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INTRACELLULAR CATALASE INHIBITION DOES NOT PREDISPOSE RAT HEART TO ISCHEMIA-REPERFUSION AND HYDROGEN PEROXIDE-INDUCED INJURIES

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The objective of this study was to determine whether inhibition of intracellular catalase would decrease the tolerance of the heart to ischemia-reperfusion and hydrogen peroxide-induced injuries. Isolated bicarbonate buffer-perfused rat hearts were used in the study. Intracellular catalase was inhibited with 3-amino-1,2,4-triazole (ATZ, 1.5 g/kg body weight, two hours prior to heart perfusion). In the ischemia-reperfusion protocol, hearts were arrested with St. Thomas' II cardioplegic solution, made ischemic for 35 min at 37°C, and reperfused with Krebs-Henseleit buffer for 30 min. The extent of ischemic injury was assessed using postischemic contractile recovery and lactate dehydrogenase (LDH) leakage into reperfusate. In the hydrogen peroxide infusion protocol, hearts were perfused with increasing concentrations of hydrogen peroxide (inflow rates 0.05-1.25 μmol/min). Inhibition of catalase activity (30.4 ± 1.8 mU/mg protein in control *vs* 2.4 ± 0.3 mU/mg in ATZ-treated hearts) affected neither pre-ischemic aerobic cardiac function nor post-ischemic functional recovery and LDH release in hearts subjected to 35 min cardioplegic ischemic arrest. Myocardial contents of lipid hydroperoxides were similar in control and ATZ-treated animals after 20 min aerobic perfusion, ischemia, and ischemia-reperfusion. During hydrogen peroxide perfusion, there was an increase in coronary flow rate followed by an elevation in diastolic pressure and inhibition of contractile function in comparison with control hearts. The functional parameters between control and ATZ-treated groups remained unchanged. The concentrations of myocardial lipid hydroperoxides were the same in both groups. We conclude that inhibition of myocardial catalase activity with ATZ does not predispose the rat heart to ischemia-reperfusion and hydrogen peroxide-induced injury.

KEY TERMS: catalase, ischemia, reperfusion injury, myocardium, hydrogen peroxide, oxidant injury

ABBREVIATIONS: ATZ, 3-amino-1,2,4-triazole; LDH, lactate dehydrogenase; GPx, glutathione peroxidase; KHB, Krebs-Henseleit buffer; RPP, rate-pressure product; MOPS, (3-[N-morpholino]propanesulfonic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; LOOHs, lipid hydroperoxides.

INTRODUCTION

It has been suggested that reactive oxygen species including the superoxide radical ($O_2^{\cdot-}$), the hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2) generated

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during myocardial ischemia and reperfusion[1,2] are responsible for cellular and tissue damage in the heart.[3,4] It has been hypothesized that the myocardial anti-oxidant defense system is overwhelmed by the sudden and increased burst of oxy-radicals during ischemia and reperfusion.[5-7] This has led to the use of antioxidant enzymes, such as catalase and superoxide dismutase as additives to cardioplegic solutions used in cardiac surgery[8,9] and as agents to limit infarct size after regional myocardial ischemia *in vivo*.[10,11]

Myocardial tissue contains low levels of catalase relative to glutathione peroxidase (GPx).[12] It has been suggested that the catalase enzyme probably plays only a minor role in the detoxification of hydrogen peroxide formed in the heart.[13,14] Myocardial catalase is reportedly sequestered in microperoxisomes and not associated with mitochondria.[15] However, the existence of catalase in the mitochondrial matrix was suggested by Nohl and Hegner.[16]

H_2O_2 consumption by rat heart mitochondria was accompanied by oxygen release suggesting a catalatic mechanism of decomposition of H_2O_2.[16] Also, Radi *et al.*[17] have recently reported that rat heart mitochondria possess an H_2O_2-metabolizing enzyme that is inhibited by cyanide. The electrophoretic mobility of the enzyme is similar to bovine and rat liver catalase and exhibits a specific immunoreactivity to anticatalase antibodies.[17] These data show that myocardium possesses a sufficient amount of catalase activity to cope with enhanced formation of H_2O_2.

