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Original Research Communication

Role of Oxidative Stress in Ischemia–Reperfusion-Induced Changes in Na^+,K^+-ATPase Isoform Expression in Rat Heart

PETR OSTADAL,[1,2] ADEL B. ELMOSELHI,[1] IRENA ZDOBNICKA,[1]
ANTON LUKAS,[1] VIJAYAN ELIMBAN,[1] and NARANJAN S. DHALLA[1]

ABSTRACT

The aim of this study was to assess whether depression of cardiac Na^+,K^+-ATPase activity during ischemia/reperfusion (I/R) is associated with alterations in Na^+,K^+-ATPase isoforms, and if oxidative stress participates in these I/R-induced changes. Na^+,K^+-ATPase α_1, α_2, α_3, β_1, β_2, and β_3 isoform contents were measured in isolated rat hearts subjected to I/R (30 min of global ischemia followed by 60 min of reperfusion) in the presence or absence of superoxide dismutase plus catalase (SOD+CAT). Effects of oxidative stress on Na^+,K^+-ATPase isoforms were also examined by perfusing the hearts for 20 min with 300 μ*M* hydrogen peroxide or 2 m*M* xanthine plus 0.03 U/ml xanthine oxidase (XXO). I/R significantly reduced the protein levels of all α and β isoforms. Treatment of I/R hearts with SOD+CAT preserved the levels of α_2, α_3, β_1, β_2, and β_3 isoforms, but not that of the α_1 isoform. Perfusion of hearts with hydrogen peroxide and XXO depressed all Na^+,K^+-ATPase α and β isoforms, except for α_1. These results indicate that the I/R-induced decrease in Na^+,K^+-ATPase may be due to changes in Na^+,K^+-ATPase isoform expression and that oxidative stress plays a role in this alteration. Antioxidant treatment attenuated the I/R-induced changes in expression of all isoforms except α_1, which appears to be more resistant to oxidative stress. *Antioxid. Redox Signal.* 6, 914–923.

INTRODUCTION

Na^+,K^+-ATPase (sodium pump) is a heterodimer protein that plays a key role in membrane potential regulation and ion transport across the sarcolemmal membrane (13, 20, 23, 29). It consists of α and β subunits; the α subunit is responsible for the catalytic activity of the enzyme and occurs in the rat heart as three isoforms: α_1, α_2, and α_3. The α_1 isoform differs from α_2 and α_3 in that it has a lower affinity to cardiac glycosides and represents the most abundant isoform in the adult heart (33). The β subunit is responsible for the proper localization and insertion of Na^+,K^+-ATPase in the sarcolemma. It is also expressed in the heart as three isoforms: the highly expressed β_1, and the other β_2 and β_3. The functional properties of the β subunit are still poorly understood, but alterations in the content of this subunit have been shown to affect the activity of the sodium pump (22). Numerous studies have examined the involvement of Na^+,K^+-ATPase in cardiac function and as the receptor mediating the positive inotropic action of cardiac glycosides (27). Yet relatively little is known regarding the regulation of different subunits of Na^+,K^+-ATPase under pathological conditions.

Considerable evidence indicates that activity of the cardiac Na^+,K^+-ATPase is decreased by different species of oxygen free radicals and oxidants. Depressed Na^+,K^+-ATPase activity is seen after the treatment of cardiac sarcolemma with hypochlorous acid (17, 19), hydrogen peroxide (H_2O_2) (21, 24, 29), and xanthine plus xanthine oxidase (XXO) (28, 32). Furthermore, the XXO-induced decrease in sodium pump activity was prevented by treatment of the sarcolemmal preparations with superoxide dismutase (SOD), either alone or in combination with catalase (CAT) (28). Ischemia and ischemia–reperfusion (I/R) injury also depress the activ-

[1]Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, and Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada.
[2]Department of Internal Medicine, University Hospital Motol, Charles University, Prague, Czech Republic.

ity of the cardiac Na^+,K^+-ATPase (4, 18). As oxidative stress has been shown to occur during development of I/R injury (4), it is likely that the depression of Na^+,K^+-ATPase activity in I/R hearts may be due to oxidative stress. In fact, the decrease in enzyme activity during I/R is attenuated by different oxyradical scavengers and antioxidants such as SOD, CAT, dimethyl sulfoxide, histidine, vitamin E, and allopurinol, and by a combination of these drugs (4, 18).

Information is lacking regarding the status of Na^+,K^+-ATPase isoforms during the development of I/R injury, as well as the mechanisms of I/R-induced depression in Na^+,K^+-ATPase activity. Thus, the present study determined changes in both Na^+,K^+-ATPase activity and isoform contents in hearts subjected to I/R. This study also assessed the possible involvement of reactive oxygen species in the mechanisms underlying alterations in sodium pump isoforms due to I/R. Changes in protein content of Na^+,K^+-ATPase isoforms, as well as Na^+,K^+-ATPase activity due to I/R, were examined in the absence or presence of SOD+CAT, a potent scavenger system for different oxygen species. In addition, hearts were perfused with XXO, a superoxide generating system, or H_2O_2, an active species of oxygen and a potent oxidant, to gain insight about the effects of oxidative stress on Na^+,K^+-ATPase isoforms. To our knowledge, this is the first report showing that the effects of reactive oxygen species mimic the alterations in Na^+,K^+-ATPase isoform content due to I/R. Furthermore, it provides evidence for a protective effect of antioxidants on the I/R-induced changes in Na^+,K^+-ATPase isoforms in the heart.

MATERIALS AND METHODS

Heart perfusion

Male Sprague–Dawley rats (280–330 g) were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). Hearts were quickly excised and perfused via the Langendorf technique with Krebs–Henseleit solution (37°C) containing the following (in m*M*): 120 NaCl, 25 $NaHCO_3$, 11 glucose, 4.7 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, and 1.25 $CaCl_2$. The solution was gassed with 95% O_2/5% CO_2 (pH 7.4) and delivered at 10 ml/min. Hearts were paced at 300 beats/min (Phipps and Bird Stimulator, Richmond, VA, U.S.A.). A water-filled plastic balloon was inserted into the left ventricle and connected to a pressure transducer (model 1050BP, Biopac System, Goleta, CA, U.S.A.) to measure the following parameters; left ventricular systolic and diastolic pressures, the left ventricular developed pressure (LVDP; difference between systolic and diastolic pressures), left ventricular end-diastolic pressure (LVEDP; adjusted to 10 mm Hg at the beginning of the experiment), and the maximum rate of left ventricular pressure development (+dP/dt) and decline (−dP/dt). Data were acquired using Acqknowledge 3.03 software for Windows (Biopac System). All hearts were stabilized for 30 min and maintained in a humidified chamber at 37°C throughout the experiment. This study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication no. 85-32, revised 1996).

