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Central involvement of Rho family GTPases in TNF-α-mediated bovine pulmonary endothelial cell apoptosis

Irina Petrache, Michael T. Crow, Michael Neuss,[1] and Joe G.N. Garcia*

Department of Medicine, Division of Pulmonary and Critical Care Medicine, Johns Hopkins University School of Medicine, 1830 Building, Room 527, Baltimore, MD 21224, USA

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Abstract

In our recent studies, we defined a critical role for increased levels of myosin light chain (MLC) phosphorylation, a regulatory event in the interaction between actin and myosin in TNF-α-induced pulmonary endothelial cell actomyosin rearrangement and apoptosis. The Rho GTPase effector, Rho kinase is an important signaling effector governing levels of MLC phosphorylation which contributes to plasma membrane blebbing in several models of apoptosis. In this study, we directly assessed the role of Rho kinase in TNF-α-induced endothelial cell microfilament rearrangement and apoptosis. Inhibition of RhoA GTPase activity by the overexpression of dominant negative RhoA attenuates TNF-α-triggered stress fiber formation, consistent with Rho activation as a key event in TNF-α-induced cytoskeletal rearrangement. Furthermore, pharmacologic inhibition of Rho kinase as well as dominant negative RhoA overexpression dramatically reduced TNF-α-induced bovine endothelial apoptosis reflected by nucleosomal fragmentation as well as caspase 7, 3, and 8 activation. These results indicate that Rho kinase-dependent cytoskeletal rearrangement is critical for early apoptotic events, possibly in the assembly of the death-inducing signaling complex leading to initiator and effector caspase activation, and suggest a novel role for Rho GTPases in endothelial cell apoptosis.

Keywords: Cytoskeleton; Acute lung injury; RhoA; Protease; DISC; TNFR1

Increased TNF-α production and evidence of augmented cellular apoptosis are important features of inflammatory processes, including acute lung injury. TNF-α directly induces pulmonary endothelial cell apoptosis through ligation of TNF-α receptor 1 (TNFR1) and via the death domain (DD) motif, subsequent recruitment of multiple adaptor proteins including TNF receptor-associated death domain protein (TRADD), receptor interacting protein-1 (RIP1), and TNF-receptor-associated factor 2 (TRAF2). TRADD, in turn, recruits Fas-associated death domain protein (FADD) to the death-inducing signaling complex (DISC), leading to the sequential recruitment and activation of the initiator caspase 8 [1,2]. Caspase 8 activation leads to downstream activation of the effector caspases (caspases 3, 6, and 7) which cleave key cellular substrates, thus silencing survival pathways and resulting in typical morphologic changes of membrane blebbing, cellular shrinkage, and apoptotic body formation.

Actin polymerization is also required for initiation of membrane blebbing and a growing number of actin-binding proteins are recognized as caspase substrates [3]. Several reports, including our own [7], have documented the capacity for TNF-α to actively produce actin cytoskeletal rearrangement in endothelium characterized by profound stress fiber and intercellular gap formation in association with increased levels of myosin light chain (MLC) phosphorylation. MLC phosphorylation is a key regulatory event for force development via actin-myosin interaction and is the result of the coordinated action of MLC kinase (MLCK) and the Rho GTPase effector, Rho kinase. Rho kinase inhibits MLC phosphatase (MYPT) activity via phosphorylation of the MYPT regulatory subunit and possibly by directly catalyzing MLC phosphorylation [4]. Both Rho kinase and MLCK

* Corresponding author. Fax: 1-443-287-3349.
E-mail address: drgarcia@jhmi.edu (J.G.N. Garcia).
[1] Present address: Department of Medicine/Cardiology, German Heart Institute, 13353 Berlin, Germany.

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have been implicated in the development of plasma membrane blebbing in apoptotic PC12 cells [5], Jurkat, and MCF-7 cells [6]. There is increasing appreciation, however, that the actin cytoskeleton may be involved not only in end-stage execution of apoptosis, but in the intracellular signaling involved in the initiating stages, as well. We previously demonstrated that MLC phosphorylation-dependent cytoskeletal changes are critical not only for cellular morphological changes (which occur downstream of caspase activation), but also for events that precede caspase activation [7]. A similar finding was reported in TNF-α-treated MDCK cells over-expressing smooth muscle MLCK [8]. We extended our previous investigation and showed that antisense MLCK as well as a kinase-dead MLCK inhibit TNF-α-induced programmed cell death. Early in the apoptotic process, MLCK activity is important for TNF-α-induced caspase 8 activation in endothelial cells [7] and co-staining for overexpressed MLCK and caspase 8 suggests a spatial interaction of the two [9]. Subsequent caspase 3 activation has been associated with enhanced enzymatic activities of MLCK [9] and Rho kinase [6] via caspase-dependent cleavage.