Recently, it was shown that enhanced postischemic recovery occurs in hearts isolated from rats challenged with heat shock[18,19] or endotoxin treatment.[20,21] In both treatment groups, myocardial catalase activity was found to be increased. This improvement in postischemic recovery was attributed to increased catalase activity in the myocardium.

Darley-Usmar *et al.*[22] have suggested that if reactive oxygen species do contribute to myocardial damage during hypoxia/reoxygenation, this effect stems from the local generation of oxidants and not through overwhelming of the cellular antioxidant defenses. Consistent with this interpretation, Chatham *et al.*[23] have also reported that high global concentrations of hydrogen peroxide are not formed during reperfusion of ischemic hearts. The role of reactive oxygen species in myocardial reperfusion injury is indeed controversial.[24,25]

The objective of this study was to investigate the importance of endogenous catalase in protecting the myocardium against intracellular hydrogen peroxide, presumably generated during ischemia-reperfusion, and exogenous hydrogen peroxide. To this end, we used isolated hearts in which 90% of the endogenous catalase activity had been inhibited. Selective inactivation of catalase in the rat myocardium was achieved by pretreating animals with 3-amino-1,2,4-triazole (ATZ), an irreversible inhibitor of catalase.[26,27]

MATERIALS AND METHODS

Isolated heart perfusion

Adult male Sprague-Dawley rats (300–350 g body weight), maintained on a standard diet, were used for this study. Rats were anesthetized with Halothane and heparinized *via* the left femoral vein (250 IU/kg). After 1 min, the heart was rapidly excised and placed in perfusion medium. Within 30 sec, the aorta was attached to a stainless steel cannula, the pulmonary artery was incised to permit adequate coronary drainage, and the heart was perfused normothermically by the

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method of Langendorff at a perfusion pressure equivalent to 12 kPa (90 mmHg). The perfusion medium used was Krebs-Henseleit buffer (KHB) having the following composition in mmol/l:NaCl, 118.5; $NaHCO_3$, 25.0; KCl, 4.8; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; glucose, 11.1 in which the $CaCl_2 \cdot 2H_2O$ content was reduced to 1.8 (pH 7.4 when gassed with 95% O_2 and 5% CO_2). During preparation of KHB, precautions were taken to prevent the precipitation of calcium by gassing the solution with 5% CO_2. St. Thomas' II solution was used as a cardioplegic solution with the following composition in mmol/l:NaCl 110; $NaHCO_3$, 10; KCl, 16; $MgCl_2 \cdot 6H_2O$, 16; $CaCl_2 \cdot 2H_2O$, 1.2 (pH was titrated to 7.8 using HCl).

Measurement of cardiac function

A water-filled latex balloon (Biomedix, Elm Grove, WI) was inserted into the left ventricle through the mitral valve and secured in place with a ligature. The balloon was connected *via* a rigid water-filled catheter to a pressure transducer (Deseret Medical Model 8148) for the measurement of left ventricular pressure and heart rate. The transducer output was amplified using a universal signal conditioner (model 20–4615–58, Gould, Cleveland, OH) and recorded on an analog chart recorder (Astromed 9500, Providence, RI). The intraventricular balloon was inflated until end diastolic pressure was 3–6 mmHg. Rate-pressure product (RPP) was determined as the product of heart rate per second and developed pressure. The coronary flow rate was measured by collecting the effluent from the right heart into a graduated cylinder and expressed as ml per min.

Tissue homogenization

The free wall of the left ventricle was excised while the heart remained on the perfusion cannula, freeze-clamped between large stainless-steel tongs precooled in liquid nitrogen, and immersed in the homogenizing buffer precooled to 4°C at 10% (w/v). The homogenizing buffer was composed of 0.3 mol/l sucrose, 1 mmol/l $CaCl_2$, 5 mmol/l MOPS, 5 mmol/l KH_2PO_4, 0.1% BSA, 100 μmol/l desferoxamine mesylate and stored under argon gas at 0–4°C. Before homogenization, 25 μmol/l butylated hydroxytoluene and 50 μmol/l phenylmethylsulfonylfluoride were added to the buffer to prevent oxidation and protease activation. Homogenization was carried out with a Polytron homogenizer (Brinkmann Instruments, Inc.) at half speed for 1–2 min. Homogenates were centrifuged at 3000 g for 17 min at 4°C (Sorvall RC3C).