Experimental protocol

The protocol for the I/R study is shown in Fig. 1A. All hearts were equilibrated for 30 min. In the control group (CT), hearts were perfused for an additional 90 min; in the ischemia group (I), global ischemia was induced by stopping the perfusion for 30 min; hearts in the I/R group were subjected to 30 min of global ischemia and 60 min of reperfusion; the hearts in the SOD+CAT group (SC) were treated with SOD+CAT for 10 min before ischemia and for 20 min after the start of reperfusion. The final concentrations of SOD (from bovine erythrocytes, ~3,500 U/mg; Sigma–Aldrich, Oakville, ON, Canada) and CAT (from bovine liver, ~25,000 U/mg; Sigma–Aldrich) in the perfusion medium were 5×10^4 and 7.5×10^4 U/L, respectively (31). To study the effect of oxidative stress, hearts were perfused in the absence (CT) and presence of the XXO mixture or H_2O_2 for 20 min (Fig. 1B). Final concentrations of xanthine, xanthine oxidase, (Sigma–Aldrich), and H_2O_2 (Fisher Scientific, Nepean, ON, Canada) were 2 m*M*, 0.03 U/ml, and 300 μ*M*, respectively. A clamp precooled in liquid nitrogen was used to freeze the hearts, which were then stored at −70°C before use.

Isolation of cardiac sarcolemma

Ventricles of three hearts were pooled, and a purified sarcolemmal membrane fraction was isolated according to Pitts

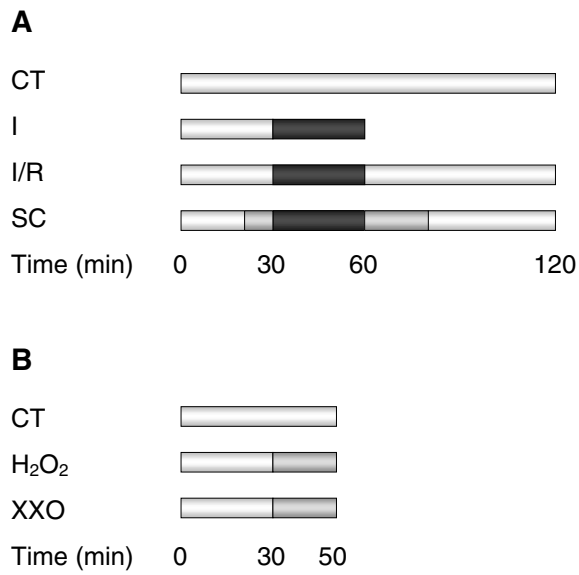

FIG. 1. Experimental protocol for isolated rat hearts perfused under different conditions. All hearts were stabilized for 30 min. (**A**) protocol for I/R study. Controls (C) were perfused for 90 min. In the ischemia group (I), global ischemia (black bars) was induced for 30 min. In the I/R group, global ischemia (30 min) was followed by 60 min of reperfusion. The treated group (SC) was subjected to I/R in the presence of SOD+CAT for 10 min before the start of ischemia and 20 min after the start of reperfusion (gray bars). (**B**) Protocol for the oxidative stress study. Control hearts (CT) were perfused for 20 min. Treated groups were subjected to infusion with 300 μ*M* H_2O_2 or 2 m*M* xanthine plus 0.03 U/ml xanthine oxidase (XXO) for 20 min (gray bars).

(26) as modified by Kaneko *et al.* (16). The final pellet was suspended in 0.25 *M* sucrose/10 m*M* histidine (pH 7.2), quickly frozen, and stored at −70°C. Marker enzyme activities (18–20) revealed a 16–18-fold purification of the membrane with respect to Na^+,K^+-ATPase activity in the heart homogenate and minimal cross-contamination with other subcellular organelles, such as mitochondria, sarcoplasmic reticulum, and myofibrils.

Measurement of ATPase activities

Na^+,K^+-ATPase activity was measured as described previously (8). In brief, sarcolemma (20 μg) was incubated for 5 min at 37°C with 1.0 m*M* EGTA (Tris) (pH 7.4), 5 m*M* NaN_3, 6 m*M* $MgCl_2$, 100 m*M* NaCl, and 10 m*M* KCl. An ATP regenerating system [2.5 m*M* phospho(enol)pyruvate and 10 IU/ml pyruvate kinase] was added to the incubation medium to maintain the ATP concentration. The reaction was initiated by adding 0.025 ml of 80 m*M* Tris-ATP (pH 7.4), and terminated after 5 min by 0.5 ml of cold 12% trichloracetic acid. Liberated phosphate was estimated according to Taussky and Schorr (30). The amounts of Mg^{2+} and ATP required to achieve the final concentration of Mg-ATP in the incubation medium were determined according to the "SPECS" FORTRAN program developed by Fabiato (11). Na^+,K^+-ATPase activity was determined as the difference between the activities with and without Na^+ and K^+, whereas Mg^{2+}-ATPase activity was determined as the difference between the activities with and without Mg^{2+} in the absence of Na^+ and K^+ in the incubation medium (7). Reactions for the measurement of ATPase activities were linear with respect to the amount of sarcolemmal protein used in this study.

