Norepinephrine-induced Apoptosis Is Inhibited in Adult Rat Ventricular Myocytes Exposed to Volatile Anesthetics

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Background: Volatile anesthetics are used to provide anesthesia to patients with heart disease under heightened adrenergic drive. The purpose of this study was to test whether volatile anesthetics can inhibit norepinephrine (NE)-induced apoptosis in cardiomyocytes.

Methods: Rat ventricular cardiomyocytes were exposed to NE (10 μM) alone or in the presence of increasing concentrations of isoflurane and halothane.

Results: Isoflurane at 1.6 minimum alveolar concentration (MAC) (4 ± 2% [SD]) and halothane at 1.2 MAC (3 ± 2%) abolished the percentage of cardiomyocytes undergoing NE-induced apoptosis (34 ± 8%), as assessed by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) (P < 0.0001). Lower concentrations of isoflurane and halothane markedly decreased the number of TUNEL-positive cells. Similarly, isoflurane at 1.6 MAC (5 ± 3%) and halothane at 1.2 MAC (6 ± 3%) prevented the increase in annexinV-staining cardiomyocytes (38 ± 7%; P < 0.0001). These findings were corroborated with a decreased quantity of NE-induced DNA laddering by volatile anesthetics. Halothane at 1.2 MAC abolished the increase in TUNEL-positive cardiomyocytes exposed to the dihydropyridine Ca²⁺-channel agonist BAY K-8644 (1 μM) (BAY K.8644 + halothane: 3 ± 2% vs BAY K.8644: 34 ± 6%; P < 0.0001) and the Ca²⁺-ionophore 4-bromo-A23187 (1 μM) (4-bromo-A23187 + halothane: 2 ± 2% vs 4-bromo-A23187: 13 ± 4%; P = 0.03). NE treatment increased caspase-9 activity to 197 ± 62% over control cardiomyocytes (P < 0.0001), whereas no caspase-8 activation was detectable. This increase in caspase-9 activity was blocked by isoflurane at 1.6 MAC and halothane at 1.2 MAC.

Conclusions: Volatile anesthetics offer significant protection against β-adrenergic apoptotic death signaling in ventricular cardiomyocytes. The authors present evidence that this protection is mainly mediated through modulation of cellular Ca²⁺ homeostasis and inhibition of the apoptosis initiator caspase-9. (Key words: Catecholamines; halothane; heart; isoflurane; programmed cell death; signal transduction.)

APOPTOSIS, the genetically programmed cell death, is executed by an energy-dependent and cell-inherent suicide machinery that requires de novo gene expression and leads to destruction and removal of cells that are no longer needed or damaged. Recent observations have indicated that apoptotic cardiomyocyte cell death plays a key role in the deterioration of myocardial function in a variety of pathologic situations. Programmed cell death has been reported to occur in myocardial infarction, heart failure, and toxic, inflammatory, and endocrine cardiomyopathies. Furthermore, experimental induction of cardiac apoptosis has been achieved by pressure overload, tachycardia, and left ventricular hypertrophy, as well as by several endogenously released excitotoxic factors, including tumor necrosis factor-α, atrial natriuretic peptide, angiotensin II, and norepinephrine (NE).

Catecholamines, although beneficial in short-term cardiovascular response, exert significant cardiac toxicity. Enhanced activation of β-adrenergic signaling results in...
Accordingly, acute accumulation of endogenously released catecholamines was reported to accelerate ischemic cardiomyocyte damage, and chronic sympathetic nervous activation was related to progressive cell death in heart failure. Volatile anesthetics are frequently used to provide general anesthesia to patients with significant heart disease under conditions of heightened adrenergic drive, a hallmark of the perioperative neuroendocrine stress response. Although volatile anesthetics are known to exert significant cardioprotection after ischemia and reperfusion, it is not clear, to date, whether volatile anesthetics modulate the cell suicide program evoked by catecholamines. The purpose of this study was to determine whether exposure to volatile anesthetics affects NE-induced apoptosis in adult cardiomyocytes in vitro and, if so, to investigate the cellular mechanisms involved.

