Thioredoxin-2 Inhibits Mitochondria-Located ASK1-Mediated Apoptosis in a JNK-Independent Manner

Rong Zhang, Rafia Al-Lamki, Lanfang Bai, Jeffrey W. Streb, Joseph M. Miano, John Bradley, Wang Min

Abstract—Apoptosis signal-regulating kinase 1 (ASK1) mediates cytokines and oxidative stress (ROS)–induced apoptosis in a mitochondria-dependent pathway. However, the underlying mechanism has not been defined. In this study, we show that ASK1 is localized in both cytoplasm and mitochondria of endothelial cells (ECs) where it binds to cytosolic (Trx1) and mitochondrial thioredoxin (Trx2), respectively. Cys-250 and Cys-30 in the N-terminal domain of ASK1 are critical for binding of Trx1 and Trx2, respectively. Mutation of ASK1 at C250 enhanced ASK1-induced JNK activation and apoptosis, whereas mutation of ASK1 at C30 specifically increased ASK1-induced apoptosis without effects on JNK activation. We further show that a JNK-specific inhibitor SP600125 completely blocks TNF induced JNK activation, Bid cleavage, and Bax mitochondrial translocation, but only partially inhibits cytochrome c release and EC death, suggesting that TNF induces both JNK-dependent and JNK-independent apoptotic pathways in EC. Mitochondria-specific expression of a constitutively active ASK1 strongly induces EC apoptosis without JNK activation, Bid cleavage, and Bax mitochondrial translocation. These data suggest that mitochondrial ASK1 mediates a JNK-independent apoptotic pathway induced by TNF. To determine the role of Trx2 in regulation of mitochondrial ASK1 activity, we show that overexpression of Trx2 inhibits ASK1-induced apoptosis without effects on ASK1-induced JNK activation. Moreover, specific knockdown of Trx2 in EC increases TNF/ASK1-induced cytochrome c release and cell death without increase in JNK activation, Bid cleavage, and Bax translocation. Our data suggest that ASK1 in cytoplasm and mitochondria mediate distinct apoptotic pathways induced by TNF, and Trx1 and Trx2 cooperatively inhibit ASK1 activities. (Circ Res. 2004;94:1483-1491.)

Key Words: thioredoxin ■ ASK1 ■ mitochondria ■ TNF-α ■ apoptosis

Thioredoxins (Trx) are cellular redox enzymes that have multiple functions in regulation of cell growth, apoptosis, and activation.1 Trx contains two redox-active cysteine residues in its catalytic center with a consensus amino acid sequence: cyst-gly-pro-cys. Trx exists either in a reduced diithiol form or in an oxidized form. It participates in redox reactions by reversible oxidation of its active center dithiol to disulfide and catalyzes disulfide exchange reactions involving many thiol-dependent processes.1 Thus, Trx system is considered to constitute an endogenous antioxidant system in addition to the glutathione and superoxide dismutase systems. Two isoforms of Trx have been identified in mammalian cells: cytosolic and mitochondrial Trx (Trx1 and Trx2, respectively). Trx1 and Trx2 are encoded by distinct nuclear genes and Trx2 contains a mitochondrial targeting signal peptide.2–4 The mitochondrion is the major source of ROS generated during physiological respiration and pathological conditions during inflammation in response to cytokines.5 Therefore, the mitochondrial antioxidant systems including Trx2, Trx2 reductase, mitochondrial Trx peroxidase, and manganese superoxide dismutase (MnSOD) are critical in regulating mitochondrial ROS-induced cytotoxicity. Consistently, Trx2-deficient cells show an accumulation of intracellular ROS and mitochondria-dependent apoptosis.3 Conversely, overexpression of Trx2 confers resistance to ROS-induced cell death.2,4 Furthermore, genetic ablation of Trx2 in mice causes massively increased apoptosis in E10.5-day embryos, leading to embryonic lethality.6 Both Trx1- and Trx2-deficient systems have been reported to prevent oxidative stress–induced cytotoxicities. It has been shown that Trx1 prevents cell apoptosis by scavenging reactive oxygen species (ROS) to protect against oxidative stress. It also acts antiapoptotically by regulating the activities of transcription factors such as NF-κB and AP-1, and by directly binding and inhibiting the activity of the proapoptotic protein apoptosis signal-regulating kinase 1 (ASK1).1,7

Apoptosis signal-regulating kinase 1 (ASK1) is one of several MAP3Ks that are activated in response to proinflammatory stimuli, ROS, and cellular stress leading to activation of MAP2K-JNK/p38 cascades.8 ASK1-induced apoptosis has
been extensively studied. ASK1 is a 170-kDa protein that is composed of an inhibitory N-terminal domain, an internal kinase domain, and a C-terminal regulatory domain. Several cellular inhibitors including Trx1 have been shown to bind to and inhibit ASK1 apoptotic activity in resting cells. Trx1 directly associates with ASK1 in the N-terminal domain of ASK1 and inhibits its kinase activity. Deletion of the N-terminal 648 amino acids of ASK1 (ASK1-∆N) causes constitutive ASK1 kinase activity, as it does in other MAP3Ks. This confirms that Trx1 inhibits ASK1 via the N-terminal inhibitory domain. Interestingly, only the reduced form of Trx1 binds to the N-terminal part of ASK1 and blocks activation of ASK1 by TNF. Neither the oxidized form (intramolecular disulfide between C32 and C35) nor the redox-inactive form (the double-mutation at catalytic sites C32 and C35) of Trx1 binds to ASK1. Apoptotic stimuli (TNF, ROS, or serum starvation) activate ASK1 in part by oxidizing Trx1 to release it from ASK1. We have recently shown that a single Cys residue in the catalytic site of Trx1 (C32 or C35) is critical for ASK1-binding. Furthermore, Trx1-C32S and Trx1-C35S constitutively bind to ASK1 even in the presence of hydrogen peroxide in vitro or of TNF in vivo, most likely because they cannot be oxidized to form a disulfide bond between the two catalytic cysteines leading to its release from ASK1. These data suggest that Trx1 is critical for the regulation of ASK1 activity.

