FOXO3a Turns the Tumor Necrosis Factor Receptor Signaling Towards Apoptosis Through Reciprocal Regulation of c-Jun N-Terminal Kinase and NF-κB

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Objective—We evaluated the full range effects of FOXO3a in endothelial cells (ECs) by microarray analysis and investigated the role of FOXO3a regulating TNF receptor signaling pathway.

Methods and Results—Human umbilical vein endothelial cells (HUVECs) were transfected with adenoviral vectors expressing constitutively active FOXO3a (Ad-TM-FOXO3a). Ad-TM-FOXO3a transfection caused remarkable apoptosis, which were accompanied with upregulation of genes related with TNF receptor signaling, such as TNF-α, TANK (TRAF-associated NF-κB activator), and TTRAP (TRAF and TNF receptor-associated protein). Furthermore, NF-κB-Ras1 (IkB-interacting Ras-like protein-1) which is known to block IkB degradation was found increased, and intranuclear translocation of NF-κB was inhibited. GADD45β and XIAP, negative regulators of c-Jun N-terminal kinase (JNK), were suppressed and JNK activity was increased. Attenuation of TNF signaling pathway either by blocking antibody for TNF receptor or by blocking JNK with DMAP (6-dimethylaminopurine) or Ad-TAM67 (dominant negative c-Jun) cotransfection, significantly reduced FOXO3a-induced apoptosis. Finally, treatment of vasculature with heat shock, an activator of endogenous FOXO3a, resulted in EC apoptosis, which was completely rescued by Ad-TAM67.

Conclusion—FOXO3a promotes apoptosis of ECs, through activation of JNK and suppression of NF-κB. These data identify a novel role of FOXO3a to turn TNF receptor signaling to a proapoptotic JNK-dependent pathway. (Arterioscler Thromb Vasc Biol. 2008;28:112–120.)

Key Words: FOXO3a • JNK • NF-κB • apoptosis

The status of the external environment influences cell survival via various signaling mechanisms. Tumor necrosis factor (TNF) is a potent cytokine that serves pleiotropic function in inflammation, cell proliferation, and apoptosis.1,2 NF-κB and c-Jun are known as 2 major transcription factors mediating the TNF receptor signaling.3 In this cascade, NF-κB plays a dominant role mediating inflammatory responses while blocking apoptosis via inhibition of the c-Jun-N-terminal kinase (JNK).4 Forkhead transcription factors are emerging as key factors, under regulation of Akt, to play various roles ranging from cellular proliferation, metabolism, apoptosis,6,7 and even adaptation to cellular stress.8,9 Regarding endothelial cells (ECs), previous studies by our group10,11 and others12 have suggested a proapoptotic role of FOXO3a, one of main forkhead transcription factors expressed in ECs. But the molecular mechanism of FOXO3a in ECs has not been fully defined yet.

In this study, we evaluated the full range effects of FOXO3a on human umbilical vein endothelial cells (HUVECs) by microarray analysis, in which we found that several genes involved in the TNF receptor pathway were upregulated. Therefore we investigated the role of FOXO3a in the TNF receptor signaling and its implication of the proapoptotic action of FOXO3a in ECs in vitro and in vivo.

Materials and Methods
For expanded methods, please see the supplemental materials, available online at http://atvb.ahajournals.org.

EC Culture and Gene Transfer Using Adenoviral Vectors
Four to 6 passage of HUVECs (Clonetics) were cultured as previously described.13

Oligonucleotide Microarray
Magic-II 10K oligonucleotide microarray (Macrogen) was used as previously described.10,14
Real-Time Quantitative RT-PCR Analysis
Changes in RNA expression of TANK (TRAF-associated NF-κB activator), and TTRAP (TRAF and TNF receptor-associated protein) were determined by real-time QRT-PCR as previously described.\textsuperscript{10}

RT-PCR and Immunoblot Analysis
Changes in RNA expression of TNF-α, κB-Ras1, GADD-45β, and XIAP were determined by RT-PCR as previously described.\textsuperscript{15}

Chromatin Immunoprecipitation (ChIP) Assay
A chromatin immunoprecipitation (ChIP) assay was performed.

