Perindopril Regulates β-Agonist-Induced Cardiac Apoptosis

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Abstract: Administration of the β-adrenergic agonist isoproterenol results in cardiac apoptosis. The effect of short-term β-adrenergic stimulation by isoproterenol on the activity of plasma, lung, and left ventricular (LV) angiotensin I–converting enzyme (ACE) activity and its association with the development of cardiac apoptosis was investigated. β-Adrenergic stimulation for 24 hours produced an early increase only in the proapoptotic proteins bax and bcl-XS without changes in the levels of the antiapoptotic protein bcl-XL. The ratio between these bcl family proteins was indicative of apoptosis and correlated with an early and significant increase (300%) in DNA laddering. However, after 5 days of the β-adrenergic stimulation, the ratio changed in favor of antiapoptotic proteins and correlated with the absence of DNA fragmentation. In addition, LV and plasma ACE activities increased markedly with isoproterenol over the study period up to 5 days. ACE activity also regulated expression of the antiapoptotic gene *bcl-XL*. The administration of perindopril (an ACE inhibitor) prevented the observed increase in bax and bcl-XS levels and attenuated (50% decrease, $P < 0.05$) the effect of isoproterenol on DNA fragmentation. Thus, early and transient cardiac apoptosis triggered by the β-adrenergic agonist isoproterenol is reversed in the presence of perindopril.

Key Words: apoptosis, ACE inhibitor, β-adrenergic agonist, heart

Cells often respond to environmental insults by initiating apoptosis.[1] Hallmarks of apoptosis include morphologic and molecular alterations, such as cell shrinkage, membrane blebbing, changes in the intracellular levels of pro- and antiapoptotic bcl-2 family proteins, activation of different caspases, and chromatin condensation.[2–4] Ultimately, DNA fragmentation is observed, and apoptotic cell death ensues.[4,5] Cardiac myocytes are essential, nonproliferating cells that die during myocardial infarction and chronic heart failure by a variety of mechanisms including apoptosis.[6–8] Although little is known of the molecular mechanisms regulating apoptosis in cardiac tissue, it has been suggested that proteins of the bcl-2 family play an important role.[8] Bcl-2 family members are divided into 2 categories, depending on whether they are considered as pro- or antiapoptotic proteins. Bcl-XL is considered the prototype of antiapoptotic family members, whereas bax or bcl-XS expression promotes apoptosis. Bcl-2 family proteins typically form homo- or heterodimers via the bcl homology domains, whereby the composition of these dimers determines cell susceptibility to induction of apoptosis. An increase or decrease in the ratio of bax or bcl-XS to bcl-XL determines whether a cell is more likely to undergo apoptosis or survive in response to a given stimulus.

The renin–angiotensin–aldosterone system (RAAS) controls many functions of the cardiovascular system. The most important hormone of the RAAS is angiotensin II (Ang II), which reportedly promotes apoptosis in vivo in cardiomyocytes and in vitro model systems.[9–12] Ang II exposure increases the bax protein levels without changing bcl-2 and decreases bad phosphorylation in cardiac myocytes.[13] Also, decreases in the bax/bcl-2 ratio have been observed in hearts exposed to ischemia-reperfusion after AT1 receptor blockage.[14] Another important component of RAAS is angiotensin I–converting enzyme (ACE). Inhibition of ACE decreases cardiomyocyte apoptosis induced by ischemia-reperfusion[15] and chronic heart failure.[16] In addition, in spontaneously hypertensive rats, increased plasma ACE activity correlates with cardiac apoptosis.[17,18] Interestingly, ACE activity has been implicated in regulation of the expression of proteins involved in both survival and apoptosis.[19] These findings raise the possibility that RAAS may affect the expression of bcl-2 family members as part of the apoptotic mechanism. Whether or not ACE inhibition regulates cardiac apoptosis induced by β-adrenergic stimulation is still unclear.

In the present study, we have investigated the effect of short-term β-adrenergic stimulation by isoproterenol on the plasma, lung, and cardiac ACE activity and its association with the onset of cardiac apoptosis. We have also analyzed whether ACE inhibition by perindopril regulated the expression of bcl-2 family members and the induction of apoptosis in cardiac cells.