Studies from ASK1 knockout mice have shown that ASK1 is a critical mediator in TNF, ROS, and stress-induced cell death. Moreover, overexpression of ASK1 induces a mitochondria-dependent apoptotic pathway. However, the underlying mechanism by which ASK1 mediates mitochondria-dependent apoptotic pathway is not fully understood. JNK, a downstream target of ASK1, has been shown to activate proapoptotic Bcl-2 family protein such as Bim/Bak/Bax, leading to release of proapoptotic factors such as cytochrome c and cell death. It is not clear whether or not the proapoptotic activity of ASK1 is solely dependent on downstream targets such as JNK. In the present study, we show that ASK1 localized in both cytoplasm and mitochondria of EC and forms a complex with Trx1 and Trx2 in resting state. Proapoptotic stimuli TNF and oxidative stress dissociate Trx1/Trx2 from ASK1, leading to enhanced mitochondria-dependent apoptosis characterized by cytochrome c release, caspase-3 activation, and nuclear fragmentation. Conversely, overexpression of Trx1 and Trx2 synergistically block TNF/ROS/ASK1-induced cell death. Furthermore, our data demonstrate that a specific interaction between ASK1 and Trx2 in mitochondria plays a pivotal role in regulation of cellular survival and apoptosis.

Materials and Methods
An expanded Materials and Methods section is provided in the online data supplement available at http://circres.ahajournals.org.

Confocal immunofluorescence microscopy, cell transfection and reporter gene assay, immunoprecipitation and immunoblotting, ASK1 and JNK kinase assays, GST-Trx pull-down assay, RNAi constructs and RNase protection assay, and quantitation of apoptosis have been described previously.

Results
ASK1 and Trx2 Form a Complex in Mitochondria That Is Dissociated in Response to TNF/ROS
To determine the underlying mechanism by which ASK1 mediates a mitochondria-dependent apoptosis, we examined subcellular localization of endogenous ASK1 by immunogold electron microscopy (EM). Human EC (HUVEC) was stained with anti-ASK1 (rabbit) and a mitochondria marker (mouse monoclonal) followed by goat anti–rabbit-5 nm gold particles and goat anti–mouse-15 nm gold particles. ASK1- and mitochondria-gold particles were visualized under EM. As shown in Figure 1A, a mitochondrial marker was detected only in mitochondria (m) but not in rough endoplasmic reticulum (rER) (left panel) or nucleus (N) (middle panel). ASK1 is stained inside mitochondria (Δ) as well cytoplasm (*). High-power views clearly show that ASK1 is colocalized with mitochondria marker (right panel). Similar results were obtained in human EC line EAhy926 (data not shown). TNF treatment (10 ng/mL for 15 minutes) does not alter the localization of ASK1 in mitochondria (data not shown).

Localization of endogenous ASK1 in EC mitochondria was further determined by indirect immunofluorescence microscopy. ASK1 showed a punctate mitochondrial staining and was colocalized with MitoTracker (online Figure S1, in the online data supplement at http://circres.ahajournals.org). Similar data were obtained in bovine endothelial cells (BAECs) and human EC cell line EAhy.926 (data not shown). To determine whether ASK1 and Trx2 forms a complex in mitochondria, cytosolic and mitochondrial fractions from HUVECs were isolated. Mitochondrial protein Trx2 reductase and Trx2 are detected only in the mitochondrial fraction, whereas Trx1 was excluded from mitochondria and detected only in the cytoplasm. However, ASK1 is detected in both mitochondrial and cytosolic fractions (Figure 1B). We next monitored formation of ASK1-Trx2 complex and its regulation by apoptotic stimuli such as TNF and hydrogen peroxide. HUVECs were left untreated or treated with TNF as described previously. Subsequently, the association of ASK1 with Trx2 in mitochondria was determined by immunoprecipitation. As previously described, Trx1 binds to cytosolic ASK1 and is dissociated from ASK1 on TNF treatment (Figure 1B). Association of Trx2 with ASK1 in mitochondria was readily detected in untreated EC cells (Figure 1B, −TNF). TNF treatment did not significantly alter the partition of ASK1 and Trx2 proteins between mitochondrial and cytosolic fractions. However, TNF significantly reduced the mitochondrial ASK1-Trx2 complex (Figure 1B, +TNF), suggesting that TNF induced a dissociation of Trx2 from ASK1. Similar results were obtained in BAECs with TNF (10 ng/mL) or H2O2 (1 mmol/L) (data not shown). These data suggest Trx2, like Trx1, binds to ASK1 in resting cells and is dissociated from ASK1 in response to TNF/ROS.

To further characterize interaction between Trx2 and ASK1, we performed an in vitro GST pull-down assay using GST-Trx2. BAEC lysates containing ASK1 protein was used for an in vitro pull-down assay. ASK1 binds to GST-Trx1 and GST-Trx2, but not GST alone (Figure 1C). Trx2 contains the two Cys located in the catalytic sites (Cys90 and Cys93).
corresponding to Cys32 and Cys35. To determine their roles in ASK1 binding, we mutated the Cys residues to generate Trx2-C90S and Trx2-C93S. We examined association of ASK1 with Trx2 proteins in the presence or absence of 1 mmol/L H2O2. ASK1 bound to Trx2-WT and Trx2-C93S (but not to Trx2-C90S) in the absence of H2O2 (Figure 1D), suggesting that Cys90 in Trx2 is critical for ASK1-binding. Addition of 1 mmol/L H2O2 disrupted the association of Trx2-WT with ASK1. In contrast, Trx2-C93S retained its association with ASK1 in the presence of H2O2 (Figure 1D).
These data indicate that Trx2-C93S, like Trx1-C32S or C35S, forms a stable complex with ASK1, which is resistant to dissociation by ROS.