In this study, we examined the hypothesis that Rho GTPases are directly involved in the regulation of TNF-α-induced actin microfilament changes and endothelial cell apoptosis. Our results reveal that Rho kinase activity increases MLC phosphorylation and is significantly involved in TNF-α-induced acto-myosin rearrangement and apoptosis in pulmonary endothelial cells. Both pharmacological and molecular inhibition of Rho GTPase and Rho kinase markedly attenuate TNFα-induced apoptosis. Our data show that the regulatory effects of Rho kinase occur early in the apoptotic pathway, before initiator caspase 8 activation, suggesting that the actin cytoskeleton directly modulates endothelial cell apoptosis, potentially via the cellular localization of specific components of the apoptotic machinery.

Materials and methods

Cell culture conditions and reagents. Bovine pulmonary artery endothelial cells (BPAEC) were obtained at 16th passage (CCL 209) from American Type Culture Collection (Rockville, MD) and experiments were performed up to passages 18–20. The cells were maintained in complete culture medium consisting of 20% bovine serum, endothelial cell growth supplement (17 μg/ml, H-neurext, Upstate Biotechnology (UBI, Lake Placid, NY), and penicillin/streptomycin (100 U/ml) (Gibco, Invitrogen, Carlsbad, CA) at 37 °C in an atmosphere of 5% CO_2 and 95% air. The Rho kinase inhibitor, Y27632, was obtained from UBI. TNF-α (biological activity of 2×10^7 U/mg) was purchased from Sigma–Aldrich (St. Louis, MO). Texas Red-X phalloidin and secondary antibodies conjugated to immunofluorescent dyes were purchased from Molecular Probes (Eugene, OR).

Constructs. The expression plasmid pRK5-TNF-R1 was obtained from Dr. David Goeddel (Genentech). Plasmids pRK5-RhoA (Q63L) and pRK5-RhoA (T19N) were obtained from Dr. Gary Bokoch (Scripps Research Institute) and were used as constitutively activated and dominant negative mutants, respectively, after introducing an N-terminal HA-epitope tag and cloning into pcDNA3.1hygro (Invitrogen, Carlsbad, CA).

Transfection and infection. Bovine lung endothelial cells were transiently transfected in 12-well tissue culture plates at 50% confluence. For each transfection, 1 μg plasmid DNA was incubated with 6 μl Fugene (Roche Molecular Biochemicals, Indianapolis, IN) in 50 μl serum-free Optimem (Gibco, Invitrogen, Carlsbad, CA) at room temperature for 15 min, followed by the addition of 450 μl of serum-free Optimem (Gibco) to each tube containing the lipid–DNA complexes. The mixture was overlaid onto the cells, which were then incubated for 4 h at 37 °C, followed by addition of complete media (1:3 volumes; 48 h).

Immunofluorescent staining of the endothelial cytoskeleton and caspases. Bovine pulmonary artery endothelial cells were cultured to confluence in 12-well dishes on coverslips coated with gelatin. After exposure to experimental conditions, endothelial cell monolayers were fixed in 3.7% formaldehyde and then permeabilized with 0.25% Triton X-100. After staining, coverslips were mounted on slides and examined under oil immersion using an Eclipse TE300 inverted microscope (Nikon, Melville, NY). Actin was visualized by Texas Red-phalloidin staining (1:200) for 1 h at room temperature and stained red. This method enabled the examination of endothelial cell morphology (cellular rounding, shrinkage), intercellular gap formation in confluent monolayers, and intracellular actin filament reorganization (stress fiber formation, cortical or perinuclear actin organization). Staining for activated caspase 7 or 3 was performed in a similar manner with 1h incubation at room temperature with anti-cleaved (active) caspase 7 antibody (BD Transduction Laboratories, Lexington, KY), or anti-cleaved caspase 3 antibody (Cell Signaling Technology, Beverly, MA), respectively, followed by 3 washes with PBS–Tween and incubation for 1 h at room temperature with an appropriate secondary antibody conjugated to immunofluorescent dyes (Alexa 546 or Texas Red). After three washes with PBS–Tween (0.1%), the coverslips were mounted and analyzed using Nikon video-imaging system consisting of the phase contrast inverted microscope connected to a digital camera and image processor. All images were recorded and saved in Adobe Photoshop.

