Both Internalization and AIP1 Association Are Required for Tumor Necrosis Factor Receptor 2-Mediated JNK Signaling

Weidong Ji,* Yonghao Li,* Ting Wan, Jing Wang, Haifeng Zhang, Hong Chen, Wang Min

Objective—The proinflammatory cytokine tumor necrosis factor (TNF), primarily via TNF receptor 1 (TNFR1), induces nuclear factor-κB (NF-κB)–dependent cell survival, and c-Jun N-terminal kinase (JNK) and caspase-dependent cell death, regulating vascular endothelial cell (EC) activation and apoptosis. However, signaling by the second receptor, TNFR2, is poorly understood. The goal of this study was to dissect how TNFR2 mediates NF-κB and JNK signaling in vascular EC, and its relevance to in vivo EC function.

Methods and Results—We show that TNFR2 contributes to TNF-induced NF-κB and JNK signaling in EC as TNFR2 deletion or knockdown reduces the TNF responses. To dissect the critical domains of TNFR2 that mediate the TNF responses, we examine the activity of TNFR2 mutant with a specific deletion of the TNFR2 intracellular region, which contains conserved domain I, domain II, domain III, and 2 TNFR-associated factor-2-binding sites. Deletion analyses indicate that different sequences on TNFR2 have distinct roles in NF-κB and JNK activation. Specifically, deletion of the TNFR-associated factor-2-binding sites (TNFR2–59) diminishes the TNFR2-mediated NF-κB, but not JNK activation; whereas, deletion of domain II or domain III blunts TNFR2-mediated JNK but not NF-κB activation. Interestingly, we find that the TNFR-associated factor-2-binding sites ensure TNFR2 on the plasma membrane, but the di-leucine LL motif within the domain II and aa338–355 within the domain III are required for TNFR2 internalization as well as TNFR2-dependent JNK signaling. Moreover, domain III of TNFR2 is responsible for association with ASK1-interacting protein-1, a signaling adaptor critical for TNF-induced JNK signaling. While TNFR2 containing the TNFR-associated factor-2-binding sites prevents EC cell death, a specific activation of JNK without NF-κB activation by TNFR2–59 strongly induces caspase activation and EC apoptosis.

Conclusion—Our data reveal that both internalization and ASK1-interacting protein-1 association are required for TNFR2-dependent JNK and apoptotic signaling. Controlling TNFR2-mediated JNK and apoptotic signaling in EC may provide a novel strategy for the treatment of vascular diseases. (Arterioscler Thromb Vasc Biol. 2012;32:2271-2279.)

Key Words: apoptosis ■ endothelial cell ■ c-Jun N-terminal kinase ■ tumor necrosis factor ■ tumor necrosis factor receptor 2

Vascular endothelial cells (EC) are among the principal physiological targets of prototypic inflammatory cytokine tumor necrosis factor (TNF).1,2 In EC, as in other cell types, TNF elicits a broad spectrum of biological effects including proliferation, differentiation, and apoptosis.3 The nature of the TNF effects depends on TNF concentration, and the type and growth state of the target cells.4 These differences in TNF-induced response are also due, in part, to the presence of 2 distinct TNF-specific plasma membrane-localized receptors, type I 155 kDa TNFR (TNFR1) and type II 75 kDa TNFR (TNFR2).5 Mice with genetic deletion of TNFR1 or TNFR2 (TNFR1-knockout [KO] and TNFR2-KO) are viable and do not show any overt phenotypic abnormalities. Data from studies using these mice suggest that TNFR1 is primarily responsible for TNF-mediated host defense and inflammatory responses.6

We have previously characterized the vascular phenotypes of TNFR1-KO and TNFR2-KO mice.7,8 These studies demonstrate that TNFR1-KO mice are normal in vascular development. However, TNFR1-KO and TNFR2-KO mice have distinct phenotype in ischemia-induced models. TNFR2, but not TNFR1, is highly upregulated in vascular endothelium in response to ischemia. TNFR1-KO mice have enhanced, whereas TNFR2-KO have reduced capacity in ischemia-induced angiogenesis and tissue recovery.7,8

Received on: May 19, 2012; final version accepted on: June 13, 2012.

