Nitric oxide induces apoptosis by activating K⁺ channels in pulmonary vascular smooth muscle cells

STEFANIE KRICK, OLEKSANDR PLATOSHYN, MICHELE SWEENEY, SHARON S. McDaniel, SHEN ZHANG, LEWIS J. RUBIN, AND JASON X.-J. YUAN. Nitric oxide induces apoptosis by activating K⁺ channels in pulmonary vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 282: H184–H193, 2002.—Nitric oxide (NO) is an endogenous endothelium-derived relaxing factor that regulates vascular smooth muscle cell proliferation and apoptosis. This study investigated underlying mechanisms involved in NO-induced apoptosis in human and rat pulmonary artery smooth muscle cells (PASMC). Exposure of PASMC to NO, which was derived from the NO donor S-nitroso-N-acetyl-penicillamine, increased the percentage of cells undergoing apoptosis. Increasing extracellular K⁺ concentration to 40 mM or blocking K⁺ channels with 1 mM tetraethylammonium (TEA), 100 nM iberiotoxin (IBTX), and 5 mM 4-aminopyridine (4-AP) significantly inhibited the NO-induced apoptosis. In single PASMC, NO reversibly increased K⁺ currents through the large-conductance Ca²⁺-activated K⁺ (KCa) channels, whereas TEA and IBTX markedly decreased the KCa currents. In the presence of TEA, NO also increased K⁺ currents through voltage-gated K⁺ (Kv) channels, whereas 4-AP significantly decreased the Kv currents. Opening of KCa channels with 0.3 mM dehydroepiandrosterone increased KCa currents, induced apoptosis, and further enhanced the NO-mediated apoptosis. Furthermore, NO depolarized the mitochondrial membrane potential. These observations indicate that NO induces PASMC apoptosis by activating KCa and Kv channels in the plasma membrane. The resulting increase in K⁺ efflux leads to cytosolic K⁺ loss and eventual apoptosis volume decrease and apoptosis. NO-induced apoptosis may also be related to mitochondrial membrane depolarization in PASMC.

mitochondrial membrane potential; calcium; artery; potassium current

IN THE PULMONARY VASCULAR SYSTEM, nitric oxide (NO) is an endothelium-derived relaxing factor (EDRF) that causes vasodilation (23, 36, 41) and inhibits vascular smooth muscle cell growth (42, 50). Inhaled NO has been used clinically to treat patients with pulmonary hypertension (41), in whom persistent vasoconstriction and excessive vascular remodeling are two major contributors to elevated pulmonary vascular resistance and arterial pressure (17). The mechanism for pulmonary vascular remodeling (e.g., medial and myointimal thickening) has been attributed to an increase in proliferation and a decrease in apoptosis of pulmonary vascular smooth muscle and endothelial cells. Therefore, the precise control of the balance of apoptosis and proliferation in pulmonary artery smooth muscle cells (PASMC) may play an important role in maintaining normal pulmonary vascular structure and function (10, 13, 18, 43).

Apoptosis is a physiological mode of cell death that is triggered by diverse external or internal signals. Dysfunction of the apoptosis process has been linked to pathogenesis of cancer, atherosclerosis, and pulmonary vascular diseases (13, 18, 43). Although apoptosis has long been recognized as a principal mechanism for the elimination of redundant, autoreactive, or neoplastic cells, it may also be a mechanism for the elimination of the “misguided” proliferative PASMC in remodeled pulmonary vasculature (43). Indeed, apoptosis in hypertrophied PASMC has been attributed to regression in medial hypertrophy, whereas inhibition of apoptosis is related to progression of pulmonary vascular medial thickening (10, 13, 42, 43). As an EDRF and a therapeutic agent, NO has been demonstrated to exert its antiproliferative effect on pulmonary vasculature by inhibiting cell proliferation (36, 42, 50) and inducing apoptosis (42, 47, 51). However, the cellular mechanisms involved in NO-induced apoptosis are still unclear.

