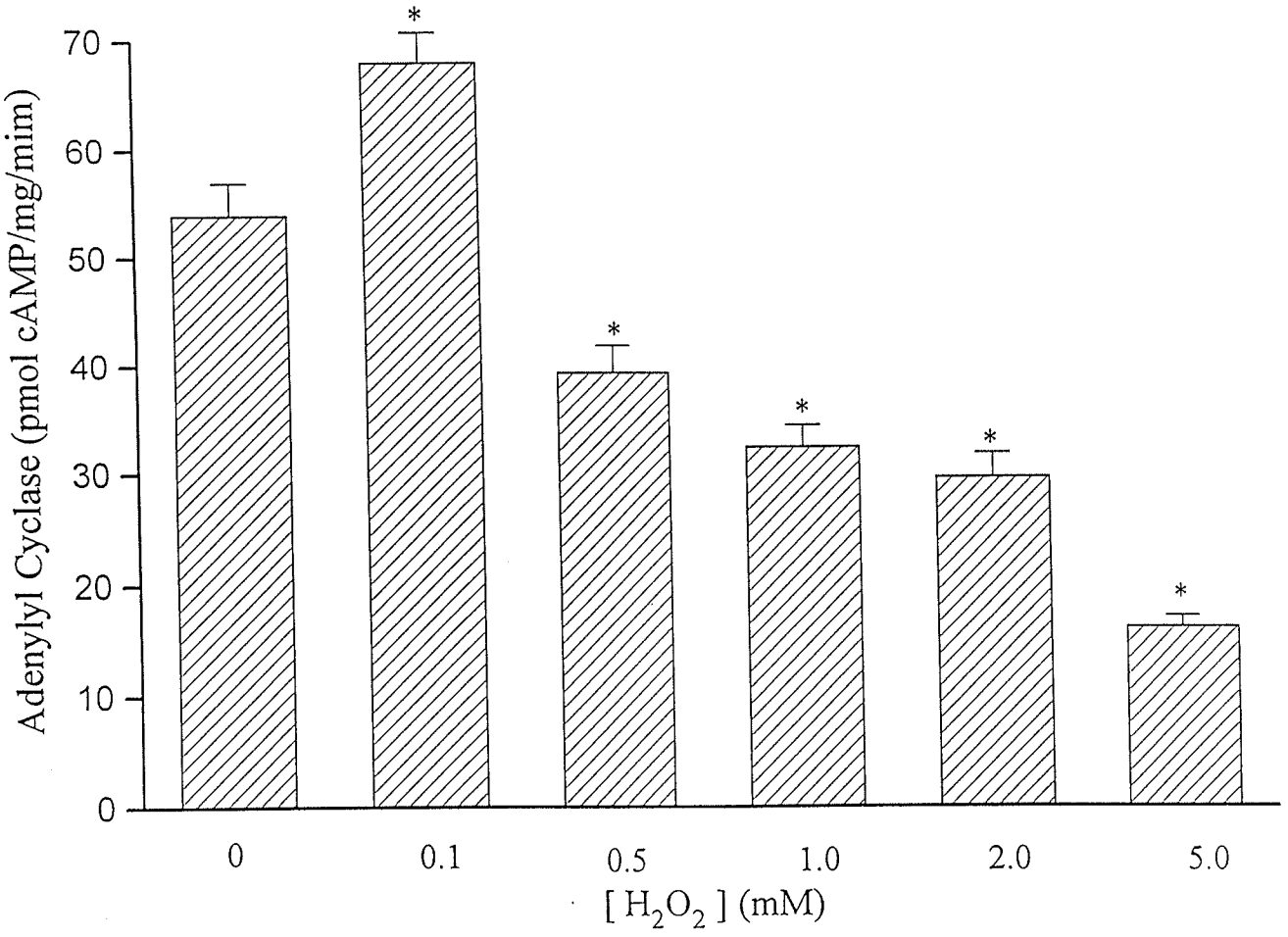

Fig. 20. Effect of different concentrations of H_2O_2 on isoproterenol-stimulated adenylyl cyclase activity in the rat heart membranes. Incubation with H_2O_2 was for 10 min at 30°C prior to the adenylyl cyclase assay; control preparations were incubated in the absence of H_2O_2. The assay medium contained 10 μM Gpp(NH)p. Each value is a mean ± SE of 6 separate membrane preparations.* Significantly different from control. ($P < 0.05$).

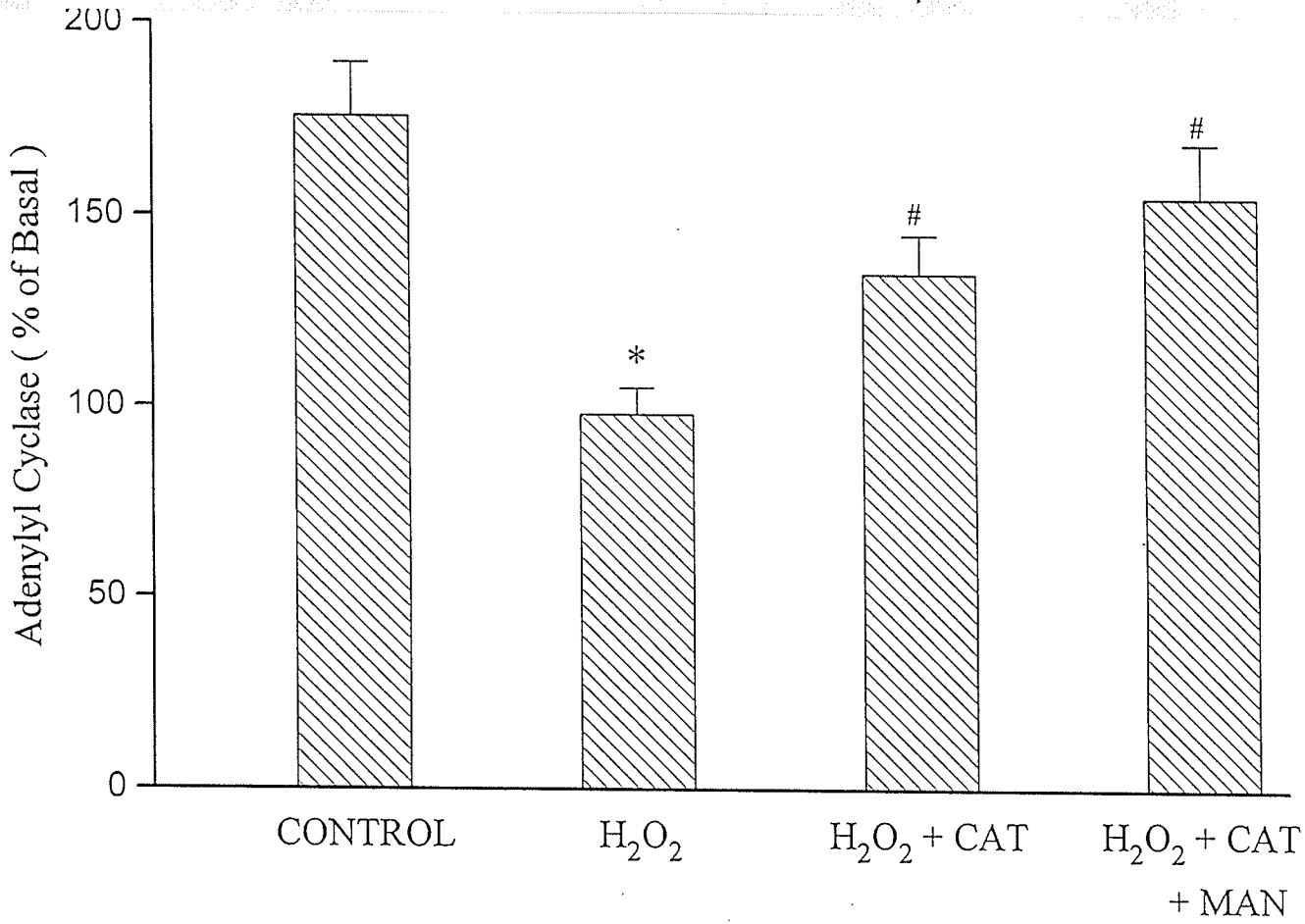

Fig. 21. Effect of H_2O_2 (1 mM) in the absence and presence of different oxyradical scavengers such as catalase (CAT) and D-mannitol (MAN) on isoproterenol-stimulated adenylyl cyclase activity in rat heart membranes. Incubation of rat heart membranes with H_2O_2 with or without scavengers was for 10 min at 30°C. The assay medium contained 10 μM Gpp(NH)p; the concentration of isoproterenol was 100 μM. The results are expressed as % of the basal activity which was determined in the control preparation in the absence of both Gpp(NH)p and isoproterenol. The concentrations of CAT and MAN were 10 μg/ml and 20mM, respectively. Each value is a mean ± SE of 6 preparations. * Significantly different from control. ($P < 0.05$); # Significantly different in comparison to the H_2O_2 group ($P < 0.05$).

Table 10. Effect of forskolin, NaF, and Gpp(NH)p on adenylyl cyclase activity in membrane preparation from control hearts and hearts perfused with H_2O_2 for 10 min.

