RhoA/Rho Kinase Up-regulate Bax to Activate a Mitochondrial Death Pathway and Induce Cardiomyocyte Apoptosis*

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The small G-protein RhoA regulates the actin cytoskeleton, and its involvement in cell proliferation has also been established. In contrast, little is known about whether RhoA participates in cell survival or apoptosis. In cardiomyocytes in vitro, RhoA induces hypertrophic cell growth and gene expression. In vivo, however, RhoA expression leads to development of heart failure (Sah, V. P., Minamisawa, S., Tam, S. P., Wu, T. H., Dorn, G. W., Ross, J. Jr., Chien, K. R., and Brown, J. H. (1999) J. Clin. Invest. 103, 1627–1634), a condition widely associated with cardiomyocyte apoptosis. We demonstrate here that adenoviral overexpression of activated RhoA in cardiomyocytes induces hypertrophy, which transitions over time to apoptosis, as evidenced by caspase activation and nucleosomal DNA fragmentation. The Rho kinase inhibitors Y-27632 and HA-1077 and the small G-protein RhoA was shown to induce apoptosis in NIH3T3 cells (12 and G13 in COS-7, Chinese hamster ovary, and HEK293 cells (16, 17). The mechanisms by which RhoA can function are not known. The current thinking proposes that RhoA can stimulate cardiomyocyte apoptosis.

There are limited data concerning involvement of RhoA in apoptosis and cell survival. The available literature is also conflicting, suggesting that cell fate in response to RhoA is cell type-specific. Most of the published data indicate that RhoA can serve a protective function (12–14). Thus, inhibiting RhoA/Rho kinase signaling by treatment with C3 exoenzyme (which ribosylates RhoA) or with the Rho kinase inhibitors Y-27632 and HA-1077 has been shown to lead to apoptosis in human umbilical vein endothelial cells (12), neuroblastoma (13), and Jurkat cells (14), consistent with the notion that RhoA is protective. In contrast to the apparent prosurvival effects of RhoA signaling in these cell types, overexpression of wild type or activated L63 RhoA was shown to induce apoptosis in NIH3T3 cells (15). RhoA was also suggested to mediate the apoptotic response to heterologously expressed and constitutively activated Gt12 and Gt13 in COS-7, Chinese hamster ovary, and HEK293 cells (16, 17). The mechanisms by which RhoA can promote cell death have not been elucidated. Whereas RhoA-mediated apoptosis was shown to be Bcl-2-sensitive, the events antagonized by overexpression of this antiapoptotic Bcl-2 family protein were not identified (16).

Bcl-2 family proteins are known to affect cell survival by regulating the permeability of mitochondria. Current thinking holds that the ratio of pro- to antiapoptotic Bcl-2 family proteins can determine cell fate. The prosurvival proteins Bcl-2 and Bcl-Xₐ bind to and sequester proapoptotic factors, such as Bak and Bak, preventing their ability to increase mitochondrial permeability (18). Activation of Bax, which occurs in response to a variety of apoptotic stimuli, leads to its oligomerization and translocation to mitochondria, where it induces cytochrome c release (19–24). The transcription factor p53 has been shown...
to directly regulate Bax expression and can induce apoptosis via activation of the mitochondrial death pathway (25).

The studies reported here are the first to examine RhoA involvement in cardiomyocyte survival, and our findings demonstrate that RhoA, acting through its effector Rho kinase, can induce cardiomyocyte apoptosis. Our work further reveals that RhoA-induced caspase activation and DNA fragmentation are initiated by an early Rho kinase-dependent increase in Bax expression, which is mediated by the transcription factor p53 and executed through Bax effects on the mitochondria.

EXPERIMENTAL PROCEDURES

**Cell Culture**—Neonatal rat ventricular myocytes were prepared from hearts of 2–3-day-old Sprague-Dawley rat pups as described previously (26). Briefly, hearts were digested with collagenase, and myocytes were purified over a Percoll gradient. Myocytes were plated at a density of 0.4×10^6 cells/6-cm dish, or 4×10^6 cells/10-cm dish in serum-containing medium overnight.

**Adenoviral Infection**—Cells were put into serum-free medium supplemented with insulin/transferrin/selenium and infected with varying titers (2–8 pfu/cell) of L63RhoA, a dominant negative p53 (Val^153) mutant defective for DNA binding and transactivation (25), a dominant negative Rho kinase, RB/PHe (TT) (27), or AdCMV control adenovirus for 16 h. Cells were subsequently washed and maintained in serum-free medium supplemented as above. The time of washing is considered as time 0 for all time course studies. Inhibitors were added at infection and replaced following virus washout unless otherwise described. The Rho kinase inhibitors Y-27632 and HA-1077, the caspase-8 and caspase-9 inhibitors, bongkrekic acid, and the Bax-inhibitory peptide V5 and negative control peptide were purchased from Calbiochem. Idun1965 was synthesized and characterized at Idun Pharmaceuticals (28). The GTPase-deficient mutant L63RhoA adenovirus was generated as described previously (4).

