Rho protein inactivation induced apoptosis of cultured human endothelial cells

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HIPPENSTIEL, Stefan, Bernd Schmeck, Phillipe Dje N’Guessan, Joachim Seybold, Matthias Krüll, Klaus Preissner, Christoph v. Eichel-Streiber, and Norbert Suttrop. Rho protein inactivation induced apoptosis of cultured human endothelial cells. Am J Physiol Lung Cell Mol Physiol 283: L830–L838, 2002. First published June 10, 2002; 10.1152/ajplung.00467.2001.—Small GTP-binding Rho GTPases regulate important signaling pathways in endothelial cells, but little is known about their role in endothelial cell apoptosis. Clostridial cytotoxins specifically inactivate GTPases by glucosylation (Clostridium difficile toxin B-10463, C. difficile toxin B-1470 (TcdB-1470)) or ADP ribosylation (C. botulinum C3 toxin). Exposure of human umbilical cord vein endothelial cells (HUVEC) to TcdB-10463, which inhibits RhoA/Rac1/Cdc42, or to C3 toxin, which inhibits RhoA, -B, -C, resulted in apoptosis, whereas inactivation of Rac1/Cdc42 with TcdB-1470 was without effect, suggesting that Rho inhibition was responsible for endothelial apoptosis. Disruption of endothelial microfilaments as well as inhibition of p160ROCK did not induce endothelial apoptosis. Exposure to TcdB-10463 resulted in activation of caspase-9 and -3 but not caspase-8 in HUVEC. Moreover, Rho inhibition reduced expression of antiapoptotic Bcl-2 and Mcl-1 and increased proapoptotic Bid but had no effect on Bax or FLIP protein levels. Caspase-3 activity and apoptosis induced by TcdB-10463 were abolished by cAMP elevation. In summary, inhibition of Rho in endothelial cells activates caspase-9- and -3-dependent apoptosis, which can be antagonized by cAMP elevation.

clostridial toxins; caspase; endothelium

RHOA, RAC1, AND CDC42 ARE MEMBERS of the Rho subfamily of the Ras superfamily of small GTP binding proteins (reviewed in Ref. 41). Although Rho GTPases were first characterized as central regulators of the actin cytoskeleton (reviewed in Refs. 25, 40), further research pointed out that they act as important molecular switches in different signaling pathways (reviewed in Ref. 26).

Rho proteins participate in the regulation of central endothelial cell functions, such as maintenance of barrier function (11, 21, 34) and cytokine and mediator expression (18, 20, 29, 34), as well as angiogenesis (15). Analysis of Rho protein function has been improved by usage of clostridial cytotoxins as highly specific molecular tools: Clostridium difficile toxin B-10463 (TcdB-10463) easily enters endothelial cells via receptor-mediated endocytosis and UDP-glucosylates RhoA (24) at threonine 37 and Rac and Cdc42 (6) at the corresponding threonine 35, thereby specifically inactivating the GTPase effector domain. C. botulinum C3 toxin inactivates RhoA, RhoB, and RhoC by ADP ribosylation (2) of asparagine 41. C. difficile toxin B-1470 (TcdB-1470) primarily glucosylates Rac1 and Cdc42, but not RhoA (6). In previous studies using these toxins, we demonstrated TcdB-10463-dependent glucosylation of Rho proteins in endothelial cells, an effect accompanied by loss of endothelial barrier function (21) and decreased PKC activation (19). Moreover, using TcdB-10463, we showed that LPS-induced activation of IL-8 expression in human endothelial cells requires both a Rho protein-dependent and -independent pathway (20).

Increasing evidence suggests a link between Rho proteins and apoptosis, but contradictory results have been reported in this context (3, 5, 12–14, 16, 31, 33). A plethora of different stimuli activates the proapoptotic machinery, consisting of caspases as central executioners of programmed cell death; the regulatory caspase-8 is directly activated by death receptors, whereas caspase-9 activation follows mitochondrial stress (for review, see Refs. 7, 10, 17). Both pathways merge by activating executioner caspase-3. Different proapoptotic (e.g., Bax, Bid) and antiapoptotic (e.g., Bcl-2, Bcl-xL, Mcl-1, FLIP) proteins participate in the regulation of apoptosis (7, 17). Although apoptosis of vascular cells, including endothelial cells, is vital for normal vasculogenesis and adaptation (8) and may play an important role in the development of various diseases (8, 27), little is known about the role of Rho proteins in the regulation of endothelial cell death.