Western blot analysis

The relative protein contents of Na^+,K^+-ATPase isoforms in various groups were determined as previously described (10, 17). Sarcolemmal membranes (20 μg of total protein/lane for α_1, α_2, α_3, and β_1; 40 μg of total protein/lane for β_2 and β_3) were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and electroblotted to polyvinylidene difluoride membranes (Boehringer Mannheim GmbH, Mannheim, Germany). Na^+,K^+-ATPase isoforms were detected using the following primary antibodies: monoclonal anti-α_1 mouse IgG (0.05 μg/ml); polyclonal anti-α_2 rabbit IgG (1:1,000); polyclonal anti-α_3 rabbit IgG (1 μg/ml); monoclonal anti-β_1 mouse IgG (0.8 μg/ml); polyclonal anti-β_2 rabbit IgG (1:1,000); and polyclonal anti-β_3 rabbit IgG (1:1,000) (Upstate Biotechnology, Lake Placid, NY, U.S.A.). Secondary antibodies consisted of biotinylated anti-mouse IgG (1:3,000) for α_1 and β_1 (Amersham Life Science, Oakville, ON, Canada) and biotinylated anti-rabbit IgG (1:3,000) for α_2, α_3, β_2 and β_3 (Upstate Biotechnology). Membranes were then incubated for 1 h with streptavidin-conjugated horseradish peroxidase (1:3,000) and processed for chemiluminiscence (ECL kit) on Hyperfilm-ECL (Amersham Life Science). An imaging densitometer (model GS-670, Bio-Rad Laboratories, Mississauga, ON, Canada) was used to scan the bands, which were then quantified using Image Analysis software (version 1.3). The amount of protein used for the western blots was in the linear range for each isoform (10). Gels were stained with Coomassie Blue after blotting, and blots were stained with Ponceau S solution to ensure uniform protein loading in all lanes. A purified microsomal preparation from rat brain (Upstate Biotechnology) was used as a positive control to identify the different Na^+,K^+-ATPase isoforms. Bands were identified based on their molecular weight using prestained standards (Bio-Rad). No standards are commercially available for any Na^+,K^+-ATPase isoform; thus absolute levels of any subunit could not be measured. To calculate the distribution of Na^+,K^+-ATPase isoforms, we determined the percentage of each subunit with respect to the total protein content for the α_1, α_2, α_3, β_1, and β_2 isoforms in each group.

Statistical analyses

All values are expressed as means ± SE. Data were analyzed using a one-way ANOVA followed by an unpaired Student's *t* test, or Dunnett's post-hoc test (for normalized data). $p < 0.05$ was considered significant.

RESULTS

Cardiac performance and ATPase activities

Hearts subjected to global ischemia for 30 min failed to generate LVDP, +dP/dt, and −dP/dt, but showed a marked increase in LVEDP compared with controls. One hour of reperfusion of the previously ischemic hearts resulted in partial recovery of contractile function, as represented by an increase in LVDP, +dP/dt, and −dP/dt. In contrast, LVEDP continued to increase during reperfusion. All contractile parameters were significantly improved, but not normalized, by SOD+CAT treatment (Table 1). The SOD+CAT treatment had no effects on LVDP, LVEDP, +dP/dt, or −dP/dt during the stabilization period. In sarcolemma isolated from the hearts subjected to ischemia, Na^+,K^+-ATPase activity was significantly decreased as compared with control hearts. Whereas further reduction was observed in the I/R group, SOD+CAT treatment led to a significant preservation of sodium pump activity and returned it to control levels (Table 1). Mg^{2+}-ATPase activity was unaffected by ischemia, I/R, or SOD+CAT treatment (Table 1).

To exclude the potential influence of differential purification of sarcolemmal preparations from different groups on Na^+,K^+-ATPase activity, we also measured marker enzyme activities in homogenates. The results in Table 2 show that the depressions in Na^+,K^+-ATPase activities in ischemic and I/R hearts, as well as changes seen upon treatment with SOD+CAT, were not associated with changes in the sarcolemmal yield. The sarcolemmal Na^+,K^+-ATPase activities showed a 16.4-, 15.9-, 15.8-, and 16.1-fold purification in comparison with heart homogenate activity in control, ischemic, I/R, and SOD+CAT-treated I/R hearts, respectively. Mitochondrial and microsomal contamination was negligible because the cytochrome *c* oxidase and rotenone-insensitive NADPH cytochrome *c* reductase activities in the sarcolemmal preparations from control and experimental hearts were low (Table 2). Also, the activities of cytochrome *c* oxidase

TABLE 1. EFFECTS OF I/R ON CARDIAC PERFORMANCE, AS WELL AS ON SARCOLEMMAL Na^+,K^+-ATPASE AND Mg^{2+}-ATPASE ACTIVITIES IN ISOLATED RAT HEARTS PERFUSED WITH OR WITHOUT SOD+CAT TREATMENT

	CT	*I*	*I/R*	*SOD+CAT*
Cardiac performance				
LVDP (mm Hg)	103 ± 13.0	3.7 ± 0.8*	34.7 ± 7.2*†	70.4 ± 4.8*‡
LVEDP (mm Hg)	3.3 ± 1.2	53.4 ± 4.6*	63.0 ± 5.6*	32.3 ± 3.4*‡
+dP/dt (mm Hg/s)	2,243 ± 126	30.4 ± 3.4*	448 ± 83.6*†	1,366 ± 99.8*‡
−dP/dt (mm Hg/s)	1,966 ± 179	31.8 ± 4.6*	271 ± 80.8*†	1,287 ± 144*‡
Enzyme activity (μmol of P_i/mg of protein/h)				
Na^+,K^+-ATPase	18.2 ± 2.1	9.5 ± 1.0*	5.6 ± 1.4*†	17.5 ± 2.0‡
Mg^{2+}-ATPase	44.9 ± 1.0	38.3 ± 4.0	44.2 ± 1.2	47.9 ± 5.6

Values are means ± SE of 12 hearts in each group. LVDP, LVEDP, +dP/dt, and −dP/dt, as well as Na^+,K^+-ATPase and Mg^{2+}-ATPase activities, were measured in control hearts perfused for 2 h (CT), ischemic hearts (I), and hearts subjected to I/R in the absence (I/R) or presence of SOD+CAT.

*$p < 0.05$ vs. control; †$p < 0.05$ vs. I; ‡$p < 0.05$ vs. I/R.

and rotenone-insensitive NADPH cytochrome *c* reductase in the sarcolemmal preparations were 0.25- and 0.32-fold of those in the control heart homogenate; no difference in the purification factor for these enzyme was seen among control, ischemic, I/R, or SOD+CAT-treated I/R hearts.