Immunoprecipitation/In Vitro JNK Kinase Assay
Immunoprecipitated proteins with anti-JNK1 antibody were incubated with anti-phospho-c-jun antibody to compare phosphorylating activity of JNK1 as previously described.\textsuperscript{16}

Electrophoretic Mobility Shift Assay
The DNA binding activity of the nuclear protein was evaluated according to the established method with modifications.\textsuperscript{17}

Evaluation of In Vivo Endothelial Denudation and Apoptosis After Heat Shock Treatment on Vessel
Male Sprague-Dawley rats, 13 weeks old, weighing about 400 g (Daehan Biolink Co, Chung-Buk, Korea) were used for animal experiment as previously described.\textsuperscript{18}

FACS Analysis of EC Apoptosis
Apoptosis was quantified by measuring the hypodiploid DNA content using flow cytometry (FACS) analysis.\textsuperscript{18}

Statistical Analysis
All data are expressed as mean±SD.

**Results**

**FOXO3a-Regulated Genes Identified by Oligonucleotide Microarray**

To identify the FOXO3a-regulated genes in ECs, we compared the gene expression profile of Ad-TM-FOXO3a–transfected HUVECs with a control vector (Ad-GFP) or Ad-DN-FOXO3a-transfected HUVECs. From our previous experiments, genes transfected by adenoviral vectors are appreciably expressed approximately 12 hours after transfection.\textsuperscript{10} Therefore, we decided to evaluate at 16, 24, and 40 hours to include both early and late response to FOXO3a activation. The activation or suppression of genes was evaluated by measuring the fold ratio between Ad-TM-FOXO3a or Ad-DN-FOXO3a–transfected HUVECs with Ad-GFP–transfected HUVECs. A cDNA was considered a putative FOXO3a-regulated target if the hybridization signal was more than 2 folds of the control.

Constitutive activation of FOXO3a increased the expression of 73 genes and decreased 59 genes out of \textasciidetilde{10} 000 genes analyzed. The Table lists some of the genes that were strongly regulated by FOXO3a. As expected from the previously reported proapoptotic action of FOXO3a, several genes associated with apoptosis or cell cycle arrest were upregulated, whereas most of the genes that were significantly downregulated were those known to be associated with extracellular matrices, heat shock proteins, and growth factors (supplemental Figure I, available online at http://atvb.ahajournals.org). From this data, we found that Fas ligand, which is a well-known effector molecule in forkhead transcription factor–induced apoptosis of neural cells and fibroblasts,\textsuperscript{19} was not upregulated in ECs, suggesting that, at least in ECs, there exists another mechanism which mediates apoptotic signaling. Interestingly, we found that, in the highly upregulated genes, there were several genes related to TNF receptor signaling pathway, including TNF-α, TANK, TTRAP, and κB-Ras1 (κB-interacting Ras-like protein-1), suggestive of a link between FOXO3a and the TNF receptor signaling.

**FOXO3a Induces and Amplifies TNF Receptor Signaling**
To validate the microarray data, we evaluated TANK and TTRAP mRNAs, which were found to be significantly upregulated (Figure 1A). The relative fold elevation compared with GAPDH was 1±0.3 at 16 hours, 122±40 at 24 hours, and 293±110 at 40 hours in case of TANK, and 1±0.3 at 16 hours, 186±60 at 24 hours, and 2999±1100 at 40 hours in case of TTRAP, respectively. Changes of TNF-α were analyzed in 2 ways; the secreted TNF-α by ELISA of the cell culture supernatant and the intracellular TNF-α by immunoblot of the cell lysates. FOXO3a activation significantly increased TNF-α in both instances (Figure 1B). Immunoblot analysis confirmed the increased protein synthesis of TANK and TTRAP, which was completely reversed by Ad-DN-FOXO3a (Figure 1C). Next, we performed ChIP analysis to investigate the transcriptional regulation of those molecules by FOXO3a and found that FOXO3a was bound to the promoter sites of TANK, whereas was not bound to either TNF-α or TTRAP (Figure 1D), of which pattern was confirmed by electrophoretic mobility shift assay using the putative FOXO3a binding site oligonucleotides (supplemental Figure II).

