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Isoflurane Inhibits Cardiac Myocyte Apoptosis during Oxidative and Inflammatory Stress by Activating Akt and Enhancing Bcl-2 Expression

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Background: **Volatile anesthetics attenuate apoptosis. The underlying mechanisms remain undefined. The authors tested whether isoflurane reduces apoptosis in cardiomyocytes subjected to oxidative or inflammatory stress by enhancing Akt and B-cell lymphoma-2 (Bcl-2).**

Methods: **Adult and neonatal rat ventricular myocytes and atrial HL-1 myocytes were exposed to hypoxia, hydrogen peroxide, or neutrophils with or without isoflurane pretreatment. The authors assessed cell damage and investigated apoptosis using mitochondrial cytochrome *c* release, caspase activity, and TUNEL assay. They determined expression of phospho-Akt and Bcl-2 and tested their involvement by blocking phospho-Akt with wortmannin and Bcl-2 with HA14-1.**

Results: **Isoflurane significantly reduced the cell damage and apoptosis induced by hypoxia, H_2O_2, and neutrophils. Isoflurane reduced hypoxia-induced mitochondrial cytochrome *c* release in HL-1 cells by 45 ± 12% and caspase activity by 28 ± 4%; in neonatal cells, it reduced caspase activity by 43 ± 5% and TUNEL-positive cells by 50 ± 2%. Isoflurane attenuated H_2O_2-induced caspase activity in HL-1 cells by 48 ± 16% and TUNEL-positive cells by 78 ± 3%; in neonatal cells, it reduced caspase activity by 30 ± 3% and TUNEL-positive cells by 32 ± 7%. In adult cardiomyocytes exposed to neutrophils, isoflurane decreased both mitochondrial cytochrome *c* and caspase activity by 47 ± 3% and TUNEL-positive cells by 25 ± 4%. Isoflurane enhanced phospho-Akt and Bcl-2 expression. Wortmannin and HA14-1 prevented the action of isoflurane (53 ± 8% and 54 ± 7% apoptotic cells *vs.* 18 ± 1% without blockers).**

Conclusions: **Isoflurane protects cardiomyocytes against apoptosis induced by hypoxia, H_2O_2, or activated neutrophils through Akt activation and increased Bcl-2 expression. This suggests that a reduction in apoptosis contributes to the cardioprotective effects of isoflurane.**

VOLATILE anesthetics, including isoflurane, produce pharmacologic preconditioning against myocardial infarction in experimental models of ischemia–reperfusion injury and also exert important cardioprotective effects in humans with coronary artery disease.[1] These drugs not only reduce cellular necrosis after prolonged coronary artery occlusion and reperfusion but may also attenuate apoptosis in response to ischemia or other forms of tissue injury.[2–4] The mechanisms by which volatile anesthetics reduce apoptosis are unknown. Apoptosis may be initiated by binding of a "death" ligand (*e.g.*, fibroblast-associated [Fas] ligand, tumor necrosis factor α) to a cell surface receptor, stress-induced alterations in B-cell lymphoma-2 (Bcl-2)–related protein homeostasis, or inhibition of the prosurvival phosphatidylinositol-3-kinase/Akt (protein kinase B) signal transduction pathway.[5–7] Our laboratory recently demonstrated that isoflurane produces protective effects during early reperfusion by activating phosphatidylinositol-3-kinase/Akt,[8] and this signaling cascade has also been implicated in hypoxic,[9] ischemic,[10] and pharmacologic preconditioning.[11] The current investigation tested the hypothesis that isoflurane attenuates apoptosis in isolated cardiac myocytes subjected to oxidative or inflammatory stress by activating Akt and enhancing expression of the antiapoptotic protein Bcl-2.

Materials and Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin, Milwaukee, Wisconsin. All conformed to the *Guiding Principles for Research Involving Animals and Human Beings*[12] of the American Physiologic Society and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.[13]

Isolation of Cells

To balance advantages and disadvantages of adult (resemble most closely the adult heart, but difficult to establish as long-term culture), neonatal (most extensively researched, but lacking some characteristics of adult cells), and HL-1 cells (reliable cell culture, but questions arising from the use of a transgenic cell line), we opted for a combination of the tree cell types in our study.

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Neonatal Rat Ventricular Myocytes. One-day-old Sprague-Dawley neonatal rats were decapitated, the hearts were excised, and ventricular myocardium was minced in ADS buffer (116 mM NaCl, 20 mM HEPES, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.6 ml/l phenol red, pH 7.35) with 0.15 mg/ml collagenase (Worthington cls II, Lakewood, NJ) and 0.52 mg/ml pancreatin (Life Technologies Inc., Grand Island, New York) and incubated on a shaker at 37°C for 20 min at 100 rpm. Tissue pieces were allowed to settle, and the supernatant was collected, suspended in 1 ml newborn calf serum (GIBCO, Carlsbad, CA), and centrifuged at 1,000 rpm for 6 min. The cell pellet was resuspended in 1 ml newborn calf serum and stored at 37°C. The procedure was repeated until all tissue was digested. The cells were then resuspended in Dulbecco's modified essential medium (DMEM) supplemented with 17% medium 199, 10% horse serum, 5% fetal bovine serum (FBS), 0.5% penicillin–streptomycin, and 20 mM HEPES at pH 7.2 and preplated for 2 h on cell culture dishes to separate ventricular myocytes from the faster-attaching nonmyocytes. The ventricular myocytes in the supernatant were collected and plated on gelatin-coated dishes. Cells were used for experiments after demonstrating confluence and rhythmic contractions (48–72 h).