**ASK1 Mutant Defective in Trx2-Binding Enhanced Apoptotic Activity Without Compromising JNK Activation**

We have previously proposed that Trx1 binds to the N-terminal domain of ASK1 via a formation of an intermolecular disulfide bridge. To define a Cys residue in ASK1 that might participate in this interaction, we generated a series of N-terminal truncation and site-specific mutant constructs of ASK1. By in vitro GST pull-down assay, we first defined the aa73–301 and aa1–73 in ASK1 are critical for association of Trx1 and Trx2, respectively (Figures 2A and 2B). We mutated the Cys residues within this region to Ser (C22, C30, C67, C120, C185, C200, 206, C225/226, and C250) and found that C30 is critical for Trx2-binding, whereas C250 for Trx1-binding (Figures 2C and 2D). ASK1-C30S lost the ability to bind Trx2, and Trx1-defective mutant (ASK1-C250S) retains ability for Trx2-binding, and vice versa (Figure 2E). These data further support our model that Trx and ASK1 form a complex via an intermolecular disulfide bridge (Cys32 or Cys35 in Trx1 with Cys250 in ASK1; Cys90 in Trx2 with Cys30 in ASK1), and TNF/ROS likely disrupt these intermolecular disulfide bonds leading to Trx oxidation and dissociation from ASK1.

We reasoned that ASK1 mutants defective in Trx-binding (ASK1-C30S for Trx2 and ASK1-C250S for Trx1) should have increased activities in apoptosis and JNK activation. To test this hypothesis, HA-tagged ASK1 constructs were transfected into BAECs. Mutations either at C30 or at C250 did not significantly alter localization of ASK1 by subcellular fractionation analyses; online Figure S2C). Similar results were obtained from subcellular localization analysis of ASK1 proteins by confocal microscopy (not shown). To determine ASK1-induced JNK activation, BAECs were transfected with ASK1 constructs. Forty eight hours after transfection, nucleus fragmentation was visualized by DAPI staining. Apoptosis rate is shown. Data are presented as mean of duplicates from two independent experiments. *P<0.05.
These data suggest that Trx1 and Trx2 are critical regulators of ASK1.

**TNF Induces Both JNK-Dependent and Independent Apoptotic Pathways**

The data that ASK1-C30S increases cell death without JNK activation suggest that ASK1 in mitochondria may mediate a JNK-independent apoptotic pathway. We first determined if TNF induces a JNK-independent pathway in ECs. ECs were treated with TNF (10 ng/mL) plus cycloheximide (CHX, 10 μg/mL) in the presence or absence of JNK-specific inhibitor SP600125 (20 μmol/L). Previously, it has been shown that JNK-dependent events in TNF-induced apoptosis include cleavage of Bid and mitochondrial translocation of Bax. We examined JNK activation, Bid cleavage, and Bax translocation in ECs. JNK activation was determined by an in vitro kinase assay. Bid cleavage was determined by Western blot with anti-Bid, which recognizes both intact and truncated Bid. Bax translocation was determined by indirect immunofluorescence microscope with anti-Bax which specifically recognizes mitochondrial form of Bax. Cytoplasm fractions from treated ECs were isolated as described and cytochrome c was determined by Western blot with anti-cytochrome c. D. SP600125 only partially inhibits TNF-induced EC apoptosis. HUVECs were treated with TNF+CHX in the presence of JNK-specific inhibitor SP600125 (20 μmol/L) or caspase-3 inhibitor z-VAD (30 μmol/L) as indicated for 6 hours. EC apoptosis was determined by DAPI staining for nuclei fragmentation. Apoptosis rate is shown. Data are presented as mean of duplicates from two independent experiments. *P<0.05.

**Mitochondria-Located ASK1 Specifically Mediates a JNK-Independent Apoptotic Pathway**

We hypothesize that cytosolic ASK1 specifically mediates JNK-dependent pathways, whereas mitochondrial ASK1 specifically mediates JNK-independent pathways. To test our hypothesis, we specifically expressed a constitutively active form of ASK1 (ASK1-ΔN) in mitochondria by fusing the Trx2 mitochondria targeting sequence to ASK1-ΔN (mtASK1-ΔN) (Figure 4A). The mitochondrial targeting sequence of Trx2 (aa1–60) is critical for its localization in mitochondria, and Trx2 with deletion of this sequence (ΔTrx2) is expressed in the cytoplasm (online Figure S3A). ASK1-ΔN lacking the N-terminal binding domain showed exclusively cytoplasmic localization (online Figure S3B). However, mtASK1-ΔN is specifically detected in mitochondria and colocalized with Trx2 (online Figure S3C). We then examined ASK1-ΔN and mtASK1-ΔN–induced JNK activation, Bax translocation, and apoptosis. Although mtASK1-ΔN shows as similar ASK1 basal activity to ASK1-ΔN as measured by an in vitro kinase assay, mtASK1-ΔN or mtASK1-ΔN-K709R (a kinase inactive mu-
tant) did not induce JNK activation (Figures 4B and 4C). ASK1-ΔN, but not mtASK1-ΔN or mtASK1-ΔN-K709R, significantly induced Bax translocation into mitochondria (Figure 4D). However, ASK1-ΔN or mtASK1-ΔN induced comparable EC apoptosis (Figure 4E). ASK1-ΔN–induced apoptosis is strongly inhibited by either caspase-3 inhibitor z-VAD or JNK-specific inhibitor SP600125. However, mtASK1-ΔN–induced apoptosis is significantly blocked by z-VAD, but not by SP600125 (Figure 4E). mtASK1-ΔN did not induce p38 activation as determined by Western blot with anti-phospho-p38, and mASK1-induced apoptosis was not inhibited by p38-specific inhibitor SB203580 (data not shown). These data strongly support that mitochondria-located ASK1 specifically induces a JNK-independent apoptotic pathway.