	Adenylyl cyclase activity (pmol/mg protein/min)		
	Control	H_2O_2	H_2O_2 + CAT + MAN
Basal	42 ± 3.0	30 ± 2.0*	39 ± 2.1#
+ Forskolin (100 μM)	350 ± 30.0	250 ± 20.0*	308 ± 23.0#
+ NaF (5 mM)	161 ± 12.1	110 ± 7.1*	141 ± 11.0#
+ Gpp(NH)p (30 μM)	180 ± 12.2	138 ± 11.0*	163 ± 13.0#

Values are the mean ± SE of 5 separate experiments. Adenylyl cyclase assays were done with membranes isolated from rat ventricles perfused with H_2O_2 in the presence or absence of catalase (CAT) and D-mannitol (MAN) for 10 min. Control hearts were perfused for 10 min with normal perfusion medium. Concentrations of H_2O_2, CAT and MAN used were 1mM, 1.0 X 10^5 U/L and 20 mM, respectively. * Significantly different from its respective control ($p < 0.05$); # Significantly different from its respective H_2O_2 group ($p < 0.05$).

Table 11. Adenylyl cyclase activity in the absence and presence of isoproterenol in cardiac membranes from rat heart perfused with H_2O_2.

	Adenylyl cyclase activity (pmol/mg protein/min)		
[Isoproterenol]	Control	H_2O_2	H_2O_2 + CAT + MAN
None	105 ± 7.0	65 ± 5.0*	97 ± 7.2#
0.1 μM	131 ± 9.1	89 ± 7.1*	119 ± 8.0#
1 μM	154 ± 11.1	104 ± 6.1*	140 ± 9.3#
10 μM	162 ± 11.0	109 ± 7.0*	145 ± 10.1#
100 μM	182 ± 12.2	113 ± 8.2*	167 ± 11.2#

Values are the mean ± S.E. of 6 separate experiments. Adenylyl cyclase assays were performed on membranes isolated from rat ventricles perfused with H_2O_2 in the absence or presence of catalase (CAT) and D-mannitol (MAN) for 10 min. Control hearts were perfused for 10 min with normal perfusion medium. Concentrations of H_2O_2, CAT and MAN used were 1 mM, 1.0 x 10^5 U/L and 20 mM, respectively. The assay medium contained 10 μM Gpp(NH)p and 0.3% ascorbic acid. *Significantly different from control at the respective concentration ($p<0.05$); # Significantly different from its respective H_2O_2 group ($p < 0.05$).

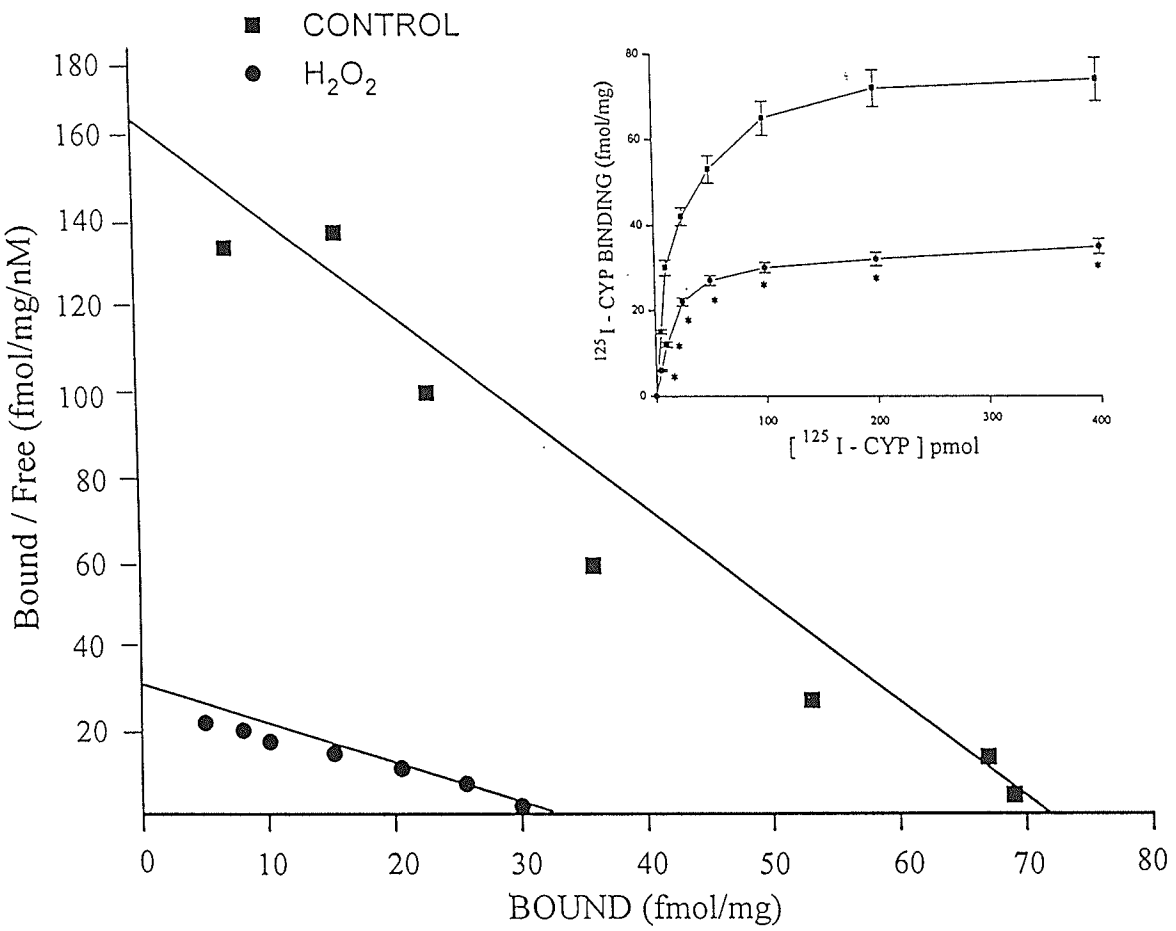

Fig. 22. Scatchard plot analysis of ^{125}I-cyanopindolol (ICYP) binding in control (■) and H_2O_2 (●) treated rat heart membranes. Data represents a typical experiment performed in triplicate. Inset: Equilibrium specific binding of ^{125}I-CYP with membranes using ICI-118,551 (100 μM), from 5-6 separate preparations. * Significantly different from control ($P < 0.05$).

treated membranes. Both the density and affinity ($^1/K_d$) of the β_1-receptors were significantly reduced in the H_2O_2-treated membranes compared to controls (Fig.22 and Table 12). Although Scatchard plot analysis of the data on ^{125}I-CYP binding to β_2-adrenoceptors also revealed a depression in density of this receptor subtype, an increase in affinity ($^1/K_d$) of β_2-adrenoceptors was evident in the H_2O_2-treated membranes (Table 12). The presence of CAT in the incubation mixture was able to greatly prevent the H_2O_2-induced alteration in the β_1- and β_2-adrenoceptors (Table 12). The presence of MAN with CAT did not afford any greater protection than that seen with CAT alone. Table 13 shows the specific binding data for ^{125}I-CYP binding (B_{max} and K_d) to the β_1- and β_2-adrenoceptors in control and H_2O_2 perfused hearts. Both the density and affinity of the β_1-adrenoceptor were depressed by 10 min perfusion with 1mM H_2O_2. The results for ^{125}I-CYP binding to β_2-adrenoceptors revealed an enhanced affinity and depressed density upon perfusing the hearts with H_2O_2 (Table 13). The presence of CAT plus MAN prevented the H_2O_2-induced alteration to the β_1- and β_2-adrenoceptors (Table 13). Agonist competition curves using isoproterenol (Fig. 23), revealed no shift, suggesting that there are no alterations to the status of the coupled β-adrenoceptors with G_s-protein in the H_2O_2-treated heart compared to control. By using two preparations for each of the control and H_2O_2-treated groups, similar results with regards to the coupling of β-adrenoceptors with G-proteins were observed in the membranes from hearts perfused with H_2O_2 (data not shown).

c. ***G-Protein activities and contents*:** Adenylyl cyclase activity due to changes in the G-protein function was determined in the presence or absence of CT, an activator of G_s-

Table 12. Binding characteristics of [^{125}I]-iodocyanopindolol to the rat cardiac membrane treated without (control) or with H_2O_2.

	β_1-adrenergic receptors		β_2-adrenergic receptors	
	K_d (pmol)	B_{max} (fmol/mg)	K_d (pmol)	B_{max} (fmol/mg)
Control	48 ± 4.1	69 ± 5.1	5.4 ± 0.4	21 ± 1.5
H_2O_2	99 ± 6.1*	32 ± 3.0*	4.2 ± 0.3*	12 ± 1.0*
H_2O_2 + CAT	59 ± 4.8#	56 ± 4.8#	4.9 ± 0.4	20 ± 1.4#
H_2O_2 + CAT + Man	60 ± 5.0#	59 ± 5.1#	4.8 ± 0.4	18 ± 1.3#

Each value is the mean ± SE of 6 separate preparations in each group. Cardiac membranes were incubated for 10 min at 37°C with the additions shown prior to assay for β_1- and β_2-adrenergic receptors. Control membranes were incubated under similar conditions without any additions. Specific binding at different concentrations of ^{125}I-cyanopindolol for β_1- and β_2-adrenergic receptors were determined in the presence of ICI-118, 551 and CGP-20712A compounds, respectively. The concentrations of H_2O_2 CAT and MAN used were, 1mM, 10 µg/ml and 20 mM respectively. * Significantly different from its respective control group ($P < 0.05$); # Significantly different from its respective H_2O_2 group ($P < 0.05$).

Table 13. Binding characteristics of ^{125}I-cyanopindolol to crude membranes isolated from rat hearts perfused with H_2O_2.