Western immunoblotting. Endothelial cell proteins were separated by SDS–PAGE, transferred to Immobilon PVDF membrane (Millipore, Bedford, MA), and immunoblotted for 1h as previously described [7] with a monoclonal anti-caspase 8 antibody (Cell Signaling Technology, Beverly, MA) that recognizes both the pro-caspase 8 and the active, cleaved caspase 8 proteins, followed by the addition of the appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000). Positive control peptide for caspase 8 was from Cell Signaling Technology. The reaction was visualized by enhanced chemiluminescence (ECL) and autoradiography (Amersham).

Nucleosome ELISA. Quantitation of apoptotic endothelial cells was performed by measurement of nucleosomal degradation as previously described [7] utilizing a Nucleosome ELISA Kit (Oncogene Research Products, Cambridge, MA). Endothelial cells were lysed and the supernatant loaded onto precoated DNA-binding protein wells. The nucleosomes were detected using anti-histone 3 biotinylated antibody, followed by streptavidin horseradish peroxidase with absorbance (450 nm) compared to lyophilized standards with designated nucleosome unit values.

Caspase activity assay. Caspase 8 activity was assayed using the ApoAlert Caspase 8 colorimetric assay kit from Clontech (Palo Alto, CA), according to the manufacturer's instructions. Endothelial cells were lysed, centrifuged, and the supernatant assayed for protease activity using the specific, chromogenic substrate, Ac-IETD-pNA with optical density measurements obtained at 400 nm every 30 min (up to 180 min) in a V_{max} microplate reader (Molecular Devices, Sunnyvale, CA). The slopes of the curves obtained were normalized to those of

blank samples (buffer and lysis buffer only). Caspase activity units were calculated by dividing the values obtained to the slope of a standard curve of absorbance of the chromogen alone (pNA).

Results

Rho kinase mediates TNF-α-induced endothelial cell actin cytoskeletal changes

Confluent unstimulated bovine pulmonary artery endothelial cells exhibit cobblestone appearance with tight intercellular contacts and predominantly peripheral cortical actin staining [7]. Consistent with our previous work [7], TNF-α challenge (20 ng/ml, 3–4 h) produces prominent actin microfilament rearrangement with dramatic increase in stress fiber formation and development of intercellular gaps (Fig. 1). TNF-α-induced endothelial cell stress fiber formation occurs in an MLC-phosphorylation-dependent fashion [9], suggesting that MLCK and Rho kinase, two enzymes that act in concert to increase MLC phosphorylation, are likely to be involved in the regulation of this process. While the role of EC MLCK has been previously assessed, to address the specific role of endothelial cell Rho kinase and its activator, RhoA, in TNF-α-induced cytoskeletal changes, we transfected endothelial cells with dominant negative RhoA (DN-RhoA), which remains in its GDP form, and thus cannot bind and activate Rho kinase [10]. Consistent with prior studies in which Rho kinase was pharmacologically inhibited [7], TNF-α-induced actin stress fiber formation is significantly attenuated by DN-RhoA (Figs. 1A and B). Confirming the role of RhoA in actin rearrangement, stress fiber formation is profoundly amplified by the transient overexpression of the constitutively active RhoA (CA-RhoA) construct (Figs. 1C and D). In control experiments, TNF-α-induced actin rearrangement is not inhibited by the empty vector control (data not shown). These results demon-