Trx2 Specifically Inhibits ASK1-Induced EC Apoptosis With no Effects on ASK1-Induced JNK Activation

To determine the effects of Trx2 on ASK1, Trx2 with a V5 epitope-tag at the C-terminus was transfected into BAECs, which have high transfection efficiency (>80%) compared with HUVECs (≈10%). The transfected Trx2, like the endogenous Trx2, showed a strong punctate mitochondrial staining, whereas Flag-tagged Trx1 displayed a smear of nonmitochondrial staining (online Figure S4A). Subcellular fractionation also showed that Trx2 is detected in the mitochondrial fraction whereas Trx1 in cytosolic fractions (online Figure S4B). We then determined the effects of Trx2 expression on ASK1-induced JNK activation as determined by an in vitro kinase assay and EC apoptosis. As previously demonstrated, overexpression of ASK1 induced JNK activation, cytochrome c release, and EC apoptosis (Figures 5A through 5C). Expression of Trx1 significantly inhibited these effects. Although no effect on ASK1-induced JNK activation was detected, expression of Trx2 significantly decreased ASK1-induced cytochrome c release and EC apoptosis (Figures 5B and 5C). In contrast, ASK-binding defective mutant Trx2-C90S diminished ability to inhibit ASK1-induced JNK activation and EC apoptosis (Figures 5B and 5C), suggesting that the inhibitory effects of Trx2 on ASK1 is dependent on the interaction between the two molecules.

Knockdown of Trx1 or Trx2 Sensitizes Cells to ASK1-Induced Apoptosis

To further characterize the roles of Trx1 and Trx2 in TNF/ASK1-induced apoptosis, we used an RNA interference (RNAi) approach (see Materials and Methods). HUVECs were infected with adenovirus expressing shRNA to human Trx1 and Trx2. The expression of endogenous Trx1 and Trx2 was specifically downregulated (75% to 80%) using appropriate shRNAs (online Figures S5A and S5B). We next examined the effects of Trx knockdown on TNF-induced JNK activation measured by an in vitro kinase assay. TNF-induced JNK activation was significantly elevated in Ad-ShTrx1, but not in Ad-ShTrx2 cells (Figure 6A). We then examined TNF-induced apoptosis in Trx1 or Trx2 knockdown cells. ECs expressing Trx1 or Trx2 shRNA were treated with TNF (10 ng/mL) in the presence of CHX (10

Figure 4. Mitochondria-located ASK1 specifically mediates a JNK-independent apoptotic pathway. A, Diagram for expression constructs of Trx2, ΔTrx2, ASK1-ΔN, mtASK1-ΔN, and mtASK1-ΔN-K709R. Mt indicates mitochondria targeting signal sequence of Trx2. Band C, JNK-dependent reporter gene was cotransfected with ASK1-ΔN, mtASK1-ΔN, and mtASK1-ΔN-K709R into BAECs. Expression and the basal ASK1 activity was determined by Western blot with anti-ASK1 and by an in vitro kinase assay using GST-MKK4 as a substrate (B), JNK activities (relative luciferase activities) are presented from mean of duplicate samples by taking vector control as 1 (C). Similar results were obtained from 2 additional experiments. D, Bax translocation. BAECs were transfected with ASK1 constructs in the presence of JNK inhibitor SP600125 (20 μmol/L) or caspase-3 inhibitor (z-VAD, 30 μmol/L). Twenty four hours after transfection, apoptotic cells (with nuclear condensation) were determined after DAPI staining. Apoptosis rate is shown. Data are presented as mean of duplicates from two independent experiments.
cytochrome c release is a common downstream regulated by Trx1 and Trx2. Thus, TNF(+CHX)−induced apoptosis (Figure 6E) were greatly increased in both Trx2- and Trx1-knockdown cells compared with the control cells. These data support that Trx2 specifically regulates the JNK-independent intrinsic apoptotic pathway induced by TNF.

**Discussion**

**Trx1 and Trx2 Regulate Distinct Apoptotic Pathways Induced by TNF and ASK1**

Although Trx1 and Trx2 are major components of the cellular antioxidant system, distinct biological functions of Trx1 and Trx2 have been demonstrated by studies using genetically
deficient mice and cells. Deletion of either Trx1 or Trx2 causes embryonic lethality, suggesting that they are not functionally redundant. In the present study, we demonstrate that Trx1 and Trx2 cooperatively regulate TNF/ASK1 apoptotic activities (Figure 7). First, Trx1 and Trx2 bind to ASK1 at different sites. Cys-250 and Cys-30 in the N-terminal domain of ASK1 are critical for association with Trx1 and Trx2, respectively. Second, Trx1 and Trx2 regulate distinct signaling events induced by TNF/ASK1 in distinct cellular compartments. ASK1 is localized in cytoplasm where it binds to Trx1 as well as in mitochondria where it associates with Trx2. We have previously demonstrated that association of Trx1 with ASK1 regulates ASK1-induced JNK activation and apoptosis in EC. Consistently, mutation of ASK1 that prevents Trx1-binding (ASK1-C250S), knockdown of Trx1, or expression of cytosolic form of ASK1 leads to a JNK-dependent apoptotic pathway (JNK activation, Bid cleavage, and Bax mitochondrial translocation). In contrast, cells expressing a mutant ASK1 with deficiency for Trx2-binding (ASK1-C30S), knockdown of Trx2 by RNA interference, or specific expression of ASK1 in mitochondria significantly increase apoptosis in a JNK-independent manner. Our data suggest that cytochrome c release and caspase-3 activation are common downstream events in JNK-dependent and JNK-independent pathways induced by TNF/ASK1. Taken together, ASK1-Trx2 complex in mitochondria represents a distinct pathway regulating vascular EC survival and death in response to proinflammatory cytokines and oxidative stress.