	Control	H_2O_2	H_2O_2 + CAT + MAN
β_1-adrenergic receptors			
K_d (pmol)	33 ± 2.3	66 ± 5.0*	43 ± 3.0#
B_{max} (fmol/mg)	71 ± 5.4	35 ± 2.8*	56 ± 3.2#
β_2-adrenergic receptors			
K_d (pmol)	13 ± 1.0	8.5 ± 0.6*	13 ± 1.0 #
B_{max} (fmol/mg)	23 ± 2.0	14 ± 1.1*	22 ± 2.0#

Each value is a mean ± SE of 6 separate experiments. Crude membranes were isolated from rat ventricles perfused with H_2O_2 for 10 min. Control hearts were perfused with normal medium for 10 min. Specific binding of ^{125}I-CYP at different concentrations with β_1- and β_2-adrenergic receptors were determined in the presence of ICI-118,551 and CGP-20712A compounds, respectively. K_d and B_{max} values were determined from Scatchard plot analysis of the data. * Significantly different from control ($P < 0.05$); # Significantly different from H_2O_2 group ($P < 0.05$).

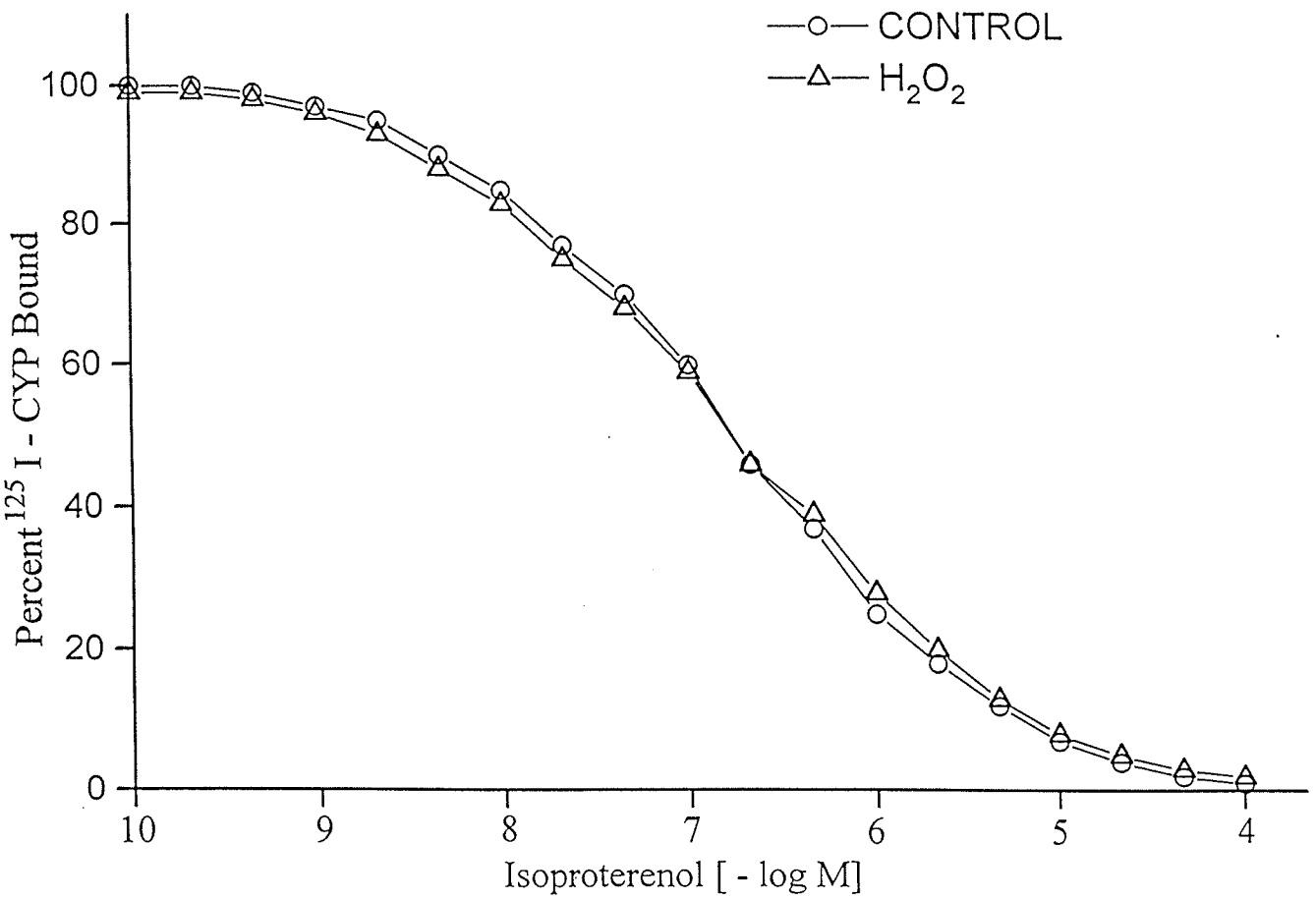

Fig. 23. Agonist competition curves for membranes for control (0) and $_{H2}O_2$-treated (△) rat heart membranes. Cardiac membranes were treated for 10 min with or withoutH_2O_2 (1mM). Data represents a typical of 3 experiment using 0.07 nM ^{125}I-CYP as a ligand and different concentrations of isoproterenol as a competing agonist.

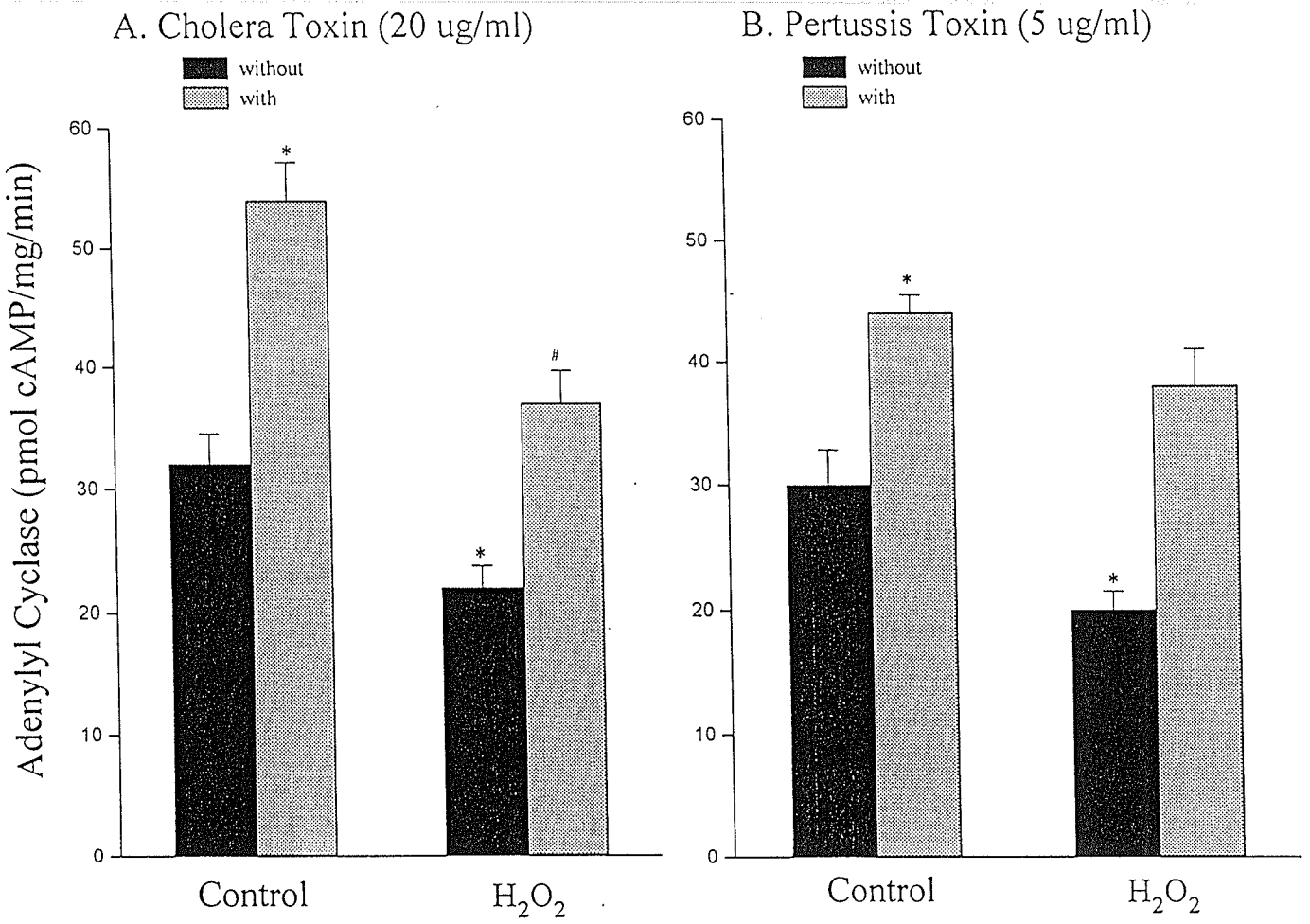

Fig. 24. Effect of cholera toxin and pertussis toxin on the adenylyl cyclase activity in control (C) and H_2O_2-treated (H) rat cardiac membranes. Membranes were treated with H_2O_2 for 10 min at 30 °C. Each value is a mean ± SE of 4 separate preparations in each group. * Significantly different from control ($P < 0.05$); # Significantly different from its respective value in the presence of toxin.

protein, and PT, an inhibitor of G_i-protein, and the results are shown in Fig. 24. The results indicate that while the CT-induced increase in adenylyl cyclase was depressed in the H_2O_2-treated membranes, the PT-induced increase in the enzymes activity was unaffected. It should be mentioned that CT-catalyzed ADP-ribosylation as well as anti G_s-protein antibody binding were seen at 45 kD and 52 kD bands (Fig. 25). While CT-stimulated ADP-ribosylation in the H_2O_2-treated preparations were depressed at both the 45 kD and 52 kD bands, G_s-protein content (as determined by anti G_s-protein antibody binding) showed an attenuation at only the 45 kD band leaving the 52 kD band unaltered. No change in the PT-catalyzed ADP-ribosylation at 40 kD and G_i-protein content (as measured by anti G_i-protein antibody binding at 40 kD) were evident due to H_2O_2 treatment (Fig. 26).

d. ***Contractile parameters*:** Perfusion of the heart with 200 μM and 500 μM H_2O_2 for 10 min was found to decrease the LVDP, +dP/dt and -dP/dt by approximately 68% and 88% respectively. A time course effect of isoproterenol (1 μM) infusion on LVDP, +dP/dt, and -dP/dt was studied in isolated rat hearts perfused with 200 μM and 500 μM H_2O_2. The results in Fig. 27 show that perfusion with H_2O_2 produced a concentration dependent decline in the isoproterenol-induced increase of LVDP, +dP/dt and -dP/dt. This depression in the contractile parameters upon H_2O_2-perfusion was normalized when CAT was present in the perfusion medium (data from 3 experiments not shown).