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.112.253666
of TNFR2 in response to TNF leads to the recruitment of TRAF2 and TRAF2-associated TRAF1, cellular inhibitor of apoptosis-1, and cellular inhibitor of apoptosis-2. We have previously identified a Bmx-binding sequence at the C terminus of TNFR2, which constitutively binds to nonreceptor tyrosine kinase Bmx, contributing to TNFR2-mediated Akt activation, EC migration, and proliferation.7,17 It has been shown that the Bmx-binding sequence also recruits TRAF2, regulating TNFR2-mediated NF-κB signaling.22,23 Recent studies suggest that TNFR2, independent of TNFR1, activates both canonical and noncanonical NF-κB pathways.18 Specifically, TNFR2 through PI3K/Akt induces phosphorylation ofIkBα kinase-β and subsequent activation of the canonical NF-κB pathway.23 Only membrane-bound TNF triggers TNFR2-mediated NF-κB-inducing kinase/IKKα-dependent noncanonical pathway.25 However, the mechanism by which TNFR2 induces JNK activation has not been investigated.

In the present study, we show that TNFR2 significantly contributes to TNF-induced NF-κB and JNK activation in vascular EC. We identify 2 sequences other than the TRAF2-binding site that are required for the formation of death-inducing signaling complex. Different from the findings in a study by Tschopp’s group, TNFR1 is a component of the intracellular death-inducing signaling complex complex. Our previous data support that an intermediate complex between complex I and complex II (likely an internalized complex I) is responsible for TNF-induced JNK activation.16–17 Because ASK1-interacting protein-1 (AIP1) is a different component than complex I, we name it the AIP1 complex. We have demonstrated that AIP1 complex is structurally and functionally different from complex I and complex II. AIP1 complex activates JNK, whereas it inhibits NF-κB signaling. Moreover, AIP1-apoptosis signal-regulating kinase-1-JNK activation induces apoptotic signaling, which is dependent on intrinsic (mitochondria-dependent) pathways.15–17 In EC, NF-κB is associated with cell survival, whereas the JNK pathway is involved in apoptosis.12

In contrast, the function and signaling of the second TNF receptor, TNFR2, is less understood. This is because of several reasons. First, TNFR2 expression is limited to specific cell types, including EC, cardiac myocytes and hematopoietic cells. Moreover, TNFR2 is often induced in response to certain stimuli, for instance, in the activation of macrophage and T lymphocytes that results in a marked increase in TNFR2 levels.6,13 TNFR2 is also highly induced in vascular EC in ischemia hind limb, as we recently demonstrated.7,8 Second, the intracellular domains of TNFR2 and interacting proteins are not well defined. Although TRAF2 was initially identified as a TNFR2-binding protein,19 subsequent studies have been focused on its role in TNFR1 signaling. Trimerization
TNFR2-KO mouse lung EC (MLEC) (1×10⁶) were treated with murine recombinant TNF (10 ng/mL) for the indicated times. Phospho- and total IκBα and JNK were determined by Western blotting with the respective antibodies (A). The quantification of the ratios of p-IκBα/IκBα and p-JNK/JNK are presented in B and C, respectively. Data are the mean±SEM from 3 independent experiments. *P<0.05 indicating statistic significance compared with the untreated vector control.

Figure 1. Tumor necrosis factor receptor 2 (TNFR2) contributes to TNF-induced nuclear factor-κB (NF-κB) and JNK-signaling pathways in endothelial cell (EC). Wild-type (WT), TNFR2-knockout (KO), and TNFR1/2-KO mouse lung EC (MLEC) (1×10⁶) were treated with murine recombinant TNF (10 ng/mL) for the indicated times. Phospho- and total IκBα and JNK were determined by immunoblotting with the respective antibodies (A). The quantification of the ratios of p-IκBα/IκBα and p-JNK/JNK are presented in B and C, respectively. Data are the mean±SEM from 3 independent experiments. *P<0.05 indicating statistic significance compared with the untreated vector control.