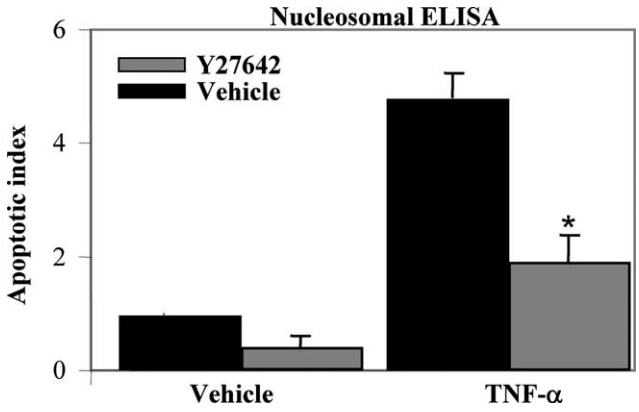

Fig. 2. Rho kinase inhibition attenuates TNF-α-induced endothelial cell apoptosis. Bar graph representing nucleosomal ELISA apoptosis results. The apoptotic index was determined by dividing the specific assay activity expressed in nucleosomal units of the TNF-α-treated cells (20 ng/ml, 18 h) to the activity of untreated cells (black bars). Pretreatment with the Rho kinase inhibitor, Y27632 (5 μM, 45 min) (gray bars), significantly attenuated TNF-α-induced bovine pulmonary endothelial cell apoptosis (* indicates statistical significance compared to TNF-α-treated cells $p = 0.005$).

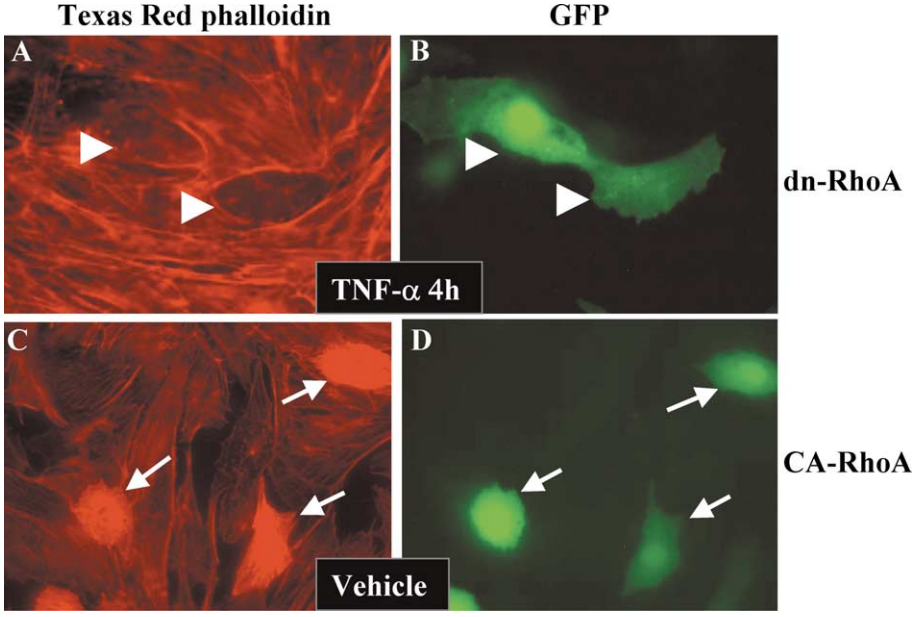

Fig. 1. TNF-α-induced endothelial cell actin cytoskeletal rearrangement is RhoA-dependent. Photomicrographs of endothelial cells co-stained for actin with Texas Red phalloidin (red) and GFP (green) to visualize RhoA transfection via fluorescent microscopy. (A and B) TNF-α (20 ng/ml, 4 h) causes dramatic increases in actin stress fiber formation, which is significantly attenuated by transfection of a dominant negative RhoA construct (DN-RhoA) (arrowhead). (C and D) Endothelial cells transiently transfected with constitutively active RhoA (CA-RhoA) (arrows) demonstrate profound actin cytoskeletal rearrangement.

strate that TNF-α-induced endothelial cell actin cytoskeletal rearrangement is Rho-dependent.

Rho kinase inhibition attenuates TNF-α-induced endothelial cell apoptosis and caspase activation

Specific inhibition of Rho kinase activity (Y27642) dramatically reduces TNF-α-induced apoptosis (by ~60%) as measured by nucleosomal ELISA (Fig. 2). Interestingly, the Rho kinase inhibitor alone reduces the baseline levels of endothelial cell apoptosis (Fig. 2).