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MATERIALS AND METHODS

Animals and Experimental Treatments

All the experiments were performed following the recommendations of the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication 85-23, revised 1985). Male Sprague-Dawley rats (80–100 g) were kept under conditions of constant temperature and exposed to 12-hour dark–light cycles. Isoproterenol (ISO, 5 mg/kg per day dissolved in 0.9% saline medium, Sigma Chemical Co, St Louis, MO) was injected SC on days 1, 5, and 10; control animals were given similar volumes of saline.[20] The ACE inhibitor perindopril (Per, 4 mg/kg, dissolved in 0.9% saline) was administrated orally once a day by gastric gavage,[21] from 2 days before isoproterenol injection until the end of the experimental period. Controls were given similar volumes of saline.

Animals were killed 24 hours after the last ISO injection. On the day of the experiment, perindopril was administrated 2 hours before rats were killed by decapitation. Hearts were rapidly removed and washed in cold 0.9% saline. After heart removal, LV was separated, weighed, and then processed for various analyses.

Determination of ACE Activity

Plasma, lung, and LV ACE activities were assayed by following the release of His-Leu from the synthetic substrate Z–Phe-His-Leu with or without enalapril as described previously.[22] A fraction of the homogenized sample or plasma was incubated with Z-Phe-His-Leu (Bachem Bioscience Inc, King of Prussia, PA) for 20 minutes (for lung and plasma) or 45 minutes (for LV) at 37°C in a shaking water bath. The reaction was stopped by adding 100 μL of ice-cold 10% TCA and 280 mM NaOH. Phthalaldehyde (0.1%) was then added to the samples and incubated for 10 minutes at 37°C before reaction was stopped with 3 M HCl. The fluorescence at 486 nm was measured following excitation at 364 nm (Fluorescence Spectrophotometer, Perkin Elmer). Plasma and tissue ACE activities were expressed as units per milliliter of plasma or per milligram of protein at 37°C, respectively. 1 U is nanomoles His-Leu produced per minute.

Determination of Bax, Bcl-XS, and Bcl-XL Protein Levels

LV samples were homogenized in cold lysis buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 100 μg/mL PMSF, 0.5 mM DTT, 0.1 mM Na_3VO_4, 1% Triton X-100, 2 μg/mL leupeptin, and 2 μg/mL aprotinin). The homogenates were centrifuged at 10,000 × *g* for 10 minutes, and supernatants were mixed with denaturing electrophoresis sample buffer and boiled. The protein content was determined by the Bradford assay[23] (Bio-Rad). Protein samples (50 μg) were separated by SDS-PAGE on 15% minigels (Bio-Rad) and transferred to nitrocellulose. Membranes were blocked with 3.5% nonfat dry milk and exposed to either rabbit polyclonal anti-rat bax (21 kDa) or rabbit polyclonal anti-rat bcl-X (Santa Cruz), which recognizes bcl-XS (26 kDa) and bcl-XL (30 kDa). Bound antibodies were detected by peroxidase-conjugated anti-rabbit IgG using an ECL System. Blots were quantified by laser scanning densitometry. Results were expressed as the ratio of protein present in samples following stimulation to control samples. All blots were controlled for equal loading by staining membranes with Ponceau Red.

Determination of DNA Fragmentation

LV was homogenized in 0.8 mM EDTA, 8 mM Tris-HCl (pH 8.0), and 4% SDS. The DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and then incubated with proteinase K (50 μg/mL) for 1 hour at 50°C. DNA was reextracted, precipitated overnight with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ice-cold ethanol followed by centrifugation at 12,000 × *g* for 15 minutes at 4°C. Pellets were resuspended in 200 μL TE buffer followed by 1 hour of incubation with DNase-free RNase A (2 mg/mL) at 37°C. Samples were reextracted, and DNA was precipitated as described above. Pellets were resuspended in TE buffer and DNA samples and then were quantified at 260 nm. Samples (20 μg) were separated by electrophoresis on 2% agarose gels and then stained with a solution containing 0.2 μg/mL ethidium bromide.