TRAF2-Binding Sites Are Required for TNFR2-Mediated NF-κB but Not JNK Activation
It has been shown that TNFR2 contains 2 TRAF2-binding sites at the C terminus, the first being the SKEE motif and the second is the last 16 aa sequence22,23 that overlaps with a Bmx-binding sequence.7,8,20 Each TRAF2 site mediates TNFR2-transduced NF-κB activation independently.22,23 Besides the TRAF2-binding consensus sequences, sequence alignment of TNFR2 intracellular regions from different species revealed 3 additional conserved domains—domain I (aa266–289), domain II (aa289–338), and domain III (aa338–380) (Figure 2A). To determine the TNFR2 domain critical for JNK activation, we constructed a series of TNFR2 truncations with deletions of these various domains (Figure 2A and 2B). Activities of TNFR2 mutants in NF-κB and JNK signaling were determined by NF-κB and JNK-reporter gene assays in TNFR2-null MLEC (Figure 2B). Overexpression of TNFR2-WT, similar to overexpression of TNFR1 or TRAF2,19 activated both NF-κB and JNK-reporter genes in a ligand-independent manner. Consistent with our previous findings, deletion of the TRAF2-binding sites (TNFR2–59) abolished TNFR2-mediated NF-κB activation (Figure 2C). To our surprise, TNFR2–59 retained the ability to activate the JNK-reporter gene, suggesting that TRAF2-binding sites are not required for TNFR2-mediated JNK signaling. However, a partial (TNFR2–84) or a complete deletion of the domain III (TNFR2–112) blunted TNFR2-dependent JNK activation. Further deletions of domain II and domain I (TNFR2–150 and TNFR2-TM) had similar effects on the JNK reporter gene (Figure 2D). These data suggest that TNFR2 utilizes different regions to transduce NF-κB and JNK signaling, and the sequence between TNFR2–84 and TNFR2–59 could be important for TNFR2-mediated JNK activation.

Domain II and Domain III Are Required for TNFR2-Mediated JNK Activation
The results indicated above using the C-terminal truncations supported a role of domain III in TNFR2-mediated JNK activation. However, the role of domain I and domain II could not be determined. To this end, each domain was then internally deleted (Δ266–289 for domain I, Δ289–338 for domain II, Δ338–355 for the first half of domain III, and Δ355–380 for the second half of domain III—the sequence located between TNFR2–84 to –59 that is critical for JNK activation). Domain II contains a di-leucine motif, which has recently been shown to be important for TNFR2 internalization.26 Therefore, we made a site-specific mutation at the di-leucine motif (L319/320A) (Figure 3A). These internal deletions had no effects on TNFR2 expression (not shown; Figure 4). Effects of these deletions/mutations on TNFR2-mediated signaling were assessed in the reporter gene assays. TNFR2 with a deletion of domain I, domain II, or domain III retained the ability to activate NF-κB reporter gene. Similar to the deletion of domain II, mutations at the di-leucine motif had no effects on TNFR2-mediated NF-κB activation (Figure 3B), consistent with a recent report.26 In contrast, a deletion of domain II or a mutation at the di-leucine motif was sufficient to abolish TNFR2 activity in JNK activation (Figure 3C). A deletion at the first half of domain III (Δ338–355) had no effects on JNK activation. However, deletion at the second half (Δ355–380), similar to TNFR2–84, diminished TNFR2-mediated JNK activation (Figure 3C). These data suggest that both di-leucine motif within domain II and the second half of domain III are critical for JNK activation. However, domain I and domain II could not be determined. To this end, each domain was then internally deleted (Δ266–289 for domain I, Δ289–338 for domain II, Δ338–355 for the first half of domain III, and Δ355–380 for the second half of domain III—the sequence located between TNFR2–84 to –59 that is critical for JNK activation). Domain II contains a di-leucine motif, which has recently been shown to be important for TNFR2 internalization.26 Therefore, we made a site-specific mutation at the di-leucine motif (L319/320A) (Figure 3A). These internal deletions had no effects on TNFR2 expression (not shown; Figure 4). Effects of these deletions/mutations on TNFR2-mediated signaling were assessed in the reporter gene assays. TNFR2 with a deletion of domain I, domain II, or domain III retained the ability to activate NF-κB reporter gene. Similar to the deletion of domain II, mutations at the di-leucine motif had no effects on TNFR2-mediated NF-κB activation (Figure 3B), consistent with a recent report.26 In contrast, a deletion of domain II or a mutation at the di-leucine motif was sufficient to abolish TNFR2 activity in JNK activation (Figure 3C). A deletion at the first half of domain III (Δ338–355) had no effects on JNK activation. However, deletion at the second half (Δ355–380), similar to TNFR2–84, diminished TNFR2-mediated JNK activation (Figure 3C). These data suggest that both di-leucine motif within domain II and the second half of domain III are required for TNFR2-mediated JNK activation.