**Western Blot Analysis**—Cells were harvested in lysis buffer (20 mM Tris, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 20 mM β-glycerophosphate) supplemented with sodium vanadate, leupeptin, aprotinin, p-nitrophenyl phosphate, phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40. Bradford analysis was performed to determine protein concentration. Equal amounts of protein (10–20 μg) were loaded onto SDS-PAGE, run, and transferred to an Immobilon membrane, and the resulting blot was probed using the following antibodies. The cleaved caspase-3, cleaved caspase-9, total Bax, phospho-Akt (Thr^308 and Ser^473), and phosphoextracellular signal-regulated kinase (p42/44) antibodies were purchased from Cell Signaling Technologies. The RhoA and total MYPT-1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Bax antibody was purchased from BD Biosciences. The Bcl-2 and Bcl-x<sub>L</sub> antibodies were purchased from Transduction Laboratories. The mouse monoclonal anti-Bax (6A7) antibody was purchased from Calbiochem. The phospho-MYPT-1 (Thr^696) antibody was purchased from U.S. Biological.

**TUNEL Staining**—Following viral infection, TUNEL staining was performed using the DeadEnd<sup>TM</sup> fluorometric TUNEL system (Promega) according to the manufacturer’s instructions. Briefly, cells were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, labeled with fluorescein-12-dUTP for 1 h, and incubated with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR) for 1 h at 37 °C to stain actin filaments. To visualize atrial natriuretic factor (ANF), cardiomyocytes were incubated in anti-ANF rabbit polyclonal antibody (Peninsula Laboratories).

**Cell Death ELISA (POD) Assay**—DNA fragmentation indicative of apoptosis was assayed using the cell death detection ELISA<sup>PLUS</sup> (Roche Applied Science) according to the manufacturer’s instructions. Briefly, lysates were incubated with anti-histone-biotin and anti-DNA-POD in a streptavidin-coated microplate for 2 h and washed, 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid substrate was added, and absorbance was measured at 405 nm.

**DNA Laddering**—Genomic DNA was isolated from RhoA or control AdCMV-infected cardiomyocytes treated with Idun1965 or vehicle 48 h following infection. Electrophoresis was performed to separate oligosomal DNA fragments, which were visualized by ethidium bromide staining.

**Activated Bax Immunoprecipitation**—Cell lysates were prepared in lysis buffer described above, and 200 μg of total protein was preclariifed with Protein G-Sepharose for 30 min at 4 °C. Samples were then incubated with 4 μg of anti-Bax monoclonal antibody (clone 6A7) at 4 °C overnight. Immunocomplexes were precipitated with 100 μl of 50% slurry Protein G-Sepharose and washed with ice-cold lysis buffer, and beads were boiled in 2× LDS buffer to elute captured protein. Proteins were resolved by SDS-PAGE and probed with total Bax antibody.

**Mitochondrial and Cytosolic Fractionation**—Cytosolic and mitochondrial fractions were prepared according to the manufacturer’s instructions (cytosol/mitochondria fractionation kit; Calbiochem). Briefly, cells were collected in ice-cold PBS, spun down, resuspended in cytosol extraction buffer mix, vortexed, and incubated on ice for 10 min. Samples were centrifuged at 2600 rpm for 10 min, and supernatant was transferred and spun at 9600 rpm for 30 min to precipitate mitochondria. Supernatant was removed as the cytosolic fraction, and the pellet was resuspended in mitochondrial extraction buffer mix as the mitochondrial fraction.

**Fluorescence-activated Cell Sorting (FACS) Analysis of Tetramethylrhodamine Ethyl Ester (TMRE) Fluorescence**—As described previously by our laboratory (29), cells were loaded with a 50 nM concentration of the fluorescent mitochondrial potential-dependent indicator TMRE (Molecular Probes) for 20 min at room temperature and collected by trypsinization (0.05% trypsin, 0.53 mM EDTA). The fluorescence intensity was monitored at 582 nm (FL-2 channel) by FACS (BD Biosciences) (15,000 cells/sample).