Therefore, we made use of clostridial cytotoxins to analyze the role of Rho proteins for the regulation of...
endothelial apoptosis. The results presented indicate that Rho inhibition reduces expression of Bel-2 and Mcl-1 but not of Bax, Bid, or FLIP. Moreover, activation of caspase-9 and -3, resulting in endothelial programmed cell death, was noted in cells with inhibited Rho proteins. Elevation of cAMP blocked TcdB-10463-related caspase-3 activation and apoptosis. Overall, our data suggest a central role of Rho GTPases for the regulation of apoptosis in human endothelial cells.

MATERIALS AND METHODS

Materials. Tissue culture plastic ware was obtained from Becton-Dickinson (Heidelberg, Germany). MCDB-131, FCS, HBSS, PBS, trypsin-EDTA solution, HEPES, Igepal CA-650, and antibiotics were from Gibco (Karlsruhe, Germany). Collagenase (CLS type II) was purchased from Worthington Biochemical (Freehold, NJ). Y-27632 was obtained from Tocris Cookson (Bristol, UK). Gelatin from porcine skin type I, leupeptin, pepstatin A, antipain, Triton X-100, PMSF, forskolin, 3,4-dichloroisocoumarin, staurosporine, cytochalasin D, rhodamine-labeled phalloidin, and Tween 20 were purchased from Sigma (Munich, Germany). All other chemicals used were analytical grade and obtained from commercial sources.

Preparation of bacterial toxins. TcdB-10463 and TcdB-1470 were purified as described previously (6, 24). C. botulinum C3 toxin and C. botulinum C2 were gifts of Professor K. Aktories (Department of Pharmacology and Toxicology, Albert Ludwig University, Freiburg, Germany).

Preparation of human umbilical cord vein endothelial cells. Cells were isolated from umbilical cord veins and identified as described previously (19, 20, 28, 37). Briefly, cells obtained from collagenase digestions were washed, resuspended in MCDB-131-5% FCS, and seeded into 6- or 96-well plates. Confluent monolayers of primary cultures only were used.

Flow cytometric analysis of endothelial annexin V binding. Rapid binding of annexin V to phosphatidyl serine was used for the identification of cells undergoing apoptosis. Endothelial annexin V binding was analyzed on a FACStarPLUS flow cytometer (Becton-Dickinson, Mountain View, CA). Human umbilical cord vein endothelial cells (HUVEC) were starved in serum-free MCDB-131 and stimulated as indicated. Cells were trypsinized and incubated simultaneously with FITC-labeled annexin V (1 \( \mu g/ml \)) and propidium iodide (PI, 2 \( \mu g/ml \), both for 30 min at 4°C in the dark). Cells were then washed twice with ice-cold PBS to remove unbound annexin and PI and resuspended in PBS. Results are displayed as percent positive cells among all gated cells.

In situ cell death detection assay. Apoptosis of endothelial cells was visualized by immunohistochemical detection of DNA strand breaks. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique was used to detect endothelial cell apoptosis (Boehringer, Mannheim, Germany). Briefly, stimulated endothelial cells cultured on chamber slides were fixed in freshly prepared paraformaldehyde (4% in PBS, pH 7.4). After blocking of endogenous peroxidase, cells were permeabilized, TUNEL reaction mixture was added, and peroxidase was used for visualization of apoptotic nuclei. Slides mounted on glass coverslips were analyzed under light microscopy and documented on Kodak TMY 400 film.

Immunofluorescence. Cells grown on gelatin-coated glass coverslips were washed twice, incubated in MCDB-131-10% FCS in a humidified atmosphere, and stimulated as indicated. Briefly, cells were fixed in freshly prepared paraformaldehyde (5% in PBS, pH 7.4), permeabilized, and washed, and DNA strand breaks were labeled by fluorescein-dUTP and analyzed by fluorescence microscopy. F-actin was visualized by marking with rhodamine-labeled phalloidin (1.4 \( \mu g/ml \)) as described previously (21, 38).

Cell death detection ELISA. A commercially available photometric ELISA was used for the detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in apoptotic endothelial cells (Boehringer Mannheim). Cells cultured in 96-well plates were washed twice, incubated in MCDB-131-10% FCS in a humidified atmosphere, and stimulated as indicated. Microtiter plates were then centrifuged, medium was removed, cells were lysed, and plates were centrifuged again. Twenty microliters of the supernatant were transferred into a streptavidin- precoated microtiter plate and incubated with the immunoreagent (anti-histone biotin, anti-DNA peroxidase) for 2 h at room temperature. After a washing, substrate solution was added, and absorbance was determined at 405 nm.