Determination of Bax and Bcl-XL mRNA Levels

Total RNA was extracted from LV using Trizol Reagent® (Gibco-BRL, Gaithersburg, MD). RNA pellets were suspended in distilled water, and the integrity of 18S and 28S RNAs was assessed after electrophoresis in 1% agarose and staining with ethidium bromide. RNA concentrations were quantified by UV spectroscopy at 260 nm, assuming 40 μg/mL for 1 absorbance unit. Reverse transcriptase polymerase chain reaction (RT-PCR) was employed to evaluate changes in the mRNA levels of *bax* and *bcl-X* genes using specific primers (RT-PCR), as previously described.[24] Briefly, 5 μg of total RNA was treated with RNase-free DNase RQ1 (Promega) to eliminate genomic DNA contamination. Reverse transcription was performed using random primers (Invitrogen, Life Technology, NY) and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, Life Technology, NY). RNA was replaced with H_2O as a negative control. The resulting cDNA was then amplified using 0.5 μM of specific primers, 1.6 mM dNTPs, 1.5 mM $MgCl_2$, and 1.5 U Taq polymerase (Life Technology, Gaithersburg, MD). Oligonucleotide primers for rat *bax, bcl-X*, and β-*actin* genes were designed using published cDNA sequences as follows: bax, 5′-GGTTTCATCCAGGATCGAGCA-3′ (sense) and 5′-TGATGGTTCTGATCAGCTCGG-3′ (antisense); bcl-X, 5′-GTCTCAGAGCAACCGGGAGCT-3′ (sense) and 5′-GTGTCTGGTCACTTCCGACTG-3′ (antisense); β-actin: 5′-GCATTGTAACCAACTGGGACG-3′ (sense) and 5′-CATGAGGTAGTCTGTCAGGTC-3′ (antisense). Portions of each cDNA sample were then amplified by PCR in a final volume of 50 μL. Amplification conditions were: denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds, and primer extension at 72°C for 60 seconds for 25 cycles for bax, 30 cycles for bcl-X, and 20 cycles for α-actin, with a final elongation step at 72°C for 10 minutes. The PCR amplification products were fractionated by electrophoresis in 1.5% agarose gel and detected by staining with ethidium bromide. Band

intensities were quantified by computerized densitometry. The bax and bcl-XL mRNA relative levels were normalized with respect to the α-actin mRNA levels.

Statistical Analysis

All data are presented as means ± SEM. The differences in each parameter were evaluated by a 1-way ANOVA of the increase or decrease of each variable measured. One-way ANOVA was followed by the Tukey test to compare the effect of different conditions on these parameters. Significance was accepted at $P < 0.05$.

RESULTS

Effect of Isoproterenol on Bax, Bcl-XS, and Bcl-XL Protein Levels

Bax, bcl-XS, and bcl-XL protein levels were determined in control and ISO-treated rats (Fig. 1). At day 1, levels of the proapoptotic proteins bax and bcl-XS were significantly higher (30%, $P < 0.05$ and 40%, $P < 0.01$, respectively) than those in control animals (Fig. 1A,B). By day 5, bax and bcl-XS protein levels in the rats treated with ISO were similar to those in controls. At day 10, only the bcl-XS protein levels in the rats treated with ISO were higher than control levels (40%, $P < 0.01$). A significant increase (100%, $P < 0.01$) in bcl-XL protein levels was observed only at day 5 in the ISO-treated animals with respect to the control group (Fig. 1C). In addition, the bax/bcl-XL ratio was compared in control and ISO-treated groups. As shown in Figure 1D, the bax/bcl-XL ratio increased by 44% after 1 day of ISO treatment ($P < 0.01$) and decreased to 46% of controls levels after 5 days of treatment ($P < 0.01$), whereas no change was apparent after 10 days.

Effect of Isoproterenol on DNA Fragmentation

DNA cleavage into nucleosome-sized fragments is a hallmark of apoptosis. An early (day 1) and significant increase (300%, $P < 0.01$) in DNA laddering was observed in the ISO-treated group as compared with control animals. However, after 5 and 10 days of ISO exposure, there was no difference in DNA fragmentation with respect to the control groups (Fig. 2).