Adult Rat Ventricular Myocytes. Adult male Wistar rats weighing between 150 and 300 g were anesthetized with thiobarbiturate (100 mg/kg, given intraperitoneally). Each heart was rapidly excised and mounted on a Langendorff apparatus. The heart was initially perfused with 11.9 g/l Joklik solution supplemented with 5.55 mM glucose and 23.8 mM NaBic, at pH 7.23. After residual blood was cleared from the heart, 0.1 mg/ml bovine serum albumin (Serologicals, Norcross, GA), 0.25–0.5 mg/ml collagenase type II (GIBCO), and 0.15–0.2 mg/ml protease XIV was added to the perfusion solution. After 20–30 min of perfusion, the ventricles were excised, minced, filtered through a 200-μm mesh, and incubated in Tyrode's solution (132 mM NaCl, 10 mM HEPES, 5 mM dextrose, 4.8 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$) for 5 min at 37°C. The cells were briefly centrifuged twice in Tyrode's solution, suspended in DMEM supplemented with 2 mg/ml bovine serum albumin, 2 mM L-carnitine, 5 mM taurine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, and subsequently plated in cell culture dishes.

HL-1 Cells. HL-1 cells, a cardiac muscle cell line derived from the AT-1 mouse atrial myocyte tumor lineage, were a gift from William C. Claycomb, Ph.D. (Professor of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, Louisiana), and maintained according to his instructions.[14] HL-1 cells were used for experimentation after reaching approximately 80% confluence.

Isolation of Canine Neutrophils. Heparinized dog blood (20 ml) was carefully layered onto 10 ml Ficoll® (Amersham Biosciences, Piscataway, NJ) and centrifuged at 2,300 rpm. The supernatant was aspirated, and the remaining neutrophil and erythrocyte layer was carefully mixed with 5% dextran. The cells were allowed to settle for 30 min, and the supernatant was subsequently centrifuged for 5 min at 1,500 rpm. The remaining erythrocytes were consecutively lysed with ice-cold H_2O and 2.7% NaCl and then centrifuged at 1,500 rpm for 5 min. The neutrophil pellet was suspended in DMEM. The isolation procedures yielded neutrophil suspensions of a purity of greater than 98% (assessed by Giemsa staining) and a viability of greater than 95% (assessed by Trypan blue exclusion). Neutrophils were activated with the chemotactic agent *N*-formyl-methionyl-leucyl-phenylalanine (100 nM) at 37°C for 15 min. Activation was confirmed microscopically through morphologic changes.

Experimental Protocols

Hypoxia. In four separate experimental groups, neonatal rat ventricular myocytes or mouse atrial HL-1 cells received no intervention, exposure to hypoxia–reoxygenation in the presence or absence of pretreatment with isoflurane, or treatment with isoflurane alone. Hypoxia was produced by 4 h of exposure of cells to 5% CO_2 and 95% N_2 in an airtight chamber in the presence of serum- and glucose-free DMEM containing 10 mM deoxyglucose to inhibit glycolysis. Reoxygenation was performed for 12 h in DMEM–10% FBS. Exposure to isoflurane was accomplished by a 30-min incubation of the cells in 0.5 mM isoflurane (approximately 1.0 minimum alveolar concentration, for rats) in DMEM–10% FBS. The isoflurane-containing medium was removed immediately before the onset of hypoxic conditions and cells washed with phosphate-buffered saline (PBS). Cells that received no intervention or isoflurane pretreatment without subsequent hypoxia were maintained in DMEM–10% FBS throughout the duration of the experiments.

Hydrogen Peroxide. In contrast to hypoxia that mimics ischemic conditions, administration of hydrogen peroxide (H_2O_2) reproduces conditions during reperfusion.[15] In eight experimental groups, neonatal rat ventricular myocytes and mouse atrial HL-1 cells received no intervention (control), H_2O_2 (50 μM), or isoflurane with or without subsequent exposure to H_2O_2. Cells were incubated in serum- and glucose-free DMEM containing 50 μM H_2O_2 and 50 μM $FeSO_4$ for 15 min. The culture medium was then changed to DMEM–10% FBS for 8 h. Exposure to isoflurane was performed as described in the protocol for hypoxia.

Activated Neutrophils. Adult rat ventricular myocytes were subjected to inflammatory stress produced by coincubation with activated canine neutrophils. In four experimental groups, myocytes received no intervention, coincubation with neutrophils alone, or isoflurane with or without subsequent coincubation with neutrophils. Myocytes were coincubated with activated neutro-

phils for 8 h, and administration of isoflurane was conducted as described for the hypoxia experiments. Using dog neutrophils instead of rat or mouse neutrophils can be problematic. Conceivable issues could be either an increased reactivity of xenogenic neutrophils[16] or, on the contrary, a decreased reaction due to a diminished recognition of surface receptors of different species.[17] However, various studies have already used this approach and have confirmed its feasibility.[18–21]

Assessment of Cell Membrane Damage and Viability

Lactate Dehydrogenase Release. Lactate dehydrogenase release into the cell culture medium was used as an indicator of membrane damage and measured spectrophotometrically according to the manufacturer's instructions (Diagnostic Chemicals Limited, Oxford, CT) at a wavelength of 340 nm. Enzymatic activity was expressed as percent change from control.