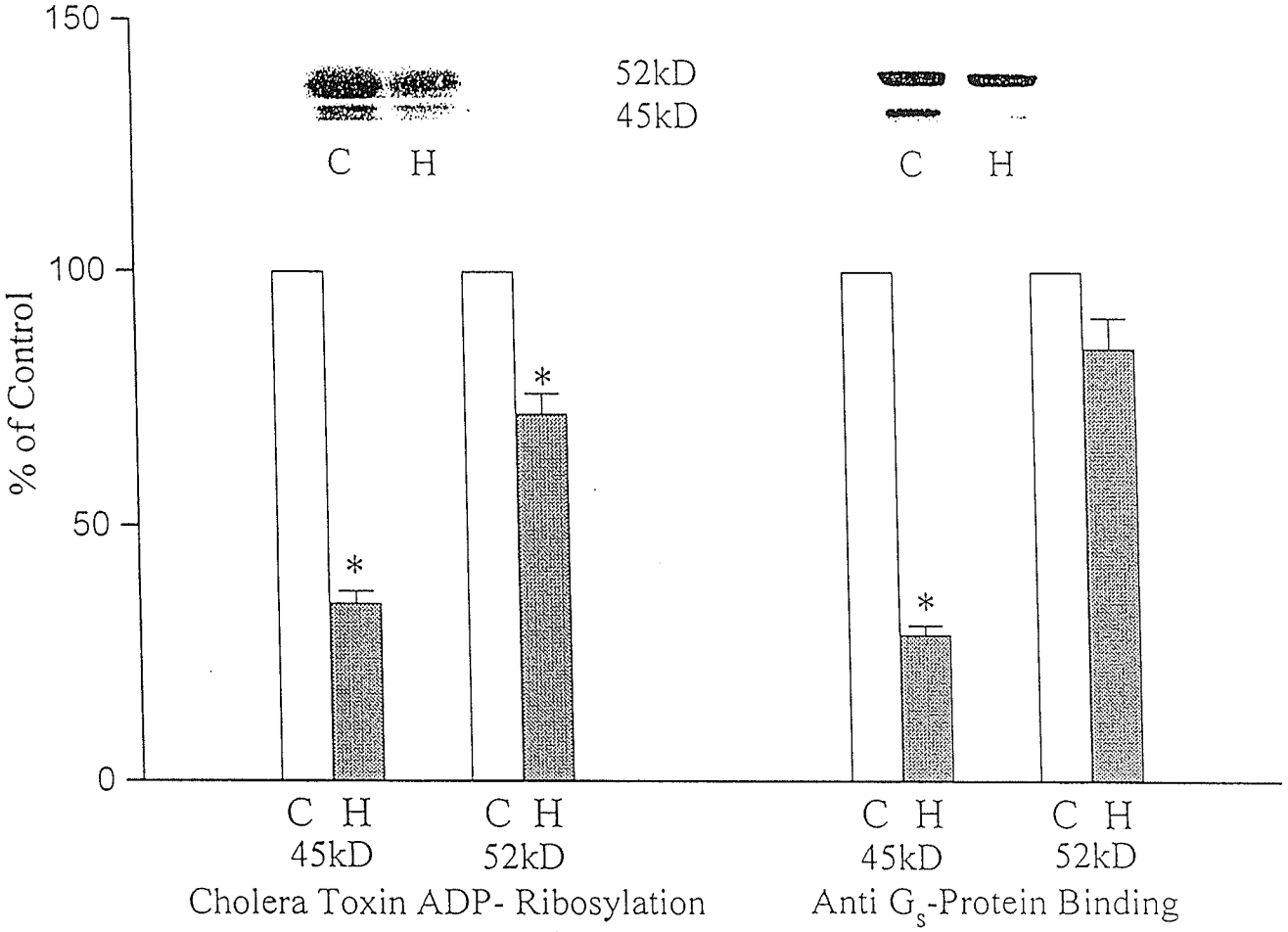

Fig. 25. Cholera toxin catalyzed ADP-ribosylation and G_s-protein immunoblots in control (C) and H_2O_2 (1mM)-treated (H) rat cardiac membranes. Upper panel shows immunoblots for the cholera toxin-catalyzed ADP-ribosylation and G_s-protein from control and H_2O_2-treated membranes. Lower panel shows barographs for the densitometric analysis of cholera toxin catalyzed ADP-ribosylation and G_s-protein immunoblots at 45 kD and 52 kD bands in control membranes and membranes treated with H_2O_2 for 10 min. Each value is the mean ± SE of 4 separate preparations. * Significantly different from control ($P < 0.05$). The concentration of cholera toxin was 20 μg/ml.

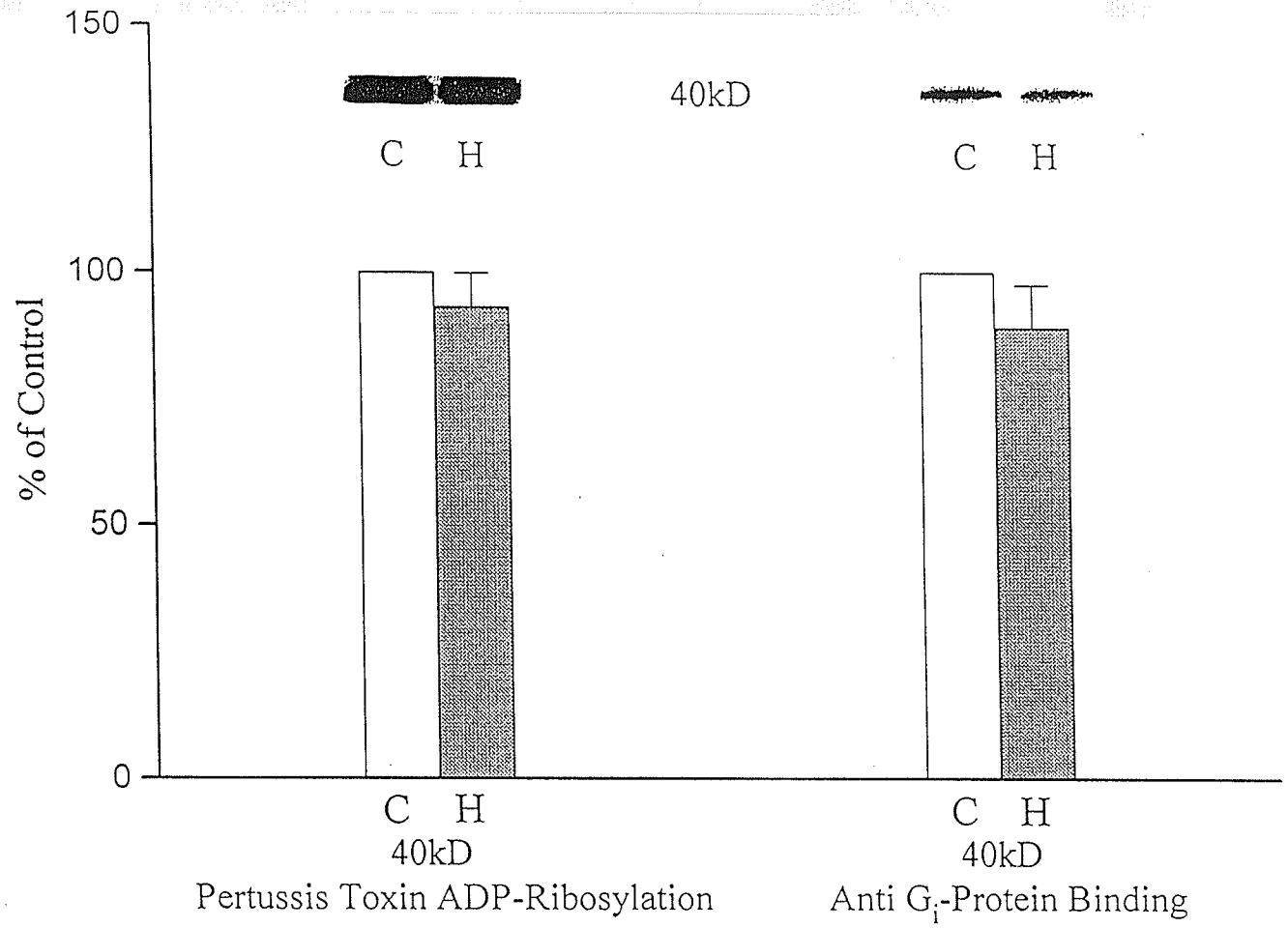

Fig. 26. Pertussis toxin-catalyzed ADP-ribosylation and G_i-protein immunoblots at 40 kD band in control (C) and H_2O_2 (1mM)-treated (H) rat cardiac membranes. Upper panel shows immunoblots for pertussis toxin-catalyzed ADP-ribosylation and G_i protein from control and H_2O_2-treated membranes. Lower panel shows barographs for the densitometric analysis of pertussis toxin catalyzed ADP-ribosylation and G_i protein immunoblots in control membranes and membranes treated with H_2O_2 for 10 min. Each value is a mean ± SE of 4 separate preparations. * Significantly different from control ($P < 0.05$).