Effect of Isoproterenol on Plasma, Lung, and Left Ventricular ACE Activities

The effects of ISO on plasma, lung, and LV ACE activities were investigated (Fig. 3). Plasma ACE activity in ISO-treated animals was significantly higher than that in saline-treated animals (115%, $P < 0.01$) throughout the entire treatment (Fig. 3A). Lung ACE activity (Fig. 3B) increased significantly after 5 and 10 days of treatment with ISO (105% and 80%, respectively, both $P < 0.01$). LV ACE activity increased markedly from day 1 through day 10 of ISO injection ($P < 0.01$, Fig. 3C).

Effect of ACE Inhibition on Isoproterenol-Induced ACE Activity

Because cardiac apoptosis detected at day 1 after ISO treatment (Figs. 1 and 2) correlated with an increase in plasma ACE and LV activity (Fig. 3), we explored whether ACE inhibition by perindopril regulated cardiac apoptosis induced by ISO. To test this, animals were pretreated with the ACE inhibitor perindopril before ISO injections. As shown in Figure 4, perindopril effectively inhibited (by 50%, $P < 0.01$) plasma ACE activity in rats treated with ISO as compared with control animals.

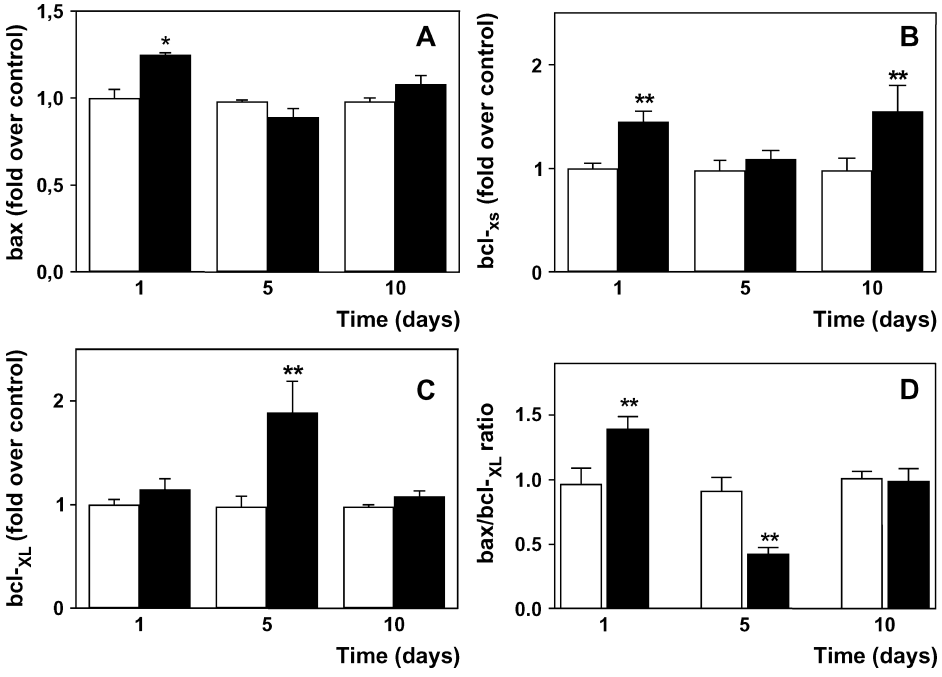

FIGURE 1. Effect of β-adrenergic stimulation on left ventricular proapoptotic and antiapoptotic protein levels of the bcl-2 family. Rats were injected SC with saline (open bars) or 5 mg/kg per day isoproterenol (black bars) for 1, 5, or 10 days. Rats were killed 24 hours after the last injection, and left ventricle extracts were prepared. Bax (A), bcl-XS (B), and bcl-XL (C) protein levels were determined by Western blotting as indicated in Materials and Methods. Panel D represents the bax/bcl-XL ratio. Results are means ± SEM (n = 3–7). *$P <$ 0.05 and **$P < 0.01$ versus controls.