Enzyme Assays

Measurement of LDH release

The coronary effluent was collected during the initial 15 min of reperfusion in a graduated cylinder kept in ice. After thoroughly mixing the contents, an aliquot of the coronary effluent was used for the determination of LDH activity with LD-L Sigma Diagnostics reagent, which measured the enzyme activity based on the oxidation of lactate. One unit (IU) of LDH activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of NADH/min at 30°C.

Catalase activity

Catalase activity was measured by monitoring the decrease in absorbance of H_2O_2 at 240 nm (ϵ_{max} for H_2O_2 = 43.6 $M^{-1}cm^{-1}$).[28] The specific activity of catalase was defined in terms of mmoles of H_2O_2 consumed/min and expressed as mU/mg

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protein in the homogenate of myocardial tissue. The reaction mixture contained 19 mmol/l H_2O_2 and 10 μl of tissue supernatant in 3.0 ml of phosphate buffer (50 mmol/l. pH 7.0).

Measurement of lipid hydroperoxides

Lipid hydroperoxides (LOOHs) were determined by a microiodometric method adapted from Hicks and Gebicki.[29] All manipulations were carried out under subdued light. Immediately after homogenization, samples were mixed with PBS containing 1 mmol/l EDTA and extracted with 2:1 (v/v) chloroform:methanol mixture. The organic phase was separated, evaporated under a stream of nitrogen, and stored at $-20°C$ until assayed. Then, the sample received 20μl of potassium iodide (1.2 g/ml in deaerated water) and 0.3 ml of deaerated 2:3 (v/v) chloroform:acetic acid and incubated for 10 min. The reaction was stopped using 0.9 ml of 0.5% cadmium acetate. The phases were separated using an Eppendorf microcentrifuge for 5 min and the upper phase was read at 353 nm. Quantitation was based on an extinction coefficient of 22.5 $mmol^{-1}cm^{-1}$, which was obtained by using enzymatically standardized cumene hydroperoxide.[30]

Experimental Time Course

Inhibition of catalase

Catalase was inhibited with ATZ administered as a 20% solution (w/v) in normal saline. Rats were anesthetized with halothane and injected with ATZ (1.5 g/kg body weight, i.p.) two hours prior to excision of the heart. Control rats received the same treatment and were injected with saline.

Ischemia-reperfusion protocol

Immediately after mounting on the cannula, hearts (n = 7/group) from ATZ-and saline-treated rats were perfused aerobically with KHB at 37°C for a 20 min equilibration period. During this period, left ventricular pressure, coronary flow rate and heart rate were continuously monitored. Hearts were then arrested with a 3 min infusion of St. Thomas' II cardioplegic solution and subjected to normothermic global ischemia for 35 min. After the ischemic duration, hearts were reperfused with KHB for 30 min. At the end of the reperfusion period, the free wall of the left ventricle was freeze-clamped for measurement of catalase activity.

Hydrogen peroxide perfusion

Isolated hearts (n = 7/group) from ATZ-and saline-treated rats were perfused aerobically for 20 min equilibration period. During this period, baseline measurements of cardiac function were taken. Hearts were infused then with increasing concentrations of H_2O_2 *via* a side arm immediately above the site of cannulation of the heart. H_2O_2 was delivered to the coronary vasculature using a Harvard syringe drive at infusion rates of 0.05, 0.1, 0.2, 0.5, 0.85, 1.25 μmol/min, for 10 min at each succeeding inflow rate. After 60 min of H_2O_2 perfusion, the free wall of the left ventricle was removed and freeze-clamped for catalase and lipid hydroperoxide measurement.

Statistical analysis

All data were presented as mean ± SEM. Statistical analysis was performed using unpaired Student *t* test. Significance was accepted at the $p < 0.05$ level.