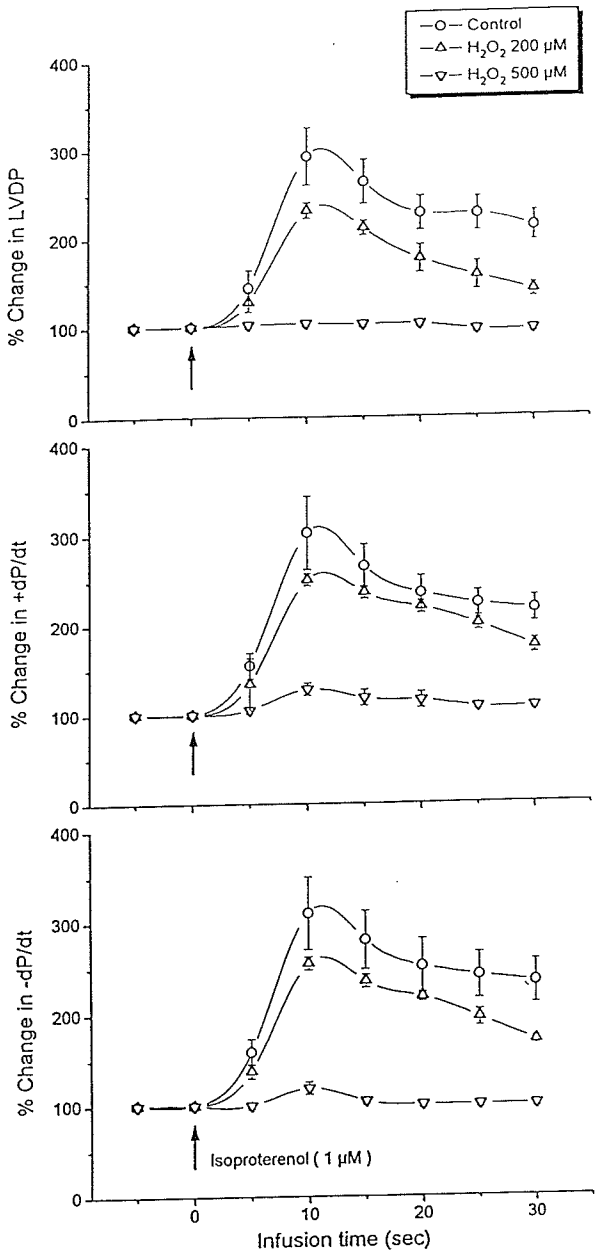

Fig. 27. Effect of infusion of isoproterenol (1 μM) on contractile parameters in control hearts and hearts perfused with H_2O_2 (200 μM and 500 μM). Hearts were perfused with H_2O_2 for 10 min before starting infusion with isoproterenol. Each value is a mean ± SE of 4-6 separate experiments. LVDP - left ventricular developed pressure; +dp/dt - rate of change of developed pressure; -dp/dt - rate of change of relaxation. The values for LVDP (mm Hg), +dP/dt (mm Hg/sec) and -dP/dt (mm Hg/sec) before starting infusion of isoproterenol were 78.2 ± 4.0, 1324 ± 64 and 1306 ± 80 for control hearts; and 33.7 ± 6.5, 572 ± 132 and 448 ± 136 for hearts perfused with 200 μM $_{H2}O_2$, and 3.6 ± 0.1, 48 ± 8 and 36 ± 6 for hearts perfused with 500 μM H_2O_2, respectively. * Significantly different from the respective control value. ($P < 0.05$).

V. DISCUSSION

1. Alterations in the β-adrenoceptor linked signal transduction in the ischemic heart

By using the isolated perfused rat heart as a model of acute global ischemia, we have shown a significant increase in the density as well as the affinity of β_1-adrenergic receptors. On the other hand, the density and affinity of β_2-adrenergic receptors remained unaltered in the ischemic heart. The observed increase in β_1-adrenoceptor density is consistent with an increase in total β-receptor density as reported by other investigators. For example, the initial observation othat ischemia increases β-adrenergic receptors density in dog heart (13) was subsequently confirmed by others (6, 12, 15, 196, 197, 228). Regional ischemia in dogs (5, 8, 10) as well as ischemia induced in guinea pig hearts (5, 196, 197, 204), rat hearts (200, 204, 229) and bovine hearts (230) has been shown to increase the β-adrenoceptor density without any changes in the affinity. However, a few studies using the canine model have shown no alteration of these receptors due to ischemia (7, 11, 231) whereas, work done with rabbit heart has revealed a depression in both density and affinity of β-adrenoceptors (232). While it is difficult to explain divergent observations reported in different experimental models, the increase in β-adrenoceptor density can be explained on the basis of externalization of β-adrenoceptors due to ischemia (196). Furthermore, the observed increase in the density of β-adrenoceptors may be due to an increase in gene expression for the receptors since Ihl-Vahl et al (229), have reported an increase in the mRNA expression for the β_1-adrenoceptors within 30 min of ischemia while the β_2-adrenoceptor mRNA expression did not change in the ischemic heart.

Regardless of the enhanced β_1-adrenoceptor density in the ischemic heart, our studies have shown a decrease in the stimulation of adenylyl cyclase activity by isoproterenol, indicating an uncoupling of β-adrenoceptors from adenylyl cyclase or a derangement at the post receptor level of the signal transduction pathway. It was evident from our study that the catalytic site of the effector enzyme itself was unaffected by ischemia since the basal and forskolin-stimulated adenylyl cyclase activities were unaltered; forskolin being a direct stimulator of adenylyl cyclase (9). Although Maisel et al, (196) using guinea pig hearts, and Freissmuth et al, (7) using dog hearts, have also reported an unaltered adenylyl cyclase during ischemia, some other investigators using a variety of animal models observed a decrease in the enzymes activity due to 45 min to 3 hr of ischemia (8-10, 200, 230). Ischemia for 30 min in rabbit hearts was found to increase the basal, forskolin- and Mn^{2+}-stimulated activities indicating an enhancement of the catalytic activity of the adenylyl cyclase enzyme (232). These inconsistencies may be ascribed to differences in experimental protocols, duration of ischemia and types of membrane preparations used in these studies. Nonetheless, the results of this study indicate that the major site of derangement during ischemia may be at the G_s-protein level. This view is supported by depressed NaF-, Gpp(NH)p- and cholera toxin-stimulated (CT) adenylyl cyclase activities in the ischemic heart. Attenuated CT-stimulated ADP-ribosylation of G_s-protein as well as reduced binding of antibodies raised to $G_{s\alpha}$ in the ischemic myocardium reinforced the depressed status of G_s-mediation of receptor-effector coupling. The observed changes in G_s-proteins seem specific since no significant alterations in G_i-protein-linked adenylyl cyclase activity, ADP-

ribosylation or antibody binding were evident in the ischemic hearts. Decreased activity and content of G_s-protein in terms of CT-stimulated ADP-ribosylation (8), and $G_{s\alpha}$-antibody binding (200, 204) as well as mRNA levels (200) in ischemic hearts of different animals are in agreement with our observations.

2. Alterations in the β-adrenoceptor linked signal transduction in the ischemic-reperfused heart

Some investigators have noted that the effects of ischemia on different components of the β-adrenergic receptor signalling pathway were reversed by reperfusion (5, 11, 233, 234). In this study reperfusion was observed to decrease the density of β_1-adrenoceptors and the proportion of coupled-receptors in the high affinity state. This is consistent with the work of Kiuchi et al. (9) and Vatner et al. (10), who showed a depression in β_1-adrenergic receptor density in the ischemic-reperfused hearts. However, it should be noted that the catalytic activity of the effector enzyme (adenylyl cyclase) as indicated by its basal and forskolin-stimulated activity as well as its G_s-protein mediated activation (NaF- and Gpp(NH)p-stimulated activity) was increased in the ischemic-reperfused hearts. The enhanced G_s-protein mediated activation of the enzyme in the ischemic-reperfused heart was also evident by an increased CT-stimulated ADP-ribosylation of G_s-protein and increased binding to G_s-proteins of antibodies against $G_{s\alpha}$; the latter probably indicates an enhanced immunosensitive state of the G_s-protein. These results however, do not concur with a few studies showing depressed basal and stimulated activities of the enzyme as well as G-protein

in a canine model of ischemia-reperfusion (9, 10). This may be due to differences in the animal species as well as the *in vivo* model used by these investigators in contrast to our *ex-vivo* model. In this regard it should be pointed out that general anesthesia and thoracotomy used in the *in vivo* canine models (9, 10) have been shown to markedly alter the physiological responses (235, 236) of the myocardium due to changes in the autonomic milieu induced by coronary artery occlusion and myocardial ischemia. Although increased PT-stimulated ADP-ribosylation of G_i-proteins as well as increased binding of antibodies against $G_{i\alpha}$ indicate an enhanced G_i-protein status, the alterations in this case are less dramatic compared to the G_s-protein and probably are not highly significant in the whole scenario. Likewise, the observed decrease in both density and affinity of β_2-adrenoceptors in the ischemic-reperfused heart was less dramatic than that seen in the β_1-adrenoceptors under this condition. Thus, the depressed isoproterenol-stimulated adenylyl cyclase activity that is observed in the ischemic-reperfused hearts may be due to the decreased β_1-adrenoceptor density and affinity as well as uncoupling of the β-adrenoceptors from the adenylyl cyclase despite the enhanced post-receptor (G_s-protein and adenylyl cyclase) status in these hearts.