**Quantitative PCR**—Real time PCR was performed using pre-optimized Taqman Gene Expression Assays to quantify Bax and glyceraldehyde-3-phosphate dehydrogenase mRNA as described by the manufacturer (Applied Biosystems).
RhoA-induced cardiomyocyte apoptosis (Fig. 1). Neonatal rat ventricular myocytes were infected with constitutively activated L63RhoA adenovirus or control AdCMV adenovirus at 4 pfu/cell. Cells were treated with 10 μM Idun1965 (Idun), a nonselective caspase inhibitor, or vehicle as described under “Experimental Procedures.” A, genomic DNA was harvested 48 h following virus washout. B, lysates were prepared 48 h following virus washout, and nucleosomal fragmentation was quantified by the ELISA-based POD assay. Values are represented as averages ± S.E. (n = 4), *** p < 0.001 versus AdCMV. #, p < 0.001 versus RhoA. C, myocytes were infected with L63RhoA or control AdCMV adenovirus at increasing concentrations, and lysates were prepared 48 h following virus washout. Nucleosomal fragmentation was quantified by POD assay. Values represent averages ± S.E. (n = 5). *** p < 0.001 versus AdCMV. D, SDS-PAGE was performed, and Western blot analysis was quantified by densitometry. Values represent averages ± S.E. (n = 3), ** p < 0.01 versus AdCMV.

**Statistical Analysis**—All results are reported as mean ± S.E. Comparisons of two groups were accomplished using unpaired Student’s t test. Experiments with more than two groups were compared by one-way analysis of variance followed by the Tukey post hoc test for comparison between groups.

**RESULTS**

Expression of activated (L63) RhoA induced DNA fragmentation as assessed by DNA laddering. This response, a hallmark of apoptosis, was completely blocked when cells were concomitantly treated with the nonselective caspase inhibitor Idun1965 (Fig. 1A). Quantitative analysis of this response by the ELISA-based POD assay showed a 3-fold increase in DNA fragmentation elicited by RhoA expression, which was fully inhibited by Idun1965 (Fig. 1B).

To further characterize the RhoA-induced apoptotic response, we infected cardiomyocytes with a range of concentrations of activated RhoA adenovirus and quantified DNA fragmentation by POD assay. At a dose half of what we used initially (2 pfu/cell), no significant increase in DNA fragmentation was detected. However, when the initial dose was doubled, the apoptotic response nearly doubled to roughly 5-fold over control (Fig. 1C). Activation of caspase-3 is a hallmark of apoptotic cell death, and caspase-3 cleavage is indicative of its activation. As determined by Western blot analysis, caspase-3 cleavage also increased dose dependently in response to increased RhoA expression, providing further evidence of RhoA-induced cardiomyocyte apoptosis (Fig. 1D).

Western blot analysis using a RhoA-specific primary antibody revealed that total RhoA levels increased ~2.5-fold in cells infected with 4 pfu/cell RhoA adenovirus for 48 h (Fig. 2, A and B). The amount of activated RhoA was investigated by pull-down assay using the RhoA binding domain of the RhoA effector rhotekin to precipitate GTP-bound RhoA as described previously (30, 31). The level of GTP-bound RhoA in cells expressing the activated protein was increased ~9-fold over control AdCMV infected cells. This increase was comparable with the increased RhoA activation observed in cardiomyocytes treated with the G-protein-coupled receptor agonist sphingosine 1-phosphate (S1P) (Fig. 2C). Thus, the cellular responses we observe occur in response to physiologically relevant, albeit sustained, increases in RhoA activity.

The involvement of RhoA in cardiomyocyte hypertrophy has been well documented, and the ability of activated RhoA to induce myofilament organization and ANF gene expression has been shown previously by our laboratory (4). Marked hypertrophy, evidenced by increases in cell size and actin myofilament organization, was observed by 24 h (Fig. 3A). Increased ANF expression was also clearly evident at this time (Fig. 3A). Apo-
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FIGURE 4. Rho kinase mediates RhoA-induced cardiomyocyte apoptosis. Neonatal rat ventricular myocytes were infected with L63RhoA, dominant negative Rho kinase (DN) at 500 viral particles/cell, or control AdCMV adenovirus and treated with 10 μM Y-27632 (Y), 10 μM HA-1077 (HA), or vehicle as described under Experimental Procedures. Cell lysates were obtained 48 h following virus washout. A, Western blot analysis was performed using specific antibodies to determine the level of phosphorylated (Thr696) MYPT-1 and total MYPT-1, respectively. B, the POD assay was used to quantify DNA fragmentation. Values represent averages ± S.E. (n = 5), **, p < 0.01 versus AdCMV. #, p < 0.01 versus RhoA. C, Western blot analysis was performed to detect levels of cleaved caspase-3 using a selective antibody that only recognizes the 17-kDa fragment. Values represent averages ± S.E. (n = 4). **, p < 0.01 versus AdCMV. #, p < 0.01 versus RhoA.