Cell shrinkage (apoptosis volume decrease) is an early characteristic feature commonly used to identify cells undergoing apoptosis. Cytosolic K⁺, a dominant cation in the cytosol with a concentration of 140–150 mM, plays a critical role in maintaining intracellular ion homeostasis and cell volume. Maintenance of a high concentration of intracellular K⁺ ([K⁺]i) is required to maintain the cytosolic ion homeostasis that is necessary to preserve normal cell volume (20, 32, 52). A sufficient [K⁺]; appears to be also required to suppress the activity of caspases and nucleases (6, 13, 18, 43).
that are believed to be the executioners of apoptosis (29).

\[ [K^+]_o \] is basically regulated by Na\(^+-\)K\(^+-\)ATPase and the K\(^+\) channels in the plasma membrane. Owing to the outwardly directed electrochemical gradient (driving force) for K\(^+\), opening of the plasmalemmal K\(^+\) channels would promote efflux or loss of cytosolic K\(^+\) and induce the apoptotic volume decrease and apoptosis. In a number of cell types, enhancement of the plasma membrane permeability to K\(^+\) ions has been associated with early responses to apoptotic stimuli (6, 7, 11, 20, 21). This study was designed to test the hypothesis that NO induces PASMC apoptosis in part by activating K\(^+\) channels in the plasma membrane. Furthermore, we also investigated whether NO affects the mitochondrial membrane potential (\(\Delta \Psi_m\)), which has been demonstrated to regulate mitochondria-dependent apoptosis (19, 29, 35, 58).

**METHODS AND MATERIALS**

**Cell preparation.** Rat PASMC were prepared from pulmonary arteries of male Sprague-Dawley rats (body wt 150–200 g) (55–57). The isolated pulmonary arteries were incubated for 20 min in Hanks’ balanced salt solution containing 1.5 mg/ml collagenase (Worthington Biochemical; Freehold, NJ). Adventitia and endothelium were carefully removed after the incubation. The remaining smooth muscle was then digested with 1.5 mg/ml collagenase and 0.5 mg/ml elastase (Sigma Chemical; St. Louis, MO) at 37°C. The cells were dispersed and plated onto 25-mm coverslips and incubated in DMEM containing 10% fetal bovine serum in a humidified atmosphere of 5% CO\(_2\) in air at 37°C. Human PASMC from normal subjects, purchased from Clonetics (BioWhittaker; Walkersville, MD), were seeded in 250-mm tissue culture dishes containing 10% fetal bovine serum in a humidified atmosphere of 5% CO\(_2\) in air at 37°C. Human PASMC from normal subjects, purchased from Clonetics (BioWhittaker; Walkersville, MD), were seeded in 250-mm tissue culture dishes containing 10% fetal bovine serum in a humidified atmosphere of 5% CO\(_2\) in air at 37°C. Human PASMC from normal subjects, purchased from Clonetics (BioWhittaker; Walkersville, MD), were seeded in 250-mm tissue culture dishes containing 10% fetal bovine serum in a humidified atmosphere of 5% CO\(_2\) in air at 37°C.

**Immunochemistry.** The cells, which were grown on 10-mm coverslips, were washed with PBS (Sigma), fixed in 95% ethanol, and stained with the membrane-permeable nucleic acid stain 4′,6′-diamidino-2-phenylindole (DAPI; Sigma). DAPI (5 \(\mu\)M) was dissolved in an antibody buffer containing 500 mM NaCl, 20 \(\mu\)M Na\(_2\)CO\(_3\), 10 \(\mu\)M BgCl\(_2\), and 20 \(\mu\)M Tris-HCl (pH 7.4). The blue fluorescence emitted at 461 nm was used to visualize the cell nuclei. The DAPI-stained cells were examined with a Nikon TE300 fluorescence microscope, and the cell nuclear images were acquired using a high-resolution fluorescence-imaging system (Solamere Technology; Salt Lake City, UT). For each coverslip, 5–10 fields (with ~20–25 cells/field) were randomly selected to determine the percentage of apoptotic cells in the total cells based on the morphological characteristics of apoptosis. The cells with clearly defined nuclear breakage, remarkably condensed nuclear fluorescence, and significantly shrunken cell body and nucleus were defined as apoptotic cells (28).

**Measurement of rhodamine fluorescence.** The cells, which were grown on 25-mm coverslips, were loaded with 10 \(\mu\)g/ml rhodamine 123 (Rh-123; Molecular Probes; Eugene, OR) incubated for 30 min at 37°C. Rh-123 is taken up selectively by mitochondria (25) and its uptake depends on \(\Delta \Psi_m\). Rh-123 fluorescence was excited at 488 nm and emission at 530 nm was measured using a GEN IV charge-coupled device camera coupled to a Nikon TE300 fluorescence microscope (Solamere Technology). In isolated mitochondria, the relationship between Rh-123 fluorescence and \(\Delta \Psi_m\) is linear at a range of 55–220 mV (14). The Rh-123 fluorescence, which is quenched...
at resting $\Delta \Psi_m$, increases with mitochondrial membrane depolarization. The cells were first superfused with PSS to establish a baseline fluorescence level. Images were then acquired once every 3 s for 5–10 min during which the perfusate was changed at selected time points. The Rh-123 images were acquired and stored in a Macintosh computer and were analyzed using QED software (Solamere Technology) and NIH Image software.