We next investigated potential mechanisms by which Rho kinase inhibition attenuates TNF-α-induced endothelial cell apoptosis. Because our studies [9] confirmed the relative weak contribution of mitochondrial activation in the execution of the TNF-α-triggered endothelial

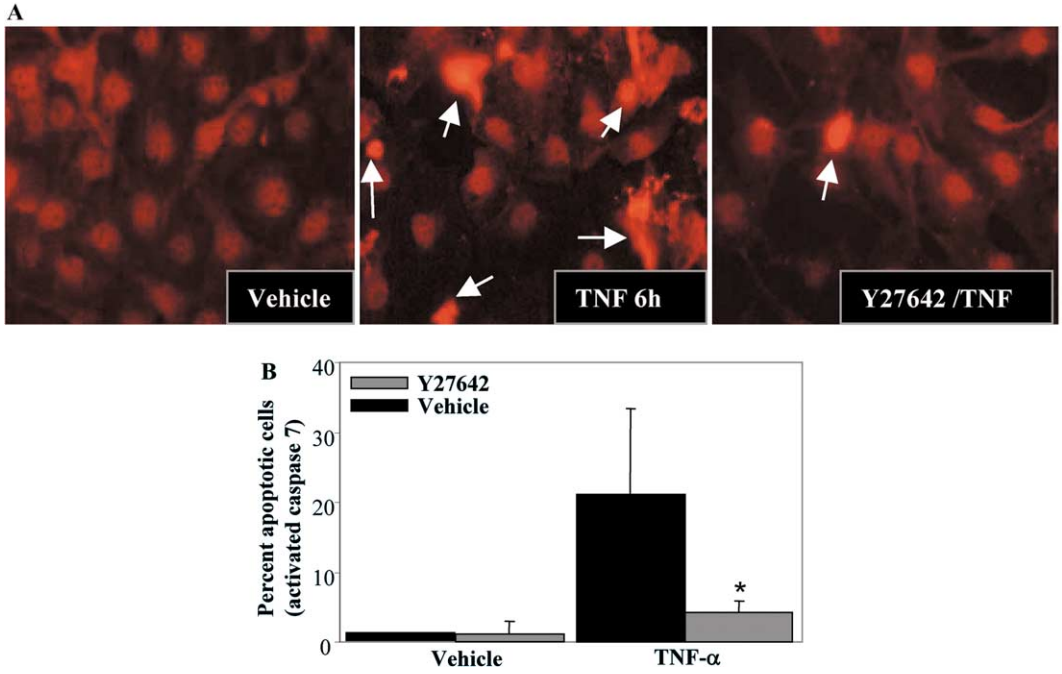

Fig. 3. Rho kinase inhibition attenuates TNF-α-induced effector caspase 7 activation in endothelial cells. (A) Fluorescence microphotographs of immunostaining for active caspase 7 in vehicle-treated cells (left image). There is increased caspase 7 activation (arrows) in response to TNF-α (20 ng/ml, 6 h) (center image) and significant inhibition of caspase 7 activation in cells pretreated with the Rho kinase inhibitor (Y27632, 5 μM, 45 min, right image). (B) The percentage of apoptotic cells as detected by immunostaining with a specific anti-active caspase 7 antibody, relative to controls ($n = 3$; * indicates statistical significance compared to TNF-α-treated cells $p = 0.03$).