Domain II and Domain III of TNFR2 Regulate TNFR2 Intracellular Localization
The cellular localization of TNFR1 determines its activity in the NF-κB and JNK-signaling pathways and in apoptosis.13,14,16 The results that mutations of the di-leucine motif diminished TNFR2-dependent JNK, but not NF-κB activity prompted us to examine TNFR2 localization. TNFR2-WT, up expression into TNFR2-KO MLEC, exhibited both cytoplasmic...
molecule mediating JNK, but not NF-κB activation in EC,15–17 and therefore we examined whether TNFR2 via its domain III associated with AIP1. To this end, AIP1-WT, TNFR2–59, and TNFR2–84 (Figure 5A) were expressed in TNFR2-KO MLEC, and association of TNFR2 with endogenous AIP1 was determined by immunoprecipitation with anti-AIP1 followed by Western blot with anti-AIP1 antibodies (Figure 5B). As expected, TNFR2-WT, but not TNFR2–59 or TNFR2–84, strongly associated with AIP1. In contrast, both TNFR2-WT and TNFR2–59 associated with AIP1, and TNFR2–59 exhibited much stronger AIP1 binding than TNFR2-WT (Figure 5B), correlating with its prominent intracellular localization and higher activity in JNK signaling. A deletion of domain III (TNFR2–84) abolished the AIP1 binding. Similar results were observed for association of TNFR2 with the AIP1 effector apoptosis signal-regulating kinase-1 (Figure 5B), an upstream MAP kinase kinase in JNK activation.

We then determined whether endogenous TNFR2 and AIP1 proteins form a complex in EC. human umbilical vein EC were untreated or treated with TNF (10 ng/mL for 15 minutes), and association of TNFR2 with AIP1 was determined in a coimmunoprecipitation assay. TNFR2 associated with AIP1 in resting EC and this association was increased in response to TNF (Figure 5C). We also examined interactions of TNFR2 and AIP1 in EC by a colocalization assay. Colocalization of TNFR2 and AIP1 was detected in untreated cells, primarily on the cytoplasmic membrane. However, TNF treatment enhanced their colocalization in intracellular vesicles (Figure III in the online-only Data Supplement). Taken together, these

**Domain III of TNFR2 Is Critical for the Association With AIP1, an Adaptor Molecule for JNK Activation**

As aa355–380 of domain III specifically mediates TNFR2-induced JNK but not NF-κB activation, we reasoned that it may be required for its association with JNK-specific intracellular signaling molecules. AIP1 is an adaptor molecule for JNK activation. Data are presented as mean±SEM of duplicate samples from 4 independent experiments. *P<0.05 indicating statistical significance compared with the vector control (normalized to 1.0). Arrows indicate that TNFR2–59 cannot activate NF-κB, but retains JNK activation. TM indicates transmembrane.

Deletions of the TRAF2/Bmx motifs (~59) enhanced TNFR2 intracellular localization (Figure 4A and 4B), indicating that the TRAF2 (and Bmx)-binding sites are required to maintain TNFR2 on the membrane. Internalized TNFR2 was detected in intracellular vesicles, where it colocalized with the endocytic marker green fluorescent protein-FYVE-1, containing 2 tandem-arranged FYVE domains of the late endosomal protein Hrs (Figure II in the online-only Data Supplement). To our surprise, a further deletion of domain III (TNFR2–84) was sufficient to block TNFR2 intracellular vesicle staining, even in the presence of the di-leucine motif (Figure 4B). This result was confirmed using the internal deletion mutants (Figure 4C and 4D). An internal deletion of aa355–380 drastically reduced TNFR2 intracellular staining. In contrast, deletion of domain I (aa266–289) or the first half of domain III (aa324–355) did not block TNFR2 internalization. As controls, a deletion or a mutation at the di-leucine motif (Δ289–338 and L319/320A, respectively) caused a loss of intracellular vesicle localization of the TNFR2 protein. These data suggest that both the di-leucine motif of domain II and aa355–380 within domain III are required for TNFR2 internalization.