Previous work has demonstrated that the β-adrenergic receptors can exist in two affinity states for agonists (237, 238) and that only the high affinity form of the receptor, which is functionally coupled to G_s-protein, is physiologically relevant (238). Thus the observed decrease in number of coupled receptors in the high affinity state in the ischemic-reperfused myocardium can significantly attenuate the signal transduction activity of an

otherwise augmented pathway. The attenuated activity of this pathway is also evident by the depressed positive inotropic effect of isoproterenol in the ischemic-reperfused hearts. It should be noted that in contrast to the ischemic-reperfused hearts, the agonist-competition experiments with β-adrenoceptors in the ischemic hearts, reveal an increase in the proportion of coupled β-adrenoceptors in the high affinity state.

Regardless of the differential effects of ischemia and reperfusion upon various components of the β-adrenoceptor-G protein-adenylyl cyclase pathway in this study, both conditions are associated with an attenuated response of adenylyl cyclase to isoproterenol. Attenuated response to isoproterenol during both ischemia and reperfusion has also been reported by others previously (7, 8, 9, 10, 200, 230). However the actual site of the signal transduction pathway that is modified to produce this attenuated response is different in these two conditions. In the case of the ischemic hearts the depressed G_s-protein status may play a crucial role whereas in the reperfused hearts the derangement appears to be at the level of the β_1-adrenoceptors. Although the β-adrenoceptors density and affinity are depressed, the enhanced adenylyl cyclase catalytic activity in ischemic-reperfused hearts may be stimulated via other pathways linked to this enzyme (eg. histaminergic receptors, dopaminergic receptors, glucagon receptors, prostacyclin receptors). The potential rise in intracellular cAMP levels through mechanisms other than β-adrenoceptors can therefore stimulate various protein kinases A-mediated phosphorylation of Ca^{2+} mobilizing components resulting in increased Ca^{2+} entry into the cell leading to Ca^{2+}- overload and promoting arrhythmias and cellular damage in the ischemic-reperfused hearts. This view is supported by the fact that

increased levels of cellular cAMP apparently to promote reperfusion arrhythmias by facilitating the development of delayed afterdepolarization (5, 239) and this effect can be prevented by both atenolol (selective β-receptor antagonist) (240) and Ca^{2+}-channel antagonist (241).

3. Mechanisms of changes in the β-adrenoceptor linked signal transduction due to ischemic-reperfusion

It should be pointed out that the beneficial effect of the antioxidants SOD plus CAT in preventing the reperfusion-induced alterations of the β-adrenoceptor-G-protein-adenylyl cyclase implicates a role of oxygen free radicals in promoting the changes observed in this study. The production of partially reduced forms of molecular oxygen during both ischemia and subsequent reperfusion, including $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$ are well documented (19, 45). It should be mentioned at this juncture that since SOD in the absence of CAT was ineffective in preventing the ischemia-reperfusion associated alterations, it is likely that H_2O_2 rather than $O_2^{\cdot-}$ may be the pertinent species of reactive oxygen involved in inducing changes in the β-adrenoceptor-linked signal transduction pathway. To this end increased production of H_2O_2 has been observed upon reperfusion of the ischemic heart, both as a dismutation product of $O_2^{\cdot-}$ (19) and by its independent generation from the mitochondria (104, 242). Arrhythmias (110), myocardial stunning and cell damage upon reperfusion of the ischemic heart have been suggested to involve free radicals (113, 243, 244). Some studies have shown that oxidative stress may alter the components of the β-adrenoceptor G-protein-adenylyl cyclase

and hence the general functioning of this pathway in the heart (37, 41, 43, 215, 216). On the other hand, the mechanisms involved in promoting the ischemia-associated modifications to the β-adrenoceptor G-protein-adenylyl cyclase pathways are less clear, since SOD and CAT are unable to prevent these alterations in the ischemic heart. This may be due to the fact that species of oxyradicals other than $O_2^{\cdot-}$ or H_2O_2 are involved in this scenario or the period of exposure of the heart to SOD and CAT as well as the concentrations of these scavengers used prior to inducing ischemia may not be adequate under our experimental conditions. Alternatively, the modifications of the β-adrenoceptor-linked signal transduction in the ischemic heart may be incurred by mechanisms unrelated to free radicals such as the depressed high energy phosphate status of the cell (6, 245, 246).

4. <u>Alterations in the β-adrenoceptor linked signal transduction due to X plus XO</u>

Treatment of cardiac membranes with X plus XO, a free radical generating system, revealed a time-dependent biphasic change in the basal activity of adenylyl cyclase, the effector enzyme for the β-adrenoceptor signal transduction pathway. Catecholamine sensitivity of the pathway as assessed by the response of adenylyl cyclase to isoproterenol indicated a hypersensitivity at 10 min and attenuation upon 30 min exposure to X plus XO. These biphasic alterations due to *in vitro* treatment of cardiac membranes were not artifactual since these were also observed in hearts perfused with X plus XO for 10 min and 30 min periods. Since X or XO alone had no effect, the alterations observed are likely due to metabolites generated by X plus XO interaction. Although Schimke et al (44) has noted a

certain time dependent biphasic alteration of isoproterenol-stimulated adenylyl cyclase activity using iron-ascorbic acid system (maximum activity was seen in less than 30 sec and thereafter the activity declined), the results in the present study, in which X plus XO was employed, showed maximal activity at 10 min and minimal activity (50% of its initial value) by 30 min. These differences in the time-course of biphasic changes may be due to the differences in the oxyradical generating systems used. Our results are also supported by functional studies which show that perfusion of hearts with X plus XO resulted in an initial augmentation of isoproterenol-stimulated contractile activity followed by a decline. Although a few studies have demonstrated an enhancement followed by a decline of cardiac contractile activity upon perfusion of hearts with X plus XO (31, 32, 247), biphasic alterations in inotropic response of the X plus XO treated hearts to isoproterenol have not been demonstrated previously. Similar changes in both the isoproterenol response of the β-adrenoceptor pathway as well as contractile response of the X plus XO-treated hearts, suggest the involvement of oxyradical induced modification of the β-adrenoceptor cascade in promoting altered inotropic response of the heart. The isoproterenol-stimulated contractile function of the myocardium was attenuated earlier than the isoproterenol stimulated adenylyl cyclase activity, and this apparent difference may be due to oxyradical-induced alterations in other subcellular mechanisms prior to the modifications in the components of the β-adrenoceptor pathway. To this end, several Ca^{2+}-translocating proteins in SL (27, 28, 31, 32, 34) and SR (25, 26) have been reported to be altered by less than 10 min exposure to oxyradicals.

In order to determine which of the three components of the β-adrenoceptor pathway are altered by X plus XO in promoting and depressing the sensitivity to catecholamines, we investigated the effect of 10 min and 30 min incubations with X plus XO on the β-receptors, adenylyl cyclase and G-proteins. The results suggest that at the level of the β-receptors the initial increase in affinity of the β_1-adrenoceptors may be more prominent at the 10 min scenario than the depressed density of these receptors, leading to a sum total of an increased responsiveness of the cascade to isoproterenol. In contrast, the depressed affinity and density at 30 min incubation with X plus XO are consistent with the attenuated isoproterenol response of the pathway at this time frame. It should be noted that both increase and decrease in β-adrenoceptor density due to oxidative stress have been reported in the literature (37, 40, 41); however, the inability of these investigator to demonstrate a biphasic change may be due to differences in the experimental design. Furthermore, the dramatic increase in affinity of these receptors upon 10 min incubation with X + XO is a novel finding which most likely is a major contributor in enhancing the activity of the pathway during the initial time period. It should also be pointed out that unlike the previous studies which reported alterations in total β-receptor status, our study has identified that alterations in the β_1-adrenoceptor may be more prominently involved in the observed biphasic alterations due to X plus XO. On the other hand, the β_2-adrenergic receptors do not exhibit biphasic alterations in that they only undergo a small decrease in density and a marginal increase in affinity upon 30 min incubation with X plus XO. The observed decrease in density and increase in affinity may likely cancel out the effect of each other leading to a minimal or insignificant

contribution of the β_2-receptors towards the modifications produced by X plus XO. The *in vitro* alterations to the β-adrenoceptor due to incubation of cardiac membranes with X plus XO were confirmed by similar alteration of the β_1- and β_2-adrenoceptor in hearts perfused by X plus XO. Also it was noted that during both *in vitro* and *ex-vivo* treatment with X plus XO, the proportion of β-adrenoceptors coupled in the high affinity state was greater after 10 min treatment in comparison to the control hearts; and this may be of some importance in promoting the enhanced status of the pathway and myocardial function at this time. On the other hand, an increased proportion of coupled receptors in the low affinity state due to the 30 min treatment with X plus XO is consistent with the attenuated isoproterenol stimulated adenylyl cyclase activity and contractility at this point. In support of this view it has been demonstrated that while β-adrenoceptors can exist in two affinity states for agonists (237, 238), it is only the high affinity form of the receptor which is coupled to G_s-protein, and is the physiologically relevant form of the receptor (238). Thus the observed increase at 10 min and decrease at 30 min in the number of coupled receptors in the high affinity state in the heart exposed to X plus XO or cardiac membranes treated with X plus XO may significantly enhance or attenuate the signal transduction activity of the pathway, respectively.