**FOXO3a Induced κB-Ras1, Suppressing NF-κB Activation**

κB-Ras1 (κB-interacting Ras-like protein-1) is known as an inhibitor of NF-κB by regulating ξB degradation.\textsuperscript{20,21} FOXO3a activation increased κB-Ras1 both in mRNA level (supplemental Figure IIIA) and in protein level (Figure 2A, upper panel), which was reversed by Ad-DN-FOXO3a. The increment of κB-Ras1 corresponded to the significant increase of ξB protein but not to the change of ξB amount (data not shown), which suggested that degradation of ξB was prevented by κB-Ras1 (Figure 2A, middle panel). The ChIP analysis also suggested that κB-Ras1 expression was transcriptionally regulated by FOXO3a (Figure 2C).

Next, NF-κB activity in response to FOXO3a overexpression was evaluated by immunofluorescent staining for p65 subunit of NF-κB after 24 hours of adenoviral transfection. NF-κB primarily remained in the cytoplasm either in Ad-GFP or Ad-TM-FOXO3a transfected HUVECs (Figure 2B, upper panels). When stimulated with TNF-α, a well-known positive regulator of NF-κB, p65 still remained in the cytoplasm in FOXO3a-transduced ECs, whereas it was swiftly translocated.
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Blank sections are not determined in microarray.
into the nucleus in the control ECs (Figure 2B, lower panels). This suppression of NF-κB activation by FOXO3a was confirmed by electrophoretic mobility shift assay, which showed reduced nuclear translocation of NF-κB in TM-FOXO3a transduced HUVECs (supplemental Figure IIIB). Because NF-κB is reported to regulate JNK via GADD45α and XIAP,22 we evaluated the expression of GADD45α and XIAP after FOXO3a activation. The expression of GADD45α and XIAP were significantly downregulated following FOXO3a activation (Figure 2D, supplemental Figure IIIC).

**FOXO3a Activates JNK, Turning TNF Receptor Pathway Toward Apoptosis**

JNK activity after FOXO3a activation was evaluated using an in vitro kinase assay. Strong activation of JNK was detected after 24 hours of TM-FOXO3a gene transduction compared with the control, which was near completely reversed by Ad-DN-FOXO3a (Figure 3A). Then, the role of the increased JNK activity on TNF-α expression was evaluated. As shown in Figure 3B, the induction of TNF-α expression by FOXO3a was completely reversed by Ad-TAM67 in ECs under stimulation of lipopolysaccharide (LPS) or heat shock. These findings indicate that the increased c-Jun after FOXO3a activation might be the regulatory mechanism of FOXO3a-induced TNF-α expression especially in the activated ECs.

Next, we performed blocking experiments of TNF receptor signaling pathway to investigate how much TNF receptor signaling to JNK contributes to proapoptotic action of FOXO3a. When TNF-α signal was inhibited by a blocking antibody against TNF receptor, FOXO3a-induced apoptosis was significantly attenuated. Moreover, when JNK was blocked either by 6-Dimethylaminopurine (DMAP), a JNK inhibitor, or by cotransfection with Ad-TAM67, apoptosis was reduced in a similar extent to TNF-α receptor blockage (Figure 3C, supplemental Figure IVA).

To elucidate the implication of native FOXO3a–JNK signaling during the stress response, HUVECs were subjected to heat shock at 42°C for 4 hours (supplemental Figure IVB). In resting state, native FOXO3a located in cytoplasm, thus remained inactive. Heat shock rapidly induced intranuclear translocation thus activation of native FOXO3a from 30 minutes, which was accompanied with increased cytotoxicity (Figure 3D). Transduction with Ad-TAM67 or DN-FOXO3a significantly reduced cytotoxicity under these conditions, suggesting the hypothesized FOXO3a - JNK signaling pathway contributes to the cytotoxicity of ECs during stress condition.
In Vivo Evidence of FOXO3a-Induced JNK-Mediated Endothelial Damage in Heat-Shock Treated Vessel