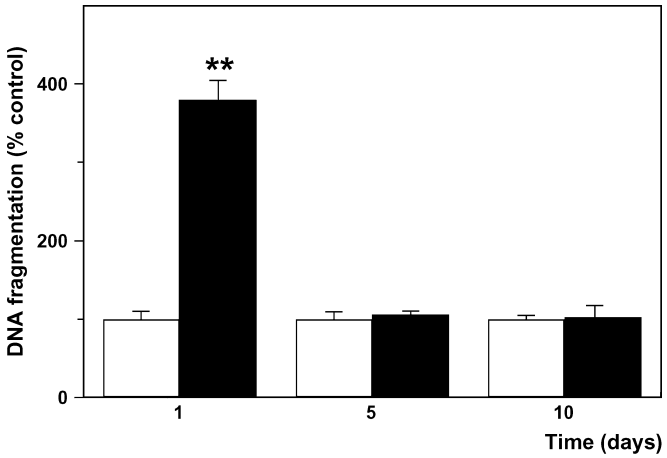

FIGURE 2. Effect of β-adrenergic stimulation on left ventricular DNA fragmentation. Rats were injected SC with saline (open bars) or isoproterenol 5 mg/kg per day (black bars) for 1, 5, or 10 days. Rats were killed 24 hours after the last injection, and DNA was isolated from left ventricles and analyzed by agarose gel electrophoresis as indicated in Materials and Methods. Results are means ± SEM (n = 4–9). $^{**}P < 0.01$ versus controls.

Effect of ACE Inhibition on Isoproterenol-Induced Cardiac Apoptosis

Cardiac apoptosis was detected only at day 1 after ISO treatment (Figs. 1 and 2). To test the hypothesis that ACE inhibition regulates the initiation of apoptosis, perindopril was administered before ISO treatment. Figure 5A–C shows that at day 1, perindopril significantly attenuated the increase in bax and bcl-XS protein levels induced by ISO. Perindopril alone also decreased bax protein levels to below the basal level (Fig. 5A). At day 5 (Fig. 5C), there was a significant increment in bcl-XL mRNA level in ISO-treated animals relative to controls (95%, $P < 0.01$). Perindopril did, however, attenuate the ISO-induced increase in bcl-XL level (32% $P < 0.05$). Also, perindopril treatment ameliorated significantly (50% decrease, $P < 0.05$) the effect of ISO on DNA fragmentation (Fig. 5D).

Effect of ACE Inhibition on Isoproterenol-Induced Bax, Bcl-XS, and Bcl-XL mRNA Levels

To establish whether or not the observed effect of perindopril on bcl-2 family protein levels is related to transcriptional regulation, we also evaluated the changes in bax, bcl-XS, and bcl-XL mRNA levels. At day 1, ISO treatment significantly increased bax mRNA (100%, $P < 0.05$) relative to control values, and perindopril presence did not prevent the increase in bax mRNA following exposure to ISO (Fig. 6A). Bcl-XS mRNA levels also did not change under any of experimental conditions (Fig. 6B). At day 5, a significant increment in bcl-XL mRNA level in ISO-treated animals relative to controls (100%, $P < 0.01$) was observed (Fig. 6C). Perindopril did, however, attenuate the ISO-induced increase in bcl-XL mRNA level (46%, $P < 0.05$).