Caspase activity. A commercially available caspase activity assay (Oligogene, Boston, MA) based on fluorometric detection of the cleavage of 7-amino-4-trifluoromethyl coumarin-labeled substrate specific for caspase-3, -8, or -9 was used for analysis of caspase activity. Briefly, cells were stimulated, collected, and lysed on ice. Cleared samples were aligned for protein content, split up into three aliquots, and incubated at 37°C for 2 h in presence of labeled caspase-specific fluorescence substrate conjugate for caspase-3, -8, or -9, respectively. We performed detection by measuring excitation at 390 nm and emission at 510 nm using a fluorometer (FluoroMax-2; ISA, Grasbrunn, Germany).

Western blotting. Endothelial cell monolayers were exposed to TcdB-10463 (0–100 ng/ml) for 6 h in a humidified atmosphere. Cells were then collected after trypsinization, washed, and lysed for 10 min on ice in a buffer consisting of 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.1% Igepal CA-650. Cell lysates were briefly centrifuged, and endothelial nuclei were harvested and lysed for 7 min on ice in 20 mM HEPES buffer, pH 7.4, containing 1% Triton X-100, 44 \( \mu g/ml \) PMSF, 2 \( \mu g/ml \) leupeptin, and 2 \( \mu g/ml \) pepstatin. After protein precipitation in methanol-chloroform, samples were resuspended in gel-loading buffer according to Laemmli, sonicated, and 20 \( \mu g \) of the sample was run in a 7.5% gel. After blotting, membranes were blocked, washed, and hybridized with a rabbit polyclonal antibody raised against poly(ADP-ribose) polymerase (PARP; Boehringer, Mannheim), followed by goat anti-rabbit antibody (Amersham, Dreieich, Germany). Membranes were blocked, washed, and hybridized with a rabbit polyclonal antibody against PARP (Upstate Biotechnology, Lake Placid, NY) and Bcl-2, Bid, Mcl-1, ERK1, or Bax by mouse, rabbit, or goat polyclonal antibodies, respectively (Santa Cruz Biotechnology, Heidelberg, Germany). Anti-FLIP mouse monoclonal antibody was a gift of P. Krammer (German Cancer Research Center, Heidelberg, Germany). Cells were treated as indicated and processed for SDS-PAGE as described above. Detection was performed by enhanced chemiluminescence (ECL; Amersham). Caspase-3, -8, and -9 precursors were detected using rabbit polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY) and Bel-2, Bid, Mcl-1, ERK1, or Bax by mouse, rabbit, or goat polyclonal antibody, respectively (Santa Cruz Biotechnology, Heidelberg, Germany). Anti-FLIP mouse monoclonal antibody was a gift of P. Krammer (German Cancer Research Center, Heidelberg, Germany). Cells were treated as indicated and processed for SDS-PAGE as described above. Detection was performed by enhanced chemiluminescence (ECL; Amersham) or by visualization of IRDye 800- or Cy5.5-labeled secondary antibodies (Odyssey infrared imaging system; LI-COR, Lincoln, NE).

Release of lactate dehydrogenase. Endothelial cell monolayers were exposed to stimuli for 24 h. Lactate dehydrogenase (LDH) activity in the supernatants was determined by the colorimetric measurement of the reduction of sodium...
pyruvate in the presence of NADH as described (19–21, 28, 37). Enzyme release was expressed as the percentage of total enzyme activity liberated from endothelial cells in the presence of 100 μg/ml mellitin.

**Statistical methods.** Depending on the number of groups and the number of different time points studied, data of Fig. 2B were analyzed by an A × B analysis of variance (ANOVA). A one-way ANOVA was used for data in Figs. 1; 2, A and C; 4; 6A; and 8, A and B. Main effects were then compared by an F-probability test. P < 0.05 was considered to be significant.