Effect of I/R and antioxidant treatment on Na^+,K^+-ATPase isoforms

To examine the basis for decreased Na^+,K^+-ATPase activity, western blot analysis was performed to determine the levels of individual isoforms. The α_1, α_2, and α_3 isoform protein levels were significantly reduced in ischemic hearts (by 28%, 31%, and 46%, respectively) and in the I/R group (by 32%, 29%, and 34%, respectively; Fig. 2). SOD+CAT treatment did not prevent the α_1 protein content reduction, but α_2 and α_3 levels were significantly higher than in the I/R group (by 21% and 20%, respectively; Fig. 2). β_1, β_2, and β_3 isoform levels were all significantly depressed in the ischemia group (by 20%, 37%, and 43%, respectively), as well as in the hearts subjected to I/R (by 25%, 55%, and 17%, respectively; Fig. 3). SOD+CAT treatment prevented the decrease of β_1, β_2, and β_3 isoform contents (by 26%, 34%, and 18%, respectively, compared with I/R hearts).

Cardiac performance, ATPase activities, and Na^+,K^+-ATPase isoform protein contents in hearts subjected to oxidative stress

To determine if oxidative stress could mimic the effects of I/R, hearts were exposed to H_2O_2 or XXO under nonischemic conditions. Both interventions induced a significant decrease in LVDP, +dP/dt, and −dP/dt and a significant increase in LVEDP (Table 3). The depression of cardiac function was associated with a marked reduction in Na^+,K^+-ATPase activity, whereas Mg^{2+}-ATPase activity remained unchanged (Table 3). To confirm that the observed changes in cardiac performance and sarcolemmal Na^+,K^+-ATPase activity in hearts perfused with H_2O_2 or XXO were due to oxidative stress, hearts were perfused with H_2O_2 or XXO in the absence or presence of SOD+CAT. The results in Table 4 show that the alterations in LVDP and LVEDP, and sarcolemmal Na^+,K^+-ATPase activity due to H_2O_2 or XXO, were attenuated by SOD+CAT. Furthermore, no difference in the sarcolemmal yield was seen among

TABLE 2. SARCOLEMMAL YIELD AND MARKER ENZYME ACTIVITIES IN PREPARATIONS OBTAINED FROM CONTROL, ISCHEMIA, I/R, AND I/R HEARTS PRETREATED WITH SOD+CAT

	CT	*I*	*I/R*	*SOD+CAT*
Sarcolemmal yield (mg of protein/g of wet heart)	1.31 ± 0.43	1.26 ± 0.35	1.36 ± 0.29	1.35 ± 0.32
Na^+,K^+-ATPase activity (μmol of P_i/mg of protein/h)	19.6 ± 1.7	10.9 ± 0.9*	7.4 ± 1.2*	17.8 ± 2.1†
Cytochrome *c* oxidase activity (nmol of cytochrome *c*/mg of protein/min)	56 ± 3.4	49 ± 4.1	58 ± 3.8	52 ± 2.8
NADPH cytochrome *c* reductase activity (nmol of cytochrome *c*/mg of protein/min)	4.1 ± 0.3	4.4 ± 0.5	4.2 ± 0.4	4.1 ± 0.3

Values are means ± SE of four hearts in each group. The NADPH cytochrome *c* reductase activity was rotenone-insensitive. CT, control; I, ischemia; SOD+CAT, SOD+CAT-treated hearts subjected to I/R.

*$p < 0.05$ vs. CT; †$p < 0.05$ vs. I/R.