Materials and Methods

Myocyte Cultures and Cell Treatments

The investigation conformed to the American Physiological Society's rules for the care and use of laboratory animals. Protocols were approved by the Institutional Animal Care and Use Committee. Ca²⁺-tolerant adult rat ventricular myocytes were isolated from hearts of male Sprague-Dawley rats (300–350 g), essentially as previously described. Twenty minutes before decapitation, animals were heparinized (500 U intraperitoneally). The hearts were quickly removed into an ice-cold HEPES-buffered solution (117 mM NaCl, 5.7 mM KCl, 1.5 mM NaH₂PO₄, 4.4 mM NaHCO₃, 20 mM HEPES, 10 mM glucose, 10 mM creatine, at pH 7.4) containing 1.8 mM Ca²⁺ and retrograde-perfused on a Langendorff apparatus for 5 min at 37°C gassed with oxygen. The perfusion solution was switched to a nominally Ca²⁺-free solution containing 0.1 mM EGTA for 7 min and then to a nominally Ca²⁺-free solution containing 0.15% collagenase B (Boehringer Mannheim, Indianapolis, IN). After 30 min of digestion, the enzymatic solution was washed out and the tissue from the left ventricle was cut into small pieces and gently swirled in the HEPES-buffered solution containing 0.125 mM Ca²⁺. Dissociated cells were filtered through a 200-μm mesh and allowed to settle for 20 min. The cells were then resuspended in the buffered solution containing 5 mg/ml bovine serum albumin and exposed to a graded series of increasing Ca²⁺ concentrations up to 1.8 mM. Each step was followed by a gentle centrifugation with less than 20 g for 2 min to separate the ventricular myocytes from nonmyocytes. The isolated myocytes were resuspended in serum-free defined culture medium consisting of Dulbecco's modified Eagle medium with 2 mg/ml bovine serum albumin, 2 mM L-carnitine, 5 mM creatine, and 5 mM taurine supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. They were cultured at a density of 100–150 cells/mm² in 60-mm plastic culture dishes (Falcon, Cockeyville, MD) or on 22-× 22-mm glass coverslips (Fisher, Pittsburgh, PA) pre-coated with laminin (1 μg/cm², Sigma, St. Louis, MO) placed in 35-mm plastic culture dishes. After 3 h, the dishes were washed to remove cells that were not attached. Purity of myocyte cultures was assessed by determining the percentage of myosin-positive staining cells after attachment on glass coverslips using immunofluorescence with a myosin heavy chain-specific antibody, MF-20. Cell viability was assessed by counting the number of adherent cells on 35-mm dishes 3 h after plating as well as at the end of the experiments. Briefly, cells were washed with Dulbecco's modified Eagle medium, and five randomly chosen fields (1 mm²) were counted at 10 × 10 magnification with a phase-contrast microscope (Leitz, Germany) in duplicate dishes. In addition, percentages of rounded cells as well as quiescent rod-shaped cells were scored. The treatment protocols and the concentrations of the reagents used were established previously. In the various protocols, for each experimental group, cells of 10 different rat hearts were used, and all experiments were performed in duplicate or triplicate dishes. I-NE (10 μM; Sigma) was added to culture dishes for 3, 6, 12, and 24 h, respectively. All dishes were supplemented with fresh ascorbic acid (0.1 mM; Sigma). A multiple gas-mixing incubator (Newage Industries, Willow Grove, PA) was used to administer volatile anesthetics to cell cultures for 3, 6, 12, and 24 h, respectively, by placing the agent-specific vaporizer (isoflurane: Airco, Madison, WI; halothane: Draeger, Lübeck, Germany) between the adjustable fresh gas supply and the interior of the incubator. A stable interior chamber atmosphere with 21% O₂, 5% CO₂, and the required anesthetic concentrations was obtained within 5 min of equilibration at 37°C. A Datex infrared gas analyzer (Puritan-Bennett, Tewksbury, MA) continuously controlled the delivered vapor concentrations of the volatile anesthetics in the incubator. To assess the NE-induced apoptosis dose-response to isoflurane and halothane, in some experiments cells were measured.
exposed to increasing concentrations of isoflurane (Mar
sam Pharmaceuticals, Cherry Hill, NJ) (0.7%, 1.2%, and
2.3% [vol/vol] corresponding to 0.4, 0.8, and 1.6 mini-
mum alveolar concentration [MAC] in rats) and halo-
thane (Halocarbon Laboratories, River Edge, NJ) (0.4%,
0.7%, and 1.5% [vol/vol] corresponding to 0.3, 0.6, and
1.2 MAC in rats) 30 min before the addition of NE. All
other experiments with isoflurane and halothane were
performed using 2.3% (vol/vol) isoflurane (1.6 MAC in
rats) and 1.5% (vol/vol) halothane (1.2 MAC in rats) 30
min before the addition of NE, BAY K-8644 (1 μM, Bi-
omol, Plymouth Meeting, PA), 4-bromo-A23187 (1 μM,
Biomol), or a combination thereof. The applied concen-
trations of isoflurane and halothane were also measured
in the media using a gas chromatograph (Perkin-Elmer,
Norwalk, CT): isoflurane 0.21 ± 0.04 μM, 0.44 ± 0.05
μM, 0.87 ± 0.05 μM; halothane 0.12 ± 0.04 μM, 0.23 ±
0.05 μM, 0.44 ± 0.04 μM. Diltiazem (1 μM; Sigma) and
propranolol (2 μM; Sigma) were added to the dishes 30
min before the addition of NE. Standard cell culture
incubators were used for experiments without volatile
anesthetics.