Similar to the isoproterenol stimulated adenylyl cyclase activity, the forskolin-, NaF- and Gpp(NH)p-stimulated activities in both *in vitro* and *ex-vivo* X plus XO-treated cardiac preparations also exhibited a biphasic pattern, the latter two indicating that G-protein mediation may be modified in a similar biphasic manner. Alterations in the basal and forskolin-stimulated adenylyl cyclase activity by X plus XO can be interpreted to suggest

changes at the level of catalytic site of the enzyme by oxyradicals. Although biphasic alteration due to oxidative stress of adenylyl cyclase catalytic activity (basal and forskolin-stimulated) as well as its G-protein mediated activity (NaF-, Gpp(NH)p-stimulated activities) have been reported previously by using iron-ascorbic acid system (44), the effect of the X plus XO system on the pathway has not been reported before. That the G-protein mediation of the pathway is altered in the biphasic manner is reconfirmed by changes in the CT-stimulated adenylyl cyclase activity and CT-stimulated ADP-ribosylation of the G_s-protein in cardiac membranes treated with X plus XO, whereby both parameters were increased by 10 min incubation and depressed by 30 min incubation with X plus XO. Increase in anti G_s protein binding to the 52 kD band and decreased binding to the 45 kD band of the G_s protein at 10 min as well as the decreased binding to both bands at 30 min may indicate an altered immunosensitivity of the protein, reinforcing the suggestion that the observed alteration may be at the level of the G_s-protein itself. Since the time periods used in this study are not compatible with *de novo* synthesis of G_s protein, the increased antibody labelling to G_s may not necessarily indicate increased content but rather may reflect an altered immunoreactivity of the G_s-protein due to oxyradical treatment. It should be noted that it is only the 52 kD band that was differentially modified by 10 min and 30 min of incubation with X plus XO indicating that this particular subunit of the G_s protein may be pertinently involved in the increased and decreased response of the pathway after 10 and 30 min treatment, respectively.

The fact that SOD only in the presence of CAT and not singly was effective in protecting the X plus XO induced alterations implies that the formation of H_2O_2 and not

superoxide radical may be involved in promoting the observed alterations during both *in vitro* and *ex-vivo* treatments with X plus XO. While low concentrations of H_2O_2 produced initially from dismutation of the primary superoxide radicals, may participate in producing the initial increase in the activity of the signalling pathway, the latter attenuated activity of the pathway may be due to continued production and accumulation of H_2O_2 resulting in its increased concentration around the environment of the membranes. Our results indicate that this may actually be the case since we noted that lower concentrations of H_2O_2 (50-100 μM) enhanced the basal and stimulated adenylyl cyclase activities while higher concentrations significantly depressed the enzymes activities. To this end, Tan et al (43) have reported an enhancement of adenylyl cyclase activity in vascular cells due to H_2O_2 at low concentrations. On the other hand, studies done by others have reported a decreased contractile activity as well as decreased activity of adenylyl cyclase due to H_2O_2 at higher concentrations (38, 41, 45, 131). Fliss et al (248), have reported that while high concentrations of H_2O_2 produce negative inotropic effects on heart muscle, low concentrations have been shown to be beneficial. Thus it is possible that H_2O_2 itself may be producing the observed biphasic alterations as a concentration dependent phenomenon. It is interesting to note that although adenylyl cyclase activation at the receptor level (isoproterenol response), the G-protein level (NaF and Gpp(NH)p responses) as well as catalytic subunit level (forskolin response) were all modified, comparable enhancement of adenylyl cyclase by X plus XO or H_2O_2 in response to all the stimulators indicate that the major site of alteration may be at the level of the catalytic subunit of the enzyme.

The observed biphasic pattern of sensitivity of adenylyl cyclase to catecholamines upon treatment with X plus XO may be of significance in pathologic conditions such as ischemia/reperfusion. The increase in cAMP levels that would result from the initial enhancement of the pathway may function as a compensatory mechanism in the recovery of contractile function of the ischemic-reperfused myocardium. To this end, it may be pointed out that SL Ca^{2+}-channels which are intimately involved in Ca^{2+}-entry and hence aid the augmentation of inotropic response due to sympathetic stimulation, are regulated by cAMP-dependent phosphorylation (249). This is only one of numerous Ca^{2+}-flux mechanisms that may potentially be triggered by cAMP-dependent protein kinase A-mediated phosphorylation. It is also conceivable that continued hyperproduction of cAMP by virtue of this same ability to promote Ca^{2+}-flux may precipitate the occurrence of intracellular Ca^{2+} overload and hence deleterious alterations in the cardiomyocytes in a concentration and time dependent manner. By the same token, the reduction of the β-adrenoceptor pathway activity due to prolonged oxidative stress may provide a feedback mechanism that attempts to salvage the heart from β-adrenoceptor hyperstimulation. Such changes in the components of the β-adrenergic pathway may well be caused by oxidative stress induced lipid peroxidation (42). Since the proteins of the β-adrenoceptor cascade are located on the membrane, it is likely that alterations in membrane fluidity due to lipid peroxidation may change the profile of the cascade. However, it is also possible that the free radicals produced during oxidative stress may directly alter the protein components of the pathway. The specificity of alterations (i.e. β_1-adrenoceptor-G_s protein-adenylyl cyclase) effected by the oxyradical treatment in this

study seem to suggest that this may be a significant mechanism. In support of this, numerous studies have cited cardiac pathophysiological conditions where oxidative stress-induced modification (especially sulfhydryl group modification) of Ca^{2+}-transport proteins led to disruption of cellular calcium homeostasis and subsequent tissue damage (250-252). In fact both the adenylyl cyclase enzyme as well as β-adrenergic receptors are known to possess sulfhydryl groups in their active site (219, 253, 254), modification of which may alter the characteristic of the protein. Schimke et al (44), have suggested that both SH group modification and lipid peroxidation may be involved in the alteration of β-adrenoceptor-adenylyl cyclase pathway. It has also been indicated that while protein modification may be the more important factor during the initial enhanced phase of the pathway, lipid peroxidation may become more relevant in producing the activity loss of the pathway in the later phase (44).

5. <u>Alterations in the β-adrenoceptor linked signal transduction due to H_2O_2</u>

Treatment of cardiac membranes with low concentrations of H_2O_2 (100 μM) was observed to augment whereas that with high concentrations of H_2O_2 (0.5 to 5 mM) produced a concentration dependent depression of the isoproterenol-stimulated adenylyl cyclase activity. An increase of the isoproterenol-stimulated adenylyl cyclase in H_2O_2 (100 μM)-treated vascular smooth muscle cells (43) and a decrease of this activity in H_2O_2 (10 mM)-treated cardiac membranes have also been reported by other investigators (38). Thus the apparent conflicting results regarding the effect of H_2O_2 on the isoproterenol-stimulated

adenylyl cyclase activity may be due to differences in the concentrations of H_2O_2 employed for treatment. Nonetheless, the depressant effect of high concentrations of H_2O_2 on the isoproterenol-stimulated adenylyl cyclase activity is not an artefact because if was evident at various concentrations of isoproterenol in cardiac membranes treated with 1 mM H_2O_2 under *in vitro* conditions or in membranes obtained from hearts perfused with 1 mM H_2O_2. Such a depression in the isoproterenol-stimulated adenylyl cyclase activity can be due to changes in the characteristics of β-adrenoceptors in cardiac membranes by H_2O_2. This view is supported by our observations showing a decrease in both the density and affinity of β_1-adrenoceptors in cardiac membranes upon treatment with high concentrations of H_2O_2 under *in vitro* conditions. Although the density of β_2-adrenoceptors in cardiac membranes treated with high concentrations of H_2O_2 under *in vitro* conditions was decreased, the affinity ($1/K_d$) of this subtype of β-adrenoceptors was increased. Such opposing effects of H_2O_2 on the density and affinity of β_2-adrenoceptors would be of doubtful significance in terms of the observed changes in the isoproterenol-stimulated adenylyl cyclase activity upon treatment with H_2O_2. Although decreased density without any changes in the affinity or vice versa with respect to the β-adrenoceptors in cardiac membranes treated with H_2O_2 in earlier studies (28, 37, 45) are at variance with the data reported in the present study, these differences appear to be due to the nature of ligand used for receptor binding studies as well as the type of membranes and experimental conditions employed. Our results for β_1- and β_2-adrenoceptors upon treating cardiac membranes with H_2O_2 under *in vitro* conditions are in agreement with those with cardiac membranes obtained upon perfusing the hearts with H_2O_2.

Furthermore, the observed changes in the β_1- and β_2-adrenoceptors are not due to any non-specific action of H_2O_2 on the membrane because Ca^{2+}-channel antagonist binding in cardiac membranes was not affected by H_2O_2 treatment under similar conditions (28). Nonetheless, changes in the isoproterenol-stimulated adenylyl cyclase due to H_2O_2 cannot be considered due to any alterations in the proportion of coupled β-adrenoceptors in the high affinity state because no shift in the competition curve was evident upon treatment of cardiac membranes with H_2O_2.