Finally, to show that the activation of FOXO3a is physiologically relevant phenomenon, we performed experiments where rat carotid arteries were exposed to heat shock with or without prior blockade of FOXO3a signaling by in vivo gene delivery of the indicated adenoviral vectors. Then, the heat-shocked vessels in organ culture were immunostained and the luminal surface of the endothelial lining of the vessel was observed en face by the confocal microscopy. Double immunostaining of VE-cadherin and activated caspase-3 visualized apoptosis of ECs on the luminal surface of blood vessel after heat shock, which was reduced by pretreatment with Ad-TAM67 and was aggravated by Ad-TM-FOXO3a (Figure 4A). In immunostaining of PECAM-1 with DAPI staining, we quantitated the endothelial damage by counting the round nuclei with PECAM-1 positivity as viable ECs on the luminal surface of vessel (Figure 4B). Heat shock treatment significantly reduced the number of viable ECs on luminal surface of vessel compared with normal temperature control group. Pretreatment with Ad-TAM67 significantly protected endothelial lining from heat shock-induced damage. Conversely, pretreatment with Ad-TM-FOXO3a significantly aggravated damage on the endothelial lining of heat-shocked vessel (Figure 4C, supplemental Figure VII).

Discussion

The important finding of our study is that the forkhead transcription factor, FOXO3a, activates JNK, while suppressing NF-κB in ECs, which turns the TNF receptor signaling from survival to apoptosis. In addition, this study has several
novel findings. First, oligonucleotide microarray showed the change of the expression of several genes, which had not been reported to be regulated by FOXO3a previously. We found that FOXO3a induced TANK, TRAP, and \(\alpha\)-B-Ras1, which might be responsible for the activation of JNK as well as suppression of NF-\(\kappa\)B. Second, inhibition of TNF receptor-JNK signaling pathway significantly attenuated the apoptosis of EC after FOXO3a activation, suggesting that the TNF receptor signaling lies in downstream of FOXO3a. This study not only shows that JNK activation and NF-\(\kappa\)B suppression are novel mechanism of FOXO3a-induced apoptosis in ECs, but also provides new insight into the role of FOXO3a in the TNF receptor signaling.

Finding Putative Genes Mediating FOXO3a-Induced Apoptosis by Oligonucleotide Microarray

By using adenoviral gene transfection and the oligonucleotide microarray, we identified several candidate genes that were regulated by FOXO3a in ECs. Among them, TNF-\(\alpha\), TRAPP, and TANK are proapoptotic molecules,23–25 of which relation to the forkhead transcription factors has not been previously reported. Another molecule upregulated by FOXO3a was \(\alpha\)-B-Ras1 (\(\alpha\)\(\kappa\)-interacting Ras-like protein-1), known as an inhibitor of NF-\(\kappa\)B activation.20,21 Among them, TANK and \(\alpha\)-B-Ras1 were found to have the forkhead factor binding motif, WAARYAAAYW (W=A or T, R=\(\kappa\) or G, Y=C or T)26 in their promoter sequences and transcriptionally regulated by FOXO3a. Though TRAP and TNF-\(\alpha\) also have similar motifs in their presumed promoter sequences, their regulation by FOXO3a was not done at transcriptional level. FOXO transcription factors can activate 2 different subsets of genes27,28: (1) genes that require FOXO DNA binding, and, intriguingly, (2) genes regulated independently of FOXO DNA binding. For example of first category, TANK and \(\alpha\)-B-Ras1 gene expression in our study were transcriptionally regulated by direct binding of FOXO3a in its promoter region. For genes of secondary category, FOXO transcription factors can cooperate with other additional transcription factors to activate transcription of genes. This second mecha-
anism may explain the discrepancy between the increased protein level in immunoblot analysis and absence of FOXO3a-DNA binding in ChIP analysis observed in cases of TTRAP and TNF-α/H9251.

Relevance of the TNF Receptor Signaling in FOXO3a-Induced Apoptosis

Because TNF-α, TRAPP, TANK, and κB-Ras1 are all known to be related with either JNK or NF-κB, the 2 main down-stream molecules of the TNF receptor pathway, we hypothesized that the TNF receptor pathway might mediate the proapoptotic action of FOXO3a. Indeed, blocking either TNF receptor or JNK significantly reduced apoptosis after FOXO3a activation. Because TNF receptor pathway is reported to be important in inflammation as well as apoptosis, the finding of this study might extend the role of forkhead transcription factor from as a downstream molecule of Akt to as a upstream molecule pivoting TNF receptor signaling. Most of these proteins have been identified as TRAF-binding proteins, eg, TTRAP and TANK. Overexpression of these factors either inhibits TRAF-mediated activation of NF-κB or potentiates TRAF-mediated apoptosis.