**Figure 2.** The tumor necrosis factor receptor associated factor-2 (TRAF2)-binding sites are required for TNFR2-mediated nuclear factor-xB (NF-xB) but not JNK activation. A, Schematic structure of TNFR2 and the truncated deletions at the C terminus. The numbers refer to the amino acid number, indicating the boundary of the extracellular and intracellular domains (I–III). A TRAF2-binding motif (SKEE) and Bmx-binding sequence are also indicated. B, Expression of TNFR2 truncates as detected by immunoblotting with anti-TNFFR2. C and D, Effects of TNFR2 truncation on NF-κB and JNK activation in reporter gene assays. A NF-κB-reporter gene or JNK-reporter gene was transfected into TNFR2-knockout (KO) mouse lung endothelial cell (MLEC) in the presence of vector control (−), TNFR2-WT or mutants. Cells were harvested for luciferase/renilla assays at 48 hours posttransfection.
data suggest that TNFR2 via its domain III binds to AIP1 (and its effector apoptosis signal-regulating kinase-1) to activate the JNK signaling.

**TNFR2-WT Inhibits, Whereas TNFR2–59 Augments, TNF-Induced Cell Apoptosis**

JNK signaling has been implicated in cell apoptosis by activating caspase-dependent pathways. As TNFR2–59 specifically activates JNK but not the NF-xB survival pathway, we used it to determine the cellular function of TNFR2-dependent JNK signaling and TNFR2-dependent apoptosis in EC. EC are normally resistant to cytotoxic actions of TNF because of NF-xB–induced gene expression of antiapoptotic proteins that inhibit cell death by both the caspase and the JNK pathways. To potentiate TNF-mediated EC death, TNF is provided in combination with either a global inhibitor of new gene transcription (such as actinomycin D) or of protein synthesis (such as cycloheximide or shiga-like toxin) or in combination with a selective inhibitor of NF-xB activation. To this end, TNFR2-KO MLEC were infected with lentivirus carrying TNFR2-WT or TNFR2–59, and cells were treated with an apoptotic stimulus (TNF plus a protein synthesis inhibitor cycloheximide). Cell apoptosis was determined by annexin V/propidium iodide staining followed by flow cytometry analysis. Expression of TNFR2-WT significantly blocked TNF+ cycloheximide-induced apoptosis, consistent with its activity in NF-xB survival signaling. In contrast, expression of TNFR2–59 strongly induced EC apoptosis, even in the absence of the apoptotic stimulus. The TNFR-59-induced responses were further augmented by TNF+ cycloheximide treatment (Figure 6A, with quantification in Figure 6B).

**Discussion**

In the present study, we have made several important findings. First, TNFR2 significantly contributes to TNF-induced NF-xB and JNK signaling in vascular EC. This is based on the studies using primary vascular EC with TNFR2 deletion or knockout. Second, by reexpressing various TNFR2 mutants into TNFR2-KO EC, we have identified the critical domains of TNFR2 involved in TNFR2-mediated NF-xB and JNK activation (Figure 6D). Specifically, the C terminal 59 residues (aa380–439) containing the TRAF2- and Bmx-binding sites are dispensable for both NF-xB and JNK activation; the adjacent domain II (aa289–338) and domain III (aa338–380) are critical for JNK activation; and the membrane proximal domain I is dispensable for both NF-xB and JNK signaling. Third, TNFR2 cellular localization correlates with its activity in NF-xB and JNK signaling. While the TRAF2/Bmx-binding sites are required for its localization on the membrane and its ability to activate NF-xB, the di-leucine motif within domain II and aa355–380 within domain III of TNFR2 are required for its internalization and its ability to activate JNK. We further show that the TNFR2 domain III is required for its association with the JNK-specific signaling molecule AIP1, providing the molecular basis for its role in JNK activation. Finally, we demonstrate that TNFR2-mediated NF-xB activity is
associated with cell survival, whereas the JNK signaling is involved in EC apoptosis.

