Inhibition of Protein Geranylgeranylation and RhoA/RhoA Kinase Pathway Induces Apoptosis in Human Endothelial Cells*

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Geranylgeranylation of RhoA small G-protein is essential for its localization to cell membranes and for its biological functions. Many RhoA effects are mediated by its downstream effector RhoA kinase. The role of protein geranylgeranylation and the RhoA pathway in the regulation of endothelial cell survival has not been elucidated. The hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor lovastatin depletes cellular pools of geranylgeranyl pyrophosphate and farnesol pyrophosphate and thereby inhibits both geranylgeranylation and farnesylation. Human umbilical vein endothelial cells (HUVECs) were exposed to lovastatin (3 μM-30 μM) for 48 h, and cell death was quantitatively determined by cytoplasmic histone-associated DNA fragments as well as caspase-3 activity. The assays showed that lovastatin caused a dose-dependent endothelial cell death. The addition of geranylgeraniol, which restores geranylgeranylation, rescued HUVEC from apoptosis. The geranylgeranyltransferase inhibitor GTGT-298, but not the farnesyltransferase inhibitor FTI-277, induced apoptosis in HUVEC. Cell death was also induced by a blockade of RhoA function by exoenzyme C3. In addition, treatment of HUVEC with the RhoA kinase inhibitors Y-27632 and HA-1077 caused dose-dependent cell death. Y-27632 did not inhibit other well known survival pathways, such as NF-κB, ERK, and phosphatidylinositol 3-kinase/Akt. However, there was an increase in p53 protein level concomitant with Y-27632-induced cell death. Unlike the apoptosis induced by TNF-α, which occurs only with inhibition of new protein synthesis, apoptosis induced by inhibitors of HMG-CoA reductase, geranylgeranyltransferase, or RhoA kinase was blocked by cycloheximide. Our data indicate that inhibition of protein geranylgeranylation and RhoA pathways induce apoptosis in HUVEC and that induction of p53 or other proapoptotic proteins is required for this process.

Apoptosis is a regulated process of programmed cell death, exhibiting characteristic morphological and biochemical hallmarks, which differ from those seen in necrosis. The function of apoptosis is to eliminate excessive cells, as well as those cells that develop improperly or sustain genetic damage. Apoptosis plays an important role both in development and in homeostasis and is a regulatory mechanism in organ growth, wound repair, tumor genesis, and immune response (1).

Endothelial cells (ECs) line vessels in every organ system and regulate the flow of nutrient substances, diverse biologically active molecules, and blood cells themselves. The regulation of EC survival and death is critical to vascular development and homeostasis. Perturbations of this balance contribute to various vascular diseases (2). Endothelial cell apoptosis is induced by diverse extrinsic and intrinsic signals, such as serum starvation (3), radiation (4), oxidative stress from hypoxia (5), lipopolysaccharide, and cytokines (6). We first reported that human ECs were rendered susceptible to tumor necrosis factor (TNF)-α-initiated death by RNA or protein synthesis inhibitors, indicating that TNF-α initiates a survival pathway that depends on protein synthesis and a death pathway that does not (6, 7).

Prenylation is an important mechanism of posttranslational modification of proteins. Prenylated proteins are modified by formation of cysteine thiethers with the isoprenoid lipids, farnesyl (C15), or geranylgeranyl (C20), at the carboxyl terminus. Geranylgeranylation and farnesylation are catalyzed by the enzymes geranylgeranyltransferase (GGTase) and farnesyltransferase (FTase), respectively. Both enzymes modify cysteines of proteins that end with the motif CAAX (C is Cys, A is an aliphatic amino acid, X is any amino acid) at their carboxyterminal. GGTase prefers leucine or isoleucine in the X position, whereas FTase prefers serine or methionine (8, 9). GGT1-298 and FTI-277 are CAAX peptidomimetics that potently and selectively inhibit GGTase I and FTase, respectively (10, 11). Prenylation is required for proper subcellular localization and biological function of these proteins (12, 13). The proteins that undergo these modifications participate in important cell regulatory functions, particularly signal transduction pathways. Protein prenylation has been shown to be involved in cell adhesion (14), cell proliferation (15), malignant transformation (11), and cell survival (16).