**Terminal Deoxynucleotidyl Transferase-mediated
Nick End Labeling Assay**

Nick-end labeling was performed in cells plated on
glass coverslips using the CardioTACS in situ Apoptosis
Detection kit (R&D Systems, Minneapolis, MN) accord-
ing to the manufacturer's instructions. A positive control
was generated by using TACS-nuclease working solution
containing DNase I. For each staining procedure, control
myocytes were treated with DNase and used as positive
control. The cells were incubated with terminal deoxynu-
cleotidyl transferase-mediated nick end labeling (TUNEL)
mixture (terminal deoxynucleotidyl transferase and biotin-
ylated nucleotides) for 1 h at 37°C in a humidified chamber.
Apoptotic nuclei were visualized with a streptavidin-
conjugated horseradish peroxidase followed by TACS Blue
staining solution at room temperature and analyzed by
fluorescence microscopy. FITC-labeled annexinV detects
phosphatidylserine translocated from the inner to the
outer cell surface, a hallmark of early apoptosis. Cells in
late apoptosis or necrosis show additional positive nu-
clear staining with propidium iodide and are indistin-
guishable from each other. The percentage of early
apoptotic cells with only green sarcolemmal staining
(annexinV-FITC excited at 450–480 nm) and of late
apoptotic and necrotic cells with both green sarcolem-
mal staining and red nuclear staining (propidium iodide
excited at 510–550 nm) was determined at 10 × 10
magnification in five randomly chosen fields in duplicate
dishes (1 mm²).

**Analysis of Genomic DNA (DNA Laddering)**

Internucleosomal DNA fragmentation was assessed using
an isotopic TACS Apoptotic DNA laddering kit (R&D
Systems). DNA samples (1 μg) from control and experi-
mental cultures were labeled by enzymatic assay with
terminal deoxynucleotidyl transferase in a buffer con-
taining 0.5 μCi [32P]dCTP (New England Nuclear, Bev-
ery, MA). After incubation for 10 min at room temper-
atum, the reaction was stopped by adding 2 μl gel
loading buffer, and the samples were analyzed on a 1.5%
tagarose gel at 100 V using TAE (tris base-acetic acid-
EDTA) buffer. The gel was fixed in 10% acetic acid,
dried, and exposed to x-ray films. Labeled 1-kb DNA
ladder was used as marker. Photographic images were
obtained, digitized, and quantified using SigmaGel (Jand-
el, Chicago, IL).

**Determination of the Enzymatic Activities of the
Upstream Caspase-8 and -9**

Determination of caspase-8 and -9 enzymatic activities
was performed using Caspase-8 and -9 Colorimetric As-
say kits (R&D Systems). Briefly, cells were washed with
phosphate-buffered saline, counted, trypsinized, and re-
suspended in lysis solution. Caspase-8 and -9-specific
substrates conjugated to the color reporter molecule
p-nitroanilide were added to the cell lysates. After 2 h of
incubation at 37°C, p-nitroanilide was quantitated spec-
trophotometrically at a wavelength of 405 nm in duplic-
ate dishes (Microplate Reader Model EL 308; BIO-TEK,
Burlington, VT). After correcting for background activity
and number of cells, enzyme activity was expressed as
percent increase in activity over control cells.

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Transmission Electron Microscopy

A separate set of experiments was performed on laminin-coated permeable membrane filters (Costar, Cambridge, MA). Monolayers were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3 and postfixed with 1% osmium tetroxide in the cacodylate buffer for 1 h at 4°C. Samples were dehydrated through ethanol and infiltrated with LR White embedding resin (London Resin Company, Berkshire, England), which was then allowed to polymerize in a 60°C oven for 72 h. Thick longitudinal sections were cut at 1 μm, stained with methylene blue, and examined by light microscopy. Thin sections (50 nm) were then prepared from selected areas and stained with uranyl acetate and lead citrate. JEM-100C (JEOL, Tokyo, Japan) was used for electron microscopy.