FIGURE 5. RhoA induces apoptosis through caspase-9 activation. Neonatal rat ventricular myocytes were infected with L63RhoA or control AdCMV adenovirus. A, cells were treated with 10 μM Y-27632 (Y) or vehicle, and lysates were obtained 48 h after virus washout. Western blot analysis was performed to detect the 17-kDa fragment of cleaved caspase-9. Values were quantified by densitometry and represent averages ± S.E. (n = 4). **, p < 0.01 versus AdCMV. #, p < 0.01 versus RhoA. 8, cardiomyocytes were treated with a caspase-8- or caspase-9-selective inhibitor or vehicle. Lysates were prepared 48 h following virus washout, and POD assay was performed to quantify DNA fragmentation. Values represent averages ± S.E. (n = 4). **, p < 0.01 versus RhoA.

Apoptosis, in contrast, was not observed until later times. No TUNEL-positive nuclei were detected at 24 h, although numerous apoptotic cells were detected at 48 h (Fig. 3B). POD and DNA laddering assays also failed to reveal signs of apoptosis until 36 h or later (data not shown). Thus, RhoA signaling initially elicits a hypertrophic response, whereas additional time is required for development of apoptosis.

To begin to elucidate the mechanism for RhoA-induced apoptosis, we examined the possibility that the RhoA effector Rho kinase was involved. Rho kinase activity was assessed by examining phosphorylation of the myosin binding subunit of myosin light chain phosphatase, MYPT-1, a well established Rho kinase-specific substrate. Western blot analysis showed increased MYPT-1 phosphorylation in cells expressing RhoA, and this was blocked by the Rho kinase inhibitor Y-27632 and adenoviral expression of a Rho kinase mutant shown previously to have strong dominant negative activity (27) (Fig. 4A). When cardiomyocytes were treated with Y-27632, or another Rho kinase inhibitor, HA-1077, at the time of RhoA infection and again after virus washout there was significant protection from RhoA-induced DNA fragmentation (Fig. 4B). Comparable protection was also observed with expression of the dominant negative Rho kinase (Fig. 4B). We also examined the involvement of Rho kinase in RhoA-induced caspase-3 activation. Myocytes infected with activated RhoA and treated with Y-27632 were assayed for caspase-3 cleavage by Western blot analysis. As shown in Fig. 4C, Y-27632 treatment completely prevented RhoA-induced caspase-3 cleavage, providing further evidence that Rho kinase mediates this apoptotic response.

We next determined which initiator caspases upstream of caspase-3 were activated by RhoA. Caspase-8 activation is typically associated with the extrinsic or death receptor pathway of apoptosis, whereas caspase-9 is involved in the mitochondrial apoptotic death pathway and is activated following mitochondrial disruption and cytochrome c release. We examined caspase-9 activation by Western blot analysis and found a significant increase in the cleaved caspase-9 product in RhoA-infected compared with control cells (Fig. 5A). This increase in caspase-9 cleavage was fully abrogated by treatment with Y-27632, indicating that both RhoA and Rho kinase are upstream of caspase-9 activation. Western analysis of cleaved caspase-8 showed no increase following RhoA overexpression (not shown). Caspase-8 and caspase-9 peptide inhibitors were
then used to selectively block the function of each respective caspase. Inhibition of caspase-8 provided no protection against RhoA-induced DNA fragmentation, whereas treatment with the caspase-9 inhibitor afforded complete protection (Fig. 5B). These data indicate that caspase-9 mediates RhoA-induced apoptosis and imply involvement of the mitochondrial death pathway.

A well characterized mechanism of cytochrome c release and caspase-9 activation is through the mitochondrial permeability transition pore (mPTP). Opening of the mPTP leads to an influx of ions into the mitochondrial matrix, resulting in loss of mitochondrial membrane potential, swelling, and rupture of the outer mitochondrial membrane and release of cytochrome c. To test whether the mPTP was involved in the RhoA-induced apoptotic response, we used a known mPTP blocker, bongkrekic acid. Treatment with bongkrekic acid did not prevent DNA fragmentation induced by RhoA overexpression (Fig. 6A). Mitochondrial membrane potential was also monitored in response to RhoA infection. The mitochondrial membrane potential-sensitive dye TMRE was used to stain for intact mitochondria, and cells were then sorted by FACS analysis, as described in our previous work (29). No change in mitochondrial membrane potential was observed in cells infected with RhoA compared with control infected cells (Fig. 6B). This contrasts with the marked loss of mitochondrial membrane potential in cells infected with constitutively activated Go_{q} (Fig. 6B), as shown previously (29). These data argue against involvement of an mPTP-dependent mechanism in RhoA-mediated caspase activation and apoptosis.