MTT Assay. Cell viability was assessed using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was converted by metabolically active cells to purple formazan. This product was then dissolved in isopropanol and quantified spectrophotometrically at a wavelength of 570 nm.

Evaluation of Apoptosis

Cytochrome *c* Release. Release of mitochondrial cytochrome *c* into the cytosol was assessed as an early indicator of apoptosis.[6] Cells were lifted from incubation plates at the conclusion of each experiment, and protein was extracted and differentially centrifuged using previously described methods.[22] Protein concentration was determined by the Lowry method (Biorad, Hercules, CA). Cytosolic protein (50 μg) was loaded on a polyacrylamide gel (Biorad Readygel Tris-HCl 4–15%) and separated by electrophoresis (100 mV for 1 h). The protein was transferred onto a nitrocellulose membrane (Biorad). Equal loading was confirmed using Ponceau S staining (Biorad). The blots were blocked in Tris-buffered saline–5% nonfat dry milk–0.5% Tween-20 for 1 h at 20°C and incubated with mouse cytochrome *c* immunoglobulin G_1 antibody (BD Biosciences, San Jose, CA) at a dilution of 1:500 in Tris-buffered saline–5% nonfat dry milk overnight at 4°C. After washing in Tris-buffered saline–0.5% Tween-20, the blot was coincubated with a 1:10,000 dilution of horseradish peroxidase–conjugated secondary anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in Tris-buffered saline–5% nonfat dry milk for 1 h. Bands were visualized by enhanced chemiluminescence (Amersham) on radiographic film and analyzed by densitometry.

Caspase Activity. Caspase activity was assessed using an *in situ* caspase detection kit (CaspaTag Caspase-3,7 *in situ* assay kit; Chemicon International, Temecula, CA) according to the manufacturer's instructions. Briefly, a sulforhodamine-labeled fluoromethyl ketone peptide inhibitor of caspases 3 and 7 (SR-DEVD-FMK) was used to covalently bind active caspases. Neonatal rat ventricular myocytes and mouse HL-1 cells were plated on laminin-coated cover slips. Adult rat ventricular myocytes remained in suspension. Nuclear DNA was counterstained with TO-PRO-3 (Molecular Probes, Eugene, OR). Images were visualized using confocal microscopy (Nikon Eclipse TE 200-U microscope with EZ C1 laser scanning software, Melville, NY) using excitation wavelengths of 543 and 633 nm and emission wavelengths of greater than 580 and 661 nm for sulforhodamine and TO-PRO-3, respectively. Sulforhodamine-positive cells were counted in eight microscopic fields at 20× magnification.

TUNEL Assay. Terminal deoxynucleotidyl transferase labels the 3′-OH terminal of DNA strand breaks that occur in apoptotic cells. Neonatal rat ventricular myocytes and mouse HL-1 cells were plated on laminin-coated cover slips. Adult rat ventricular myocytes remained in suspension for the experiments and were subsequently allowed to dry on microscope slides. All cells were fixed in 1% paraformaldehyde and postfixed in ethanol–acetic acid. TUNEL staining was performed according to the manufacturer's specifications (ApopTag® Apoptosis Detection Kit; Serologicals Corporation, Norcross, GA). Apoptotic bodies were visualized with fluorescein isothiocyanate. Images were visualized using a confocal microscope with excitation and emission wavelengths of 488 and 519 nm, respectively. Nuclear DNA was counterstained using TO-PRO-3 as described above. TUNEL-positive cells were counted in eight microscopic fields at 20× magnification. To confirm the involvement of the PI3Kinase/Akt pathway and Bcl-2 in isoflurane preconditioning, 100 μM wortmannin (a blocker of Akt phosphorylation;[23] Biomol International LP, Plymouth Meeting, PA) or 50 μM HA14-1 (ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate, a blocker of Bcl-2;[24] Sigma-Aldrich, St. Louis, MO) was added into the cell culture medium of neonatal cells for 15 min before and during administration of isoflurane and hypoxia.

Immunohistochemistry and Western Blotting of Phospho-Akt and Bcl-2

Neonatal rat ventricular myocytes were plated on laminin-coated cover slips, and experiments with hypoxia and H_2O_2 were performed as described above. Cells were fixed in 4% paraformaldehyde, permeabilized in either 1% TritonX-100 (Sigma-Aldrich) or methanol (−20°C), and incubated for 30 min at 37°C with primary monoclonal antibodies phospho-Akt (Ser 473; Cell Signaling, Beverly, MA) at a dilution of 1:200 in PBS or Bcl-2 (Chemicon) at a dilution of 1:100 in PBS. Incubation with corresponding biotinylated secondary antibodies (Santa Cruz Biotechnology; 1:200 dilution) was con-

ducted for 30 min at 37°C. After washing with PBS, cells were incubated for 15 min at 37°C with 1:500 fluorescein isothiocyanate followed by 1:1,000 TO-PRO-3 for 5 min at room temperature, washed again with PBS, and mounted on microscopic slides. Images were visualized using confocal microscopy as described above. Total fluorescence for phospho-Akt, Bcl-2, and TO-PRO-3 was measured with fixed intensity thresholds. Changes in fluorescence were quantified as the percent change in relation to the total number of cells.