**RESULTS**

Prenylation of Rho proteins is required for membrane binding and Rho-dependent signaling. We used 50 and 100 μM lovastatin for 24 h to inhibit 3-hydroxy-3-methylglutaryl (HMG)-CoA-reductase, thereby blocking protein prenylation (Fig. 1). Lovastatin exposure induced significant apoptosis in human endothelial cells that was reduced by simultaneous addition of the HMG-CoA reductase product mevalonic acid (100 μM) (Fig. 1). The effect of lovastatin on endothelial apoptosis was ~30% of the effect of the well-known strong proapoptotic agent staurosporine (1 μM). No increased LDH release was noted within the time frame tested, i.e., there was no sign for endothelial cell necrosis (data not shown).

To address more specifically the role of the small GTP-binding Rho proteins RhoA, Rac1, and Cdc42, we made use of TcdB-10463 (Fig. 2). These toxins induced dose (0.01–10 ng/ml)- and time (4–8 h)-dependent programmed cell death in human endothelial cells and were more effective than 100 ng/ml TNF-α, as shown by apoptosis ELISA (Fig. 2, A and B). In addition, this specific inhibition of Rho proteins was as effective as treatment of endothelial cells with the programmed cell death-inducing kinase inhibitor staurosporine (Fig. 1). Within the time frame and for the doses tested, no LDH release was measured (data not shown). Moreover, FACS analysis demonstrated that inhibition of Rho proteins by TcdB-10463 (0.1–10 ng/ml) (Fig. 2C), as well as exposure to 100 ng/ml TNF-α for 4 h (data not shown), resulted in a dose dependently enhanced annexin V binding to endothelial cells. About 35% of all gated endothelial cells treated with 10 ng/ml TcdB-10463 showed annexin V binding. Because PI was still excluded in these experiments, the data pattern is compatible with cell apoptosis but not necrosis (Fig. 2C).

Western blot analysis revealed cleavage of PARP after 6 h of cell incubation with 1–100 ng/ml TcdB-10463 as a sign for increased caspase activity (Fig. 2D).

In situ TUNEL staining of endothelial cell monolayers revealed an increase in DNA fragmentation after 8 h of inhibition of RhoA, Rac, and Cdc42 by TcdB-10463 (10 ng/ml) (Fig. 3B) and, to a lesser extent, after stimulation with 100 ng/ml TNF-α (Fig. 3C) compared with control cells. In contrast, no DNA fragmentation was noted after inactivation of Rac1 and Cdc42 (but not RhoA) by cell exposure to 100 ng/ml TcdB-1470 (Fig. 3D).

To characterize the individual GTPases involved in endothelial cell apoptosis, different clostridial toxins (TcdB-10463, TcdB-1470, and C. botulinum C2 and C3 toxin) were used. These toxins inactivate specific small GTP binding proteins (TcdB-10463, TcdB-1470, C. botulinum C3 toxin) or interfere with actin assembly (C. botulinum C2 toxin) (Fig. 4).

Inactivation of RhoA, Rac1, and Cdc42 by TcdB-10463 (10 ng/ml) or inactivation of RhoA, RhoB, and RhoC by C. botulinum C3 toxin (200 μg/ml) resulted in significantly increased endothelial programmed cell death (Fig. 4). However, inhibition of Rac, Cdc42, Rap, and Ral by 100 ng/ml TcdB-1470 did not affect apoptosis (Fig. 4). Because all toxins used altered endothelial cell microfilament system, we checked this by exposing endothelial cell monolayers to C. botulinum C2 toxin, a highly selective tool that specifically ADP-ribosylates G-actin, resulting in marked actin depolymerization. C2 toxin-induced modification of endothelial cell microfilaments (50 ng/ml C2 toxin, 8 h) was accompanied by a small, yet significant increase in cell apoptosis (Fig. 4). Depolymerization of endothelial F-actin by 1 μg/ml cytochalasin D displayed no increase in endothelial cell apoptosis (Fig. 4). Because p160ROCK plays an important role in Rho-dependent microfilament alterations, we inhibited this kinase with 10 μM of the specific inhibitor Y-27632. Blocking of p160ROCK by Y-27632 did not result in endothelial apoptosis within the time frame tested (Fig. 4). No increase in LDH activity was noted in endothelial cells treated as described above for the dose and time frame tested (data not shown).