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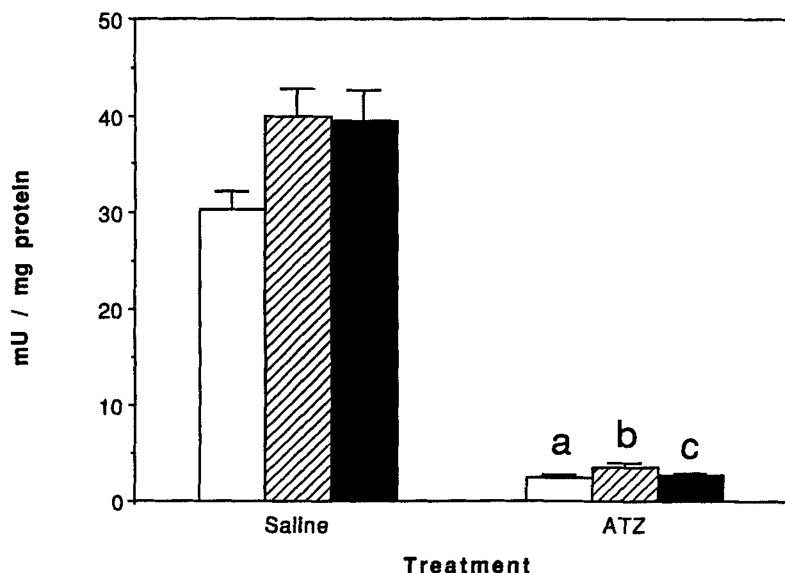

FIGURE 1 Catalase activity in aerobic (□) and ischemic (▨), and ischemic-reperfused (■) myocardium. Rats were injected with saline or ATZ 2 hr prior to excision of the heart. Hearts were freeze-clamped and homogenized as described in Methods. a, b, c – $p < 0.05$ compared with aerobic perfusion, ischemia, and ischemia-reperfusion, respectively (saline vs ATZ).

Ethical Considerations

Animals used in this study received humane care in compliance with "Guiding Principles in the Care and Use of Animals" approved by the Council of the American Physiological Society, and the "Guide for the Care and use of Laboratory Animals" prepared by the National Academy of Science and published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

RESULTS

Catalase inhibition and aerobic cardiac function

The myocardial catalase activity after 20 min of aerobic perfusion of hearts from saline-and ATZ-treated rats is shown in Figure 1. The ATZ-treated animals had only 10% of myocardial catalase activity compared with control (saline-treated) rats. Despite extensive inhibition in catalase activity (≃90%), there was virtually no difference in the aerobic function and myocardial LOOHs content of control and ATZ-treated hearts (see Tables I and II).

Catalase inhibition and ischemia-reperfusion injury

The functional recovery of RPP (Figure 2, lower panel) after cardioplegic ischemic arrest remained the same in control and ATZ-treated hearts, while the recovery of developed pressure was slightly better in the ATZ-treated group at 30 min of reperfusion (Figure 2, upper panel) ($P < 0.05$). Also, there was no difference in LDH release during reperfusion between control and ATZ-treated groups (Figure 3). Myocardial catalase activity was slightly increased after ischemia and reperfusion in the control group as compared with aerobic perfusion (Figure 1), but failed to reach a level of