For Western blotting, cells were lifted from incubation plates at the conclusion of each experiment, and protein was extracted following a protocol published by Weiland *et al.*[25] for the analysis of Bcl-2 and following Krieg *et al.*[26] for phospho-Akt. Western blotting was performed as described above for cytochrome *c* using primary antibodies against phospho-Akt or Bcl-2 with corresponding horseradish peroxidase–conjugated secondary antibodies.

Statistical Analysis

Statistical analysis of data within and between groups was performed with analysis of variance followed by Student-Newman-Keuls test. Data are expressed as mean ± SD and were considered statistically significant at $P < 0.05$.

Results

Hypoxia

Exposure to hypoxia significantly ($P < 0.05$) increased lactate dehydrogenase release from and reduced MTT conversion in neonatal rat ventricular myocytes and mouse HL-1 cells (fig. 1). Isoflurane improved these indicators of cell membrane damage and viability in response to hypoxia. Exposure to hypoxia enhanced cytochrome *c* release in mouse HL-1 cells as compared with untreated controls (fig. 2). Treatment with isoflurane did not alter cytochrome *c* translocation, but reduced release of cytochrome *c* in the mouse HL-1 cells subsequently exposed to hypoxia. An increase in the percentage of cells with active caspases 3 and 7 was observed in neonatal rat ventricular myocytes and mouse HL-1 cells exposed to hypoxia. This effect was also attenuated by isoflurane. Similarly, administration of isoflurane before hypoxia reduced the percentage of TUNEL-positive cells as compared with hypoxia alone. Isoflurane in the absence of hypoxia did not alter caspase activity or TUNEL staining.

Hydrogen Peroxide

Similar to hypoxia, exposure to H_2O_2 increased lactate dehydrogenase release and MTT conversion in neonatal rat ventricular myocytes and mouse HL-1 cells as compared with control experiments. Pretreatment with isoflurane attenuated these H_2O_2-induced changes in markers of cellular injury and viability (data not shown). Caspase 3 and 7 activities increased in neonatal rat ventricular myocytes and mouse HL-1 cells exposed to H_2O_2 as compared with controls (fig. 3). Isoflurane reduced caspase activity in cells exposed to H_2O_2. Similarly, pretreatment with isoflurane decreased the percentage of TUNEL-positive cells resulting from H_2O_2 exposure as compared with those that did not receive the volatile agent.

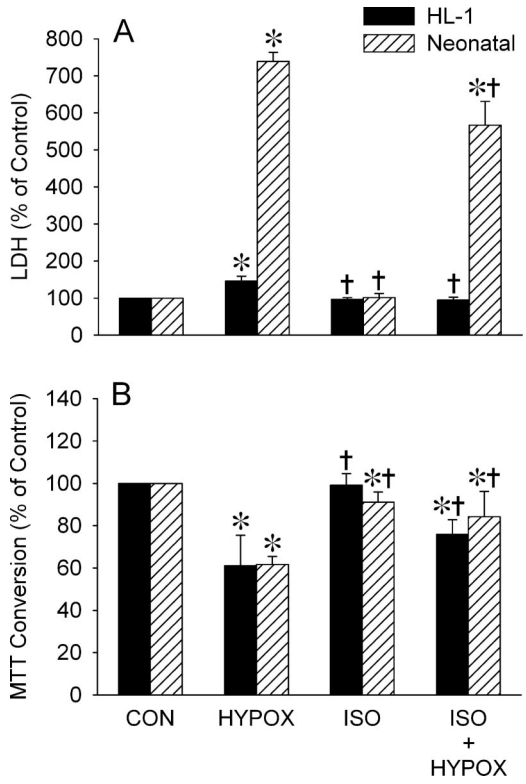

Fig. 1. Lactate dehydrogenase (LDH) release (*A*) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) conversion (*B*) expressed as percent of control in mouse HL-1 cells (*solid bars*) and neonatal rat ventricular myocytes (*hatched bars*) under control (CON) conditions, after exposure to hypoxia (HYPOX), and after pretreatment with isoflurane in the presence (ISO + HYPOX) or absence (ISO) of hypoxia. * Significantly ($P < 0.05$) different from CON; † significantly ($P < 0.05$) different from HYPOX, n = 5.

Activated Neutrophils

An increase in cytochrome *c* release, caspase 3 and 7 activity, and TUNEL-positive cells was observed in adult rat ventricular myocytes exposed to activated neutrophils (fig. 4). Pretreatment with isoflurane attenuated the actions of activated neutrophils on these indices of apoptosis.