Statistical Analysis

Data are expressed as mean ± SD. Student unpaired t test was used to compare control with NE-treated cells. Analysis of variance with post hoc Scheffe test for multiple comparisons was performed to determine statistical significance of multiple treatments. P < 0.05 was considered significant (StatView Software, Abacus Concepts, Berkeley, CA).

Results

Norepinephrine-induced Apoptosis

More than 99% of cells stained positively with the MF-20 antibody, indicating an essentially pure cardiomyocyte population. The few irregular contractions present immediately after cell isolation nearly disappeared completely after attachment. During the course of the experiments, the majority of myocytes remained viable and attached to culture dishes in control cultures over a 24-h period (85 ± 6% of baseline, n = 10 in duplicates). Administration of NE significantly decreased the number of adherent cells to 76 ± 4%, 70 ± 4%, and 65 ± 6% of baseline after 6, 12, and 24 h, respectively, of NE exposure (P < 0.001 vs time control, n = 10 in duplicates). In addition, after 6, 12, and 24 h, in control cultures the majority of myocytes retained their distinctive cylindrical shape, with only 2 ± 2%, 3 ± 2%, and 4 ± 4% of adherent cells, respectively, showing a rounded morphology and disorientation of cross-striations. However, these morphologic changes occurred in 60 ± 8%, 75 ± 6%, and 80 ± 6% of adherent cells after 6, 12, and 24 h, respectively, of NE exposure (P < 0.0001 vs time control, n = 10 in duplicates). No changes in the number of adherent cells and the cell morphology were observed after 3 h of NE exposure when compared with control dishes.

Relative to control plates (3 ± 2% of TUNEL-positive cells), a significant increase in TUNEL-positive cells was observed after 6 h of NE exposure (15 ± 3%, P < 0.0001 vs time control, n = 10 in triplicates) (fig. 1A). Treatment with NE for 12 h increased the percentage of TUNEL-positive cells to 34 ± 8% (P < 0.0001 vs time control). NE exposure for 24 h caused only a small further increase in TUNEL-positive myocytes (36 ± 5%, P > 0.45 vs NE exposure for 12 h; fig. 1A).
APOPTOTIC CARDIOPROTECTION AND VOLATILE ANESTHETICS

Volatile Anesthetics Decrease Norepinephrine-induced Apoptosis

Isoflurane and halothane dose-dependently inhibited NE-induced apoptosis, as assessed by TUNEL staining (n = 10 in triplicates; fig. 1B). Isoflurane at 0.4 MAC and halothane at 0.3 MAC markedly inhibited NE-induced apoptosis (P < 0.001 vs NE), whereas isoflurane at 1.6 MAC and halothane at 1.2 MAC completely blocked apoptotic cell death when compared with control plates. The majority of rounded cells and also some with cylindrical shape showed positive TUNEL reaction (figs. 2A–E). Optimal concentrations of volatile anesthetics (isoflurane at 1.6 MAC and halothane at 1.2 MAC) and the optimal time interval of 12 h were chosen in all subsequent experiments to facilitate exploration and delineation of the apoptotic response between the various experimental groups.

The percentages of adherent myocytes exhibiting the early apoptotic pattern of annexinV/propidium iodide labeling (positive sarcolemmal staining with annexinV and concomitant nuclear staining with propidium iodide: An+/PI-) were significantly increased in NE-treated dishes (An+/PI-: 28 ± 4%; An+/PI+: 10 ± 3%) when compared with control dishes (An+/PI-: 3 ± 2%; An+/PI+: 2 ± 2%; P < 0.0001, n = 10 in duplicates). Similarly to diltiazem and propranolol, isoflurane at 1.6 MAC and halothane at 1.2 MAC reduced the percentages of single- and double-staining adherent cells to levels observed in control dishes (figs. 3A–C).

The isolated DNA, which was size-fractionated by electrophoresis, exhibited a typical DNA ladder pattern. Clearly increased intensity of low molecular DNA fragments (180-1,000 base pairs) was observed in DNA from myocytes treated with NE (P < 0.0001 vs control).