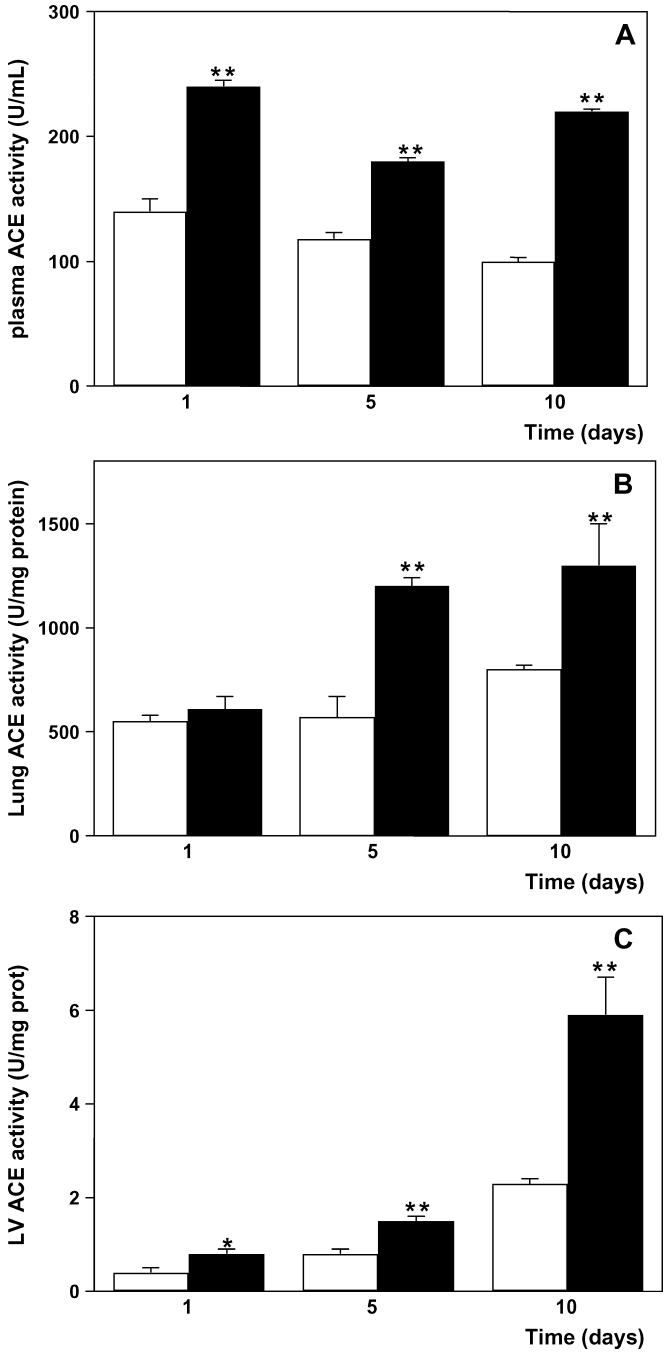

FIGURE 3. Time course of the effect of β-adrenergic stimulation on the activity of angiotensin-converting enzyme (ACE) in plasma, lung, and left ventricle. Rats were injected SC with saline (open bar) or 5 mg/kg/d isoproterenol (ISO, black bar) for 1, 5, and 10 days. Rats were killed 24 hours after the last injection, and plasma (A), lung (B), and left ventricle (C) ACE activities were determined as described in Materials and Methods. Results are means ± SEM from 5–13 rats. $^{**}P < 0.01$ versus saline.

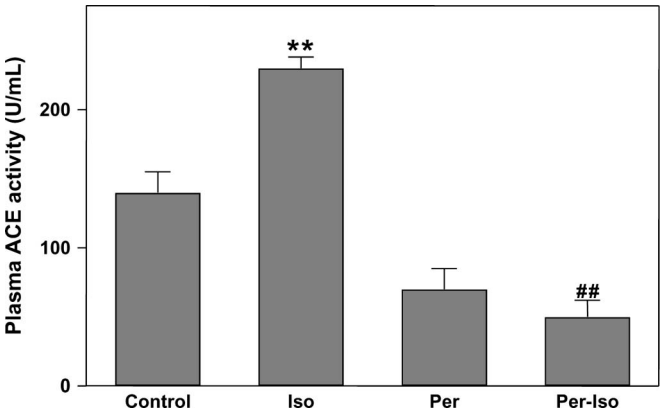

FIGURE 4. Effect of ACE inhibition on plasma ACE activity. Perindopril (Per) 4 mg/kg or saline (control) was administered orally once a day by gastric gavage beginning 2 days before treatment with isoproterenol (ISO; 5 mg/kg per day) for 1 day. Plasma ACE activities were determined 24 hours after the isoproterenol treatment as described in Materials and Methods. Results are means ± SEM from 4–6 rats. $^{**}P < 0.05$ versus control and $^{\#\#}P < 0.01$ versus ISO.

DISCUSSION

The main findings of the present study are that perindopril inhibited early and transient cardiac apoptosis triggered by β-adrenergic stimulation. Isoproterenol apoptotic effects were associated with increased bax and bcl-XS protein levels. Also, evidence is provided indicating that the ACE inhibitor regulated expression of the antiapoptotic gene bcl-XL.