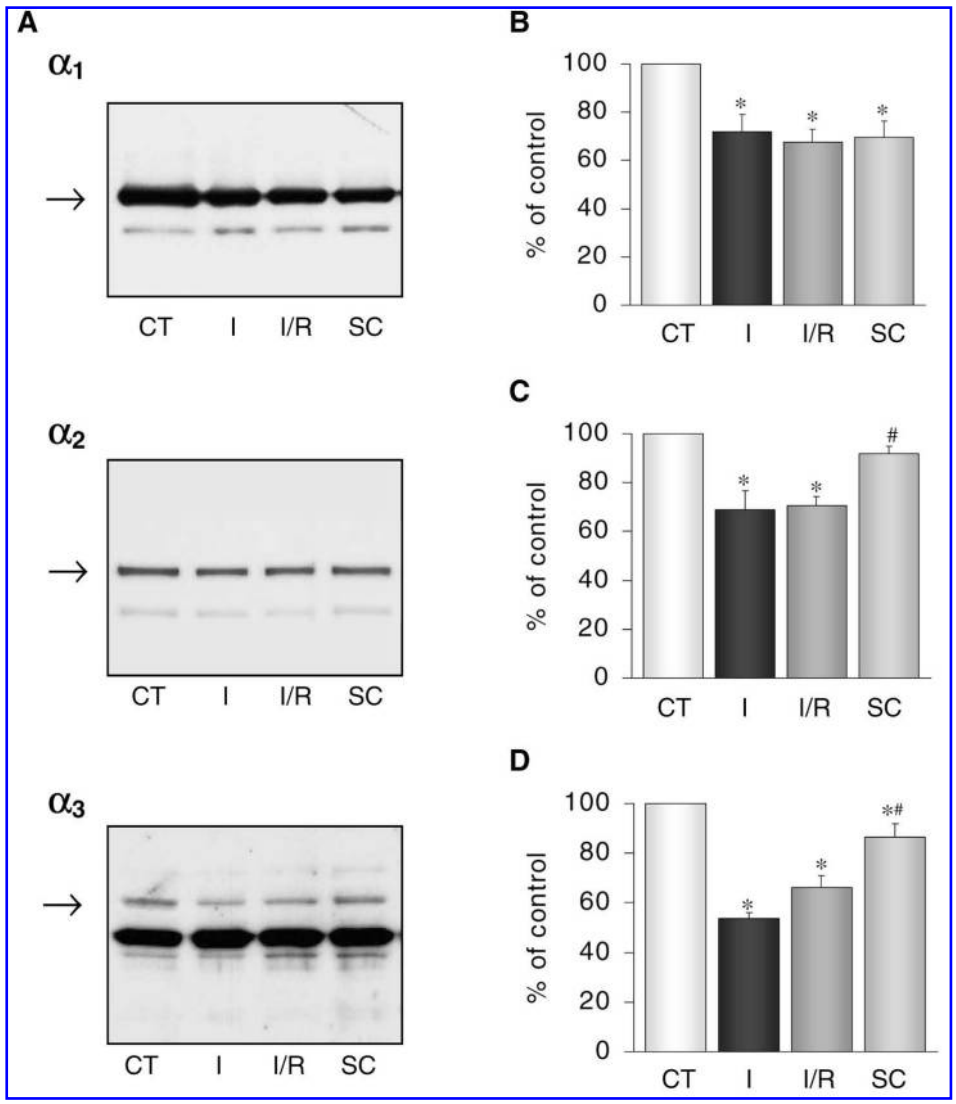

FIG. 2. Effects of I/R in the absence or presence of SOD+CAT on Na^+,K^+-ATPase α isoform protein contents in isolated rat hearts. The figure shows western blot autoradiograms for α_1, α_2, and α_3 isoforms (**A**), α_1 isoform content analysis (**B**), α_2 isoform content analysis (**C**), and α_3 isoform content analysis (**D**) in control hearts (CT) and hearts subjected to ischemia (I), I/R, and SOD+CAT treatment (SC). The α_1, α_2, and α_3 isoforms were located at ~110 kDa. The arrows indicate the isoform band measured. $^*p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. I/R.

TABLE 3. EFFECTS OF H_2O_2 AND XXO STRESS ON CARDIAC PERFORMANCE, AS WELL AS ON SARCOLEMMAL Na^+,K^+-ATPASE AND Mg^{2+}-ATPASE ACTIVITIES

	CT	*H_2O_2*	*XXO*
Cardiac performance			
LVDP (mm Hg)	123 ± 12.6	14.5 ± 1.1*	12.8 ± 1.5*
LVEDP (mm Hg)	1.8 ± 0.5	83 ± 4.6*	84 ± 2.9*
+dP/dt (mm Hg/s)	2,701 ± 286	77 ± 5.6*	66 ± 4.2*
−dP/dt (mm Hg/s)	2,223 ± 169.4	91 ± 5.5*	83 ± 6.6*
ATPase activity (μmol of P_i/mg of protein/h)			
Na^+,K^+-ATPase	18.1 ± 2.1	4.5 ± 1.4*	3.0 ± 1.4*
Mg^{2+}-ATPase	47.0 ± 2.2	39.4 ± 1.2	40.6 ± 2.0

Values are means ± SE of 12 hearts in each group. LVDP, LVEDP, +dP/dt, and −dP/dt, as well as Na^+,K^+-ATPase and Mg^{2+}-ATPase activities, were measured in control hearts perfused for 50 min (CT) and in hearts subjected to 20 min of infusion with H_2O_2 or with XXO.

$^*p < 0.05$ vs. control.