Furthermore, studies using TUNEL stain and rhodamine-labeled phalloidin to visualize endothelial

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**Fig. 1.** Inhibition of geranylgeranylation by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor lovastatin induced human endothelial cell apoptosis. Human umbilical cord vein endothelial cells (HUVEC) were treated with 50 or 100 μM lovastatin or 100 μM mevalonic acid or both for 24 h. Inhibition of geranylgeranylation by lovastatin induced endothelial cell apoptosis. Co-incubation with the HMG-CoA reductase product mevalonic acid blocked this effect. Lovastatin was less effective in inducing endothelial apoptosis than 1 μM staurosporine within the same time frame. Apoptosis was analyzed by an ELISA detecting cytoplasmatic histone-associated DNA fragments. Data presented are means ± SE of 4 separate experiments.
Fig. 2. Inhibition of RhoA, Rac1, and Cdc42 by Clostridium difficile toxin B-10463 (TcdB-10463) induced dose- and time-dependent apoptosis of human endothelial cells. Cells were incubated with 0.01–10 ng/ml TcdB-10463 for 8 h (A) or with 10 ng/ml TcdB-10463 (B) for varying periods of time; 100 ng/ml TNF-α (open symbols) were used for 8 h. Apoptosis was quantified in an ELISA detecting cytoplasmatic histone-associated DNA fragments. TcdB-10463 (≥0.1 ng/ml) induced significant apoptosis. C: increased binding of annexin V (solid bars) and exclusion of propidium iodide (PI, open bars) in TcdB-10463-exposed HUVEC were measured after incubation with 0.1–10 ng/ml TcdB-10463 for 4 h by FACS analysis, indicating apoptosis but not necrosis. D: after incubation of cells with 1–100 ng/ml TcdB-10463 for 6 h, nuclear cell extracts were separated on SDS-PAGE, and the 85-kDa cleavage product of poly(ADP ribose) polymerase (PARP) was detected by Western blotting. Note that Rho protein inhibition results in PARP cleavage as a marker of HUVEC apoptosis. Data presented are means ± SE of 4 separate experiments in A and B and of 3 separate experiments in C, and in D a representative gel (out of 3) is shown.

Fig. 3. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) stain demonstrated apoptosis in endothelial cells with inhibited Rho proteins. Histochemical analysis of TcdB-10463-related HUVEC apoptosis: confluent endothelial cell monolayers were incubated for 8 h with solvent alone (A), 10 ng/ml TcdB-10463 (B), 100 ng/ml TNF-α (C), or 100 ng/ml C. difficile toxin B-1470 (TcdB-1470, D), inhibiting Rac1 and Cdc42, but not RhoA, and DNA strand breaks were visualized by immunohistochemical detection with the TUNEL technique. Nuclei of apoptotic cells are indicated by black arrows. Representative fields of HUVEC monolayers (out of 3) are shown (magnification ×400).

Fig. 4. RhoA, but not Rac1 and Cdc42, proteins are essential for endothelial cell survival. Inactivation of RhoA, Rac1, and Cdc42 by TcdB-10463 (10 ng/ml, 8 h) and of RhoA, -B, and -C by C. botulinum C3 toxin (200 ng/ml, 24 h), as well as blocking of Cdc42 and Rac1 by TcdB-1470 (100 ng/ml, 8 h), was carried out in endothelial cell cultures. F-actin was depolymerized by C. botulinum C2 toxin (50 ng/ml, 8 h) or cytochalasin D (1 μg/ml), and p160ROCK kinase was inhibited by 1 μM of the specific inhibitor Y-27632. Results indicate that inhibition of RhoA (TcdB-10463, C. botulinum C3 toxin) but not of Rac1 and Cdc42 (TcdB-1470) or F-actin depolymerization (C. botulinum C2 toxin, cytochalasin D) or p160ROCK (Y-27632) induced endothelial cell apoptosis. Apoptosis was quantified in an ELISA detecting cytoplasmatic histone-associated DNA fragments. Data presented are means ± SE of 3 separate experiments. Cl. bot., Clostridium botulinum.
F-actin have demonstrated that impairment of the endothelial cell microfilament system alone is not sufficient to induce apoptosis. Although inhibition of Rho proteins by 10 ng/ml TcdB-10463 disrupted endothelial F-actin and induced apoptosis (Fig. 5, C and D), exposure of cells to cytochalasin D resulted in loss of endothelial F-actin but did not increase apoptosis, as demonstrated by TUNEL stain (Fig. 5, E and F). Moreover, inhibition of p160ROCK by 1 μM Y-27632 also displayed no increase in endothelial apoptosis (Fig. 5, G and H). However, treatment of endothelial cells with staurosporin resulted in programmed cell death and marked alterations of the microfilament system (Fig. 5, I and J).