Our results show that β-adrenergic stimulation by ISO causes an imbalance between pro- and antiapoptotic proteins and thus triggers cardiac apoptosis. Recently, Goldspink et al showed that ISO induced early and transient apoptosis (3–6 hours) followed by necrosis and an increase in levels of the proapoptotic protein bax.[25] Likewise, early apoptosis has been reported in the initial phase after aortic stenosis.[26] These events were of short duration and clearly preceded the development of cardiac hypertrophy,[26] suggesting that cardiomyocyte apoptosis can be activated by perturbing the redox state. Our results also agree with those of Prabhu et al,[27] who demonstrated that ISO increases myocyte bcl-XS expression via β_1-adrenergic receptor activation. In conclusion, our results indicate that ISO-dependent apoptosis is the consequence of an increase in the ratio between proapoptotic bax and bcl-XS to antiapoptotic bcl-XL proteins.

Subsequently, the effect of ACE inhibitors on ISO-induced apoptosis was analyzed. Perindopril administration 2 days before ISO had no effects on either plasma ACE activity or apoptosis (Figs. 4 and 5). Our data are in agreement with those of Diez et al, who showed that cardiomyocyte apoptosis is related to exaggerated ACE activity in LV of spontaneously hypertensive adult rats.[17] An imbalance between increased Ang II and decreased nitric oxide secondary to an increment in ACE activity may be involved in the induction of cardiac apoptosis by isoproterenol.[28] In a model for ischemia-reperfusion damage to the heart, ischemia-reperfusion was shown to completely suppress bax and bcl-XL protein expression in the center of the ischemic region. However, bcl-XL staining remained unaffected in the ischemic area after ACE inhibitor treatment,[15] suggesting that RAAS may affect the expression of bcl-2 family members as an apoptotic mechanism. Our results showing increased bcl-XL protein levels at day 5 are consistent with the notion that ACE activity may control the expression of bcl-2 family members. The same authors suggested that inhibition of ACE reduces myocardial infarction and apoptosis in part via the bradykinin B_2 receptor. This antiapoptotic effect of ACE inhibitor was attributed to changes in the expression of bcl-XL.[15] Our results suggest that the exposure to isoproterenol promotes apoptosis at early time points, whereas ACE inhibition blocks this effect.

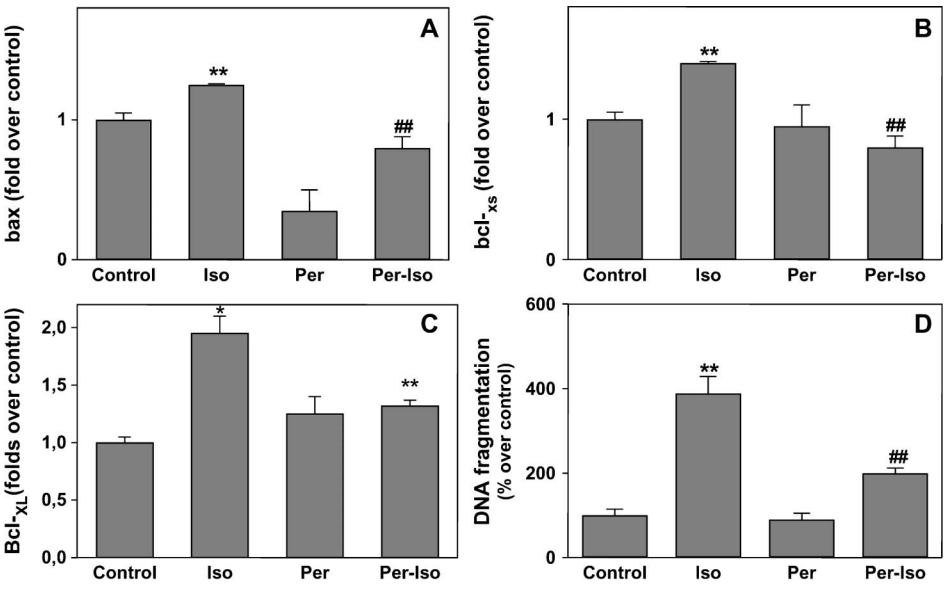

FIGURE 5. Effect of ACE inhibition on left ventricular bcl-2 family protein and DNA fragmentation triggered by isoproterenol. Perindopril (Per) 4 mg/kg or saline (control) was administered orally once a day by gastric gavage beginning 2 days before treatment with isoproterenol (ISO) 5 mg/kg per day for 1 day. Bax (A) and bcl-XS (B) were determined at day 1, and bcl-XL (C) was determined at day 5; protein levels and DNA fragmentation levels (D) were determined 24 hours after the treatment, as described in Materials and Methods. Results are means ± SEM from 3 rats. $^{**}P < 0.05$ versus control and $^{\#}P < 0.05$ versus ISO.