Figure 4. In vivo pathophysiologic significance of FOXO3a in heat shock treated blood vessel. A, Confocal microscopic en face view of luminal surface of blood vessels immunostained with antibodies against VE-cadherin and activated caspase-3 with DAPI. White arrows indicate apoptotic endothelial cells. B, Confocal microscopic en face view of luminal surface of blood vessels immunostained with antibody against PECAM-1 plus DAPI before and after heat shock treatment. Viable ECs are PECAM-1 positive (red) cells with round nucleus. In contrast, vascular smooth muscle cells below endothelium are PECAM-1 negative cells with elongated nucleus. C, Quantitative data of viable ECs on luminal surface of blood vessels.
activation of JNK. TANK is reported to be a regulatory molecule potentiating both NF-κB and JNK, thus amplifying TNF receptor signaling. On the other hand, TTRAP is known to inhibit NF-κB in a dose-dependent and stimulus-dependent manner, thus turning TNF receptor pathway signals toward JNK activation. This fits in with the results of the present study, where both TANK and TTRAP were found to be upregulated by FOXO3a activation. Taken together, expression of not only TANK but also TTRAP after FOXO3a activation may not only potentiate but also turn TNF receptor pathway signals toward JNK. Another regulatory mechanism of JNK by NF-κB is through GADD45β and XIAP. In our study, GADD45β and XIAP expression was found to be decreased after FOXO3a activation, which could also result in JNK activation. We do not make any conclusion on whether induction of TANK and TTRAP or attenuation of GADD45β and XIAP plays a major role in activation of JNK by FOXO3a. However, we think it is important that activation of FOXO3a results, at the end, in an overall increase in JNK activity, resulting in EC apoptosis. To the best of our knowledge, activation of JNK by forkhead transcription factors is a novel finding which has not been previously reported.

In Vivo Pathophysiologic Significance of FOXO3a-Induced Endothelial Damage

Using in vivo animal experiment, we confirmed in vivo pathophysiologic significance of FOXO3a-induced JNK-mediated endothelial damage in heat shock–treated rat carotid artery. In this experiment, we demonstrated that heat shock, an activator of endogenous FOXO3a, significantly induced endothelial damage, and that such damage was completely prevented by pretreatment of Ad-TAM67 (dominant-negative c-jun). These findings suggest that pathophysiologic stress like heat shock may induce FOXO3a and JNK, leading to endothelial damage and vasculopathy.

Conclusion

As summarized in Figure 5, our study demonstrates that FOXO3a plays a pivotal role in switching the TNF receptor pathway toward apoptosis by suppressing NF-κB and activating c-Jun. This novel finding provides insight into endothelial signaling of TNF receptor pathway regulated by FOXO3a.

Acknowledgments

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Disclosures

None.

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FOXO3a Turns the Tumor Necrosis Factor Receptor Signaling Towards Apoptosis Through Reciprocal Regulation of c-Jun N-Terminal Kinase and NF-κB
Hae-Young Lee, Seock-Won Youn, Ju-Young Kim, Kyung-Woo Park, Chang-II Hwang, Woong-Yang Park, Byung-Hee Oh, Young-Bae Park, Kenneth Walsh, Jeong-Sun Seo and Hyo-Soo Kim

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Supplemental figure 1. Functional grouping of genes regulated by FOXO3a in oligonucleotide microarray
Supplemental figure 2. Differences of binding to FOXO3a protein among promoter sequences of TNF-a, TTRAP, and TANK (EMSA)

In order to confirm the ChIP results, we performed Electrophoretic mobility shift assay (EMSA) using the putative FOXO3a binding site oligonucleotides sequences from TNF-a, TTRAP, and TANK gene as presented in Supplemental figure 4-6. We observed a supershift (thus binding with FOXO3a) in case of TANK. But the supershift was not observed either in TNF-α or in TTRAP, which is consistent with ChIP analysis results.
Supplemental figure 3.

A. RT-PCR for κB-Ras1.

16 hour 24 hour
κB-Ras1
GAPDH
Ad-GFP Ad-TM-FOXO3a Ad-GFP Ad-TM-FOXO3a

B. Ad-GFP + + - + + + + +
Ad-TM-FOXO3a - - + - - - - -
TNF-α - + + + + + + +
NFκB competitor - - - S25 S100 NS
NFκB antibody - - - - - - p65 p50

EMS A for p65 subunit of NF-κB. FOXO3a overexpression significantly suppressed NF-κB binding activity. NF-κB protein-oligonucleotide complexes are indicated by the arrows, while the free oligonucleotides by the broken arrow. NF-κB protein-antibody complexes are indicated by the asterisks (∗)
Supplemental figure 4.