One surprising finding in our study is that the TRAF2-binding sites are not required for, and in fact inhibit, TNFR2-mediated JNK activation. The C-terminal TNFR2 contains 2 TRAF2-binding sites, 1 is the canonical TRAF2 motif (SKEE), which has been shown to be critical for TRAF2 binding and NF-κB activation. The second site, overlapping with the Bmx-binding sequence, is located at the C-terminal 16 aa residues. The role of this second TRAF2-binding site in TNFR2-mediated NF-κB signaling has also been characterized.22,23 However, the mechanism by which TNFR2 induces JNK activation has not been investigated. Our present study suggests that a deletion of both TRAF2-binding sites (TNFR2–59) diminishes TNFR2-mediated NF-κB but augments TNFR2-mediated JNK signaling. On the basis of previous and current findings, we propose the following possible models by which the TRAF2-binding sites inhibit TNFR2-mediated JNK signaling. (1) The TRAF2-binding sites regulate TNFR2 intracellular localization. The TRAF2/Bmx-binding sites appear to be important in maintaining TNFR2 on the plasma membrane, leading to NF-κB but not JNK activation. However, deletion of this sequence (TNFR2–59) causes TNFR2 intracellular localization and specific JNK activation. Previous work has demonstrated that TRAF2 regulates TNFR2 membrane localization.30 It has also been shown that Bmx is localized on the plasma membrane.20,31,32 It will be interesting to determine whether TRAF2 and Bmx proteins cooperatively regulate TNFR2 membrane localization and internalization. (2) NF-κB inhibits JNK signaling. In TNFR1 signaling, NF-κB induces gene expression of antiapoptotic proteins that inhibit both the JNK and the caspase pathways. The best characterized factor is FLICE inhibitory protein (cellular FLICE inhibitory protein)33 and A20.34,35 Cellular FLICE inhibitory protein competes with procaspase 8 for binding to Fas-associated death domain protein and prevents the autocatalytic activation of caspase 8 (and 10) by the TRADD/RIPK1/TRAF2/Fas-associated death domain protein complex. A20 exhibits both deubiquinating and E3 ubiquitin ligase activities, and is thought to remove the signaling scaffold of lysine 48-linked ubiquitin polymer that forms on RIPK1 as part of the NF-κB and JNK activation pathways, replacing them with a lysine 68-linked ubiquitin polymer that targets RIPK1 for proteosome-mediated degradation.34,36 As a result, both JNK and caspase activation are inhibited. The importance of NF-κB–induced protective proteins is demonstrated by the potentiation of TNF-mediated EC death when TNF is provided in combination with either a global inhibitor of new gene transcription (such as actinomycin D) or of protein synthesis (such as cycloheximide or shiga-like toxin) or in combination with a selective inhibitor

**Figure 4.** Both domain II and domain III of tumor necrosis factor receptor 2 (TNFR2) regulate TNFR2 intracellular localization. A and C, Schematic structure of TNFR2 truncation and internal deletion are shown. B and D, Effects of truncations and deletions on TNFR2 cellular localization. TNFR2-knockout (KO) mouse lung endothelial cell (MLEC) were transfected with various TNFR2 mutants, and the localization of the TNFR2 protein was determined by indirect fluorescence microscopy with anti-TNFR2 followed by Alexa Fluor-488 donkey anti-goat IgG. TNFR2-KO cells showed no staining (not shown). Representative images from 5 cells for each truncate are shown.
JNK inhibition had no effects on the cellular localization of TNFR2-WT or TNFR2–59, a mutant TNFR2 exhibits strong internalization and JNK activation (Figure IV in the online-only Data Supplement). These data suggest that JNK activation is likely a consequence of TNFR2 internalization. Interestingly, the domain III of TNFR2 contains serine/threonine-rich sequences that are potentially phosphorylated. We have previously shown that AIP1 via one of the polylysine clusters binds to a serine-rich sequence on apoptosis signal-regulating kinase-1. It is conceivable that internalized TNFR2 via serine/threonine-rich sequences forms a complex with AIP1, leading to JNK activation.