Many of the prenylated proteins are small G-proteins, which include the Ras, Rho, and Rac families. Prenylation of small G-proteins with farnesyl or geranylgeranyl groups is essential for their localization to cell membranes and hence for their biological functions. RhoA is geranylgeranylated (17), whereas

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1 The abbreviations used are: EC, endothelial cell; TNF, tumor necrosis factor; GGTase, geranylgeranyltransferase; HMG, 3-hydroxy-3-methylglutaryl; FTase, farnesyltransferase; HUVEC(s), human umbilical vein endothelial cell(s); GGOH, geranylgeraniol; ERK, extracellular signal-regulated kinase; PI, phosphatidylinositol; CNF-1, cytotoxic necrotizing factor-1; FTI, farnesyltransferase inhibitor; GGTI, geranylgeranyltransferase inhibitor; NF-κB, nuclear factor κB.
H-Ras is selectively farnesylated (12). Ras family proteins are involved in the regulation of cell growth, differentiation, and apoptosis. The major function of RhoA is to regulate the assembly and organization of the actin cytoskeleton. When cells are stimulated, the activated RhoA binds to specific effectors to exert its biological function. A variety of putative RhoA effectors have been identified (18, 19). Among these effectors, the serine/threonine kinase, named RhoA kinase, has been found to mediate multiple RhoA effects, such as formation of actin stress fibers and focal adhesion (20), EC barrier dysfunction (21), vascular smooth muscle cell DNA synthesis and migration (22), and angiogenesis (23).

Recently, a specific RhoA kinase inhibitor, named Y-27632 ([+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride] was developed (24). Y-27632 has been shown to inhibit the RhoA kinase family 100 times more potently than other kinases, including protein kinase C, cAMP-dependent kinase, and myosin light chain kinase. Subsequently, Y-27632 has been widely used as a RhoA kinase-inhibitor to evaluate the involvement and roles of RhoA kinase in a variety of systems (20–23). In addition, 1-(5-isooquinolinesulfonyl) homopiperazine (HA-1077), which is structurally unrelated to Y-27632, has been recently identified as a potent inhibitor of RhoA kinase (25–27).

Many of the previous studies on the RhoA pathway have focused on adhesion and cytoskeletal reorganization. Much less is known about the participation of RhoA and its effector RhoA kinase on cell survival. The limited published data on this are controversial due to different approaches and cell types (28–31). In the present study, we directed our focus on the role of protein prenylation in general and the RhoA pathway more specifically in the regulation of EC survival. We demonstrate that inhibition of protein geranylgeranylation by lovastatin and GGTI-298 or inhibition of RhoA pathway by exoenzyme C3 and Y-27632 induces apoptosis in HUVEC. Unlike the apoptosis induced by TNF-α, which requires the inhibition of new protein synthesis, apoptosis induced by these inhibitors is dependent on de novo protein synthesis. In addition, there is an increase in p53 protein level concomitant with cell death. These results suggest that induction or activation of p53 and other proapoptotic proteins is required for apoptosis induced by inhibition of protein geranylgeranylation or by the RhoA/RhoA kinase pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media and supplies were obtained from Bio-Whittaker (Walkersville, MD). Lovastatin, exoenzyme C3, and HA-1077 were purchased from Calbiochem (La Jolla, CA). RhoA kinase inhibitor, Y-27632, was kindly provided by Welfide Corporation (Osaka, Japan). Ac-DEV-D-AMC was purchased from BACHEM (Torrance, CA). Anti-p53 antibody was purchased from BD PharMingen (San Diego, CA). Anti-1xBo was purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). Anti-phospho-Akt, anti-phospho-ERK, anti-phospho-IxBo, anti-Akt, and anti-ERK antibodies were purchased from New England Biolabs, Inc. (Beverly, MA).

**Cell Culture**—Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described (32). Cells were used in passages two through five.

**Immunoblot Analysis**—After experimental treatment, the confluent HUVEC monolayers were washed with ice-cold phosphate-buffered saline, lysed with ice-cold RIPA lysis buffer (50 μl Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 μl NaCl, 1 μl EDTA, protease inhibitor mixture tablet (Roche Molecular Biochemicals), 1 μg/ml pepstatin, 1 μg/ml type 1 Dnase, 1 μg/ml NaF, 1% sodium deoxycholate, and 0.1% Tween 20) and then incubated with primary antibodies at 1:1000 dilution for 1 h. The blots were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin. Immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences) and exposed to Kodak X-Omat Film.