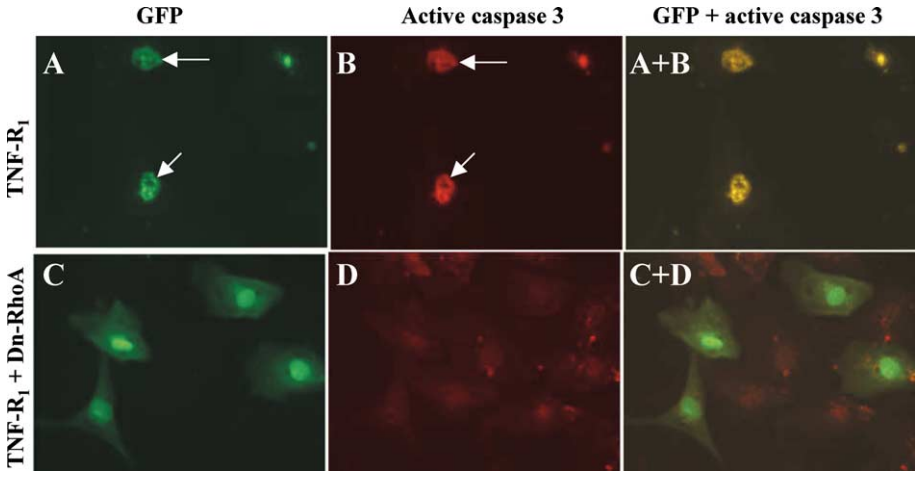

Fig. 4. Transient transfection with dominant negative RhoA inhibits TNFR-1-induced endothelial cell caspase 3 activation. Shown are photomicrographs of endothelial cells co-transfected with TNFR-1 and DN-RhoA, as described in Materials and methods, and then co-stained for active caspase 3 (red) and GFP (green) and visualized with fluorescent microscopy. Transfected cells overexpressing TNFR-1 (A) exhibit clear activation of caspase 3 (B) as marked with arrows. The co-localization is depicted in the overlay (A + B). Co-transfection of TNFR-1 and DN-RhoA (C) blocks caspase 3 activation (D) with the merged overlay seen in (C + D).

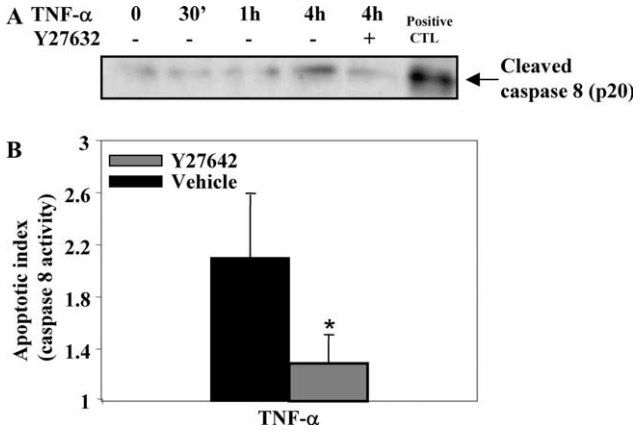

Fig. 5. Rho kinase inhibition attenuates TNF-α-induced initiator caspase activation in endothelial cells. (A) Immunoblot with anti-caspase 8 antibody illustrating that caspase 8 cleavage by TNF-α (20 ng/ml, 4 h) is inhibited by the Rho kinase inhibitor, Y27632 (5 μM, 45 min). Similarly, in (B) Rho kinase inhibition reduces caspase 8 activity as assessed by a colorimetric caspase 8 activity assay (* indicates statistical significance $p = 0.04$) (see Materials and methods).

cell apoptosis [11], we focused on the effect of the Rho kinase on caspase cascade activation. Our results indicate that TNF-α-stimulated Rho kinase activity is essential to the activation of the effector caspases, caspase 7 and caspase 3. For example, pretreatment with Y27642 potently reduces TNF-α-induced caspase 7 cleavage (fourfold reduction) as detected by immunofluorescence (Fig. 3). Consistent with these results, transfection with the dominant negative RhoA construct dramatically reduced caspase 3 activation elicited by TNF-R1 overexpression (Fig. 4). Finally, Rho kinase inhibition (Y27642) effectively inhibited caspase 8 activation, as assessed by immunoblotting with a caspase 8 antibody that recognizes the active, cleaved form of the protease (Fig. 5) and by a colorimetric caspase 8 activity assay (Fig. 5). Together, these results demonstrate that reductions in Rho kinase activity inhibit TNF-α-induced endothelial cell apoptosis and effector caspase activation by modulating the initiator caspase activation.

Discussion

TNF-α effects on the pulmonary endothelium are pleiotropic and include cytokine secretion, ROS production, apoptosis, and increases in vascular permeability [7,12]. TNF-α-induced cellular activation also triggers endothelial actin cytoskeletal rearrangement with intercellular gaps and stress fiber formation, where the motor behind actin cytoskeletal changes is myosin, an ATPase capable of generating mechanical force by promoting translational movement across the actin fibers [13]. Myosin II is the main non-muscle class of myosin consisting of two sets of myosin heavy chains (200 kDa) and two sets of MLC (16–20 kDa) and is regulated by MLC phosphorylation. TNF-α increases the level of MLC phosphorylation in endothelium, likely representing a combined effect of MLCK and Rho kinase, as assessed by earlier biochemical inhibitory studies [7]. Our previous work also suggested that activation of MLCK by TNF-α is critical for the execution of the programmed cell death, as pharmacological and molecular inhibition of MLC phosphorylation significantly attenuated TNF-α-induced apoptosis [7,9]. In this report, we investigated the role of the Rho family GTPase effector Rho kinase, a key regulator of endothelial cytoskeletal rearrangement, in TNF-α-induced apoptosis.