**Chemicals.** The NO donor, S-nitroso-N-acetyl-penicillamine (SNAP; Sigma), was prepared as a 100 mM stock solution in DMSO; aliquots of the stock solution were diluted 100–1,000 times into the culture media or PSS for experimentation. SNAP is a stable NO generator with a half-life of 37 ± 4 h (16). The concentration of SNAP in solution is linearly related to the concentration of NO; for example, 0.01, 0.1, and 1.0 mM SNAP concentrations correspond to 0.013, 0.13, and 1.3 μM NO concentrations (22). Sodium nitroprusside (SNP; Sigma), diethylenetriamine NONOate (DETA-NONOate; Cayman Chemical; Ann Arbor, MI), TEA, iberiotoxin (IBTX; Sigma), and dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one, DHEA; Calbiochem-Novabiochem; San Diego, CA) (15) were directly dissolved in the culture media or PSS on the day of use. In high-K$^+$ PSS or culture medium, NaCl in PSS or the customized DMEM (Cellgro; MediaTech; Herndon, VA) was replaced mole by mole with KCl to maintain the osmolarity of the solution.

**Statistics.** The composite data are expressed as means ± SE. Statistical analysis was performed using paired or unpaired Student's t-test or ANOVA and post hoc tests (Student-Newman-Keuls) where appropriate. Differences were considered to be significant when $P < 0.05$.

**RESULTS**

NO induces apoptosis in PASMC. Treatment of human and rat PASMC for 20 h with NO, which was derived from the NO donor SNAP (0.01–1 mM), induced apoptosis with its typical characteristics of nuclear shrinkage, condensation, and breakage as well as formation of apoptotic bodies (Fig. 1A) and positive staining with TUNEL reagent (Fig. 1B). The apoptotic effect of SNAP on human PASMC was dose dependent with an EC$_{50}$ of $\sim 8 \mu$M (Fig. 1C), which corresponds to $\sim 10$ nM of NO (22). The apoptotic effect of SNAP on human PASMC was also time dependent (Fig. 1D). Three hours after incubation of the cells in media containing 0.1 mM SNAP, the percentage of apoptotic nuclei significantly increased from 4.3 ± 1.3% to 16.5 ± 2.2%, and the apoptotic effect appeared to be maximized (to 34.2 ± 2.9%) at 18–24 h (Fig. 1D). The EC$_{50}$ for 0.1 mM SNAP-induced apoptosis in human PASMC was at $\sim 4.7$ h.

Similar apoptotic effects of other NO donors were also observed in both human and rat PASMC. For...

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**Fig. 1.** Apoptotic effect of nitric oxide (NO) on pulmonary artery smooth muscle cells (PASMC). A: representative 4′-6′-diamidino-2-phenylindole (DAPI)-stained nuclei of human (h) and rat (r) PASMC before (control) and after treatment with 0.1 mM S-nitroso-N-acetyl-penicillamine (SNAP) for 20 h. Nuclear shrinkage, condensation, and breakage of single cells are shown (right). B: rPASMC double-stained with DAPI and transferase-mediated nick-end labeling (TUNEL) for quantitative measurements before (control) and after treatment with SNAP. C: dose-response curve for the apoptotic effect of SNAP concentration ([SNAP]) on hPASMC. Data are means ± SE; $n = 28$–32 fields of cells from 5 coverslips for each data point. Calculated NO concentrations ([NO]) derived from SNAP in solution are shown according to Ichimori et al. (22). D: time course of the apoptotic effect of 0.1 mM SNAP on hPASMC. Data are means ± SE; $n = 15$ fields of cells from 4 coverslips for each data point.
example, DETA-NONOate (0.1 mM) and SNP (0.1 mM) increased the percentage of apoptotic cells from 5.6 ± 1.1% to 26.4 ± 1.9% \((P < 0.01; n = 21)\) and from 5.9 ± 1.2% to 28.0 ± 2.3% \((P < 0.001; n = 12)\), respectively.