Inhibiting RhoA, Rac1, and Cdc42 by exposure of HUVEC to 10 ng/ml TcdB-10463 increased caspase-3 and -9 but not caspase-8 activity time dependently, as shown by the caspase activity assay (Fig. 6A). Caspase activation was confirmed by Western blot analysis of procaspase cleavage (Fig. 6B). Cleavage of procaspase-3 and -9 but not procaspase-8 was noted in TcdB-10463-treated cells, indicating the involvement of the mitochondrial pathway in TcdB-10463-related apoptosis. To further explore the role of mitochondrial factors in TcdB-10463-induced programmed cell death, we analyzed the expression of antiapoptotic Bcl-2 and Mcl-1, as well as proapoptotic Bax, Bid, and FLIP proteins, in cells with blocked Rho proteins (Fig. 7). Western blot analysis demonstrates a reduction of Bcl-2 and Mcl-1 protein levels, whereas Bax and FLIP protein expression was not affected in TcdB-10463-exposed endothelial cells (Fig. 7). Interestingly, levels of proapoptotic Bid protein seem to be increased in cells without Rho function. We observed no cleaved Bid protein for the dose and time frame tested. Besides Bcl-2 and Mcl-1, staurosporine treatment of endothelial cells also reduced expression of FLIP. Moreover, Bid protein levels were increased in staurosporine-exposed cells, as well as in cells with inhibited Rho proteins (Fig. 7).

Because cAMP is known to interfere with mitochondria-dependent apoptosis, forskolin and the phosphodiesterase isoenzyme 4-specific inhibitor RP-73401 (both 1 μM) were used to increase endothelial cAMP levels (Fig. 8). Elevation of cAMP blocked TcdB-10463-related programmed cell death (Fig. 8A) and caspase-3

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Fig. 5. Microfilament alterations are not sufficient for induction of endothelial cell apoptosis. Immunofluorescence analysis of endothelial cell apoptosis (left, TUNEL stain) and F-actin (right, rhodamine-labeled phallolidin). Cells were untreated (A, B), or RhoA, Rac1, and Cdc42 were inhibited by TcdB-10463 (10 ng/ml, 8 h; C, D). E, F: cytochalasin D (1 μg/ml, 8 h) was used for F-actin depolymerization. G, H: p160ROCK was blocked by 1 μM Y-27632 (8 h); I, J: staurosporine (1 μg/ml, 8 h)-treated cells served as positive controls. Although cytochalasin D exposure (E, F) as well as TcdB-10463 incubation (C, D) of endothelial cells both resulted in a breakdown of microfilaments (D, F) in these cells, only TcdB-10463-induced apoptosis (C) p160ROCK inhibition induced moderate alteration of endothelial microfilaments (H) but not significant apoptosis (G). Stauromycin treatment resulted in massive endothelial programmed cell death (I). Representative fields of HUVEC monolayers (out of 3) are shown (magnification ×1,000).
activation, as shown by the caspase-3 activity assay (Fig. 8B) and procaspase-3 Western blot (Fig. 8C).

DISCUSSION

The results presented here indicate that inhibition of Rho proteins reduced protein levels of Bcl-2 and Mcl-1 and increased expression of proapoptotic Bid protein, whereas Bax and FLIP levels remained unchanged. Furthermore, blocking of Rho proteins induced caspase-9- and -3-dependent apoptosis of cultured human endothelial cells. On the basis of studies using clostridial toxins to inhibit well-defined subsets of GTPases, RhoA seems to be essential for endothelial cell viability. cAMP elevation blocked TcdB-10463-related caspase-3 activation and apoptosis.