**Cell Death Detection Enzyme-linked Immunosorben Assay—**We used a cell death detection enzyme-linked immunosorben assay kit (Roche Diagnostics, Indianapolis, IN) to quantitatively determine cytoplasmic histone-associated DNA fragments associated with apoptotic cell death. Briefly, after treatment, cells were lysed with 200 μl of lysis buffer and incubated for 30 min at room temperature. Then 20 μl of supernatant was transferred into the streptavidin-coated microtiter plate, and 80 μl of the immunoreagent was added to each well. After incubation at room temperature for 2 h, the solution was decanted, and each well was rinsed three times with incubation buffer. A cell death detection enzyme-linked immunosorben assay kit (Roche Diagnostics). 100 μl of cell lysates were transferred into a 96-well plate, and additional 100 μl of incubation buffer with 100 μM Ac-DEVD-AMC were added to each well. After 2.5 h of incubation at 37 °C, the fluorescence of the cleaved substrate was measured by a spectrofluorometer (Cytofluor 4000) with an excitation wavelength at 360 nm and an emission wavelength at 460 nm.

**RESULTS**

**Lovastatin Induces HUVEC Death—**Previous studies showed that lovastatin as well as other HMG-CoA reductase inhibitors induced apoptosis in various cell types (15, 16). To determine whether lovastatin had a similar effect on EC, HUVECs were cultured in medium containing various concentrations of lovastatin for 48 h, and cell death was then assayed by cytoplasmic histone-associated DNA fragments. As shown in Fig. 1A, lovastatin caused a dose-dependent increase in cell death with a 9-fold increase compared with control at 30 μM. Because lovastatin inhibits protein geranylgeranylation by depleting cellular pools of geranylgeranyl pyrophosphate, we next determined whether the addition of geranylgeraniol (GGGOH), which restores geranylgeranylation, would rescue cells from apoptosis. HUVECs were coincubated with 30 μM lovastatin and 5 μM GGOH for 48 h. As shown in Fig. 1, B and C, treatment of HUVECs with GGOH almost completely reversed cell death induced by lovastatin. However, farnesol, which restores farnesylation, did not rescue cells from apoptosis (data not shown). These results suggest that inhibition of protein geranylgeranylation accounts for lovastatin-induced cell death.

**Inhibition of Protein Geranylgeranylation, but Not Farnesylation, Induces HUVEC Death—**To confirm that lovastatin induced apoptosis by inhibiting protein geranylgeranylation, we next examined whether the GTGTase inhibitor, GGTI-298, mimicked the effect of lovastatin. HUVECs were treated with a range of concentrations of GGTI-298 or the PTase inhibitor FTI-277, and histone-associated DNA fragmentation was measured after 48 h. As shown in Fig. 2A, treatment of these cells with GGTI-298 caused a significant increase of cell death at 10 and 30 μM. In contrast, cells treated with FTI-277 at the same concentrations showed very little increase in cell death compared with control cells. The above results indicate that inhibition of protein geranylgeranylation but not farnesylation induces cell death in HUVEC.

**Inhibition of the RhoA/RhoA Kinase Pathway Induces HUVEC Death—**Because RhoA is a geranylgeranylated protein, the inhibition of protein geranylgeranylation should affect the RhoA pathway. We hypothesized that the apoptosis induced by lovastatin and GGTI-298 was mediated by RhoA and its effector, RhoA kinase. Closidium botulinum exoenzyme C3 catalyzes the specific ADP-ribosylation and inactivation of RhoA, RhoB, or RhoC and has been used to probe Rho function. Treatment of HUVECs with 30 μg/ml C3 for 24 h caused a

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Y-27632 or HA-1077 induced a cell death in a concentration-dependent manner with a 5–7-fold increase in cell death at the concentration of 30 μM for both inhibitors. These data show that inhibition of RhoA or RhoA kinase reduced endothelial cell survival.