A. Apoptotic FACS analysis counting hypodiploid cells. HUVECs were cultured in the presence of serum for 24 hours following transduction with indicated adenoviral vectors and / or designated blocking agents. TNF-α R Ab, neutralizing antibody of TNF receptor; DMAP, 6-Dimethylaminopurine; Ad-TAM67, adenoviral vector expressing a dominant negative c-Jun mutant. Results are expressed as the mean ± SEM of triplicate assays.

B. Immunofluorescent staining for FOXO3a after heat incubation (42°C). In resting state, native FOXO3a located in cytoplasm, thus remaining inactive (Upper left). However, heat shock treatment rapidly translocated native FOXO3a into nucleus, indicating activation of native FOXO3a (Lower left and right).
Supplemental figure 5. Promoter sequences of TNF-α

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TNF-a promoter sequence used in EMSA (Tm : 63C)
Forward: GATTA\textcolor{red}{G}AAG\textcolor{red}{G}AAACACAGACCACAGACACTGG (29mers)
Reverse: CCAGGTCTGTGCTCTGTTTCTTTCTCTCTAATC (29mers)

Putative FOXO3a binding site in TNF-a promoter sequence
:\textcolor{red}{g}AAAG\textcolor{red}{g}AAACA

Thick underlined green two small letters are miss-matched sequences.
Supplemental figure 6. Promoter sequences of TTRAP

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TTRAP promoter sequence used in EMSA (Tm : 64.5C)
Forward: GGTGGAGTGAGAAGCAAATGGAAATCTG (28mers)
Reverse: CAGATTTCCATTTGCTTCTCACTCCACC (28mers)

Putative FOXO3a binding site in TTRAP promoter sequence
: AgAaGCAAAATg

Thick underlined green three small letter are miss-matched sequences.
Supplemental figure 7. Promoter sequences of TANK

TANK promoter sequence used in EMSA (Tm : 55.1C)
Forward: AGACAACTATAATCAAATTGGTTTTTACC (29mers)
Reverse: GGTAAAAACCAATTTGATTATAGTTGTCT (29mers)

Putative FOXO3a binding site in TANK promoter sequence :TAA\text{\textcolor{green}{\textbf{t}}}CAAAATT

Thick underlined green one small letter is miss-matched sequence.
Supplemental figure 8. Disruption of endothelial lining in blood vessel following in vivo FOXO3a activation

A. Plain microscopic photographs of the harvested arterial segments

B. IF image for PECAM-1 & DAPI (Magnification x 40)

C. IF image for PECAM-1 & DAPI (Magnification x 100)

D. Scanning electron microscopy of luminal surface of the harvested arterial segments (Magnification x 350)

Red color: PECAM-1 visualizing endothelial lining, Arrow head
Blue color: DAPI visualizing nuclei
Materials and methods

EC culture and gene transfer using adenoviral vectors

Four to six passage of HUVECs (Clonetics™) were cultured in endothelial growth medium (EGM bullet kit, Clonetics) with 10% fetal bovine serum as previously described1.

To evaluate the role of FOXO3a, an adenoviral vector expressing constitutively-active triple-mutant FOXO3a (Ad-TM-FOXO3a) was constructed as previously described17. Briefly, Ad-TM-FOXO3a was constructed by replacing three phosphorylation sites, Thr32, Ser253, and Ser315 with alanine residues, thus unphosphorylatable by Akt. For blocking experiment, an adenoviral vector expressing dominant-negative form (Ad-DN-FOXO3a), of which transactivation domain from the C terminus was deleted, was used2. As a control, an adenoviral vector expressing green fluorescence protein (Ad-GFP) was used. Adenovirus vector expressing a dominant-negative c-Jun mutant (Ad-TAM67) was a generous gift from Harris Perlman (Northwestern University Medical School, Chicago, Ill)3.