Phospho-Akt and Bcl-2 Expression

A decrease in phospho-Akt expression was observed in neonatal rat ventricular myocytes exposed to H_2O_2 but not hypoxia (figs. 5 and 6). Isoflurane enhanced phospho-Akt expression in the presence or absence of sub-

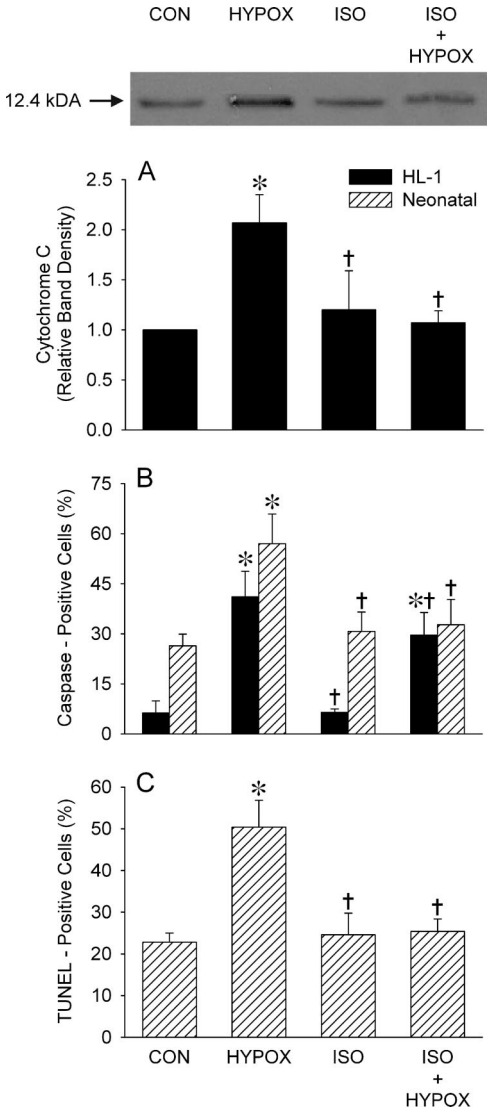

Fig. 2. The effect of hypoxia and isoflurane on cytochrome *c* translocation (*A*, n = 4, histogram and representative Western blot), the percentage of cells demonstrating caspase activity (*B*, n = 6), and TUNEL-positive staining (*C*, n = 6) in mouse HL-1 cells (*solid bars*) and neonatal rat ventricular myocytes (*hatched bars*). Control (CON) conditions, after exposure to hypoxia (HYPOX), and after pretreatment with isoflurane in the presence (ISO + HYPOX) or absence (ISO) of hypoxia. * Significantly ($P < 0.05$) different from CON; † significantly ($P < 0.05$) different from HYPOX.

sequent exposure to hypoxia or H_2O_2. Expression of Bcl-2 increased in response to hypoxia (fig. 5). In contrast, a decrease in Bcl-2 expression occurred in response to H_2O_2. Isoflurane enhanced Bcl-2 expression in the presence or absence of subsequent exposure to hypoxia or H_2O_2.

Inhibition of Akt Phosphorylation and Bcl-2

Wortmannin or HA14-1 alone did not cause any increase in TUNEL-positive neonatal cells (fig. 7) but prevented the attenuation of hypoxia-induced apoptosis through isoflurane preconditioning.

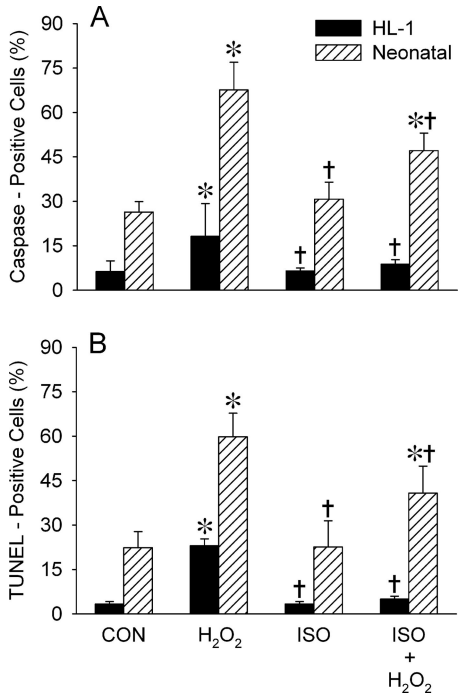

Fig. 3. The effect of H_2O_2 and isoflurane on caspase activity (*A*) and TUNEL-positive staining (*B*) in mouse HL-1 cells (*solid bars*) and neonatal rat ventricular myocytes (*hatched bars*). Control conditions (CON), after exposure to hydrogen peroxide (H_2O_2), and after pretreatment with isoflurane in the presence (ISO + H_2O_2) or absence (ISO) of H_2O_2. * Significantly ($P < 0.05$) different from CON; † significantly ($P < 0.05$) different from H_2O_2, n = 10.

Discussion

A limited number of previous studies have suggested that volatile anesthetics may be capable of protecting against apoptotic cell death induced by various forms of cellular stress including hypoxia. Isoflurane attenuated norepinephrine-induced apoptosis in adult cardiac myocytes.[2] Isoflurane and other volatile anesthetics also protected against apoptosis produced by oxygen or glucose deprivation in cultured cortical neurons.[3,4] The current results confirm and extend these previous findings and indicate that pretreatment with isoflurane attenuates mitochondrial cytochrome *c* translocation into the cytosol, inhibits caspase activation, and reduces DNA fragmentation (TUNEL staining) induced by hypoxia and reoxygenation in neonatal rat ventricular myocytes and mouse atrial HL-1 myocytes *in vitro*, concomitant with decreases in myocardial damage (lactate dehydrogenase release) and maintenance of cellular viability (MTT conversion). These data support the contention that administration of isoflurane before an ischemic event *in vivo* may preserve myocardial integrity by reducing not only necrosis but also apoptosis.