**Activation of Caspase-3 by Lovastatin, GGTI-298, and Y-27632 Treatment in HUVEC**—Because caspase-3 plays a key role in various forms of apoptosis, we investigated whether caspase-3 was involved in the cell death induced by these inhibitors. HUVECs were treated withLovastatin, GGTI-298, or Y-27632 at 30 μM for 24 h, and caspase-3 activity was then determined by use of a fluorogenic tetrapeptide substrate, Ac-DEVD-AMC. Fig. 3 shows that 24 h of treatment with the inhibitors resulted in a 6-fold increase in caspase-3 activity. The same result was obtained when cells were treated with 10 ng/ml TNF-α and 1 μg/ml cycloheximide. Treatment of HUVECs with 30 μM FTI-277 showed no substantial increase in caspase-3 activity (data not shown). Thus, apoptosis induced by these inhibitors involved caspase-3.

**De Novo Protein Synthesis Is Required for the Apoptosis Induced by Lovastatin, GGTI-298, and Y-27632 but Not for TNF-α**—Apoptosis is affected differentially by protein synthesis inhibition depending on the cell type and stimulus. Our previous studies showed that TNF-α induced HUVEC cell death only in the presence of cycloheximide or actinomycin D (6, 7). These results were confirmed as shown in Fig. 4. The role of new protein synthesis in lovastatin-induced apoptosis was examined. HUVECs were coincubated with cycloheximide and lovastatin for 48 h. In contrast to TNF-α, lovastatin-induced cell death, demonstrated by both cell death enzyme-linked immunosorbent assay (Fig. 4A) and microscopic examination (Fig. 4B), was completely abrogated by cycloheximide at the concentration of 1 μg/ml. Cycloheximide also prevented cell death induced by GGTI-298 and Y-27632 (Fig. 4).

**Y-27632 Increases p53 Protein Level**—The p53 tumor suppressor gene is crucial in some forms of apoptosis. Several studies have shown that p53 is involved in RhoA-regulated survival pathways (34, 35). We examined whether p53 protein level was increased in HUVEC after inhibition of RhoA/RhoA kinase pathway. Fig. 5 shows that a minimal level of p53 protein was present in untreated HUVECs. However, treatment of HUVECs with Y-27632 at 30 μM caused a significant increase in p53 protein level. Cycloheximide at 1 μg/ml abrogated apoptosis induced by Y-27632 and prevented the induction of p53 (Fig. 5). These results show a correlation between cell death induced by RhoA kinase blockade and p53 induction.

**RhoA Kinase Inhibitor Y-27632 Does Not Affect the Activation of NF-κB, ERK, and Akt**—NF-κB, ERK, and PI 3-kinase/Akt are well known survival pathways in EC as well as in other cell types (6, 36–38). We next investigated whether Y-27632 inhibited activation of these pathways. The activation of NF-κB was evaluated by immunoblot using phospho-IκBα and IκBα antibody. As shown in Fig. 6A, the cytoplasmic IκBα, but not phospho-IκBα, was detected in untreated HUVEC. Treatment with TNF-α for 15 min resulted in phosphorylation of IκBα as well as IκBα degradation. However, pretreatment with Y-27632 for 30 min did not block IκBα phosphorylation and degradation induced by TNF-α. The activation of ERK and Akt by TNF-α was assessed by immunoblot using phospho-protein-specific antibodies. The phospho-ERK and phospho-Akt protein levels were dramatically increased after stimulation by TNF-α for 15 min, and preincubation with Y-27632 had no effect on level of these phosphorylated proteins (Fig. 6B and C). These data suggest that Y-27632 induced-apoptosis is not mediated by inhibition of well identified survival pathways, including NF-κB, ERK, and PI 3-kinase/Akt pathways.

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**Fig. 1. Lovastatin induces endothelial cell death.** A, HUVECs were treated with various concentrations of lovastatin for 48 h, and cell death was quantitatively determined by cytoplasmic histone-associated DNA fragments. B, HUVECs were incubated with 30 μMLovastatin in the presence or absence of 5 μM GGOH for 48 h, and cell death was determined as above. C, cells were treated as in B and then visualized by phase contrast microscopy. Magnification was ×400, medium control (panel A),Lovastatin (panel B), GGOH (panel C), andLovastatin and GGOH (panel D). Results are the means ± S.D. of triplicate wells in a single experiment and are representative of three separate experiments.