In addition to changes in the characteristics of β_1-adrenoceptors, the evidence presented in this study indicates that alterations in both the catalytic activity of adenylyl cyclase and the function of G_s-proteins may also explain the observed depression in the isoproterenol-stimulated adenylyl cyclase in heart membranes upon treatment with high concentrations of H_2O_2. In this regard, adenylyl cyclase activities in the absence (basal) and presence of forskolin, which is known to stimulate the catalytic subunit of the enzyme directly (255), were found to decrease upon treatment of cardiac membranes with H_2O_2. Although the adenylyl cyclase activities in the presence of both NaF and Gpp(NH)p, which are known to activate the enzyme through their interaction with G-proteins (256), were also depressed upon treating the cardiac membranes with H_2O_2, the results in Table 1 indicate that the depressions in the effects of these agents (26 to 28%) were comparable to that of forskolin (25%). It should be noted that changes in the adenylate cyclase activities in the absence or presence of different stimulants in cardiac membranes treated with H_2O_2 under *in vitro* conditions were similar to those seen upon *ex-vivo* treatment of hearts with H_2O_2

(Table 2) and thus cannot be regarded as artefacts of experimental conditions employed in this study. The involvement of G_s-proteins in the observed depression of the isoproterenol-stimulated adenylyl cyclase by H_2O_2 is suggested from our observations that the adenylyl cyclase activity in the presence of CT, an activator of G_s-proteins (224), unlike that in the presence of PT, an inhibitor of G_i-proteins (224), was inhibited by treatment of cardiac membranes with H_2O_2. Furthermore, the CT-catalyzed ADP-ribosylation at both 45 kD and 52 kD bands of G_s-proteins, unlike the PT-catalyzed ADP-ribosylation at 40kD band of G_i-proteins, was also depressed by H_2O_2 treatment. The inability of some investigators (38, 43) to detect changes in CT-catalyzed ADP-ribosylation upon treating vascular smooth cells and cardiac membranes with H_2O_2 may be due to differences in tissue specificity and experimental design used in these studies. On the other hand, our results are supported by the fact that the immunoreactivity of the 40 kD band to antibodies for G_i-proteins was unaltered whereas that of the 45 kD band to antibodies for G_s-proteins was markedly depressed upon treatment of cardiac membranes with H_2O_2. It should be noted from Fig. 7 that the decrease in the anti G_s-protein binding at 52 kD band, unlike that at 45 kD band, was not significant. Furthermore, the depressant effect of H_2O_2 on CT-catalyzed ADP ribosylation at 52 kD was of lesser magnitude in relation to that seen at the 45 kD band for G_s-proteins in the H_2O_2-treated membranes. It thus appears that the site of action of H_2O_2 for reducing G_s-protein functions may primarily be at the 45 kD band of the G_s-proteins.

A depression in the β_1-adrenoceptor-G_s-protein-adenylyl cyclase activity due to H_2O_2 can be seen to decrease the formation of cAMP (36, 257, 258) and this may attenuate the

positive inotropic effect of catecholamines in the heart. In fact perfusing the hearts with H_2O_2 was found to depress the increase in LVDP, + dp/dt and -dp/dt due to isoproterenol infusion. However, it should be pointed out that the depression in the isoproterenol-induced changes in contractile parameters upon perfusing the hearts with 500 mM H_2O_2 was of greater magnitude in comparison to the depression observed in the β_1-adrenoceptor-adenylyl cyclase complex in cardiac membranes treated with 1 mM H_2O_2 *in vitro* or obtained from hearts perfused with 1 mM H_2O_2. The greater sensitivity of H_2O_2 in attenuating the inotropic responses of hearts to isoproterenol may be due to the fact that other cellular mechanisms regulating the intracellular Ca^{2+}, which may be involved in contractile responses, are also affected by H_2O_2 (27, 28, 30, 45). The observed depression in the β_1-adrenoceptor-G_s-protein-adenylyl cyclase complex cannot be considered to participate in raising the intracellular concentration of Ca^{2+} in cardiomyocytes exposed to H_2O_2 as on the contrary it will decrease both the influx of Ca^{2+} as well as release of Ca^{2+} from the intracellular Ca^{2+}-stores (36). Nonetheless, the results presented in this study indicate that the effects of H_2O_2 on the β-adrenoceptor-adenylyl cyclase complex are probably of a direct nature and probably are not mediated through the highly reactive hydroxyl radicals generated by the interaction of H_2O_2 with redox-active transitional metals (19, 45). This is evident from our observations that CAT was able to prevent the alterations effected by H_2O_2 and these beneficial effects of CAT were not further enhanced by the presence of MAN. Although the exact mechanisms by which H_2O_2 alters the β-adrenoceptor ternary complex are unclear, it seems appropriate to indicate that the reduced β_1-adrenoceptor-G_s protein-adenylyl cyclase activity may be

caused by the effect of H_2O_2-induced lipid peroxidation (19, 45) on the physical state of the membrane. This view is based on the fact that the trans-membrane β-adrenoceptor signal transduction has been observed to be depressed by a reduction in membrane fluidity (259), and lipid peroxidation has been reported to decrease the membrane fluidity (260). The nature of the modifications of β_1-adrenoceptor-G_s-protein-adenylyl cyclase may be suggestive of changes incurred at the level of protein components of the β-adrenoceptor complex. This view is supported by the fact that aldehydes (malondealdehyde and 4-hydroxyneonatal) formed during lipid peroxidation have been shown to influence the function of β-adrenoceptor-adenylyl cyclase system in SL by reacting with NH_2- and/or SH-groups of these components (42, 260). In fact both the adenylyl cyclase enzyme as well as β-adrenergic receptors are known to possess sulfhydryl groups in their active sites (219, 254), modification of which may alter the characteristics of these proteins.

6. Overall significance of the study

Irrespective of the exact mechanisms for the observed changes, this study indicates that both 30 min ischemia and 60 min reperfusion of the ischemic heart attenuates the activation of adenylyl cyclase by isoproterenol. Although these changes may reflect an uncoupling of the β-adrenoceptor signal transduction system, the etiology of the uncoupling seems to be different in the two conditions. While ischemia is associated with an uncoupling at the level of G_s-protein by a mechanism that may or may not involve oxyradicals, reperfusion is associated with a depression in the density and affinity of β_1-adrenoceptors

and appears to involve an oxygen radical-mediated mechanism. It seems pertinent to point out that although previous studies have reported alterations in β-adrenoceptor during ischemia and to a lesser extent during reperfusion, this study reports that these changes in the ischemic heart are limited to alterations in the β_1-adrenoceptors whereas those in the ischemic-reperfused hearts are due to alterations in both β_1- and β_2-adrenoceptors. With regards to the effect of X plus XO, it is likely that low concentration of H_2O_2 produced from the dismutation of the primary superoxide radicals may promote compensatory alterations to the β-adrenoceptor pathway during early period, such as in the early phase of ischemia-reperfusion. However, with its continued production and accumulation, as in the case of prolonged ischemic-reperfusion, it may inflict disruptive effects upon the β-adrenoceptor pathway leading to the characteristic loss of the inotropic response in this condition. The inhibitory effect of high concentrations of H_2O_2 on the β-adrenoceptor-G-$_s$-protein-adenylyl cyclase pathway may be of pathophysiological significance because the concentrations of H_2O_2 comparable to those used in this study have been reported to occur in the myocardium during ischemia-reperfusion injury (46, 261). Although the methods used to generate the free radicals in this study were fairly artificial, the parallel alterations in both *in vitro* treated membrane and in membranes from hearts perfused with the oxyradicals indicate that the results obtained are valid and not artefactual. Also the correlation between the alteration in the isoproterenol response of the β-adrenoceptor pathway and isoproterenol response of contractile function indicates a significance of the findings in pathological conditions such as ischemic heart disease where oxygen radical generation and loss of inotropic response are

well documented.

From the foregoing discussion and the salient features of the observations made in this study (Table 14), it should be pointed out that neither the free radical generation nor the formation of H_2O_2 appears to be involved in promoting the alterations in β-adrenoceptor linked signal transduction in the ischemic heart. This may be due to the fact that either non-radical species, such as accumulated metabolites, or species of reactive oxygen other than $O_2^{\cdot-}$ or H_2O_2 may be involved during this period. However, it appears that in the reperfused heart, both superoxide radicals and H_2O_2 may be partially involved. Although the responses to catecholamines and the β-adrenoceptor mechanisms are attenuated in the ischemic-reperfused heart (262, 263) as well as hearts treated with X plus XO or H_2O_2, the changes in the pattern of abnormalities in the β-adrenoceptors, G-proteins and adenylyl cyclase system in the ischemic-reperfused hearts are not exactly similar to those seen upon oxyradical treatment. This may be due to the involvement of free radicals and active species of oxygen and oxidants other than those used in this study, in the ischemic myocardium. Nonetheless, this study provides a comprehensive knowledge regarding changes in the β-adrenoceptor signal transduction pathway due to ischemia-reperfusion injury and thus hopefully will be of some clinical relevance in improving the therapeutic value of procedures such as angioplasty or coronary by-pass surgery.

Table 14. Summary of the alterations incurred in different components of the β-adrenergic signal transduction pathway due to ischemia, reperfusion and oxyradical treatments

	β_1		β_2		G_s	G_i	Adenylyl Cyclase				
	K_d	B_{max}	K_d	B_{max}			Basal	Forskolin	NaF	Gpp(NH)p	Isoproterenol
Ischemia	⇑	⇑	--	--	⇓	--	--	--	⇓	⇓	⇓
Reperfusion	⇓	⇑	⇓	⇓	⇑	--	⇑	⇑	⇑	⇑	⇓
X + XO (10 min)	⇑	⇓	--	--	⇑	--	⇑	⇑	⇑	⇑	⇑
X + XO (30 min)	⇓	⇓	⇑	⇑	⇓	--	⇓	⇓	⇓	⇓	⇓
H_2O_2 (100 μM)	ND	ND	ND	ND	ND	ND	⇑	⇑	⇑	⇑	⇑
H_2O_2 (≥ 200 μM)	⇓	⇓	⇓	⇓	⇓	--	⇓	⇓	⇓	⇓	⇓

⇑ = increased in comparison to control; ⇓ = decreased in comparison to control; -- = unaltered in comparison to control; ND = not determined.

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