The current results demonstrate for the first time that isoflurane activates Akt and enhances Bcl-2 protein expression in ventricular myocytes exposed to hypoxia and reoxygenation. Akt has been shown to play a major

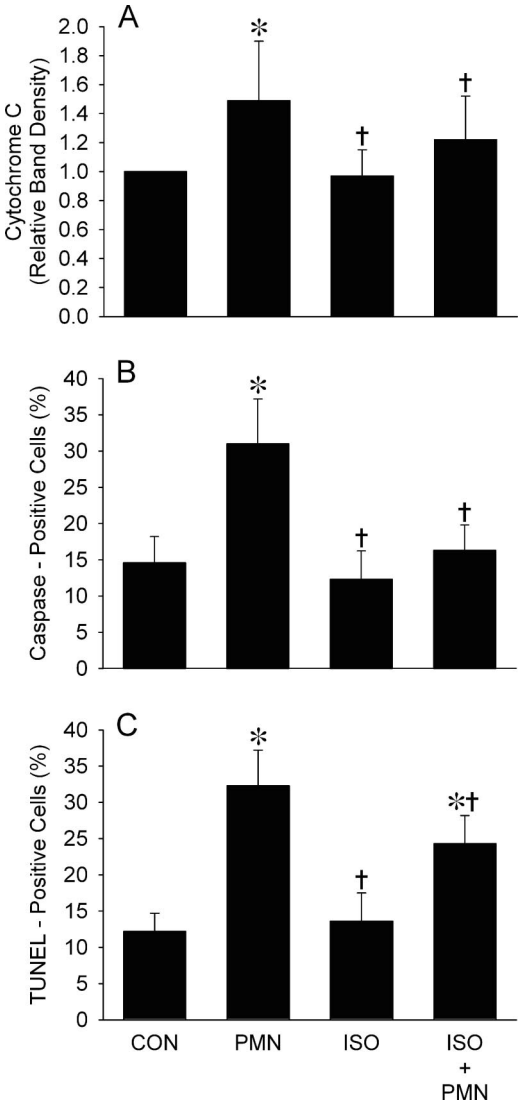

Fig. 4. The effect of activated neutrophils and isoflurane on cytochrome *c* release (*A*, n = 5, histogram and representative Western blot), the percentage of cells with caspase activity (*B*, n = 5), and TUNEL-positive staining (*C*, n = 10) in adult rat ventricular myocytes. Control conditions (CON), after exposure to activated canine neutrophils (PMN), and after pretreatment with isoflurane in the presence (ISO + PMN) or absence (ISO) of exposure to activated neutrophils. * Significantly ($P < 0.05$) different from CON; † significantly ($P < 0.05$) different from PMN.

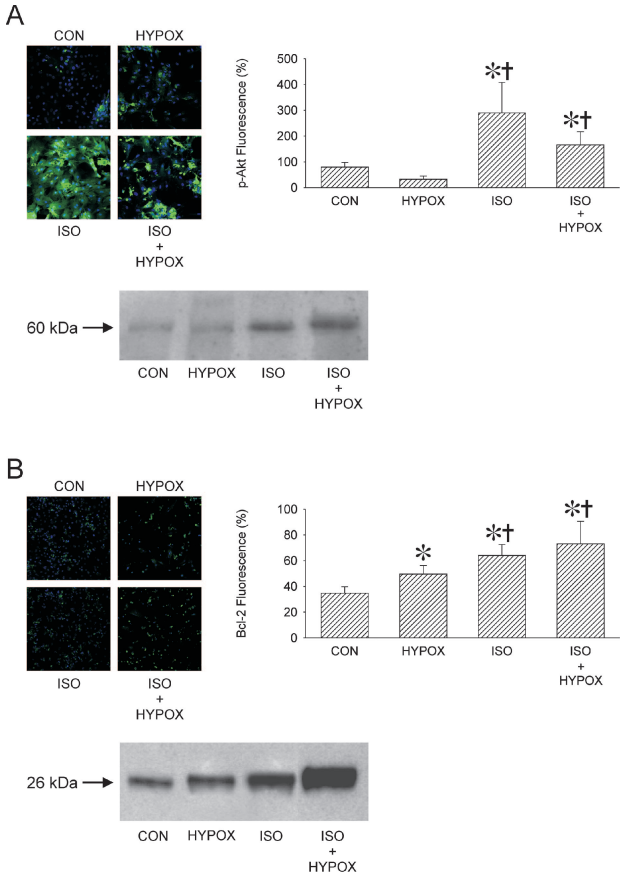

Fig. 5. Representative immunohistochemistry photomicrographs, corresponding histograms, and representative Western blots depicting phospho-Akt (*A*) and Bcl-2 (*B*) expression in neonatal rat ventricular myocytes under control (CON) conditions, after exposure to hypoxia (HYPOX), and after pretreatment with isoflurane in the presence (ISO + HYPOX) or absence (ISO) of hypoxia. * Significantly ($P < 0.05$) different from CON; †significantly ($P < 0.05$) different from HYPOX, n = 4. Western blotting was repeated at least twice, with similar results as in the blots shown in the figures.