Inhibitory effects of 40 mM K\(^+\), TEA, IBTX, and 4-AP and augmenting effect of DHEA on NO-induced apoptosis. Approximately 3–6% apoptotic cells were detected in both rat and human cell cultures under control conditions. Treatment of rat PASMC with SNAP (0.1 mM) for 24 h caused 35–45% of the cells to undergo apoptosis (Fig. 2). Increasing the extracellular K\(^+\) concentration from 5 to 40 mM or adding the KC\(_a\) channel blockers TEA (1 mM) or IBTX (100 nM) and the KC channel blocker 4-AP (5 mM) to the culture media significantly inhibited the SNAP-mediated apoptosis (Fig. 2). In rat PASMC, 40 mM K\(^+\), TEA, or IBTX inhibited the SNAP-induced apoptosis by 65–68% (Fig. 2A). In human PASMC, 40 mM K\(^+\), TEA, IBTX, or 4-AP reduced the SNAP-induced apoptosis by 35 ± 5%, 54 ± 5%, 55 ± 3%, or 59 ± 5%, respectively (Fig. 2B). In contrast, treatment of the cells with 0.3 mM DHEA, an endogenous steroid that has been shown to activate KC\(_a\) channels (15), induced PASMC apoptosis (Fig. 2, A and B) and further enhanced the SNAP-induced apoptosis (by 1.37-fold; see Fig. 2B). These results indicate that NO-induced apoptosis is regulated by the transmembrane K\(^+\) gradient and permeability. The inhibitory effects of 40 mM K\(^+\), TEA, IBTX, and 4-AP on NO-induced apoptosis imply that an increased K\(^+\) efflux through sarcolemmal KC\(_a\) channels is involved in initiating apoptosis in PASMC. The additive effect of DHEA on SNAP-induced apoptosis suggests that apoptosis induced by NO and DHEA may result from different mechanisms (i.e., parallel but independent processes).

Augmenting effect of NO on KC\(_a\) channel activity in PASMC. In human PASMC, a large-amplitude single-channel K\(^+\) current \((I_{KC})\) was observed in cell-attached membrane patches with a symmetrical K\(^+\) gradient (Fig. 3A, left). Slope conductance \((g_K)\) of the channels responsible for the current was 249 pS (Fig. 3A, right), which is consistent with the conductance (200–250 pS) of MaxiK or BK channels described in PASMC (1, 38). Extracellular application of the NO donor SNAP (0.1 mM for 2–5 min) significantly and reversibly increased the activity of the large-conductance KC\(_a\) channels in human PASMC. The steady-state open probability \((NP_{open})\) of the currents was increased by 53-fold (from 0.00576 to 0.31497) (Fig. 3Bb), whereas the time constant \((\tau_{open})\) for the open-times distribution curve (one exponential fitting curve) was increased by 1.8-fold (from 0.625 to 1.121 ms) (Fig. 3Bc). However, SNAP negligibly affected the single-channel conductance of Ca\(^2+\)-activated K\(^+\) currents \([I_{K(Ca)}]\) in human PASMC (Fig. 3C).

To characterize the pharmacological properties of the large-conductance \(I_{K(Ca)}\) we tested the effects of TEA and IBTX on the large-amplitude single-channel \(I_{K(Ca)}\) in human PASMC. Extracellular application of TEA (1 mM) reversibly reduced the single-channel \(I_{K(Ca)}\) in outside-out membrane patches (Fig. 4A) and the whole cell \(I_{K(Ca)}\) (Fig. 4B) in human PASMC dialyzed with a pipette solution containing a low concentration (0.1 mM) of EGTA. It was noticed that extracellular application of 1 mM TEA predominantly decreased the whole cell currents that are noisy at positive potentials (Fig. 4B), which is a characteristic of \(I_{K(Ca)}\) in vascular smooth muscle cells (3, 38). Furthermore, extracellular application of 100 nM IBTX markedly decreased the single-channel \(I_{K(Ca)}\) (Fig. 4C); the averaged \(NP_{open}\) in the cell-attached membrane patches were 0.066566 ± 0.013964 \((n = 16)\) under control conditions (-IBTX, in which no IBTX was included in the pipette solutions) and 0.000439 ± 0.000028 \((n = 21); P < 0.001\) when the pipette (extracellular) solutions contained 100 nM IBTX (+IBTX).