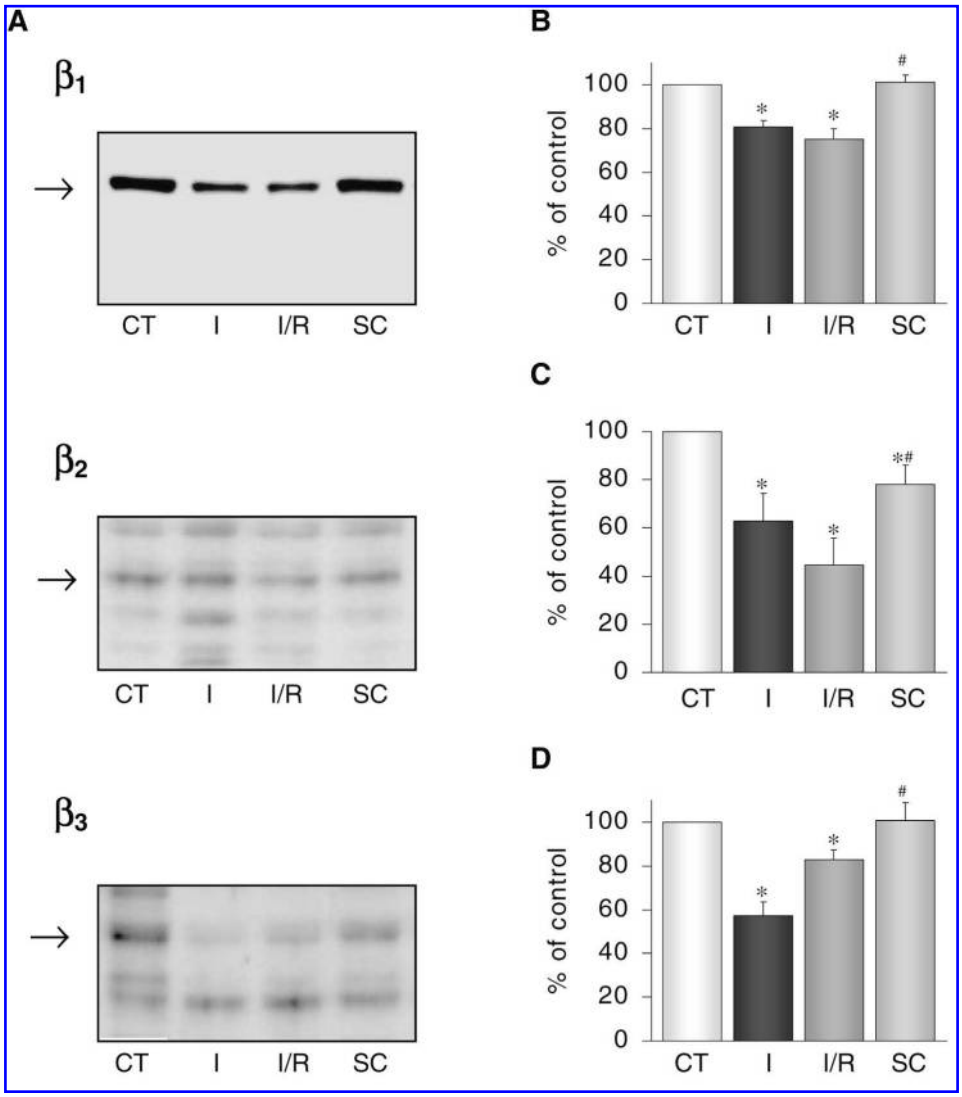

FIG. 3. Effects of I/R in the absence or presence of SOD+CAT on Na+,K+-ATPase β isoform protein contents in isolated rat hearts. The figure shows western blot autoradiograms for β_1, β_2, and β_3 isoforms (**A**), β_1 isoform content analysis (**B**), β_2 isoform content analysis (**C**), and β_3 isoform content analysis (**D**) in control hearts (CT), and hearts subjected to ischemia (I), I/R and SOD+CAT treatment (SC). The β_1 and β_2 bands were located at ~55 and ~45 kDa, respectively and the β_3 band was at ~40 kDa. The arrows indicate the isoform band measured. *$p < 0.05$ vs. control; #$p < 0.05$ vs. I/R.

control and experimental groups (Table 4), indicating that the results are not confounded by differential purification of the sarcolemmal preparation.

To examine whether the depression in sarcolemmal Na+,K+-ATPase activity upon perfusion with H_2O_2 or XXO was associated with changes in the enzyme isoforms, assessment of isoform protein content was performed by western blot analysis. Whereas α_1 was not affected by H_2O_2 and even significantly increased by XXO (by 24%), both α_2 and α_3 levels were decreased in H_2O_2 perfused hearts (by 53 and 68%, respectively) and in the XXO group (by 51% and 67%, respectively; Fig. 4). All β isoforms were significantly depressed by both H_2O_2 and XXO: β_1 by 25% and 16%, respectively, β_2 by 57% and 51%, respectively, and β_3 by 38% and 39%, respectively (Fig. 5).

DISCUSSION

Myocardial ischemia is a serious, life-threatening condition. Thus, early reperfusion would seem a crucial medical target in the treatment of myocardial infarction. However, reperfusion can also induce damage to the myocardium if it is not instituted within a certain time period. In fact, depression of cardiac function as a result of ischemia or I/R is well known from clinical medicine and experimental studies (1, 4). Our results show a significant alteration in contractile parameters in hearts subjected to I/R or the oxyradical generating systems, H_2O_2 and XXO (4, 31). Similarly, our data confirm previous studies (4) showing an improvement in cardiac performance of I/R hearts after treatment with the oxyradical scavenging system, SOD+CAT. The observed decrease in

TABLE 4. ATTENUATION OF H_2O_2- OR XXO-INDUCED CHANGES IN CARDIAC PERFORMANCE AND SARCOLEMMAL Na^+,K^+-ATPASE BY SOD+CAT TREATMENT IN THE ISOLATED RAT HEART

	CT	*H_2O_2*	*SOD+CAT-treated H_2O_2*	*XXO*	*SOD+CAT-treated XXO*
Cardiac performance					
LVDP (mm Hg)	116 ± 7.5	21.2 ± 2.4*	80 ± 4.7†	28.4 ± 3.6*	76 ± 2.5†
LVEDP (mm Hg)	3.1 ± 0.4	75 ± 5.9*	26.1 ± 2.3†	78 ± 4.4*	31.2 ± 2.8†
Sarcolemma					
Yield (mg of protein/g of wet heart)	1.33 ± 0.36	1.34 ± 0.41	1.41 ± 0.33	1.37 ± 0.24	1.40 ± 0.29
Na^+,K^+-ATPase activity (μmol of P_i/mg of protein/h)	18.4 ± 1.7	5.2 ± 1.2*	13.4 ± 1.7†	6.9 ± 0.5*	12.2 ± 1.4†

Values are the means ± SE of four hearts in each group. Control hearts (CT) were perfused for 50 min. After a 30-min period of stabilization, hearts for the H_2O_2 or XXO group were perfused for 20 min with H_2O_2 or XXO. For SOD+CAT groups, hearts were first perfused with SOD+CAT for 10 min and then H_2O_2 or XXO was added in the continued presence of SOD+CAT for 20 min.
*$p < 0.05$ vs. CT; †$p < 0.05$ vs. oxidant alone.