Fig. 2. (A) Control myocytes (12 h). Note rod-shaped morphology. (B) Norepinephrine (NE)-treated myocytes with rounded morphology (12 h). (C) NE + halothane-treated myocytes (12 h, halothane at 1.2 MAC). Note preservation of rod-shaped morphology under NE treatment. (D) Representative myocytes treated with NE alone with positive TUNEL reaction seen at higher magnification than A–C. (E) Representative myocytes treated with NE + halothane showing negative TUNEL reaction, same magnification as D (bar = 75 μm for A–C, 25 μm for D and E).

Fig. 3. AnnexinV/propidium iodide staining was performed to assess phosphatidylserine translocation from the inner to the outer cell surface. Myocytes grown on coverslips were exposed to norepinephrine (NE; 10 μM) alone or in presence of diltiazem (DIL; 1 μM), propranolol (PRO; 2 μM), isoflurane (ISO; 1.6 MAC), and halothane (HAL; 1.2 MAC) for 12 h and subjected to annexinV/propidium iodide staining as described in Methods. (A) Mean percentages of adherent cells with the early apoptotic pattern of labeling (An+/PI-) as well as the late apoptotic (or necrotic) pattern of labeling (An+/PI+) at 12 h and subjected to annexinV/propidium iodide staining as described in Methods. (A) Mean percentages of adherent cells with the early apoptotic pattern of labeling (An+/PI-) as well as the late apoptotic pattern of labeling (An+/PI+). Data are mean ± SD. *P < 0.0001 versus control (CTL), tP < 0.0001 versus NE, not significant versus CTL. (B) Early apoptotic pattern of annexinV/propidium iodide staining: An+/PI-. (C) Late apoptotic pattern of annexinV/propidium iodide staining: An+/PI+ (bar = 25 μm).
Fig. 4. DNA fragmentation in myocytes as assessed by laddering. Total genomic DNA was isolated, labeled with \([^{32}P]dCTP\), and size-fractionated by electrophoresis. For the various groups, the intensity of the signals corresponding to low-molecular-weight DNA is expressed as percent increase in absorption compared with control (CTL). Norepinephrine (NE)-induced increase in DNA fragmentation is similarly blocked by treatment with diltiazem (DIL; 1 µm), propranolol (PRO; 2 µm), isoflurane (ISO; 1.6 MAC), and halothane (HAL; 1.2 MAC). Data are mean ± SD. \(*P < 0.0001\) versus CTL, ?not significant versus CTL. M indicates 1-kb marker.

Similarly to diltiazem and propranolol, isoflurane at 1.6 MAC and halothane at 1.2 MAC reduced the quantity of fragmented DNA to control levels as assessed by densitometry (n = 10 in duplicates; fig. 4). Diltiazem, propranolol, and the volatile anesthetics alone did not affect the quantity of apoptosis as assessed by TUNEL staining, annexinV/propidium iodide staining, and DNA laddering when compared with control dishes.

Transmission electron microscopy disclosed frequent apoptotic events with various degrees of changes of nuclear and cytoplasmic components in NE-treated cells (fig. 5). These included clustering and condensation of mitochondria with malalignment of cristae (figs. 5B and 5F), margination and condensation of nuclear chromatin (fig. 5b), loss of myofibrillar cross-striations (fig. 5D), and membrane blebbing (fig. 5E). Consistent with the smaller percentage of apoptotic cells in isoflurane-, halothane-, diltiazem-, and propranolol-treated dishes, characteristic morphologic apoptotic events were only sparsely observed in these dishes (figs. 5A and 5C).

Fig. 5. Electron micrographs in representative myocytes treated with norepinephrine (NE; 10 µm; B, D, E, F) and NE (10 µm) + halothane (1.2 MAC; A and C). (A) Normal morphology in NE + halothane-treated myocyte. Characteristic morphologic events of apoptosis were only sparsely observed in NE + halothane-treated myocytes. (B) Increased electron density of cytoplasmic organelles with clustering of mitochondria was associated with considerable cell shrinkage. Note condensation of mitochondria and margination of chromatin at the inner surface of the nuclear envelope (arrowhead). (C) NE + halothane-treated myocyte with clearly visible myofibril band pattern. (D) Surveys revealed random orientation of myofibrils with loss of myofibril bands in NE-treated myocytes (arrowhead). (E) Excessive membrane blebbing of the cell surface was observed in NE-treated myocytes. (F) High magnification micrograph of a mitochondrion with malalignment of cristae (arrowhead). Bar = 10 µm for A and B, 1.0 µm for C and D, 5 µm for E, and 0.5 µm for F.
Modulation of the apoptotic response by the Ca\textsuperscript{2+}-channel activator BAY K-8644 and the Ca\textsuperscript{2+}-ionophore 4-bromo-A23187. Myocytes were exposed to BAY K-8644 (1 μM) or 4-bromo-A23187 (1 μM) in the presence or absence of norepinephrine (NE; 10 μM) and/or halothane (HAL; 1 MAC). HAL effectively decreased the percentages of myocytes undergoing Ca\textsuperscript{2+} channel activator- and Ca\textsuperscript{2+}-ionophore-mediated apoptosis. Data are mean ± SD. *P < 0.0001 versus control, tP < 0.05 versus respective treatment without the protective effects of halothane.