What is the in vivo relevance of TNFR2-mediated JNK signaling? Dr DiCorleto’s laboratory has shown that TNFR2 is essential for TNF-induced E-selectin, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1 expression as well as for TNF-induced leukocyte-EC interaction in vivo. More importantly, TNFR2 is also required for progression of atherosclerosis in mouse models. It is conceivable that TNFR2-mediated NF-κB and JNK synergistically regulate expression of the adhesion molecules as we demonstrated previously. TNFR2-mediated JNK activation could be detected in ischemic hind limb at day 3 postsurgery, correlating with ischemia-induced apoptosis. This transient JNK activation under ischemia is likely because of decreased expression of TRAF2 in response to ischemia. The JNK activation and cellular apoptosis may be an early event during the adaptive response, which is required for revascularization and ischemic tissue regeneration. Therefore, modulation of TNFR2-mediated JNK signaling in EC may provide a novel strategy for the treatment of vascular diseases such as atherosclerosis, coronary artery disease, and peripheral arterial disease.
None.

This work was supported by grants from National Institutes of Health grants R01HL65978 and R01HL109420.

We thank Dr Jordan S. Pober for discussion.

**Sources of Funding**

This work was supported by grants from National Institutes of Health grants R01HL65978 and R01HL109420.

**Disclosures**

None.

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Arterioscler Thromb Vasc Biol. 2012;32:2271-2279; originally published online June 28, 2012; doi: 10.1161/ATVBAHA.112.253666

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/32/9/2271

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SUPPLEMENTAL METHODS

**Plasmids.** Mammalian expression plasmids for TNFR2 and TNFR2-59 have been described previously. Other TNFR2 mutants were generated by the Quickchange site-specific mutagenesis kit (Stratagene, La Jolla, California, USA) to create a stop codon at the indicated site following the manufacturer’s instructions. Mutations were confirmed by DNA sequencing and by immunoblotting for protein expression.

**Cells and cytokines.** Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, Calif.) or Yale Core facility. Lung microvascular endothelial cells (MLEC) isolated from TNFR2- or TNFR1/2 double knockout mice (The Jackson Laboratory) were isolated according to a procedure described previously. The isolated cells were assessed for EC phenotype, including morphology and expression of von Willebrand Factor and PECAM-1. EC were used at passages 1 to 5. Human and murine recombinant TNF and polyclonal antibodies against TNFR1 and TNFR2 were from R&D Systems (Minneapolis, Minn.). MLEC were reconstituted with various TNFR2-WT or mutants by transfection with electroporation as described previously.

**Transfection and reporter assay.** Transfection of MLEC cells was performed with Lipofectamine 2000 (Invitrogen Corp., San Diego, California, USA) according to manufacturer’s protocol. Cells were cultured at 90% confluence in 6-well plates and were transfected with a total of 2 µg of plasmid constructs as indicated. Cells were harvested at 24 to 36 h post-transfection. Luciferase activity followed by renilla activity was measured three times in duplicate using a Berthold luminometer (EG&G Wallac, Gaithersburg, Maryland, USA). All data were normalized as relative luciferase light units/renilla unit.

**Immunoprecipitation and immunoblotting.** MLEC cells were washed with cold phosphate-buffered saline (PBS) and were harvested in a membrane lysis buffer (30 mM Tris [pH 8], 10 mM NaCl, 5 mM EDTA, 10 mM of polyoxyethylene-8-lauryl ether, 1 mM O-phenanthroline, 1 mM indocetamide, 10 mM NaF, 5 mM orthovanadate, 10 mM sodium pyrophosphate). Anti-TRAF2, anti-RIPK1, anti-JNK1, anti-P38, and anti-IκBα (rabbit polyclonal) were purchased from Santa Cruz Biotechnology. Anti-caspase-3, anti-phospho-IκBα and anti-phospho-JNK (rabbit polyclonal) were purchased from cell signaling. Anti-TNFR2 recognizing the extracellular region of TNFR2 (goat) was from R&D.