Rho kinase/ROK/ROCK II acts both by inhibition of the regulatory subunit of the myosin-specific phosphatase (MYPT) and potentially via direct MLC phosphorylation [14,15]. Rho kinase is a key target of activated Rho (Ras homology, Rho) GTPases, which are active when GTP bound and has a key role in the regulation of cytoskeletal organization and cellular motility [4]. Our data provide several lines of evidence that TNF-α-induced endothelial cell MLC-phosphorylation and actin rearrangement is Rho kinase dependent. Inactivation of Rho kinase by overexpression of dominant negative RhoA (GDP RhoA that cannot activate Rho kinase) inhibited TNF-α-induced stress fiber formation, while a constitutively active RhoA (GTP form) resulted in enhancement of cytoskeletal rearrangement in response to TNF-α, implicating Rho kinase as an essential mediator of TNF-α-induced MLC phosphorylation and actin rearrangement in endothelial cells.

Previous reports identified the involvement of MLCK and Rho kinase in apoptotic membrane blebbing [5,6], a process which may be dissociated from the execution phase of apoptosis. For example, Rho kinase was recently found as a substrate for caspase 3 and the cleaved fragment assumes a constitutively active function producing increased membrane blebbing in Jurkat cells [6]. We found that both biochemical inhibition of Rho kinase with Y27642 and transfection of dominant negative RhoA resulted in significant reductions in caspase activation and apoptosis in response to TNF-α stimulation or transient transfection with activated TNFR-1. These results suggest an essential role for the Rho kinase-dependent cytoskeletal rearrangement, not mainly as an effector but as a direct participant in the assembly of the intracellular death activating pathway, such as the caspase pathway or mitochondria-regulated pathways. Since the role of the mitochondria-dependent apoptotic mechanisms is not prominent in our model of TNF-α-triggered endothelial cell apoptosis [11], we explored the role of Rho kinase on caspase cascade activation. TNF-α-induced apoptotic signals are transmitted via TNFR-1 which leads to the assembly of the death-inducing signaling complex (DISC), activates procaspase 8 (through proximity-induced oligomerization), and subsequently initiates the caspase cascade (caspases 3 and 7). We

demonstrated that Rho kinase is critical for the activation of the initiator caspase 8 and of the downstream effector caspase 3. Therefore, Rho kinase may regulate compartmental actomyosin rearrangement that is critical to the initiator caspase activation. Recent reports substantiate the hypothesis that the cytoskeleton acts as an apoptosis facilitator. Siegel et al. [16] showed that recruitment of death effector filaments by death effector domain containing proteins triggers efficient caspase 8 recruitment and activation. Moreover, MLCK-dependent actin rearrangement is important in initiator caspase 8 activation and possibly recruitment in endothelial cells [7,9] and may facilitate the recruitment of the TNFR-1 to the plasma membrane in MDCK cells [8]. Interestingly, Rho kinase overexpression induced caspase-independent, but cytoskeletal-dependent membrane blebbing and chromatin condensation in MCF-7 cells, suggesting the presence of caspase-independent pathways regulated by Rho-GTPases [17]. Moreover, excessive inhibition of Rho kinase (Y27632, 30 μM) is associated with induction of apoptosis in human umbilical vein endothelial cells [18], which leads us to speculate that the downstream effects of Rho kinase are dictated by precise levels and localization of intracellular pools of Rho kinase.

In summary, we have examined the role of the actin/myosin microfilament regulator Rho kinase in TNF-α-induced endothelial cell apoptosis. We demonstrated that Rho kinase is critical for TNF-α-induced apoptosis signaling in endothelial cells, regulating both initiator and effector caspase activation. The mechanisms by which Rho kinase-mediated cytoskeletal changes modulate the apoptotic process remain to be demonstrated, however, our work suggests a role for Rho kinase in upstream events, involving recruitment of the DISC and initiator caspase activation.

Acknowledgments

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