Previous reports have provided evidence that the proapoptotic Bcl-2 family protein Bax can translocate to the outer mitochondrial membrane. At the outer mitochondrial membrane, Bax, perhaps in concert with the proapoptotic protein Bak, can form a pore, which allows the release of cytochrome c from the intermembrane space and activation of downstream caspases. To test the hypothesis that RhoA increased the ratio of proapoptotic to antiapoptotic Bcl-2 family proteins, we performed Western blot analysis for Bax, Bak, Bcl-2, and Bcl-xL proteins. Levels of Bax protein were increased ~4-fold over control following 48 h of expression of activated RhoA, whereas levels of Bcl-2 and Bcl-xL, prosurvival mediators, were unchanged. We also detected no change in Bak protein expression (Fig. 7A). The RhoA-induced increase in Bak protein was completely abrogated by treatment with Y-27632 and expression of the dominant negative Rho kinase construct (data not shown), suggesting upstream Rho kinase involvement (Fig. 7B). Bax mRNA was also measured in response to RhoA expression. Using real time PCR, we determined that Bax message was increased ~2-fold at 24 h and 2.5-fold at 48 h, suggesting transcriptional activation of this gene (Fig. 7C).

To investigate whether RhoA expression increases levels of activated Bax protein, we used the mouse monoclonal anti-Bax (6A7) antibody, which recognizes only the active conformation of Bax, to immunoprecipitate Bax from cell lysates. There was a marked increase in activated Bax in cells expressing RhoA compared with control infected cells (Fig. 8A). This increase was inhibited by treatment with the Rho kinase inhibitor Y-27632 (Fig. 8B) and decreased in cells expressing dominant negative Rho kinase construct (data not shown). These results suggest that RhoA-induced increase in Bax activity is through Rho kinase-dependent pathway.

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FIGURE 6. RhoA does not signal through mPTP to elicit cardiomyocyte apoptosis. Neonatal rat ventricular myocytes were infected with L63RhoA or control AdCMV adenovirus. A, cardiomyocytes were treated with 50 µM bongkrekic acid (BA) or vehicle, and lysates were prepared 48 h following virus washout. POD assay was performed, and DNA fragmentation was quantified. Values represent averages ± S.E. (n = 4). ***, p < 0.001 versus AdCMV. * p < 0.01 versus AdCMV. B, cardiomyocytes were infected with L63RhoA, AdCMV, or activated Go_{q} (Q209L). 48 h after virus washout, cells were loaded with 50 nM TMRE for 20 min, collected by trypsinization, and subjected to FACS analysis. Data are from a representative experiment replicated three times.

FIGURE 7. RhoA induces Rho kinase-dependent up-regulation of Bax. Neonatal rat ventricular myocytes were infected with L63RhoA or control AdCMV adenovirus. A, Western blot analysis was performed on lysates collected 48 h after virus washout to determine Bcl-2 family protein expression. B, cells were treated with 10 µM Y-27632 (Y) or vehicle prior to Western analysis. Values represent averages ± S.E. (n = 4). ***, p < 0.001 versus AdCMV. #, p < 0.01 versus RhoA. C, RNA was prepared from AdCMV- and L63RhoA-infected myocytes at 0, 24, and 48 h following virus washout, and real-time PCR was performed. Bax mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA. Values represent averages ± S.E. (n = 9). ***, p < 0.001 versus time-matched AdCMV.
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To better understand the role of Bax in RhoA signaling, we examined the kinetics of Bax protein expression. A roughly 1.5-fold increase in Bax protein, albeit not statistically significant, was seen as early as 12 h after virus washout. By 24 h, Bax protein levels were more than 2-fold greater than control, and a 4-fold increase was apparent at 48 h (Fig. 10A). These data indicate that Bax up-regulation is an early response to RhoA activation. As demonstrated above (Figs. 4 and 5), Rho kinase inhibitors prevent RhoA-induced DNA fragmentation and caspase activation. Interestingly, however, when the addition of the Rho kinase inhibitor was delayed until 12 h after virus washout (time denoted by an *arrow* in Fig. 10A), it failed to protect against development of apoptosis (Fig. 10B). The delayed addition of the Rho kinase inhibitor also failed to prevent the RhoA-induced increase in Bax expression (Fig. 10C). These data suggest that increased Bax expression is initiated within a 12-h time window and is necessary to achieve RhoA- and Rho kinase-induced apoptosis.