**ASK1 Mediates Multiple Apoptotic Pathways**

ASK1 can be activated by various proapoptotic stimuli including death receptors, DNA damaging agents, oxidants, and cellular stresses such as growth factor deprivation and endoplasmic reticulum (ER) stresses caused by protein aggregation, suggesting that ASK1 may mediate multiple apoptotic pathways. Indeed, it has been shown that ASK1 induced cytochrome c release and activation of caspase-9 and caspase-3, but not of caspase-8. In response to ER-stress such as protein aggregation, TRAF2 and ASK1 are recruited by an ER-transmembrane sensor IRE1 to form IRE1-TRAF2-ASK1 complex leading to JNK activation and apoptosis. In this study, we show that ASK1 is localized in both cytoplasm and mitochondria of vascular EC where it induces distinct apoptotic signaling events. Because we did not observe significant translocation of ASK1 from cytoplasm to mitochondria during apoptosis, we propose that ASK1 compartmentalization is critical for responses to various proapoptotic stimuli. Thus, cytoplasm-located ASK1 may mediate death receptor (Fas), Daxx, or ER stress-induced extrinsic apoptotic pathway. In contrast, ASK1 located in mitochondria may mediate cell death via an intrinsic apoptotic pathway in response to TNF, ROS, or DNA damaging agents.

**Function of ASK1-Trx2 Complex in Mitochondria**

Our data from immunogold EM clearly showed that ASK1 is localized in mitochondria in resting cells. This is different from JNK protein that translocate into mitochondria during apoptosis. ASK1 is colocalized with Trx2 and forms a complex with Trx2 in mitochondria, suggesting that ASK1 likely resides inside mitochondria matrix where Trx2 is located. The mechanism by which ASK1 in mitochondria mediates TNF-induced apoptosis is not understood. Trx is one of the major systems combating against oxidative stress. It is conceivable that Trx1 in cytoplasm and Trx2 in mitochondria inhibit ASK1 activity by either scavenging ROS or by a direct interaction with ASK1. A direct association of Trx1 or Trx2 with ASK1 appears to be critical for regulation of ASK1 activity. It has been shown that TNF induces mitochondrial ROS production in HUVECs primarily occurs at the ubisemiquinone site. It is likely that ROS generated in the mitochondria in response to TNF induce dissociation of Trx2 from ASK1 leading to its activation. Interestingly, we have previously shown that TNF can be delivered into mitochondria of ECs where TNF receptors are present.

ASK1 appears to directly target on regulators participated in cytochrome c release. It has been shown that Trx2 forms complexes with cytochrome c. Thus, ASK1 may directly regulate the formation and dissociation of Trx2-cytochrome c complex. In support of this model, Trx2-knockdown cells show sensitized cytochrome c release in response to TNF. The nature of ASK1-Trx2-cytochrome c complex and its regulation needs to be further investigated. Another possibil-

![Figure 7. Model for regulation of ASK1-mediated apoptosis by Trx1 and Trx2.](http://circres.ahajournals.org/)}
ity is that ASK1 may target Bcl-2/Bax family proteins, which have been implicated in regulation of mitochondrial voltage-dependent anion channel and release of cytochrome c. Alternatively, ASK1 may target other mitochondrial components such as the mitochondrial permeability transition pore (PTP). This is supported by the data that overexpression of Trx2 showed increased mitochondrial membrane potential and sensitivity toward rotenone, an inhibitor of complex I of the respiratory chain.

In summary, our data demonstrate that Trx1 and Trx2 differentially regulate ASK1-mediated apoptotic events (Figure 7). It is conceivable that Trx1 and Trx2 cooperatively execute their antiapoptotic function against ASK1-mediated cell death. Our study should provide novel therapeutic approaches to treat apoptosis-associated cardiovascular diseases.

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Supplemental Materials (Zhang et al., Thioredoxin-2 inhibits mitochondria-located ASK1-mediated apoptosis in a JNK-independent manner).

MATERIALS AND METHODS

Constructs Mammalian expression plasmids for V5-epitope tagged (at the C-terminus) Trx2 and cytosolic form of Trx2 lacking the mitochondria targeting sequence (aa1-60) (ΔTrx2) were generously provided by Dr. Dean P. Jones (Emory University). Trx1 and ASK1 constructs were described previously. A mitochondrial form of ASK1-ΔN (mtASK1-ΔN) was generated by inserting Trx2 mitochondrial targeting sequence (aa1-60) to the N-terminus of ASK1-ΔN. The mutant ASK1 (CS mutants), Trx2-C90S and Trx2-C93S were constructed by site-directed mutagenesis using Quickchange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol, and mutations at the desired sites were confirmed by DNA sequencing. pShag vector were from Dr. Gregory J. Hannon (Cold Spring Harbor Laboratory, NY). The adenoviral Gateway vector (pAd/PL-DEST) and ViraPower Adenoviral Expression System were purchased from Invitrogen (Invitrogen Corp., San Diego, California, USA).

Antibodies. A rabbit polyclonal antibody against Trx1 or Trx2 was generated by immunizing rabbits with GST-Trx1 or GST-Trx2 protein through Cocalico Biologicals Inc. (Reamstown, Pennsylvania, USA). We obtained anti-ASK1 (H300), anti-Bax (N20) and anti-caspase 8 from Santa Cruz Biotechnology. Anti-cytochrome c and anti-β-tubulin were purchased from BD Pharmingen. Anti-Bid and anti-phospho-p38 were purchased from Cell Signaling. Anti-V5 was from Invitrogen, anti-HA was from Roche and anti-Flag was from Sigma.
**Cells, cytokines and inhibitors.** Human umbilical vein endothelial cells (HUVECs) and bovine aortic ECs (BAECs) and were purchased from Clonetics Corp. (San Diego, California, USA). Human umbilical vein EC (HUVEC) were cultured in modified M199 culture medium, containing 20% v/v heat-inactivated bovine fetal calf serum (FCS), 100µg/ml heparin sodium salt, 30 µg/ml endothelial cells growth supplement, 2 mM L-glutamine, 60 units/ml penicillin, and 0.5 µg/ml streptomycin at 37°C, in 5% CO₂ on gelatin-coated tissue culture plastic as described previously. Cells were used at passages 2-4. Human recombinant TNF was from R&D Systems Inc. (Minneapolis, MN) and used at 10 ng/ml. Caspase-3 inhibitor (z-VAD), JNK-specific inhibitor SP600125 and p38 inhibitor SB203580 were purchased from Calbiochem.