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2–3-fold increase in histone-associated DNA fragmentation (Fig. 2B). We next examined whether inhibition of RhoA kinase, the immediate downstream effector of RhoA, caused HUVEC death. Two structurally different RhoA inhibitors, Y-27632 and HA-1077, were used to address this question. HUVECs were incubated in the absence or presence of inhibitors at concentrations ranging from 3 to 30 μM for 24 h. Fig. 2, C and D show that treatment of HUVECs for 24 h with either
DISCUSSION

Prenylation of protein is an important posttranslational modification, which is required for cellular localization and biological function of small G-proteins. The addition of isoprenoid lipid farnesyl or geranylgeranyl is mediated by the enzymes FTase and GGTase, respectively. Lovastatin and related drugs are inhibitors of HMG-CoA reductase, an early and rate-limiting enzyme in the sterol synthesis pathway. The inhibitors reduce the level of isoprenoids including geranylgeranyl pyrophosphate and farnesyl pyrophosphate by depleting cellular pools of the precursors, which are substrates for GGTase and FTase, respectively. Several studies have shown that lovastatin, as well as other HMG-CoA reductase inhibitors, inhibit both geranylgeranylation and farnesylation in various cell types. In this study, we found that lovastatin caused dose-dependent cell death in HUVEC (Fig. 1A), which is consistent with other reports showing cell death induced by lovastatin (15, 16). GGOH, but not farnesol, reversed the effect of lovastatin, suggesting that a protein(s) modified by a geranylgeranyl group is required for EC survival (Fig. 1, B and C).

Next, we used an alternative approach to demonstrate that protein geranylgeranylation is required for EC survival. FTI-277 and GGTI-298 are CAAX peptidomimetics that potently and selectively inhibit FTase and GGTase, respectively. These inhibitors have been widely used to evaluate the biological functions of prenylated proteins and have been shown the specific inhibition of protein farnesylation or geranylgeranylation in various cell types (10, 11, 14, 15). We used these inhibitors to assess the importance of protein farnesylation and geranylgeranylation in HUVEC survival. Consistent with a selective effect on protein prenylation, the inhibitors also showed a different effect on EC viability. GGTI-298 induced cell death in a dose-dependent manner as measured by histone assay, whereas FTI-277 had little effect on cell viability (Fig. 2A). Caspase-3 activity was also increased after 24 h of treatment with GGTI-298 (Fig. 3), correlating with cell death measured by histone assay. These results suggest that the inhibition of protein geranylgeranylation induces caspase-3-mediated apoptosis. Protein geranylgeranylation has been shown to be involved in survival in vascular smooth muscle cells (15) and rat cortical neurons (16), as well as human lung adenocarcinoma A549 cells (39). Our results are consistent with these other studies.

Rho subfamily proteins require geranylgeranylation for their function, and several have been implicated in the regulation of cell survival (28, 30). Our studies showed that 48 h of treatment with lovastatin or GGTI-298 induced EC cell death, which is consistent with the half-life of Rho proteins. We further show that cell death was induced by exoenzyme C3 transferase (Fig. 2B), which is a highly specific inhibitor of RhoA, RhoB, and RhoC, whereas other Rho subfamily members, Rac and Cdc42, are poor substrates (40, 41). These results indicate that the Rho pathway is involved in lovastatin- and GGTI-298-induced cell death.

RhoA kinase is an immediate effector of RhoA, and it has been implicated in most of the identified functions of RhoA (18). Recently, a new synthetic compound, Y-27632, has been widely used as a specific inhibitor of RhoA kinase to identify the roles of RhoA pathway in a variety of systems (21–24). Here we used Y-27632 to determine the role of RhoA kinase in cell viability. Treatment with Y-27632 at concentrations from 3 to 30 μM induced EC death (Figs. 2C and 4). Y-27632 also increased caspase-3 activity, consistent with caspase-mediated apoptosis (Fig. 3). Another RhoA kinase inhibitor HA-1077, which is structurally unrelated to Y-27632, was used to confirm the effect of inhibition of RhoA kinase on cell viability. HA-1077

![Graph A](image1.png)

**Graph A**

**Graph A**

**Graph B**

**Graph C**

**Graph D**

![Graph D](image2.png)