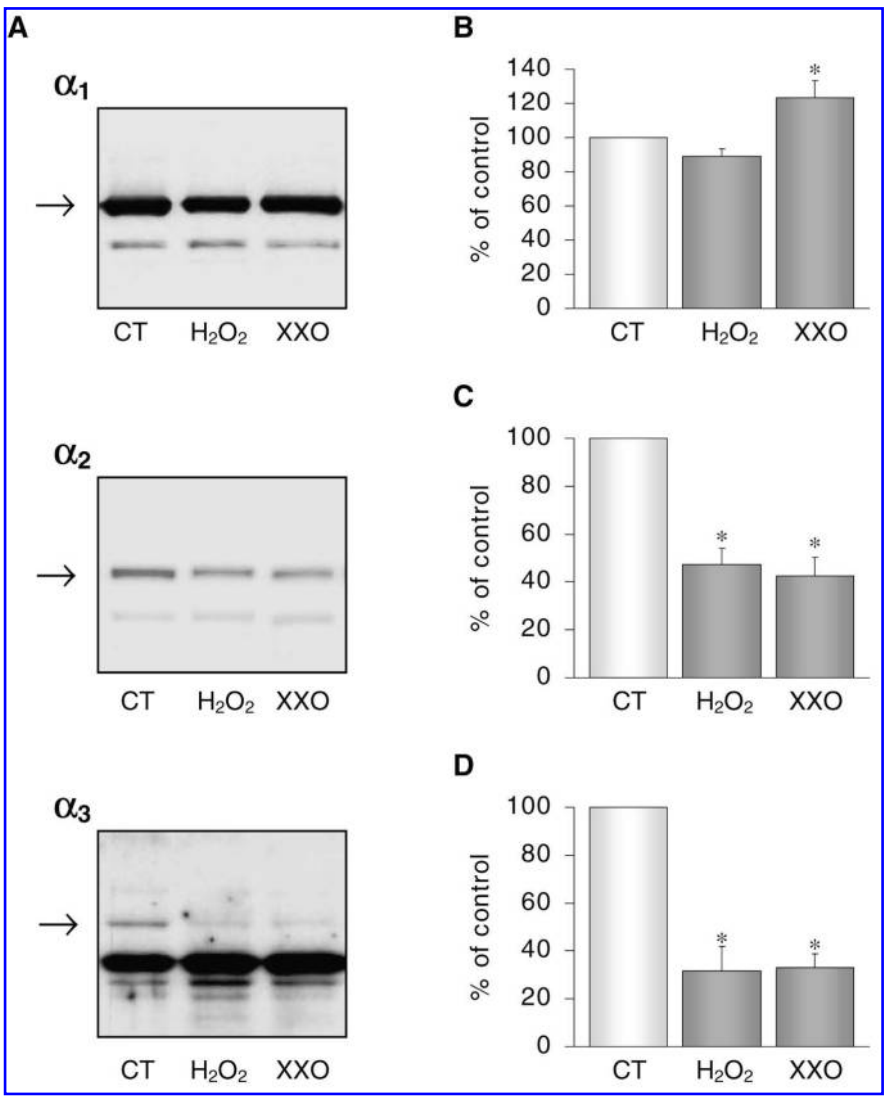

FIG. 4. Effects of H_2O_2 and XXO on Na^+,K^+-ATPase α isoform protein contents in isolated rat hearts. The figure shows western blot autoradiograms for α_1, α_2, and α_3 isoforms (**A**), α_1 isoform content analysis (**B**), α_2 isoform content analysis (**C**), and α_3 isoform content analysis (**D**) in control hearts (CT), hearts treated with H_2O_2, and hearts subjected to treatment with XXO. The α_1, α_2, and α_3 isoforms were located at ~110 kDa. The arrows indicate the isoform band measured. *$p < 0.05$ vs. control.

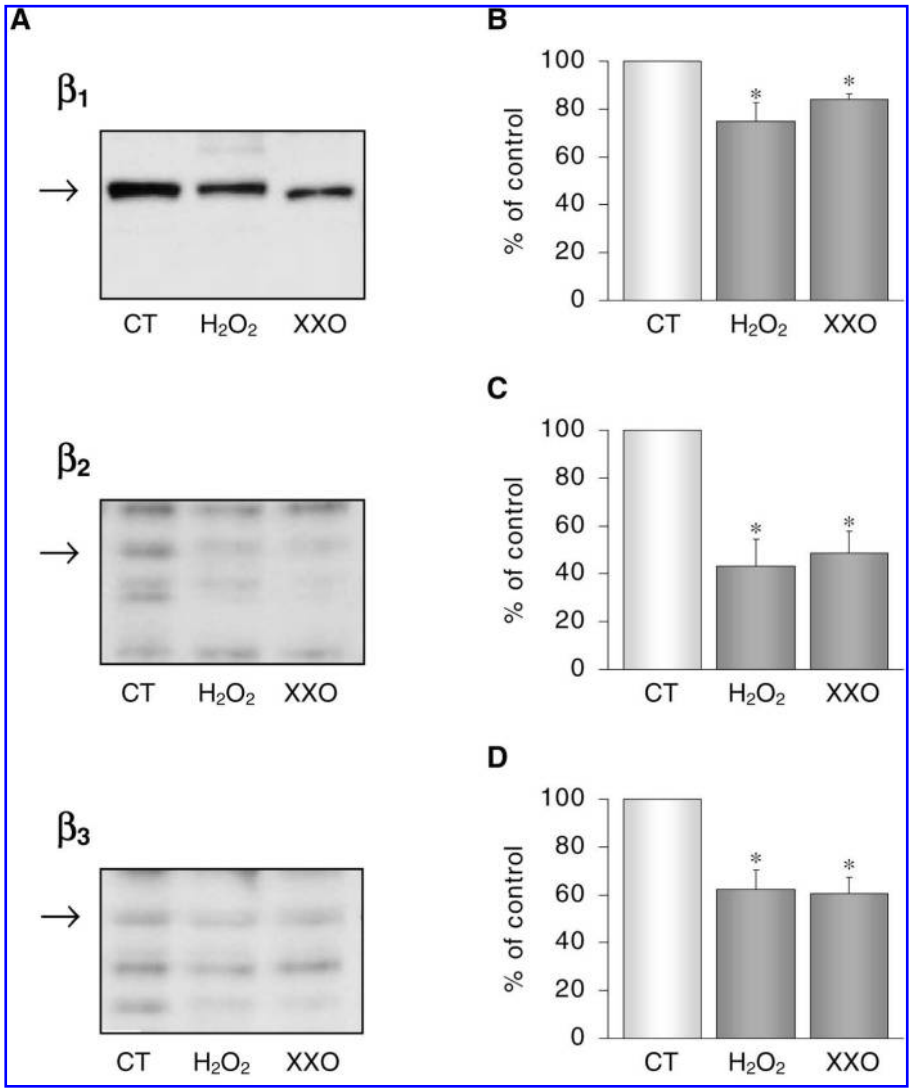

FIG. 5. Effects of H_2O_2 and XXO on oxidative stress on Na^+,K^+-ATPase β isoform protein contents in isolated rat hearts. The figure shows western blot autoradiograms for β_1, β_2, and β_3 isoforms (**A**), β_1 isoform content analysis (**B**), β_2 isoform content analysis (**C**), and β_3 isoform content analysis (**D**) in control hearts (CT), hearts treated with H_2O_2, and hearts subjected to treatment with XXO. The β_1 and β_2 bands were located at ~55 and ~45 kDa, respectively, and the β_3 band was at ~40 kDa. The arrows indicate the isoform band measured. *$p < 0.05$ vs. control.