**Indirect immunofluorescence confocal microscopy.** Fixation, permeabilization, and staining of cultured EC were performed as described previously. Alexa Fluor 488 (green) or 594 (red)
conjugated-secondary antibodies (Molecular Probes, Eugene, OR) were used. Confocal immunofluorescence microscopy was performed using an Olympus confocal microscope (La Jolla, CA), and the acquired images were transferred to Photoshop CS5 (Adobe Systems, San Jose, CA) to generate the final figures.

**Analysis of the apoptosis rate by Annexin V/Propidium iodide staining and flow cytometry.**
The apoptosis rate was measured by flow cytometry according to the instructions provided by the annexin V-FITC kit. In brief, after treatment with CHX for one hour and TNF for 24 hours, cells were harvested and washed once with PBS, trypsinized, and resuspended briefly in serum containing medium to quench the trypsin. Cells were centrifuged and washed once in PBS, counted, and 100,000 cells were added to the annexin binding reagents for 15 min in the dark. Propidium iodide was included in the reaction to allow the identification of cells in late apoptosis. Cells were then subjected to analytic flow cytometry on a FACSort (BD Biosciences, San Jose, CA). The fluorescence signal was recorded on the FL1 (green) or FL3 (red) channel and analyzed by using CellQuest software. The apoptotic percentage of 10,000 cells was determined, and all the experiments reported in this study were performed in duplicates.

**Statistical Analysis.** All data are expressed as means ± SEM. Statistical differences were measured by the nonparametric Mann-Whitney test. A value of $p<0.05$ was considered as statistically significant.
SUPPLEMENTAL FIGURES

Supplemental Fig.I.
A. Characterization of TNFR2-KO and TNFR1/2-KO EC. WT, TNFR2-KO and TNFR1/2-KO MLEC (1X10^6) were harvested. Expression of TNFR1 and TNFR2 was determined by immunoblotting with anti-TNFR1 and anti-TNFR2. β-actin was used as a loading control.

B. TNFR2 contributes to TNF-induced NF-κB and JNK signaling pathways in human EC. HUVEC (1X10^6) were transfected with a control siRNA or TNFR2 siRNA. 48 h post-transfection, cells were treated with human TNF (10 ng/ml) for the indicated times. TNFR2 knockdown was verified by immunoblotting with anti-TNFR2. β-actin was used as a loading control. Phospho- and total IκBα and JNK were determined by immunoblotting with the respective antibodies. Representative images from three independent experiments are shown.

Supplemental Fig.II. Internalized TNFR2 was localized in endocytic vesicles. TNFR2-KO MLEC were transfected with TNFR2-WT or TNFR2-59 together with an endocytic marker GFP-FYVE-1, containing two tandem-arranged FYVE domains of the late endosomal protein Hrs. Localization of TNFR2 was determined by indirect immunofluorescence with anti-TNFR2 followed by Alexa Fluor-599 (red) donkey anti-goat IgG. Co-localization of TNFR2 with GFP-FYVE was visualized under microscope and merged images are shown on the right.

Supplemental Fig.III. Colocalization of endogenous TNFR2 with AIP1 in EC. HUVEC were untreated or treated with human TNF (10 ng/ml) for 15 min. Localization of AIP1 and TNFR2 was determined by indirect immunofluorescence with anti-AIP1 (rabbit) and anti-TNFR2 (goat) followed by Alexa Fluor-488 (green) donkey anti-rabbit IgG and Alexa Fluor-599 (red) donkey anti-goat IgG. Representative images are shown. Merged images are shown on the right.

Supplemental Fig.IV. JNK inhibition had no effects on TNFR2 localization. TNFR2-KO MLEC were transfected with TNFR2-WT or TNFR2-59. Cells were treated with DMSO (control) or JNK inhibitor (SP600125, 20 µM) for 1 h as we described previously 4, 5. Localization of TNFR2 was determined by indirect immunofluorescence with anti-TNFR2 followed by Alexa Fluor-488 (green) donkey anti-goat IgG. Co-localization of TNFR2 with DAPI (blue) was visualized under microscope and merged images are shown.

REFERENCES:


Wan et al., Supplemental Fig.I

A

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Wan et al., Supplemental Fig.III
DMSO | JNK inhibitor
---|---
TNFR2-WT |  
TNFR2-59 |  

Wan et al., Supplemental Fig.IV