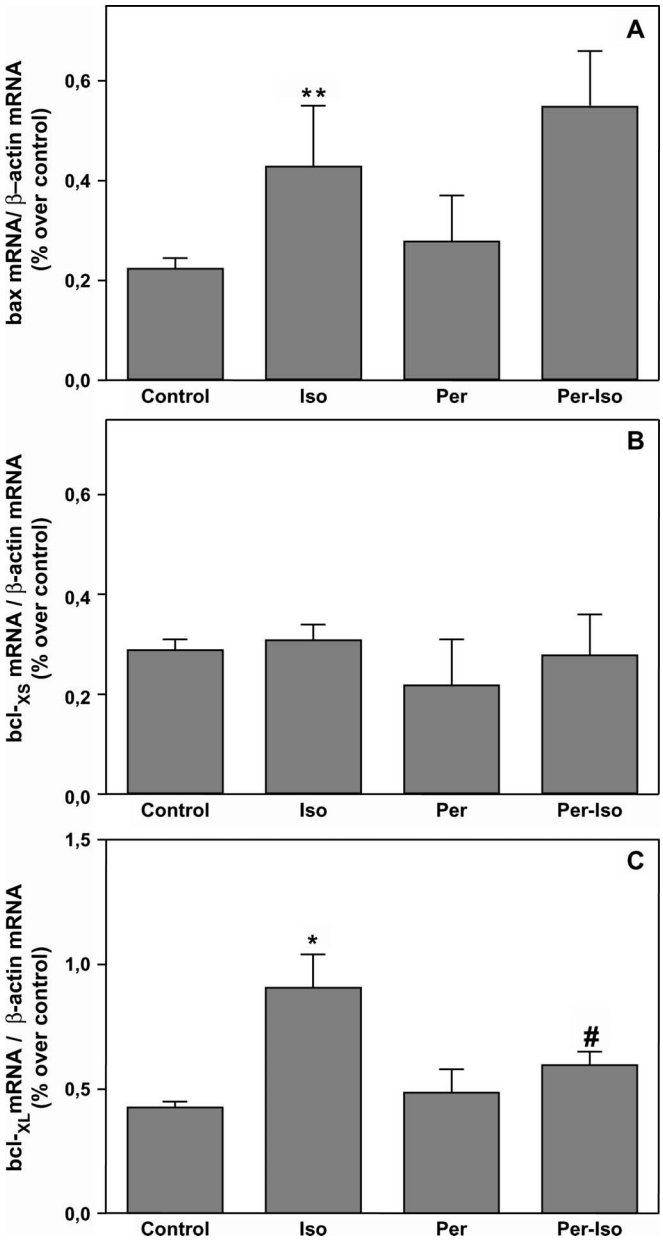

FIGURE 6. Effect of β-adrenergic stimulation and ACE inhibitor on left ventricular proapoptotic and antiapoptotic mRNA levels of Bcl-2 family. Perindopril (Per) 4 mg/kg or saline (control) was administered orally once a day by gastric gavage beginning 2 days before treatment with isoproterenol. Rats were injected SC with saline (open bars) or isoproterenol 5 mg/kg per day (black bars) for 1 (for bax and bcl-XS mRNA) or 5 (for bcl-XL) days. Total RNA was isolated 24 hours after the last injection from left ventricles, and bax, bcl-XS, bcl-XL, and β-actin mRNA levels were determined by reverse transcription (RT)-polymerase chain reaction (PCR) as indicated in Materials and Methods. The bax, bcl-XS, and bcl-XL mRNA relative levels were normalized by comparison with the β-(actin mRNA levels. Results are means ± SEM (n = 5). *$P < 0.05$, **$P < 0.01$ versus controls, and #$P < 0.05$ versus ISO.

In conclusion, our data indicate that ACE inhibitor attenuates early cardiac apoptosis triggered by β-adrenergic stimulation.

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