Na^+,K^+-ATPase activity during ischemia and I/R also agrees with previous reports (3, 4, 10). We, and others, have shown that this effect can be simulated by H_2O_2 and XXO (2, 17), and that SOD+CAT treatment offers nearly complete protection of Na^+,K^+-ATPase activity against I/R-induced damage (4, 18). I/R affected sodium pump activity significantly more than ischemia itself, as has been described previously (4). This is in contrast to the effects on global cardiac function and suggests that the Na^+,K^+-ATPase probably does not participate in the marked recovery of cardiac contractility during early reperfusion of previously ischemic heart. The mechanism underlying the marked depression of Na^+,K^+-ATPase activity under conditions of oxidative stress or I/R is not fully understood. A possible involvement of the oxidation of sulfhydryl groups and lipid peroxidation of sarcolemmal membrane has been suggested (12, 17). Consistent with other reports (2, 3), we did not observe any significant influence on Mg^{2+}-ATPase activity by either ischemia, I/R, or oxyradical generating systems. Thus, Na^+,K^+-ATPase appears to be more susceptible to the effects of these interventions than other membrane protein systems. Our data show similar effects of I/R and oxidants, as well as beneficial effects of SOD+CAT in I/R hearts, and support the theory that oxidative stress is involved in the pathogenic mechanisms leading to I/R-induced contractile dysfunction and suppression of Na^+,K^+-ATPase activity (1, 4). Ischemic preconditioning, which is considered to attenuate oxidative stress, also attenuated the I/R-induced changes in contractile and Na^+,K^+-ATPase activities (10).

We observed that ischemia and I/R reduce the protein content of all α and β Na^+,K^+-ATPase isoforms in the rat heart. However, H_2O_2 and XXO also depressed α_2, α_3, β_2, and β_3; β_1 content was decreased slightly, but still significantly, and α_1 was not affected by H_2O_2 and even markedly increased

with XXO. Moreover, antioxidant treatment with SOD+CAT prevented the depression of all isoforms but α_1. These results suggest that individual Na^+,K^+-ATPase isoforms have markedly different sensitivities to oxygen free radicals. While oxidative stress appears to participate in I/R-induced changes in α_2, α_3, and all β isoforms, α_1 seems to be more resistant to oxygen radicals and oxidants. Thus, pathways not involving oxygen radicals may be involved in I/R-induced changes in the α_1 Na^+,K^+-ATPase isoform. Our study provides data on the effects of ischemia and I/R on all the Na^+,K^+-ATPase isoforms. Another study to date that reported different sensitivities of isoforms to oxidative stress dealt with the β_1 and α isoforms only. Kato *et al.* (17) reported that β_1 exhibits significantly greater sensitivity to hypochlorous acid than α_1 and α_2 in porcine heart sarcolemmal Na^+,K^+-ATPase. Huang *et al.* (15) showed a marked reduction of cardiac α_2 protein levels, but only a marginal reduction of α_1 protein levels as a result of oxidative stress in copper-deficient rat. The same group also reported that α_3 in ferret heart tissue treated with H_2O_2 appears to be significantly more sensitive to oxidative stress than α_1, and rat axolemma α_2 and α_3 have markedly higher sensitivities to H_2O_2 compared with rat kidney α_1 (14). Our results support the findings that α_1 is more resistant to oxidative stress than other isoforms (14, 15, 17). Precise mechanisms of reactive oxygen species-induced changes in the Na^+,K^+-ATPase and the reasons for differences among individual isoforms are still not clear, but different sulfhydryl group content and sulfhydryl group oxidation may be involved (17).

Our finding that the I/R-induced decrease in Na^+,K^+-ATPase activity was more dramatic than the reduction in the level of its isoforms raises the possibility that changes in protein content may not fully explain the inhibition in enzyme activity. However, it can be argued that inhibition of Na^+,K^+-ATPase activity may not depend solely on the changes in one specific isoform, but may be due to changes in multiple isoforms. It is also likely that the observed changes in Na^+,K^+-ATPase isoforms may be a consequence of increased proteolysis, because hearts subjected to I/R or oxidants (XXO or H_2O_2) exhibit intracellular Ca^{2+} overload and activation of proteases (4–6). In fact, we have shown that pretreatment of hearts with leupeptin, a protease inhibitor, attenuates I/R-induced changes in sarcolemmal Na^+,K^+-ATPase isoforms (9). Although I/R produces changes in gene expression for Na^+,K^+-ATPase isoforms in the heart (10), it is an unlikely mechanism for the changes in protein contents due to the short duration of I/R used in our study. Also, the observed changes in all isoforms except the α_1 isoform may explain the depression of Na^+,K^+-ATPase activity in hearts following I/R or H_2O_2 and XXO perfusion, because both the α and β isoforms contribute to enzyme activity. Thus, our studies provide direct and indirect evidence for the role of oxidative stress in I/R-induced alterations in Na^+,K^+-ATPase and its individual isoforms. Furthermore, we showed that antioxidant treatment with SOD+CAT could improve Na^+,K^+-ATPase activity and levels of all isoforms except α_1. Complete recovery of Na^+,K^+-ATPase activity in the I/R hearts after treatment with SOD+CAT was not associated with full recovery of all isoforms. Thus, it appears that optimal enzyme activity may not require the full levels of each isoform (*i.e.,* some isoforms may be present in overabundance). Whereas oxidative stress appears to play an important role in I/R-induced changes in α_2, α_3, and all β isoforms, the α_1 isoform seems to be more resistant to oxygen free radicals, and oxidative stress probably plays only a marginal role in the alteration of this isoform during reperfusion. Therefore, antioxidants may bring advances in management of acute coronary syndromes, as well as ischemia and reperfusion injury linked to cardiac surgery.

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ABBREVIATIONS

CAT, catalase; −dP/dt, maximum rate of left ventricular pressure decline; +dP/dt, maximum rate of left ventricular pressure development; H_2O_2, hydrogen peroxide; I/R, ischemia–reperfusion; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; SOD, superoxide dismutase; XXO, xanthine plus xanthine oxidase.

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Address reprint requests to:
Dr. Naranjan S. Dhalla
Institute of Cardiovascular Sciences
St. Boniface General Hospital Research Centre
351 Tache Avenue
Winnipeg, Manitoba, R2H 2A6 Canada

E-mail: nsdhalla@sbrc.ca

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