The transcription factor p53 is a known inducer of apoptosis and can activate transcription of various proapoptotic factors, including Bax (25, 36–38). To further investigate the up-regulation of Bax, we tested the effect of blocking p53 function on Bax protein expression. Adenoviral expression of a mutant p53 construct, shown previously to act as a dominant negative in *in vivo* as well as in *in vitro* (39), significantly inhibited RhoA-induced Bax protein up-regulation (Fig. 11A). Further, blocking p53 function with the dominant negative construct markedly inhibited RhoA-induced DNA fragmentation (Fig. 11B).

Cardiomyocyte hypertrophy initially develops as a compensatory mechanism to increased cardiac demand but often transitions to heart failure, a condition for which the importance of apoptotic cell loss has been established. To determine whether enhanced RhoA expression could contribute to this process, we tested the effect of activated RhoA on hypertrophied cardiomyocytes. Phenylephrine (PE), although not an efficacious activator of RhoA, is a robust hypertrophic agonist in neonatal rat
ventricular myocytes (40). To test the effect of hypertrophy on the ability of RhoA to induce Bax up-regulation and cardiomyocyte apoptosis, we pretreated myocytes with PE for 24 h and then infected the cells with control AdCMV or RhoA adenovirus. Our data show that PE pretreatment, which induces hypertrophy, synergistically increases RhoA-induced Bax up-regulation (Fig. 12A). Interestingly, myocytes that were initially hypertrophied were partially protected against RhoA-induced cell death (Fig. 12B). PE has been shown previously to activate numerous signaling pathways known to afford protection in cardiac myocytes. As shown in Fig. 12C, a 36-h PE treatment increased phosphorylation of two known protective molecules, extracellular signal-regulated kinase (p42, p44) and Akt, which may account for the ability of PE treatment to attenuate RhoA-induced apoptosis.

**DISCUSSION**

We previously demonstrated that expression of RhoA in cardiomyocytes induces cellular hypertrophy and hypertrophic gene expression (4). These observations are confirmed by the increases in cell size and ANF expression shown here following 24 h of RhoA expression. Although an initial hypertrophic response was observed, cardiomyocytes expressing RhoA for 36–48 h became frankly apoptotic, as indicated by TUNEL

![FIGURE 10. Bax up-regulation is an early RhoA/Rho kinase-mediated event that is sufficient to cause apoptosis. Neonatal rat ventricular myocytes were infected with L63RhoA or control AdCMV adenovirus. Cells were harvested 48 h prior to infection with L63RhoA or control AdCMV adenovirus. Myocytes were harvested 12 h following virus washout, and lysates were prepared. Western blot analysis was performed to detect Bax protein. Values are averages ± S.E. (n = 3). *, p < 0.05 versus vehicle AdCMV. B, POD analysis was performed to quantify DNA fragmentation. Values are averages ± S.E. (n = 3).**, p < 0.001 versus vehicle AdCMV. C, Western blot analysis was performed to determine Bax protein expression in cells treated as described in B.

![FIGURE 11. RhoA-induced Bax up-regulation and apoptosis are mediated by p53. Neonatal rat ventricular myocytes were co-infected with L63RhoA or control AdCMV adenovirus and dominant negative p53 adenovirus (8 pfu/cell). Myocytes were harvested 48 h following virus washout, and lysates were prepared. Western blot analysis was performed to detect Bax protein. Values are averages ± S.E. (n = 7). ***, p < 0.001 versus AdCMV. #, p < 0.001 versus RhoA. B, POD analysis was performed to quantify DNA fragmentation. Values are averages ± S.E. (n = 9). ***, p < 0.001 versus AdCMV. #, p < 0.001 versus RhoA.

![FIGURE 12. Phenylephrine treatment accentuates RhoA-induced Bax up-regulation but attenuates RhoA-induced apoptosis. A and B, neonatal rat ventricular myocytes were treated with 100 μM phenylephrine or vehicle for 24 h prior to infection with L63RhoA or control AdCMV adenovirus. Myocytes were harvested 12 h following virus washout, and lysates were prepared. Western blot analysis was performed to detect Bax protein. POD analysis was performed to quantify DNA fragmentation. Values are averages ± S.E. (n = 3). ***, p < 0.001 versus vehicle AdCMV. #, p < 0.001 versus RhoA. C, Western blot analysis was performed to detect phospho-p42/44, phospho-Akt (Thr308 and Ser473), and α-actinin.

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staining, DNA laddering, and fragmentation, and caspase activation. All of these responses were blocked by treatment with either of two Rho kinase inhibitors, Y-27632 and HA-1077, as well as expression of dominant negative Rho kinase. Remarkably, although DNA fragmentation and caspase activation were not evident for 36–48 h, a critical event occurs during the first 12-h window. Thus, when the addition of Rho kinase inhibitors is delayed until 12 h after virus washout, DNA fragmentation is not prevented. The effects of Rho kinase that ultimately lead to apoptosis must therefore be elicited at the time of hypertrophy to initiate the subsequent sequence of apoptotic events.