**Fig. 2.** Blockade of the RhoA/RhoA kinase pathway induces cell death in HUVEC. HUVECs were treated with the various inhibitors, and cell death was quantitatively determined by cytoplasmic histone-associated DNA fragments. Results are the means ± S.D. of triplicate wells in a single experiment and are representative of three separate experiments. A, GGTI-298 and FTI-277 for 48 h. B, 30 μg/ml exoenzyme C3 for 24 h. C, Y-27632 for 24 h. D, HA-1077 for 24 h.
has been demonstrated to inhibit purified RhoA kinase with an
IC50 value of ~2 μM and to prevent RhoA kinase-mediated
inhibition of myosin phosphatase in smooth muscle cells (25).
Increased synthesis of phosphatidylinositol 4,5-bisphosphate
induced by overexpression of RhoA and constitutively active
RhoA kinase was reversed by HA-1077 (42), and HA-1077
specifically blocked the stress fiber and focal adhesion forma-
tion induced by the active form of RhoA or RhoA kinase in NIH
3T3 cells (43). Like Y-27632, treatment of HUVECs with HA-
1077 induced dose-dependent cell death (Fig. 2D). Thus, a
blockade of RhoA/RhoA kinase pathway by multiple reagents
induced EC apoptosis, demonstrating that the RhoA/RhoA ki-
nase pathway is a survival pathway in EC. Of note, the fact
that Y-27632 did not promote TNF-α-induced EC death sug-
gests that the RhoA/RhoA kinase pathway is not a component
of the TNF-α-induced survival pathway in EC (data not
shown).

Support for a role for RhoA/RhoA kinase as cell survival
signaling comes from studies in other cell types. Overexpression
of a constitutively active form of RhoA protein enhanced resistance of HeLa cells to the cytotoxic effects of Clostridium
difficile toxins (44). Activation of RhoA by cytotoxic necrotizing
factor-1 (CNF-1) inhibited epithelial cell apoptosis induced by
UVB (30). Bobak et al. created a novel system that could rapidly
and efficiently produce high intracellular levels of C3
transferase in intact cells and found that the inactivation of
RhoA by C3 exoenzyme induced apoptosis in murine L929 cells
(28). Thymocytes from C3 transferase-transgenic mice that
lack RhoA function showed cell survival defects (45). Treatment
of rats with Y-27632 also significantly increased apoptotic smooth muscle cells in neointima (46). In contrast to these
studies demonstrating a survival function for RhoA/RhoA ki-
nase, Lacal and co-workers showed that the overexpression of
constitutively active forms of RhoA in NIH 3T3 cells induced
apoptosis upon serum deprivation but not in the presence of
serum (29). There are several possible reasons for the conflict-
ing results of Lacal and co-workers and our studies. First, they
overexpressed constitutively active RhoA, whereas we studied
the endogenous enzyme. Second, in our system, HUVECs were
cultured in serum-containing medium, which is a physiological
condition, whereas they studied serum withdrawal. However,
we observed that serum deprivation increased apoptosis in-
duced by both Y-27632 and HA-1077 (data not shown). Finally,
the regulation of cell survival by the RhoA/RhoA kinase path-
way may be dependent upon the cell type. NIH 3T3 cells, which
were used by Lacal and co-workers, are an immortalized mu-
rine cell line, whereas HUVECs examined in our study are
human primary cells.

RhoA/RhoA kinase plays a critical role in the regulation of
the assembly and organization of the actin cytoskeleton (18),
which are required for cell spreading and focal adhesion. A
previous study showed that increased endothelial cell spreading
promoted cell survival and growth (47). Therefore, inhibi-
tion of cell spreading as well as adhesion may account for the
reduction of cell survival produced by disrupting the RhoA/
RhoA kinase pathway. Consistent with this notion, Y-27632
has been reported to block formation of stress fibers and cell
spreading in various cell types including EC (21). In addition,
Y-27632 inhibited phosphorylation of focal adhesion kinase and
the assembly of focal contacts (21, 26, 48). Moreover, activation
of RhoA by CNF-1 increased the expression of focal adhesion
kinase and promoted cell spreading (49). Recently, RhoA ki-
nase activation was shown to be required for apoptotic mem-
brane blebbing in NIH 3T3 and Jurkat cells (50, 51). Notably,
although Y-27632 treatment inhibited membrane blebbing in-
duced by the apoptosis stimulus, it did not prevent the bio-
chemical processes of apoptosis, such as caspase activation,
release of cytochrome c from mitochondria, or exposure of phos-
phatidylserine on the outer plasma membrane. Thus, mem-
brane blebbing appears to be a concomitant morphological
change rather than a cause of apoptosis. Consequently, it does
not appear that RhoA kinase is a proapoptotic pathway in these
cells. Although our results indicate that RhoA is involved in the
regulation of HUVEC survival, we cannot rule out the possi-
ability that, in addition to RhoA, other geranylgeranylated pro-
teins are also involved.