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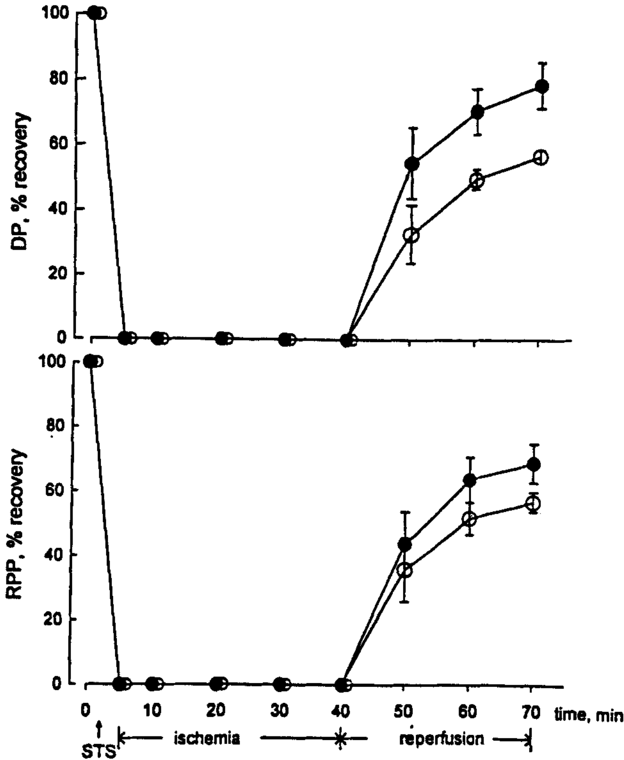

FIGURE 2 Functional recovery of saline-(○) and ATZ-treated (●) hearts after 35 min cardioplegic ischemic arrest. DP - developed pressure; RPP - rate-pressure product; STS - St. Thomas' II cardioplegic solution.

TABLE I
Baseline functional measurements

Parameter	Control (n = 7)	ATZ-treated (n = 7)
Coronary flow (ml/min)	9.8 ± 0.4	9.4 ± 0.6
Developed pressure (mmHg)	134 ± 6	128 ± 6
Heart rate (beats/min)	271 ± 12	304 ± 12
Rate-pressure product (mmHg/sec)	600 ± 22	643 ± 36

Values are derived from isolated rat hearts after 20 min of aerobic perfusion and presented as mean ± SEM.

significance. The amount of LOOH formed during ischemia and reperfusion in the control and ATZ-treated groups was almost the same (Table II).

Catalase inhibition and hydrogen peroxide-induced injury

During perfusion of isolated hearts with increasing concentrations of H_2O_2, diastolic pressure remained at the baseline level for 40 min (Figure 4, upper panel) and then started to rise, reaching 18 ± 3 and 21 ± 3 mmHg at 60 min in control and ATZ-treated groups, respectively. There was an increase in coronary flow rate that reached its maximum at 50 min of H_2O_2 perfusion (147 ± 7 and 159 ± 5% of

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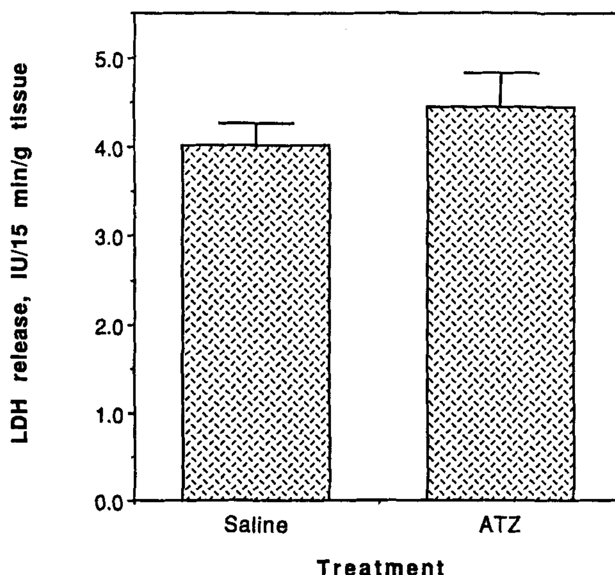

FIGURE 3 Lactate dehydrogenase (LDH) leakage from saline- and ATZ-treated hearts following 35 min cardioplegic ischemic arrest. Activity of LDH was measured in the coronary perfusate collected during the initial 15 min period of reperfusion.