role in cell survival signaling pathways by activating endothelial nitric oxide synthase, phosphorylating and inactivating proapoptotic proteins (*e.g.*, Bad, Bax), and inhibiting caspase formation.[7] Previous studies have demonstrated that phosphatidylinositol-3-kinase–mediated activation of Akt attenuates apoptosis during hypoxic[9,10,27] and pharmacologic preconditioning.[11] We have recently demonstrated that administration of isoflurane immediately before and during early reperfusion after prolonged coronary artery occlusion increased phosphorylation of Akt concomitant with a myocardial protective effect in rabbits.[8] The current findings support these previous observations and indicate that isoflurane reduces hypoxia-reoxygenation–induced apoptosis by activating Akt. This protection was reversed by wortmannin, an inhibitor of Akt phosphorylation. The antiapoptotic protein Bcl-2 has been identified in the outer mitochondrial membrane.[28] Bcl-2 may attenuate cellular injury by inhibiting cytochrome *c* translocation[29] and preventing deleterious calcium release from the endoplasmic reticulum.[30] An increase in Bcl-2 concomitant with a decrease in the proapoptotic protein Bax was observed during ischemic preconditioning in isolated rat hearts.[31] The protective effects of hypothermia against apoptosis during ischemia–reperfusion injury were also associated with enhanced Bcl-2 protein translation.[32] The current results support these previous findings and demonstrate that isoflurane-induced protection against apoptotic cell death produced by hypoxia occurs concomitantly with increases in Bcl-2 protein expression and is inhibited by previous administration of the Bcl-2 inhibitor HA14-1. Pretreatment with isoflurane attenuated hypoxia-induced cytochrome *c* release from mito-

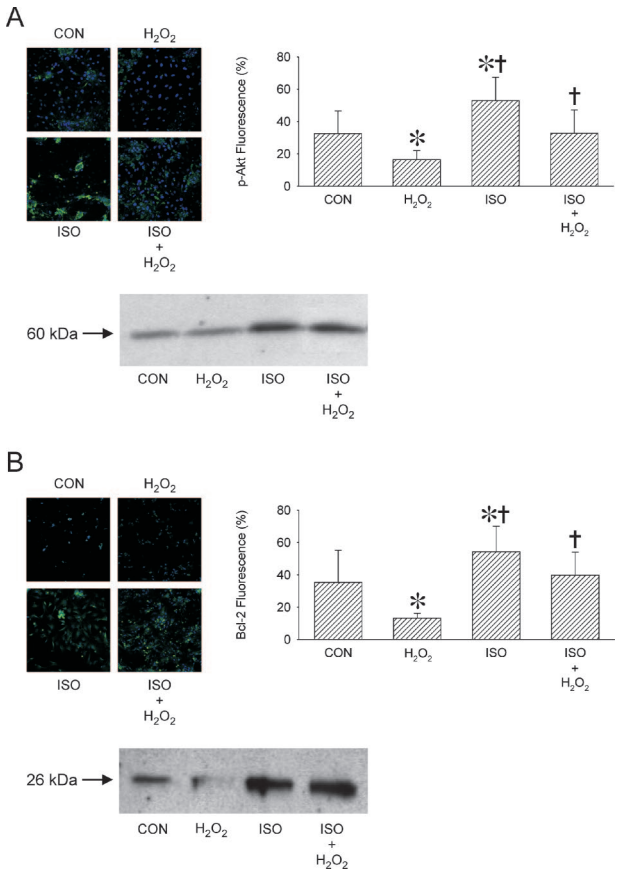

Fig. 6. Representative immunohistochemistry photomicrographs, corresponding histograms, and representative Western blots depicting phospho-Akt (*A*) and Bcl-2 (*B*) expression in neonatal rat ventricular myocytes under control (CON) conditions, after exposure to hydrogen peroxide (H_2O_2), and after pretreatment with isoflurane in the presence (ISO + H_2O_2) or absence (ISO) of hydrogen peroxide. * Significantly ($P < 0.05$) different from CON; † significantly ($P < 0.05$) different from H_2O_2, n = 4. Western blotting was repeated at least twice, with similar results as in the blots shown in the figures.

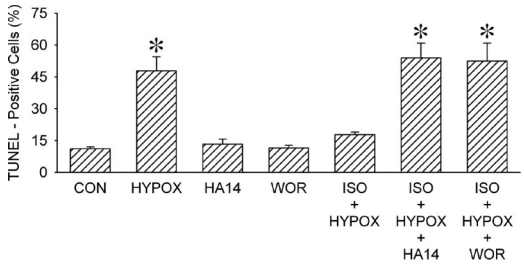

Fig. 7. The effect of hypoxia, isoflurane, HA14-1, and wortmannin on TUNEL-positive staining in neonatal rat ventricular myocytes. Control conditions (CON), after exposure to hypoxia (HYPOX), after exposure to HA14-1 and wortmannin (HA14, WOR), after pretreatment with isoflurane in the presence of hypoxia (ISO + HYPOX), and after pretreatment with isoflurane in the presence of hypoxia, HA14-1, and wortmannin, respectively (ISO + HYPOX + HA14, ISO + HYPOX + WOR). * Significantly ($P < 0.05$) different from CON, HA14, WOR, ISO + HYPOX, n = 5.

chondria, and it is likely that this action may be mediated by the observed increases in Bcl-2. Confirmation of this hypothesis will require further investigation. Various studies have reported conflicting evidence on cellular expression of Bcl-2 and phospho-Akt under hypoxia, reporting an increase, a decrease, or no change after hypoxia.[9,33–36] Our data, reporting an increase in Bcl-2 after hypoxia, could indicate an endogenous protective response of the cell against hypoxia. This protective response is enhanced through isoflurane preconditioning.