**Immunogold electron microscopy.** The cells were dislodged gently using a rubber policeman, fixed in 4% formaldehyde in 0.1M PIPES (Sigma, UK) buffer pH 7.5 for 30 minutes at 4°C, washed three times for 2 minutes each in 0.1M PIPES containing 2mM CaCl₂ and centrifuged at 210 x g. The cells pellets were then collected and processed for freeze substitution and low temperature embedding for immunocytochemistry as described. Briefly, HUVEC were cryoprotected in 30% propylene glycol for 1 hour at 4°C, and were frozen in melting propane cooled in liquid nitrogen, substituted against methanol containing 0.1% uranyl acetate at -90°C for 24 hours, at -70°C for 24 hours, and at -50°C for 24 hours. Cells were then impregnated with Lowicryl HM 20 resin over a period of 3 days and the resin was polymerized by ultraviolet irradiation at a temperature of -50°C. Ultrathin sections (40-50 nm) were cut on a Leica Ultracut-S (Leica, Vienna, Austria) ultramicrotome and mounted on Formvar-coated nickel grids. The grids were incubated, section down, for 30 min at room temperature in blocking buffer containing 10% FCS (fetal calf serum) in 0.1M Tris/HCl buffer pH 7.5 (TBS) to suppress non-specific
antibody binding. Excess blocking buffer was removed and sections were incubated overnight at room temperature, with rabbit polyclonal anti-human ASK1 (H-300; sc-7931; Santa Cruz Biotechnology) or anti-human Trx2 antibody diluted at 1:5 in blocking buffer at a final concentration of 40 µg/ml. After rinsing extensively with TBS, the grids were incubated for 1 hour at room temperature with goat anti-rabbit-5 nm colloidal gold particles (British Biocell, Cardiff, UK) diluted 1:100 in the blocking buffer. For co-localization of ASK1 and mitochondria, grids were incubated with anti-ASK1 (rabbit polyclonal) and anti-mitochondria (mouse monoclonal anti-p65 in mitochondria, MAB1273, Chemicon with 1:5 dilution) followed by goat anti-rabbit-5 nm colloidal gold particles and goat anti-mouse-15 nm colloidal gold particles. After thorough rinsing in TBS and in double distilled water, the grids were contrast-stained with uranyl-acetate and lead citrate for 3 seconds each. Grids were then viewed in a Philip CM 100 electron microscope at an accelerating voltage of 80 kV. Controls included omission of the primary antibody or use of non-immune serum as negative controls.

Confocal immunofluorescence microscopy. Fixation, permeabilization, and staining of cultured HUVEC and BACE were performed as described previously. For mitochondria visualization, cells were stained with 20 nM tetramethylrhodamine methyl ester (TMRM) (Molecular Probes, Eugene, OR) for 30 min at 37°C and washed twice with phosphate buffered saline (PBS). For indirect fluorescence microscopy, the fluorescein isothiocyanate (FITC)-conjugated anti-IgG and tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated anti-IgG were purchase from Molecular Probes. Confocal immunofluorescence microscopy was performed using an Olympus confocal microscope.

Cell transfection and reporter gene assay. Transfection of HUVEC was performed by the DEAE-dextran method as described previously and transfection of BAECs was performed by Lipofectamine
2000 according to manufacturer’s protocol (Invitrogen Corp., San Diego, California, USA). For reporter gene assay, cells were cultured at 90% confluence in 6-well plates and were transfected with total of 2.5 µg plasmid constructs including reporter gene (1 µg), renilla gene (0.5 µg) and various transgenes (1 µg) as indicated. Cells were harvested at 36-48 h post-transfection and cell lysates (10 µl) were measured for luciferase activity followed by renilla activity twice in duplicates with Promega reagents (Promega Corp., Madison, Wisconsin, USA) using a Berthold luminometer (EG&G Wallac, Gaithersburg, Maryland, USA). All data were normalized as relative luciferase light unit/renilla unit.

**Preparation of subcellular fractions.** Subcellular fractions from HUVEC and BAEC were prepared as described \(^1\). To obtain the cytosolic fraction, cells were washed with PBS and resuspended in 50 µl of 250 mM sucrose and 70 mM Tris (pH 7.0) with protease inhibitors mixture (Roche Diagnostics Corp., Indianapolis, Indiana, USA). 10 µl of 4 mg/ml digitonin was added followed by incubation at room temperature for 2 min. 2 µl of Cells were stained with trypan blue followed by direct observation with light phase contrast microscopy to obtain lysis of 90–95% of cells. This approach allows preparation of cytosol that is essentially free of mitochondrial contamination \(^1\). After centrifugation at 600 x g for 2 min at room temperature, the supernatant was collected as the cytosolic fraction. To prepare the mitochondrial fraction, cells were washed once with PBS, resuspended in ice-cold hypotonic buffer (10 mM NaCl, 1.5 mM CaCl\(_2\), 10 mM Tris-Cl, pH 7.5, with protease inhibitors mixture, and kept on ice for 10 min. MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mM Tris, pH 7.6) was then added, and cells were homogenized using a Dounce homogenizer with 30 strokes. Disruption of plasma membrane was monitored by trypan blue staining. After removing the nuclear fraction by two successive centrifugations at 3000 x g for 10 min, the supernatant was centrifuged at 12,000 x g for 10 min. The pellet was collected as the mitochondrial portion and resuspended in lysis buffer (50 mM
HEPES, pH 7.0, 500 mM NaCl, and 1% Nonidet P-40, supplemented with a mixture of protease inhibitors. After measuring the protein concentrations (Bio-Rad reagents), the recombinant protein contents in both fractions were analyzed by Western blot analysis for cytochrome c (BD PharMingen, San Diego, California, USA), Trx1, Trx2 and ASK1 as indicated.