TABLE II
Myocardial contents of lipid hydroperoxides (LOOHs)

	Perfusion	Ischemia	Reperfusion	H_2O_2
Saline treated	1.60 ± 0.10	1.47 ± 0.17	1.29 ± 0.07	1.53 ± 0.07
ATZ treated	1.61 ± 0.19	1.65 ± 0.13	1.98 ± 0.21	1.61 ± 0.13

Values are derived from three measurements per heart (n = 3–7) and presented as nmoles LOOH/mg protein (mean ± SEM).

baseline in control and ATZ-treated groups, respectively). Heart rate remained unchanged during H_2O_2 perfusion (data not shown). RPP was somewhat elevated above the baseline level until 40 min, and then decreased to 74 ± 12 and 77 ± 10% of baseline for control and catalase-inhibited groups, respectively (Figure 4, lower panel). There was no difference between control and ATZ-treated groups with respect to myocardial function during H_2O_2 perfusion up to a concentration of 0.85 μmol/min. At longer infusion times (> 50 min), there was a slight decrease in myocardial function in both control and ATZ-treated hearts. There was no LDH release into the perfusate in either group until 50 min of H_2O_2 perfusion. During the last 10 min of H_2O_2 perfusion, LDH activities were 0.38 ± 0.23 IU/10 min/g and 0.63 ± 0.35 IU/10 min/g in control and ATZ-treated groups, respectively ($p > 0.05$ n.s.). Although the catalase activity in ATZ-treated hearts at the end of H_2O_2 perfusion period was only 8% of control value, the amount of lipid hydroperoxides formed in the myocardium was unaffected by H_2O_2 treatment (Figure 5) (Table II).

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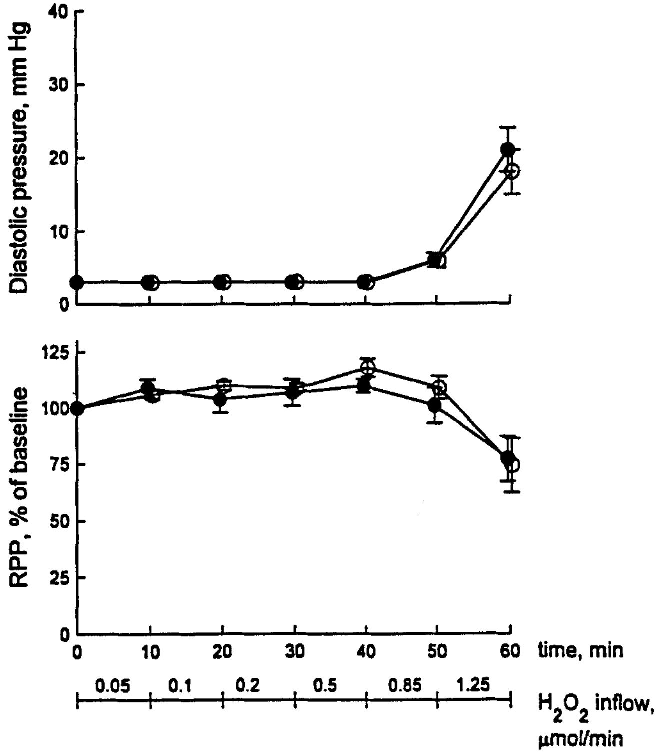

FIGURE 4 Functional activity of isolated saline-(○) and ATZ-treated (●) hearts during perfusion with hydrogen peroxide. H_2O_2 was infused in the coronary vasculature at a concentration rate of 0.05–1.25 μmol/min. RPP – rate-pressure product.

DISCUSSION

The present study demonstrates that rat hearts in which 90% of the endogenous catalase activity has been inhibited, showed no difference in myocardial function as compared with control hearts to both ischemia-reperfusion- and hydrogen peroxide-induced injuries. The concentrations of lipid peroxides in hydrogen peroxide-perfused myocardium were similar in ATZ-treated and control rat hearts.

The rationale for using catalase, alone or in combination with superoxide dismutase, to improve postischemic recovery and limit infarct size *in vivo*[8-11] is based on the premise that reactive oxygen species contribute to myocardial ischemia-reperfusion injury.[1-4] Catalase is a tetrameric protein with a molecular weight of 240,000 daltons, and, therefore, it cannot be expected to cross the membrane readily. It is likely that much of the enzyme remains within vascular space or, probably, enters endothelial cells *via* a pinocytotic mechanism.