Mechanisms of Apoptotic Cardioprotection

Additional experiments with halothane at 1.2 MAC were performed to explore the mechanisms underlying the apoptotic cardioprotection by volatile anesthetics (n = 10 in duplicates; fig. 6). Myocytes were exposed to the dihydropyridine Ca\textsuperscript{2+}-channel agonist BAY K-8644 and the Ca\textsuperscript{2+}-ionophore 4-bromo-A23187 in the presence and absence of halothane and/or NE. TUNEL-positive staining cells were evaluated after 12 h of exposure. Although treatment with the Ca\textsuperscript{2+}-channel activator was more effective in increasing the percentage of TUNEL-positive cells (34 ± 4%) when compared with treatment with the Ca\textsuperscript{2+}-ionophore 4-bromo-A23187 (13 ± 4%; P < 0.0001), both treatments significantly increased the percentage of TUNEL-positive staining cells (BAY K-8644 vs control, P < 0.0001; 4-bromo-A23187 vs control, P < 0.001). Furthermore, addition of NE potentiated the apoptotic response (BAY K-8644: 34 ± 6% vs BAY K-8644 + NE: 90 ± 5%, P < 0.0001; 4-bromo-A23187: 13 ± 4% vs 4-bromo-A23187 + NE: 42 ± 4%, P < 0.0001). Conversely, halothane effectively decreased the percentage of apoptotic cells in Ca\textsuperscript{2+}-channel activator-mediated apoptosis as well as in Ca\textsuperscript{2+}-ionophore-mediated apoptosis (BAY K-8644 + halothane: 3 ± 2% vs BAY K-8644: 34 ± 6%, P < 0.0001; 4-bromo-A23187 + halothane: 2 ± 1% vs 4-bromo-A23187: 13 ± 4%, P = 0.03). This protective effect continued to exist with concomitant NE treatment (fig. 5).

Norepinephrine treatment increased caspase-9 activity by 197 ± 62% when compared with control cells (P < 0.0001, n = 10 in duplicates; fig. 7A). Similarly to diltiazem and propranolol, isoflurane at 1.6 MAC and halothane at 1.2 MAC decreased caspase-9 activity to levels not significantly different from control. No significant increase in caspase-8 activity over control dishes was detectable in any of the treatment regimens (n = 10 in duplicates; fig. 7B).

Fig. 6. Modulation of the apoptotic response by the Ca\textsuperscript{2+}-channel activator BAY K-8644 and the Ca\textsuperscript{2+}-ionophore 4-bromo-A23187. Myocytes were exposed to BAY K-8644 (1 μM) or 4-bromo-A23187 (1 μM) in the presence or absence of norepinephrine (NE; 10 μM) and/or halothane (HAL; 1.2 MAC). HAL effectively decreased the percentages of myocytes undergoing Ca\textsuperscript{2+} channel activator- and Ca\textsuperscript{2+}-ionophore-mediated apoptosis. Data are mean ± SD. *P < 0.0001 versus control, tP < 0.05 versus respective treatment without the protective effects of halothane.