**Immunoprecipitation and immunoblotting.** HUVECs or BAECs after various treatments were washed twice with cold PBS and lysed in 1.5 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.75% Brij 96, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM PMSF, 1 mM EDTA) for 20 min on ice. Protein concentrations were determined with a Bio-Rad kit. For immunoprecipitation to analyze protein interaction in vivo, 400 µg of cell lysate supernatant were precleared by incubating with 5 µg normal rabbit serum and protein A/G agarose (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) on rotator at 4˚C overnight. The lysates were then incubated with 5 µg of the first protein-specific antiserum (e.g. anti-Trx1 or anti-Trx2 antibody) for 2 h with 50 µl of protein A/G agarose. Immune complexes were collected after each immunoprecipitation by centrifugation at 14,000 x g for 10 min followed by 4 washes with lysis buffer. The immune complexes were subjected to SDS-PAGE followed by immunoblot (Immobilon P, Millipore, Milford, MA) with the second protein (e.g., ASK1)-specific antibody (Santa Cruz Biotechnology Inc.). The chemiluminescence was detected using an ECL kit according to the instructions of the manufacturer (Amersham Life Science, Arlington Heights, Illinois, USA). For detection of Flag-tagged proteins (Trx1 and ASK1-N, anti-Flag M2 antibody (Sigma-Aldrich, St Louis, Missouri, USA) was used for Western blot. For detection of HA-tagged proteins (ASK1) and V5-tagged proteins (Trx2), anti-HA (Roche Diagnostics Corp) and anti-V5 antibodies (Invitrogen) was used for Western blot, respectively.
**ASK1 and JNK kinase assays.** ASK1 and JNK assays were performed as described previously\(^2,3\) using GST-MKK4 and GST-c-Jun (1-80) fusion protein as a substrate, respectively. Briefly, total 400 µg cell lysates were immunoprecipitated with 5 µg of antibody against ASK1 or JNK1 (Santa Cruz). The immunoprecipitates were mixed with 10 µg GST-MKK4 or GST-c-Jun (1-80) suspended in the kinase buffer (20 mM Hepes, pH 7.6, 20 mM MgCl, 25 mM β-glycerophosphate, 100 µM sodium orthovanadate, 2 mM DTT, 20 µM ATP) containing 1 µl (10 µCi) of [γ-32P] ATP. The kinase assay was performed at 25°C for 30 min. The reaction was terminated by the addition of Laemmli sample buffer and the products were resolved by SDS-PAGE (12%) followed by protein transferring to a membrane (Immobilon P). The phosphorylated GST-MKK4 or GST-c-Jun (1-80) was visualized by autoradiography. The membrane was further used for Western blot with anti-ASK1 or anti-JNK1.

**GST-Trx pull-down assay.** GST fusion protein preparation and GST pull-down assay were performed as described previously\(^2,3\). Briefly, GST-Trx fusion proteins expressed in *Escherichia coli* XL-1 blue were affinity purified on glutathione-Sepharose beads. 400 µg of cell lysates expressing ASK1 were incubated overnight at 4°C with 10 µg of GST-Trx bound to glutathione-Sepharose in the lysis buffer containing either 1 mM DTT or 1 mM H\(_2\)O\(_2\). The beads were washed 4 times with the lysis buffer before the addition of boiling Laemmli sample buffer. Bound ASK1 proteins were resolved on SDS-PAGE and detected by Western blot with anti-ASK1 (for full-length ASK1) or anti-Flag antibody (for Flag-tagged ASK1-N).

**Adenoviral expression of RNAi for ShTrx.** A short hairpin RNA (shRNA) targeted to human Trx1 (+53 to +80 bp from ATG) or Trx2 (+22 to +49 bp from ATG) was selected based on the program
double-strand oligonucleotides encoding the shRNA were synthesized and cloned into the pShag vector. The sequence of the oligonucleotides used to create pSh-Trx1 were: \textbf{Trx1-A}: CAG TCT TGC TCT CGA TCT GCT TCA CCA TGA AGC TTG ATG GTG AGG CGG ATC GGG AGC AAG ATT GCT TTT TTT T; \textbf{Trx1-B}: GAT CAA AAA AAA GCA ATC TTG CTC CCG ATC CGC CTC ACC ATC AAG CTT CAT GGT GAA GCA GAT CGA GAG CAA GAC TGC G; \textbf{Trx2-A}: CTT CCT GGA GAT GAC AGA GGC CAG GAA CGA AGC TTG GTT CTT GGC CTC TGT CGT CTC CGG GGA GCC CTT TTT T; \textbf{Trx2-B}: GAT CAA AAA AGG GCT CCC CGG AGA CGA CAG AGG CCA AGA ACC AAG CTT CGT TCC TGG CCT CTG TCA TCT CCA GGA AGC G. The oligonucleotides were synthesized by Integrated DNA Technology Inc. (Skotie, Illinois, USA) and were annealed/cloned into pShag vector \textsuperscript{4}. The clones were confirmed by DNA sequencing. Expression of short hairpin RNA (ShRNA) for Trx is under control of the U6 promoter. The U6-sh-Trx DNA fragment was subsequently cloned into pAd-PL-DEST by Gateway LR Clonase Enzyme Mix (Invitrogen). The cloning and viral preparation were performed according to the manufacturer’s protocol.

\textbf{RNase protection assay.} Total RNA from cultured cells was extracted with a Total RNA Isolation Kit (Ambion Inc., Austin, Texas, USA). RNase protection assays (RPA) were performed using the HybSpeed RPA kit (Ambion Inc.), per manufacturer’s directions. Each U6-hairpin cassette was cloned into \textit{Not I}-\textit{EcoR V} cut pBluescript II (Promega), linearized with \textit{Nde I}, and radiolabeled using the Maxiscript T7 polymerase in vitro transcription kit (Ambion) per manufacturer’s instructions to generate antisense riboprobes for use in RPA. Briefly, 10 \(\mu\)g of RNA per sample were hybridized with each probe, digested with an RNase A/T mixture, and separated on 8\% acrylamide/ 8M Urea gel followed by autoradiography as described previously \textsuperscript{3,10}. 
**Caspase 9 activity assay:** Caspase 9 activity was measured with a Caspase 9 peptide substrate acetyl-ASP-Glu-Val-Asp-7 amido-4-methylcoumarin (Ac-DEVD-AMC) (Sigma) as described previously for caspase 3\(^3\). Briefly, BAEC were harvested in a lysis buffer (25 mM Hepes, pH 7.4, 5 mM CHAPS, 5 mM DTT) and incubated on ice for 15-20 min followed by a centrifugation at 14,000 x g for 10-15 min at 4°C. For each reaction, 5 µl (200 µg) of cell lysate was incubated with 200 µl of 16 µM Caspase 9 peptide substrate acetyl-Val-Glu-His-Asp-7 amido-4-methylcoumarin (Ac-VEHD-AMC) in the assay buffer (25 mM Hepes, pH 7.4, 5 mM EDTA, 0.1% CHAPS, 5 mM DTT). The reaction was incubated in the dark for 1-1.5 h and fluorescence was measured in a fluorescence plate reader. The measured fluorescence was used as an arbitrary unit.