Similar to the low dose of TEA, extracellular application of 100 nM IBTX also reversibly decreased the whole cell \(I_{K(Ca)}\) (data not shown). These results indicate that the large-amplitude single-channel \(I_K\) and the noisy whole cell \(I_K\) were actually the K\(^+\) currents \([I_{K(Ca)}]\) resulting from K\(^+\) efflux through the MaxiK channels (3). The remaining currents during appli-
of 0.1 mM SNAP. Averaged before (control), during (SNAP), and after (wash) 5-min application (Fig. 3). The steady-state open probability: unitary current recordings (B), channel (see Figs. 3 and 4), NO also activates the 4-AP-sensitive Kc channel in human PASMC.

Augmenting effect of DHEA on Kc channel activity in PASMC. DHEA is an endogenous steroid that has been demonstrated to activate MaxiK channels (15). Consistently, we also observed that extracellular application of 0.1 mM DHEA reversibly increased whole-cell and single-channel I(KCa) in human PASMC (Fig. 5). These results suggest that the proapoptotic effect of DHEA and its enhancing effect on NO-induced apoptosis (see Fig. 2) are likely due to the DHEA-induced increase in I(KCa) (Fig. 6).

NO depolarizes the ΔΨm in PASMC. Mitochondrial membrane depolarization has been demonstrated to induce cytochrome c release (2, 18, 29, 58), which subsequently activates cytosolic caspases and induces apoptosis (33). Whether the NO-induced apoptosis (see Fig. 1) is related to ΔΨm depolarization was examined using the cationic dye Rh-123.

Extracellular application of the NO donor SNAP (0.1 mM) caused a 19-fold increase in Rh-123 fluorescence intensity (which increases when ΔΨm decreases) in PASMC (Fig. 7). The Rh-123 fluorescence intensity started to increase almost immediately after superfusion with SNAP and gradually reached a plateau after 4 min (Fig. 7, A and B). Extracellular application of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a protonophore that dissipates the H+ gradient across the inner membrane of mitochondria, rapidly increased the Rh-123 fluorescence intensity (Fig. 7C). Although the time courses were quite different, the maximal increases in Rh-123 fluorescence intensity induced by SNAP and FCCP were similar (118 ± 7% and 129 ± 8%, respectively; P = 0.38; Fig. 7, B and C). Pretreatment of the cells with 40 mM K+ and 100 nM
IBTX had little effect on the SNAP-induced increase in Rh-123 fluorescence (Fig. 7D), which suggests that the antiapoptotic effects of 40 mM K\(^+\) and IBTX are not related to \(\Delta \Psi_m\).

**DISCUSSION**

Intracellular K\(^+\) homeostasis plays an important role in regulating the physiological balance between proliferation and apoptosis (6, 7, 11, 21). A high concentration (140–150 mM) of cytosolic free K\(^+\) ions is required under normal conditions to maintain cell volume (32). Thus an increase in K\(^+\) efflux or a net K\(^+\) loss would lead to an apoptosis volume decrease and apoptosis (6, 7, 11, 34, 52). In addition to controlling cell volume, a high \([K^+]_i\) has been demonstrated to apply a tonic suppressive force on the activity of caspases, a set of cytosolic cysteine proteases that are known as the central executioners of the apoptotic pathway (6, 7, 11, 21, 29). High \([K^+]_i\) has also been demonstrated to suppress the nuclease activity that is responsible for characteristic DNA fragmentation during the apoptotic pathway downstream of the caspase activation (21). When K\(^+\) efflux is enhanced due to the activation of sarclemmal K\(^+\) channels, the net loss of cytoplasmic K\(^+\) would relieve the tonic suppression of cytosolic caspases and nucleases and cause apoptosis.

The NO-induced increase in K\(^+\) currents would result in a net K\(^+\) loss and lead to the apoptosis volume decrease and apoptosis (34, 52). The observations from this study indicate that decrease of K\(^+\) efflux by pharmacological blockade of K\(^+\) channels or by reducing the K\(^+\) electrochemical gradient (using 40 mM K\(^+\)) attenuated the NO-mediated apoptosis, whereas increase of K\(^+\) efflux by activating K\(^+\) channels (using DHEA) or augmenting the K\(^+\) permeability (using valinomycin; data not shown) (28) enhanced the NO-mediated apoptosis. These results support the contention that cytosolic K\(^+\) loss due to increased K\(^+\) efflux through plasmalemmal K\(^+\) channels plays an important role in inducing the apoptosis volume decrease and apoptosis (6, 7, 9, 11, 53, 54). In PASM, NO activated both K\(_{Ca}\) and Kv channels, whereas low doses of TEA and IBTX selectively blocked the large-conductance K\(_{Ca}\) channels and 4-AP blocked the Kv channels. The inhibitory effects of TEA, IBTX, and 4-AP and the augmenting effect of DHEA (which activates K\(_{Ca}\) channels) on the NO-mediated apoptosis suggest that the increased K\(^+\) efflux through opened K\(_{Ca}\) channels is a critical mechanism involved in initiating apoptosis volume decrease and apoptosis in PASM.