There are currently two major paradigms for apoptotic cell death: the receptor-mediated “extrinsic” pathway and the mitochondrial mediated “intrinsic” pathway. The mitochondrial death pathway is initiated by cellular signals that ultimately impact upon the mitochondria, leading to the release of cytochrome c and caspase activation (18). Our finding that caspase-9 is activated downstream of RhoA/Rho kinase and that a caspase-9-selective inhibitor is able to block RhoA-induced DNA fragmentation implicates a mitochondrial death pathway in RhoA-induced apoptosis. The lack of caspase-8 activation and a lack of protection by inhibition of caspase-8 further support the notion that a mitochondrial rather than a death receptor (extrinsic) pathway is involved. Our finding that expression of activated RhoA induces cytochrome c release (data not shown) provides additional support for a mitochondrial pathway. There are currently two known mechanisms of cytochrome c release from mitochondria, the first involving the opening of the mPTP and the second dependent on Bcl-2 family-mediated pore formation. Cells expressing RhoA for up to 48 h showed no loss of inner mitochondrial membrane potential as assessed using the mitochondrial membrane potential sensitive dye, TMRE, and FACS analysis. This contrasted with data obtained in parallel using cells infected with constitutively activated Goαq, which dramatically decreased mitochondrial membrane potential (29, 41). Bongkrekic acid, an mPTP blocker previously shown to protect against Goαq-induced apoptosis (41), did not prevent RhoA-induced apoptosis, further suggesting that the mPTP was not involved.

An alternative mechanism of mitochondrial mediated caspase activation and apoptosis involves altered expression or activity of Bcl-2 family proteins. There is general agreement that a balance between pro- and antiapoptotic Bcl-2 family proteins can directly regulate the mitochondria and determine cell fate (42). An initial screen found that the amount of Bax protein present in the cell was increased ~4-fold over control levels in response to activated RhoA. In contrast, Bcl-2, Bcl-xL, and Bak protein levels were unaffected by expression of RhoA. The time course of Bax expression revealed a trend toward increased protein at 12 h, a significant 2-fold increase by 24 h, and maximal expression at 48 h. Bax mRNA levels were also significantly increased at 24 h. Rho kinase was implicated in Bax up-regulation, since the increase in Bax protein expression was inhibited by Y-27632 and dominant negative Rho kinase. Remarkably, although inhibition of Rho kinase with Y-27632 could fully abrogate RhoA-induced up-regulation of Bax protein, this response was not prevented when Y-27632 addition was delayed until 12 h after virus washout. These data suggest that Bax up-regulation is an early RhoA/Rho kinase-mediated event that, once initiated, is sufficient to lead to apoptosis at later times.

Two published studies demonstrated that RhoA-mediated apoptosis was Bcl-2-sensitive (16, 17), but the apoptotic pathway inhibited by Bcl-2 was not elucidated. Prosurvival Bcl-2 family proteins are known to antagonize Bax-mediated mitochondrial membrane permeabilization (35), cytochrome c release, and cell death (34). Thus, our finding that RhoA-induced apoptosis involves the up-regulation of Bax is consistent with, and can explain the protective effect of, increased Bcl-2 expression observed in these earlier studies. Further, adenoviral expression of either Bcl-2 or Bcl-xL prevented RhoA-induced cardiomyocyte apoptosis (data not shown), presumably by antagonizing Bax function.

Activation and mitochondrial translocation of Bax appear to be required for its apoptotic activity. Bax is present in the cytosol of unstimulated cells, and following its activation and change in conformation induced by apoptotic insult (43), it translocates to mitochondria (19, 32). Death signals cause Bax to oligomerize at the outer mitochondrial membrane (20, 21, 44), where this complex can form a pore allowing the release of cytochrome c and other apoptogenic factors into the cytosol (23, 24). We therefore also examined Bax activation and association with mitochondria. Our data provide evidence that in addition to up-regulating expression of Bax, RhoA expression leads to an increase in activated Bax and to an increase in Bax protein associated with the mitochondria. Bax translocation has not been extensively documented in cardiomyocytes, although recent evidence suggests that this can occur in neonatal rat ventricular myocytes in response to simulated ischemia/reperfusion both acutely (3 h) and chronically (36 h) (45, 46). Further, the ability of bax ablation to prevent decreased cardiac function induced by ischemia/reperfusion in mice demonstrates its role in cardiac injury (47). Importantly, our studies using the Bax inhibitor peptide provide evidence that Bax function is critical to subsequent caspase activation, DNA fragmentation, and apoptosis. Our finding that sustained RhoA activation (48 h) can also increase mitochondrial distribution of Bax, in combination with previous data demonstrating increased RhoA activity following myocardial infarction (48, 49), suggests that RhoA activation could play a role in ischemia/reperfusion-induced cell death.