The current findings further demonstrate that isoflurane attenuates apoptosis produced by H_2O_2 *in vitro*. Similar to the findings during hypoxia–reoxygenation experiments, pretreatment with isoflurane reduced H_2O_2-induced cytochrome *c* release, caspase activation, and structural damage to DNA in cultured atrial and ventricular myocyte cell lines. In addition, preconditioning with isoflurane also enhanced Akt phosphorylation and Bcl-2 protein expression in myocytes exposed to H_2O_2. The first minutes of reperfusion are critical for salvaging ischemic myocardium, but paradoxically, reperfusion also exacerbates ischemic damage by releasing large quantities of cytotoxic reactive oxygen species that disrupt intracellular homeostasis, depress contractile function, and cause cell membrane damage.[37] In the current investigation, apoptosis was examined in an experimental model that simulated reperfusion injury by exposing cultured cells to H_2O_2.

A variety of preconditioning stimuli, including brief ischemia, mitochondrial adenosine triphosphate–sensitive potassium channel openers, and opioids, are known to facilitate small "priming" bursts of reactive oxygen species that initiate downstream signaling events and produce protection from subsequent ischemia.[38] Our laboratory recently demonstrated that isoflurane directly increases superoxide anion formation *in vivo* independent of an ischemic episode, and scavengers of reactive oxygen species attenuated isoflurane-induced reductions in myocardial infarct size in rabbits.[39,40] These reactive oxygen species originated from complex III of the mitochondrial electron transport chain,[41] and selective inhibition of this complex abolished the cardioprotective effects of the volatile agent *in vivo*. Cytochrome *c* facilitates electron transport between complexes III and IV of the transport chain, and loss of this crucial enzyme from the mitochondria enhances cellular destruction by inhibiting respiration.[6] Isoflurane-induced retention of cytochrome *c* in myocytes exposed to hypoxia or H_2O_2 may contribute to reduced apoptosis by preservation of mitochondrial respiration and allowing for the production of small quantities of reactive oxygen species implicated in cardioprotection to occur. Interestingly, opening of the mitochondrial permeability transition pore by proapoptotic proteins (*e.g.*, Bad, Bax) has been proposed as a central mechanism by which mitochondrial loss of cytochrome *c* occurs during apoptotic cell death.[7] A recent study by Piriou *et al.*[42] demonstrated that another volatile anesthetic (desflurane) closes the mitochondrial permeability transition pore. Whether en-

hanced Bcl-2 protein expression mediates isoflurane-induced reductions in cytochrome *c* translocation through closure of the mitochondrial permeability transition pore will require further investigation to ascertain.

Neutrophils play a central role in the pathogenesis of myocardial reperfusion injury.[43] These leukocytes are activated during reperfusion, adhere to coronary vascular endothelium, infiltrate the ischemic zone through diapedesis, and cause damage by releasing a variety of cytotoxic substances as a result of direct contact with myocytes.[44] Previous studies have shown that activated neutrophils produce apoptosis in cardiac myocytes,[45] pulmonary epithelium,[46] and bronchial smooth muscle cells.[47] The current findings confirm that activated neutrophils produce apoptosis in adult rat ventricular myocytes as indicated by enhanced mitochondrial cytochrome *c* release, caspase activation, and DNA fragmentation. The apoptotic actions of activated neutrophils on cardiac myocytes were attenuated by isoflurane. Direct incubation of neutrophils with volatile anesthetics has been shown to reduce the ability of these leukocytes to adhere to endothelium[20,21] and produce cardiac dysfunction.[48] However, our study revealed that cardiomyocytes themselves are protected by their own isoflurane-inducible mechanism. Other evidence indirectly suggests that volatile anesthetics may exert protection against apoptosis by modulating nitric oxide metabolism or activation of the p38 mitogen-activated protein kinase (p38 MAPK). Isoflurane has been shown to enhance nitric oxide synthesis and inducible nitric oxide synthase expression,[49–51] and nitric oxide attenuates neutrophil-induced myocyte injury.[52] Thus, isoflurane may have reduced ventricular myocyte apoptosis resulting from exposure to activated neutrophils through a nitric oxide–dependent mechanism. Another study demonstrated that neutrophil-induced apoptosis was partly mediated by p38 MAPK in rat neonatal cardiac myocytes.[45] Zheng and Zuo[53] recently reported that isoflurane preconditioning produces neuroprotection by activating p38 MAPK, and Itoh *et al.*[54] also observed that volatile agents were capable of differentially modulating proinflammatory cytokine-induced p38 MAPK activation. These data suggest that activation of p38 MAPK may also play a role in isoflurane-mediated attenuation of neutrophil-induced apoptosis. However, further investigation will be required to define whether either nitric oxide metabolism or p38 MAPK activation is involved in this process.

In summary, the current study indicates that pretreatment with isoflurane protects atrial and ventricular myocytes against apoptosis produced by hypoxia–reoxygenation, exposure to H_2O_2, and coincubation with activated neutrophils. These protective effects were mediated by activation of the prosurvival protein Akt and enhanced expression of the antiapoptotic protein Bcl-2 *in vitro*. The results suggest that a reduction in apoptotic cell death as well as a decrease in myocyte necrosis contributes to the cardioprotective effects of isoflurane.

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