The efflux of K\(^+\) is obviously insufficient to cause a volume change in cells, because it must be accompanied by an efflux of an anion to achieve a net reduction in the amount of solute within the cell (34, 52). In

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**Fig. 4.** Inhibitory effects of TEA and IBTX on \(I_{K(Ca)}\) in hPASMC. **A:** unitary current recordings (a) and all-points amplitude histograms (b) from an outside-out membrane patch in asymmetrical K\(^+\) gradient before (cont), during (TEA), and after (wash) extracellular application of 1 mM TEA. Patch membrane potential was held at +30 mV. Averaged \(NP_{av} \pm s\) values were 0.31308, 0.00010, and 0.40872 before, during, and after application of TEA, respectively. Current levels ranging from −40 to +80 mV in 20-mV increments (holding potential, −70 mV) in PASMC before, during, and after extracellular application of 1 mM TEA. Leakage and capacitive currents were subtracted. **B:** representative families of superimposed currents elicited by test potentials ranging from −40 to +80 mV in 20-mV increments (holding potential, −70 mV) in PASMC before, during, and after extracellular application of 1 mM TEA. Leakage and capacitive currents were subtracted. **C:** unitary current recordings from cell-attached membrane patches using the control pipette (extracellular) solutions that did not contain IBTX (−IBTX) and the pipette solutions that contained 100 nM IBTX (+IBTX). Current records were obtained from three PASMC using IBTX-free pipette solutions (a, b, and c) and three cells using IBTX-containing pipette solutions (a′, b′, and c′). Patch membrane potential was held at +70 mV.
smooth muscle cells, \( \text{Cl}^- \) ion is a dominant anion in the cytosol with a concentration of 50–100 mM and a calculated \( \text{Cl}^- \) equilibrium potential of \(-25 \text{ to } -9 \text{ mV} \) (27). Therefore, the NO-mediated membrane hyperpolarization, which is due to activation of \( K_{\text{Ca}} \) and \( K_v \) channels, would increase \( \text{Cl}^- \) efflux by opening \( \text{Cl}^- \) channels and would facilitate the apoptotic volume decrease (cell shrinkage) (34, 52). Indeed, the apoptotic volume decrease and apoptosis are significantly attenuated by blocking either \( K^+ \) or \( \text{Cl}^- \) channels, which suggests that the volume decrease is achieved by a parallel efflux of \( K^+ \) and \( \text{Cl}^- \) and is regulated concurrently by activities of \( K^+ \) and \( \text{Cl}^- \) channels (30, 34, 39, 52). Further studies are needed to investigate whether NO-mediated apoptosis is associated with an increase in \( \text{Cl}^- \) efflux in human and rat PASMC.

The results from this study also demonstrated that NO induced \( \Delta \Psi_m \) depolarization. Although it is still controversial whether \( \Delta \Psi_m \) depolarization is required to trigger apoptosis (29, 31), it has been shown that there is a direct relationship between \( \Delta \Psi_m \) depolarization and the release of cytochrome c and other apoptosis-inducing factors (2, 19, 29, 58). By activating caspase-9 (which subsequently activates the effector caspases-3, -6, and -7) in the cytosol, the cytochrome c release is a control point in the mitochondria-dependent apoptosis (25, 33). Our results showed that 40 mM...
K\textsuperscript+ and IBTX, which inhibit K\textsuperscript+ efflux by lowering the K\textsuperscript+ electrochemical gradient across the plasma membrane and blocking K\textsubscript{Ca} channels, were unable to prevent the NO-induced ΔΨ\textsubscript{m} depolarization but significantly inhibited the NO-induced apoptosis. These findings suggest that NO-mediated K\textsuperscript+ loss through activation of K\textsubscript{Ca} channels works downstream or independent of the mitochondria to trigger apoptosis in PASMC.