The tumor suppressor p53 has a well documented role in activating gene expression leading to apoptosis. p53-regulated proapoptotic target genes include puma, noxa, bid, and bax (36–38, 50). Our finding that RhoA increased Bax mRNA and our studies using a dominant negative mutant of p53, which acts to inhibit endogenous p53 function (39), implicate p53 in RhoA-induced increases in Bax protein and DNA fragmentation. These data suggest that p53 mediates the apoptotic response and provides a link between RhoA/Rho kinase and Bax up-regulation.

There is a growing literature indicating that inhibiting RhoA/Rho kinase has salutary effects in cardiovascular disease. It has been reported that RhoA/Rho kinase expression and activity are up-regulated in diabetes (51), hypertension (48, 49), and myocardial infarction (52, 53). Recent reports in the literature...
suggest that treatment with the Rho kinase inhibitors Y-27632 and fasudil (HA-1077) affords protective cardiac effects in vivo. Rho kinase inhibition was able to decrease myocardial fibrosis in response to infarct in the rat (54). Inhibition of Rho kinase with Y-27632 was also shown to reduce infarct size and apoptosis resulting from 30-min ischemia followed by 150 min (rat) or 24 h (mouse) of reperfusion (53, 54). Further, Rikitake et al. (55) found decreased fibrosis in ROCK-1 (Rho kinase) haploinsufficient mice following myocardial infarct and pressure overload. These data all demonstrate salutary effects of blocking Rho kinase activity, although it remains unclear whether the beneficial effect of blocking Rho kinase in these in vivo models reflects the inhibition of Rho kinase activity in the cardiomyocyte or in other peripheral cell types, such as fibroblasts, neutrophils, or endothelial cells. However, a recent report by Chang et al. (56) describes the importance of caspase-3 cleavage in activation of ROCK-1 and apoptosis in cardiomyocytes. We demonstrate here that sustained RhoA and Rho kinase activity in cardiomyocytes can lead to caspase-3 activation and apoptosis. Taken together, these findings suggest that Rho kinase can function both upstream and downstream of caspase-3 to induce cardiomyocyte apoptosis. Further, they provide an explanation for the salutary cardiac effects of Rho kinase inhibitors in vivo.

Based on our findings, we propose that RhoA activation in cardiomyocytes signals through its effector Rho kinase to induce p53-mediated increases in Bax mRNA, total and activated Bax protein, and Bax protein present at mitochondria. Why this up-regulation of Bax expression occurs early and does not induce apoptosis until later times is still unclear. It is possible that other genes are concomitantly up-regulated, possibly prosurvival genes that allow the cell to balance the apoptotic actions of Bax with survival pathways. Interestingly, in this regard, we find that the hypertrophic agonist PE acts synergistically with RhoA to induce Bax up-regulation but does not exacerbate RhoA-induced apoptosis. Indeed, PE attenuates RhoA-induced apoptosis, probably due to its ability to activate protective molecules, such as extracellular signal-regulated kinase and Akt. This paradigm suggests a balance between prosurvival and proapoptotic signaling events in the hypertrophic cardiomyocyte. This balance may occur in RhoA-infected cells as well, since our preliminary studies suggest that RhoA expression activates the survival molecule Akt at early times. Over time, however, Bax up-regulation appears to be sufficient to shift the balance in Bcl-2 family protein expression, induce mitochondrial permeabilization, initiate activation of caspase-9 and -3, and ultimately induce cardiomyocyte DNA fragmentation and cell death (schema in Fig. 13). We are currently generating inducible lines of cardiac specific RhoA transgenic mice as model systems that will allow us to temporally control RhoA expression and signaling in the cardiomyocyte in vivo. This should avoid the lethality observed with our previous RhoA transgenic lines (11) and provide a means to further explore the salutary and deleterious cardiac effects of RhoA in a physiologic context.

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**FIGURE 13. Model of the pathway by which RhoA induces apoptosis.** RhoA/Rho kinase induce p53 mediated up-regulation of Bax expression and activation. Bax translocates to and permeablizes the mitochondria, allowing the release of apoptogenic mediators and subsequent caspase-9 and caspase-3 activation, culminating in cardiomyocyte apoptosis.

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