It has been recently shown that during postischemic reperfusion, exogenously added catalase prevented the accumulation of H_2O_2-induced polymer product on the luminal surfaces of endothelial cells.[31] In some studies catalase alone failed to protect the ischemic myocardium,[8] while in others, catalase afforded protection

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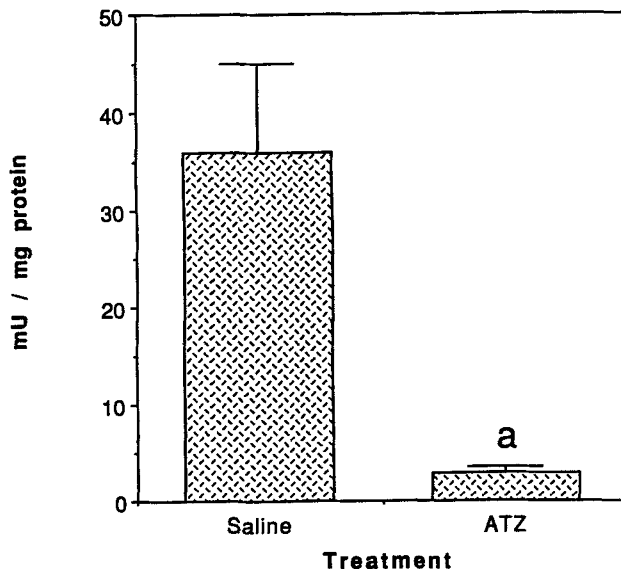

FIGURE 5 Catalase activity and saline- and ATZ-treated hearts after 60 min perfusion with hydrogen peroxide. Rats were injected with saline or ATZ 2 hr prior to excision of the heart. Hearts were freeze-clamped and homogenized as described in Methods. a – $p < 0.05$ compared with saline-treated groups.

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against ischemia-reperfusion and hypoxia-reoxygenation injuries.[32-34] In the present study, inhibition of 90% of intracellular catalase activity does not predispose the heart to ischemia-reperfusion injury, and it seems unlikely that a protective mechanism of the exogenous catalase may, therefore, involve an intracellular locus of action.

Recently, induction of intracellular catalase was suggested to be a primary reason for myocardial protection in heat shock- and endotoxin-treated rats.[18-21] Both hyperthermic and endotoxin treatments increased myocardial catalase activity that was associated with improved postischemic recovery of isolated rat hearts. Inhibition of catalase activity with ATZ abolished the protective effects of heat- and endotoxin-induced stress.[18-21] The present data clearly show that, despite extensive inhibition of myocardial catalase activity, rat hearts are not very susceptible to H_2O_2 infusion nor to H_2O_2 produced during myocardial ischemia and reperfusion injury. The potential role of other protective mechanisms need to be considered as well. It is plausible that other defensive system(s) capable of repairing H_2O_2-induced damage may have been induced in hearts in which the catalase activity is inhibited.

The GPx activity in the liver and heart was found to be increased as a result of catalase inhibition.[14,35] The activity of Se-dependent GPx was markedly decreased in animals maintained on a Se-deficient diet, however, the activity of non-Se-dependent GPx was found to increase significantly.[36] Inhibition of myocardial catalase activity did not increase peroxidation in myocardial tissue, whereas reduction in GPx activity (produced either by low-Se diet or cadmium supplementation) enhanced myocardial lipid peroxidation.[13,14]

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Finally, the lack of exacerbation of injury to catalase-depleted myocardium by either ischemia/reperfusion or hydrogen peroxide may be attributed to non-specific effects of ATZ. ATZ has been shown to inhibit oxygen radical generation from cyclooxygenase *in vitro* and *in vivo* in brain,[37] although the contribution of cyclooxygenase-derived free radicals in ischemia/ reperfusion in the heart has not been fully established. Alternatively, it could also be argued that the 10% catalase activity still present in ATZ-treated myocardium is enough to cope with the excess H_2O_2. This may, however, be very unlikely in light of the finding (Figure 4) that a similar dose-response behavior was observed in control and ATZ-treated hearts during H_2O_2 treatment.

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