The tumor suppressor gene p53 has been implicated in ap-
optosis in response to direct genomic damage (radiation and
chemotherapeutic agents) as well as physiological stimuli (hy-
poxia and oxidative stress) (52). p53-induced apoptosis is me-
diated by both transcription-dependent and -independent path-
ways. Several studies demonstrated that p53 plays a critical
role in cell death induced by inhibition of the RhoA pathway or
protein geranylgeranylation. Thymocytes from p53−/− mice were
shown to be resistant to C3 transferase-induced death (34).
While inhibition of Rac1 and Cdc42 by dominant-negative
mutants efficiently triggered apoptosis in adherent fibroblasts,
cell death was not observed in p53−/− cells (35). In the present
study, we found that Y-27632 increased endogenous p53 pro-
tein levels. In addition, the induction of p53 protein as well as
cell death induced by Y-27632 was abrogated by 1 μg/ml cyclo-
heximide (Figs. 4 and 5). Induction of p53 by blockade of the
RhoA/RhoA kinase pathway may be due to inhibition of cell
adhesion signaling. Ligation of integrin αvβ3 in EC suppressed
p53 activity and increased the Bcl-2:Bax ratio, thereby promot-
ing cell survival (53). In contrast, blocking integrin αvβ3 liga-
Fig. 3. Caspase-3 activity by lovastatin, GGTI-298, and Y-27632.
HUVECs were treated with 30 μM lovastatin, 30 μM GGTI-298, 30 μM Y-27632,
or 10 ng/ml TNF-α plus 1 μg/ml cyclo-
heximide for 24 h. Caspase-3 activity was
measured by using Ac-DEVD-AMC as a
substrate. Results are the means ± S.D.
of triplicate wells in a single experiment
and are representative of three separate experiments.
tion with integrin antagonists induced p53 activation and inhibited Bcl-2 expression (53). Both CNF-1, an activator of RhoA, and constitutively active RhoA induced Bcl-2 expression (54, 55). Our previous study also showed that endogenous p53 level correlated with cell death in hypoxic HUVEC, and overexpression of p53 protein via adenoviral vector was sufficient to induce apoptosis in HUVEC (5). Taken together, we conclude that induction of p53 or other proapoptotic proteins is required.
RhoA/Rho Kinase Pathway Promotes Endothelial Cell Survival

Y-27632 is an inhibitor of RhoA kinase, which is a serine/threonine kinase. Phosphorylation of serine/threonine residues is involved in the activation of other kinases, such as NF-κB, ERK, and PI 3-kinase/Akt. Moreover, activation of these pathways is antiapoptotic in various cell types, including endothelial cells (6, 36–38). A previous study demonstrated that activation of NF-κB is regulated by RhoA (59). We therefore investigated whether Y-27632 inhibited activation of these serine/threonine kinases. Fig. 6 shows that the phosphorylation of IkBα, ERK, and Akt by TNF-α was not inhibited by Y-27632. These results suggest that Y-27632 specifically inhibits RhoA kinase rather than other serine/threonine kinases activated by TNF-α. It is therefore unlikely that NF-κB, ERK, and PI 3-kinase/Akt are the downstream targets of the RhoA/Rho kinase survival pathway.

In summary, we demonstrate a role of geranylgeranylated proteins, specifically RhoA, and RhoA kinase in the regulation of EC survival. Unlike the apoptosis induced by TNF-α, which requires the inhibition of de novo protein synthesis, apoptosis induced by inhibition of protein geranylgeranylation and RhoA kinase requires de novo protein synthesis. Apoptosis induced by inhibition of this pathway may involve the induction of p53 or other proapoptotic proteins. These findings further demonstrate the different mechanisms involved in the regulation of EC survival and apoptosis.

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