Fig. 7. Caspase-8 and -9 activities. After correction of background activity and number of cells, enzymatic activity was expressed as percent increase in activity over control cells. (A) Caspase-9 activity. Norepinephrine (NE) induced a significant increase in caspase-9 activity, which was inhibited by treatment with diltiazem (DIL; 1 μM), propranolol (PRO; 2 μM), isoflurane (ISO; 1.6 MAC), and halothane (HAL; 1.2 MAC). Data are mean ± SD. *P < 0.0001 versus control (CTL), †not significant versus CTL. (B) Caspase-8 activity. Caspase-8 was not activated in NE-treated cultures.
Discussion

The major new finding of this study is that isoflurane and halothane dose-dependently decreased NE-induced apoptotic cell death in adult rat cardiomyocytes. The potency of volatile anesthetics in inhibiting apoptotic cell death was mimicked by the β-adrenergic receptor antagonist propranolol and the voltage-dependent Ca\(^{2+}\)-channel blocker diltiazem, and paralleled modulation of free intracellular Ca\(^{2+}\) concentration by the dihydropyridine Ca\(^{2+}\)-channel activator BAY K-8644. However, the anti-apoptotic effect was preserved under the treatment with the Ca\(^{2+}\)-ionophore 4-bromo-A23187, which indicates that mechanisms other than depression of Ca\(^{2+}\) influx through L-type Ca\(^{2+}\)-channels may also have a role in the apoptotic protection by volatile anesthetics. The results of this study also demonstrate that caspase-9, a specific cysteine protease, is activated during NE-induced apoptosis, and that caspase-9 activation is effectively inhibited by volatile anesthetics.

In contrast to necrosis, apoptosis is a strictly regulated and ordered removal of damaged cells with near-to-intact plasma membrane and virtually no inflammatory response. Cardiomyocyte apoptosis represents the major form of myocardial damage, significantly contributing to infarct size in rats, and constitutes a pivotal event in the variety of pathologic circumstances, including heart failure and various forms of cardiomyopathies. Recent observations from cardiomyocytes in vitro emphasize the apoptogenic potential of several endogenously released factors, including tumor necrosis factor-α, atrial natriuretic peptide, angiotensin II, and NE. Although cardiotoxic effects of catecholamines have been known for many years, their toxicity was not related to enhanced β-adrenergic signaling until latterly by Mann et al. Apoptotic cardiomyocyte death by activation of the β-adrenergic pathway was reported in NE-stimulated adult rat cardiomyocytes and in isoproterenol-treated rat hearts. Enhanced activation of the β-adrenergic signaling by overexpression of G\(_{s}\)α in hearts of transgenic mice was also found to induce apoptosis. Furthermore, deleterious effects of endogenously released catecholamines during ischemia are well established and may trigger apoptosis. Accordingly, decreased apoptosis has been reported in carvedilol- and propranolol-pretreated rabbit hearts subjected to ischemia/reperfusion injury.

The results of this study now demonstrate that NE-stimulated apoptosis is dose-dependently inhibited in adult rat ventricular myocytes exposed to volatile anesthetics. Hallmarks of apoptotic events were assessed on various apoptotic execution sites, including the sarcolemmal, enzymatic, and nuclear level, and the results were internally consistent. Importantly, even lower concentrations of isoflurane and halothane, i.e., less than 1 MAC in rats and humans, effectively decrease NE-induced apoptotic cell death. Because the number of apoptotic cardiomyocytes was increased after only 6 h of NE exposure, the observed apoptotic cardioprotection by volatile anesthetics may be clinically relevant under intraoperative conditions of heightened adrenergic drive, particularly in patients with preexisting heart disease.

Apoptosis involves a commitment phase followed by an execution phase. Numerous studies have implicated increases in intracellular free Ca\(^{2+}\) in apoptotic cell death. β-Adrenergic receptor stimulation leads to protein kinase A-mediated phosphorylation of L-type Ca\(^{2+}\)-channels, thereby increasing the mean open time of single channels and the number of channels available for voltage-dependent activation. Massive cytosolic Ca\(^{2+}\) elevations subsequently trigger an increase in mitochondrial membrane permeability, which is followed by liberation of holocytochrome c and apoptosis-inducing factor into the cytosol. These factors finally activate caspases, the executioners of apoptotic cell death. Caspases cleave critical cellular substrates, including lamin and fodrin, precipitating the dramatic morphologic changes of apoptosis. Not all models of apoptotic cell death use the same caspases. Caspase-9, which is highly expressed in heart tissue, is an upstream initiator caspase directly activated by apoptogenic mitochondrial factors. The findings of this study confirm the pivotal role of increased intracellular Ca\(^{2+}\) on apoptosis and extend the previous observations by showing that NE initiates the apoptotic cascade by activation of caspase-9 in cardiomyocytes. Interestingly, caspase-8, the other major initiator caspase, was not activated in NE-treated cultures.