Increases in K\textsuperscript+ concentration significantly attenuate the cytochrome c-induced caspase-3 activation in cell lysates, which suggests a direct linkage between cytosolic K\textsuperscript+ ions and caspase-3 activity (11, 21). In isolated nuclei from HeLa cells, a decrease in K\textsuperscript+ concentration from 140 to 80 mM markedly increases apoptosis (11). In lymphocytes, the potent apoptosis inducer staurosporine decreases [K\textsuperscript+]\textsubscript{i} from 140 to 55 mM, whereas a decrease in K\textsuperscript+ concentration in assay buffer from 150 to 80 mM significantly increases DNA degradation in isolated thymocyte nuclei (21). Furthermore, decreasing K\textsuperscript+ concentration from 140 to 80 mM causes a 2.5-fold enhancement of the cytochrome c-mediated increase in caspase-3 activity (21). These observations suggest that a decrease in K\textsuperscript+ concentration is associated with an increase in caspase activity in the presence of apoptosis inducers. Thus a net loss of cytosolic K\textsuperscript+ ions due to the NO-induced activation of

Fig. 7. NO depolarizes the mitochondrial membrane potential (ΔΨ\textsubscript{m}) in PASMC. A: pseudocolor images (a) show rhodamine 123 (Rh-123) fluorescence, which was used to estimate the relative change of ΔΨ\textsubscript{m} in rPASMC before (control) and during application of 0.1 mM SNAP. A three-dimensional plot (b) shows the time course of the Rh-123 fluorescence changes in a line (~375 pixels) drawn through a peripheral area of the cell superfused with the solution including SNAP. B and C: time course of the relative changes of Rh-123 fluorescence measured in the perinuclear areas of the cells before and during application of 0.1 SNAP and 5 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). D: summarized data (means ± SE) show the SNAP-induced relative changes of Rh-123 fluorescence (percentage increase of the fluorescence intensity) in PASMC bathed in solutions with 5 or 40 mM K\textsuperscript+ (n = 19–63 cells) and in solutions with (+) or without (−) 100 nM IBTX (n = 21 cells).

Fig. 8. Schematic diagram illustrates the proposed mechanisms involved in the proapoptotic and vasodilative effects of NO on the pulmonary vasculature. Mito, mitochondria; cyt-c, cytochrome c; 40K, 40 mM K\textsuperscript+; VDCC, voltage-dependent Ca\textsuperscript{2+} channels; PVR, pulmonary vascular resistance; and PAP, pulmonary arterial pressure; circled −, inhibition; circled +, activation.
K⁺ channels would further the caspase-mediated apoptosis.

Clearly, further study is necessary to determine the influence of K⁺ loss or K⁺ channel activation on the individual caspases in the cascade and the interactions between them. It remains to be investigated whether NO-mediated activation of KᵥATP channels in the mitochondrial inner membrane (24, 45) contributes to induction of apoptosis. Owing to the inwardly directed electrochemical gradient of K⁺ across the mitochondrial inner membrane, activation of mitochondrial K⁺ channels would lead to K⁺ influx from the intermembrane space to the matrix, which could subsequently cause ΔΨₘ depolarization, matrix swelling, outermembrane damage, loss of mitochondrial function, and release of cytochrome c (4, 5). Furthermore, it would be very interesting to investigate whether KᵥCa and Kᵥ channels are distributed in the mitochondrial membranes (37, 46) and directly participate in the regulation of mitochondrial function and cytochrome c release in human and rat PASMC.

In summary, the results from this study suggest that at least two mechanisms are involved in NO-induced apoptosis in PASMC: 1) activation of KᵥCa and Kᵥ channels, which leads to the loss of cytoplasmic K⁺ ions and apoptosis volume decrease; and 2) ΔΨₘ depolarization, which leads to the release of apoptosis-inducing factors. It has been well documented that NO also induces membrane hyperpolarization by activating K⁺ channels and causes vasodilation by cGMP-dependent and -independent mechanisms in vascular smooth muscle cells (1, 56). Therefore, the therapeutic effects of NO in patients with pulmonary hypertension may involve, at least in part, 1) vasodilation (36, 41) due to membrane hyperpolarization as a result of activated K⁺ channels, and 2) inhibition of pulmonary vascular remodeling (44) due to PASMC apoptosis that results from activated sarcolemmal K⁺ channels and ΔΨₘ depolarization (Fig. 8).

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