Rho proteins were identified as central regulators of cell function, ranging from regulation of the cell cytoskeleton to different important signaling pathways (reviewed in Refs. 26, 40, 41). Studying Rho protein function used to be difficult. Available tools, such as C. botulinum C3 toxin, which ADP-ribosylates Rho proteins at Asn41 (2), enter mammalian cells poorly, and constitutively activated p21Rho must be microinjected into target cells (26, 41). Overexpression of GTPases has been a useful method for studying Rho protein function, although it has also some limitations (8, 26, 32, 35, 40, 41). TcdB-10463, which glucosylates Rho proteins at threonine 35/37, thereby rendering them functionally inactive, easily enters mammalian cells and turns out to be a highly selective and powerful alternative tool with which to study Rho protein function (22-24). For example, the toxin was used to demonstrate the requirement of Rho proteins for maintenance of endothelial barrier function (21), PKC activation and translocation (19), phospholipase D activation (36), or myosin light chain phosphorylation (30). Using TcdB-10463, we recently demonstrated that LPS induces two parallel signaling pathways.
shown in SE of 4 separate experiments. A representative gel (out of 3) is more effective than unspeci
cification of various proteins, including small GTPases involved in apoptosis, we made use of various
TcdB-10463-induced apoptosis in cultured intestinal
Rho/tyrosine kinase and one Rho independent, MKK6/ p38 MAPK dependent), leading to IL-8 expression in human endothelial cells (20).
In the present study, TcdB-10463 dose- and time
dependently induced apoptosis of cultured human en
dothelial cells as shown by the detection of cytoplasmic histone-associated DNA fragments, increased binding of annexin V to the cells’ outer membrane, PARP pro
teolysis, and DNA strand breaks. The amount of apop
tosis observed in cells without Rho function was equal to that induced by the strong apoptosis inducer staurosporine, underlining the importance of functional intact Rho proteins for maintenance of endothelial cell viability. Within the time frame studied, there was no increase in PI uptake or in LDH release, indicating the absence of cell necrosis. Fiorentini et al. (13) reported TcdB-10463-induced apoptosis in cultured intestinal cells, suggesting that the results obtained are not limited to endothelial cells. Inhibition of protein prenylation with respect to endothelial apoptosis.
In an attempt to further characterize the different GTPases involved in apoptosis, we made use of various clostridial toxins (TcdB-10463, TcdB-1470, and C. bot
luminum C3 toxin). Endothelial cells loaded with C. botulinum C3 toxin, which inactivates RhoA, RhoB, and RhoC by ADP-ribosylating asparagine 41, also underwent apoptosis. Using a novel Sindbis virus
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nosis, suggesting that loss of F-actin alone is not sufficient to induce programmed cell death of endothelial cells. Moreover, blocking of the Rho effector protein p160ROCK kinase by the specific inhibitor Y-27632 did not result in endothelial apoptosis.

It is of interest to note that cAMP-elevating agents (adenyl cyclase activation/phosphodiesterase inhibition) (39) blocked TcDB-10463-related caspase-3 activation and apoptosis. Whether mitochondria-anchored PKA-dependent phosphorylation and inactivation of Bad or alternative cAMP-dependent pathways contribute to the rescue of endothelial cells against TcDB-10463 cell death remains to be determined (42).

We demonstrate here that inhibition of Rho proteins reduced the expression of antiapoptotic Bcl-2 and Mcl-1 proteins and increased protein levels of proapoptotic Bid but had no effect on Bax or FLIP levels. Rho inhibition induced caspase-9- and -3-dependent programmed cell death of cultured human endothelial cells. Studies using different clostridial toxins inactivating well-defined subsets of GTPases showed that functional, active RhoA is necessary to prevent endothelial apoptosis. Elevation of cAMP content blocked TcDB-10463-related caspase-3 activation and apoptosis. Further studies are required to define the exact position of Rho GTPases in antiapoptotic signaling pathways.

The technical assistance of S. Tannert-Otto, V. Johnston, and K. Mörh is greatly appreciated. We acknowledge the help of Dr. M. Germer (Bad Nauheim) with the annexin V assay. We also thank the Delivery Services staffs at the hospitals Ev. Waldkrankenhaus Spandau (Berlin) and Humboldt Krankenhaus Reinickendorf (Berlin) for help in collecting umbilical cords. Parts of this work will be included in the M. D. theses of Bernd Schmeck and Philippe Dje N’guessan in collecting umbilical cords. Parts of this work will be included in the M. D. theses of Bernd Schmeck and Philippe Dje N’guessan. We demonstrate here that inhibition of Rho proteins reduced the expression of antiapoptotic Bcl-2 and Mcl-1 proteins and increased protein levels of proapoptotic Bid but had no effect on Bax or FLIP levels. Rho inhibition induced caspase-9- and -3-dependent programmed cell death of cultured human endothelial cells. Studies using different clostridial toxins inactivating well-defined subsets of GTPases showed that functional, active RhoA is necessary to prevent endothelial apoptosis. Elevation of cAMP content blocked TcDB-10463-related caspase-3 activation and apoptosis. Further studies are required to define the exact position of Rho GTPases in antiapoptotic signaling pathways.

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