Volatile anesthetics are potent cardio depressant agents with favorable cardioprotective effects. Volatile anesthetics affect hydrophobic sites in cell membranes, protein–protein and lipid–protein interactions. At a cellular level, volatile anesthetics efficiently change intracellular Ca\(^{2+}\) homeostasis by blocking sarcolemmal Ca\(^{2+}\) entry, inhibiting the Na\(^{+}\)–Ca\(^{2+}\) exchange channel, and decreasing the Ca\(^{2+}\) content in the sarcoplasmic reticulum. Because changes in Ca\(^{2+}\) homeostasis play a pivotal role in events associated with myocardial injury, changes in cellular Ca\(^{2+}\) homeostasis by volatile anesthetics may explain, in part, the observed anti-apoptotic effect. However, the anti-apoptotic effect of halothane was also present in L-type Ca\(^{2+}\)-channel-independent ap-
optosis stimulated by the $\text{Ca}^{2+}$ ionophore 4-bromo-A23187. Thus, mechanisms other than pure inhibition in slow $\text{Ca}^{2+}$ inward current through L-type $\text{Ca}^{2+}$ channels may be involved. These may include increasing the resistance of the mitochondrial membrane to noxious stimuli, since volatile anesthetics significantly affect mitochondrial $\text{Ca}^{2+}$ uptake and release, a key event in apoptosis.

Volatile anesthetics interfere with signal transduction pathways, but there are conflicting reports on their effect on $\beta$-adrenergic signaling. Halothane has been reported to antagonize $\beta$-adrenergic responses by inhibiting adenylate cyclase activity in dog, rat, and mouse cardiomyocytes. Lynch reported to antagonize $\alpha$-adrenergic responses by inhibiting adenylate cyclase activity produced by isoproterenol. Conversely, halothane-mediated disruption of the $\beta$-adrenoceptor-$G_i$ protein interaction was postulated to enhance $\beta$-adrenergic signaling in rat and human cardiomyocytes. However, the reported potentiation of $\beta$-adrenoceptor stimulation was only examined on a short-term basis and did not overcome the negative inotropic effects of halothane. The conflicting results may be also attributed to methodologic differences and species differences. Moreover, sympathoadrenergic regulation of cardiac $\text{Ca}^{2+}$ channels by NE involves both $\beta$- and $\alpha$-adrenoceptors, and $\alpha$-adrenergic activation may inhibit $\beta$-adrenergic-stimulated L-type $\text{Ca}^{2+}$ channel currents. Although not specifically investigated in this study, volatile anesthetics may activate adenosine type 1 receptors, which attenuate catecholamine-induced increases in cyclic adenosine monophosphate. Finally, desensitization of cardiac $\beta$-adrenoceptors by volatile anesthetics or as an adaptive mechanism due to continuous $\beta$-adrenergic stimulation may play an important role in the present study.

Under the experimental conditions used, cardiomyocytes were quiescent, and addition of NE did not restore contraction. It is therefore unlikely that the observed anti-apoptotic effect of volatile anesthetics is mediated by a decreased oxygen demand or an improved oxygen supply-to-demand ratio. Caution should be exerted in extrapolating these in vitro observations to in vivo models. Although most volatile anesthetics are known to decrease sympathetic nervous system activity, recent studies provide evidence that desflurane induces intramyocardial catecholamine release in rat ventricular papillary muscle. It is possible that this effect may diminish the postulated anti-apoptotic properties of desflurane. Nonetheless, it is tempting to speculate that some of the previously reported beneficial therapeutic effects of volatile anesthetics may be explained by their inhibition of the apoptotic death signaling in cardiomyocytes. Because the time course for apoptotic cell death is only a few hours, apoptosis may be an important mechanism for loss of viable cardiomyocytes and myocardial dysfunction in the immediate perioperative period. Further studies should evaluate whether other classes of anesthetics, including barbiturates, propofol, or opioids, exert similar anti-apoptotic effects or, indeed, potentiate apoptotic death signaling in cardiomyocytes.

In conclusion, the results of this study indicate that volatile anesthetics offer significant protection against apoptotic cell death stimulated by the $\beta$-adrenergic pathway. The presented experimental data also suggest that the anti-apoptotic effect of volatile anesthetics is governed by modifying cellular $\text{Ca}^{2+}$ homeostasis and by inhibiting activation of the cell death initiator caspase-9 in adult rat cardiomyocytes.

References

endogenous catecholamines in the ischemic myocardium of the rat.