**Quantitation of apoptosis.** Cell killing assayed was performed as described previously with a modification \(^3,10\). For apoptosis assays, cell nuclei were stained with DAPI (2.5 µg/ml), and apoptotic cell (nucleus fragmentation) were counted under UV microscope \(^3,10\).

**Statistical analysis.** Data are presented as means (±SD). For Western blot, kinase activity, apoptosis and reporter gene assays, experiments were performed at least twice with duplicates. Analysis of densitometry was performed using NIH Image 1.60. Results were then normalized for comparison among different experimental groups by arbitrarily setting the value of control cells to 1.0. Statistical analyses were performed with StatView 4.0 package (ABACUS Concepts). Differences were analyzed by unpaired 2-tailed Student \(t\) test. Values of \(p<0.05\) were taken as significant.

**Legends to supplemental figures**
**Fig.S1. Co-localization of endogenous ASK1 in mitochondria of HUVEC.** HUVEC were stained with 20 nM tetramethylrhodamine methyl ester (TMRM) for 30 min at 37°C and washed twice with phosphate buffered saline (PBS) followed by indirect immunofluorescence microscopy. Staining was performed with anti-ASK1 antibody (rabbit polyclonal) followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (left). Mitotracker is shown in red (middle). The merging picture is shown on the right.

**Fig.S2. Subcellular localization of ASK mutants.** ASK1-WT, C250S or C30S were transfected into BAEC and subcellular fractionations were prepared. ASK1 protein in cytosolic (cyt) and mitochondrial (mit) fractions was determined by Western blot with anti-HA. As a control, endogenous Trx2 protein was determined by Western blot with anti-Trx2.

**Fig.S3. Localization of Trx2, ΔTrx2, ASK1-ΔN and mtASK1-ΔN.**

**a.** Localization of the wild-type Trx2 and a truncated Trx2 lacking the mitochondria targeting sequence (ΔTrx2) in EC. V5-tagged Trx2 and ΔTrx2 were transfected into BAEC. Trx2 and ΔTrx2 protein localization was determined by immunofluorescence microscope with anti-V5. **b-c.** Localization of ASK1-ΔN and mtASK1-ΔN. ASK1-ΔN or mtASK1-ΔN was co-transfected with V5-tagged Trx2 into BAEC. Co-localization of ASK1-ΔN (b) and mtASK1-ΔN (c) with Trx2 was determined by immunofluorescence microscope with anti-ASK1 (followed by anti-rabbit IgG conjugated with FITC) and anti-V5 (followed by anti-mouse IgG conjugated with TRITC). **d.** Distribution of Trx2, ΔTrx2, ASK1-ΔN and mtASK1-ΔN. Cytosolic and mitochondrial fractions were prepared and protein expression was determined by Western blot with anti-V5 (Trx2 and ΔTrx2) or anti-ASK1 (ASK1-ΔN and mtASK1-ΔN).
FigS4. Localization and distribution of Trx1 and Trx2. a. Localization of Trx1 and Trx2. BAEC were transfected with Flag-Trx1 and Trx2-V5. Localization of Trx1 and Trx2 was visualized by indirect confocal microscopy using FITC-conjugated anti-Flag and anti-V5 respectively. b. Trx2 is detected in mitochondrial whereas Trx1 in cytosolic fractions. Cytosolic (cyt) and mitochondrial (mit) fractions were prepared as described. Trx1 and Trx2 were determined by Western blot with anti-Flag and anti-V5, respectively.

FigS5. Characterization of Trx1 and Trx2-knockdown by shRNA. Expression of Trx1 ShRNA specifically knockdown Trx1 protein (a) whereas Trx2 ShRNA knockdown Trx2 protein (b). HUVEC were infected with adenovirus (multiple of infection = 100) harboring Shag, shTrx1 or shTrx2. Total RNA was isolated and expression of Trx shRNA was evaluated by RNase protection assay (RPA), using in vitro transcribed RNA spanning the Trx1 shRNA or Trx2 shRNA sequences as a probe (~ 400bp). The protected shRNA corresponding to Trx1 or Trx2 (~29 nt) were detected by PAGE, indicating that the transcribed shRNA was processed to siRNA (top panels). RNA from Shag was used as a control. Total cell lysates from EC were used to determine Trx1 (a) and Trx2 (b) expression by Western blot with anti-Trx1 and anti-Trx2, respectively. β-tubulin expression was used as a control. The relative levels of Trx are shown below each lane. Relative levels of Trx expression are shown (setting Shag vector as 1.0).

Reference:


**Fig. S2**

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(anti-HA)

**Fig. S3**

a-c: see separate Fig. S3a, b, c

d.  

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(anti-Trx2)

(anti-HA)

ΔTrx2 (IB: V5)

ASK1-ΔN

mtASK1-ΔN
FigS4a
Fig. S4

a. see separate Fig. S4a

b.

Fig. S5

a. Shag ShTrx1

ShTrx1 (29 nt) (RPA: Trx1)

Trx1 (IB: Trx1)

β-tubulin (IB: tubulin)

b.

Shag ShTrx2

ShTrx2 (29 nt) (RPA: Trx2)

Trx2 (IB: Trx2)

β-tubulin (IB: tubulin)