For gene transduction, HUVECs were infected with the indicated adenoviral vectors at 25MOI for 24 hours, at which concentration the transfection efficiency reached more than 90% efficiency, as showed in our previous paper4.
**Oligonucleotide microarray**

To investigate the downstream gene expression profiles of FOXO3a, we used Magic-II 10K oligonucleotide microarray (Macrogen™, Seoul, Korea) using a previously described method\(^4\,^5\). Briefly, HUVECs were transfected with Ad-GFP, Ad-TM-FOXO3a, or Ad-DN-FOXO3a at 25MOI in the presence of 1% FBS. Total RNAs were isolated, reversely transcribed to cyanine 3- or cyanine 5-labeled cDNA and hybridized to the oligonucleotide microarray. The cyanine fluorescence was scanned using a laser confocal microscope, and images were analyzed using an ImaGene v3.0 program to calibrate relative ratios and confidence intervals used for significance determinations. The transcriptome patterns between HUVECs infected with the indicated adenoviral vectors were compared in a triplicate and replicate experimental design. Data were normalized by Lowess Nonlinear regression\(^6\), and analyzed using hierarchical clustering (Cluster v2.20) and display programs (Tree View v1.60) developed by Eisen et al. (1998)\(^7\).

**Real-time quantitative reverse transcription PCR analysis**

Changes in RNA-expression of TANK and TTRAP were determined by real-time
QRT-PCR as previously described. Primers and probes used were as follows: TANK (forward primer: 5’-GGA CCC ATC TGA TGC ACC TTT-3’; reverse primer: 5’-GCC ACT TAG TAC CAC CGA GTC ACT-3’; probe: 5’-6FAM-CAA TCC GAG GAC CAC AGC AGC CC-TAMRA-3’), and TTRAP (forward primer: 5’-ATG CCG CAG TGG CTC AGT-3’; reverse primer: 5’-TAG GAG TTC AGA GCC CTT TCC A-3’; probe: 5’-6FAM-CTT CCT GGC CGA GAA CGC TGG G-TAMRA-3’). Fold changes in gene expression were determined using the Ct method. To standardize the quantification of the genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from each sample was quantified and the selected genes were normalized to GAPDH.

RT-PCR and immunoblot analysis

Changes in RNA expression of TNF-α, κB-Ras1, GADD-45β, and XIAP were determined by RT-PCR as previously described. Primers used were as follows: TNF-α (forward primer: 5’-CAG AGG GAA GAG TTC CCC AG ; reverse primer: CCT TGG TCT GGT AGG AGA CG-3’), κB-Ras1 (forward primer: 5’-TTT GTG GAT TGT TAT CTG TGG GG; reverse primer: CGA TCT GTA ACA GTC ACC TCC C-3’), GADD-45β (forward primer: 5’-TGA CTT TGG AGG AAT TCT CGG C-3’; reverse primer: 5’-ATG AAT GTG GAT TCG TCA CCA GCA CGC AGT-3’), and XIAP (forward primer:
5'-CTT GAG GAG TGT CTG GTA A-3'; reverse primer: 5'-GTG ACT AGA TGT CCA CAA GG-3'). Total RNAs were isolated by the Trizol (Invitrogen) method, reverse-transcribed using the reverse transcription system (Promega), and amplified with the primer of each molecule and GAPDH. Because the basal level of TNF-α expression in endothelial cells is much lower than that of macrophages, which might be too small to observe the difference between blocking experiments, we performed blocking experiment both in the basal condition as well as after stimulation using LPS and/or heat shock⁹.

Immunoblot analysis was performed by modification of the procedure described previously¹⁰. The primary antibodies used in this study are as follows: anti-TNF-α (SantaCruz Biotechnology, INC), anti-TANK (SantaCruz Biotechnology, INC), anti-TTRAP (Zymed Laboratory), anti-GADD45β (SantaCruz Biotechnology, INC), anti-XIAP (SantaCruz Biotechnology, INC), anti-IκBα (SantaCruz Biotechnology, INC), anti-IκBβ (SantaCruz Biotechnology, INC), and anti-κB-Ras1 (SantaCruz Biotechnology, INC) at a dilution of 1:500. As a secondary antibody, anti-mouse IgG HRP (Promega) or anti-rabbit IgG HRP (Promega) was used at a dilution of 1:2000. Immunoblot was quantified using chemiluminescent detection reagents ECL (Amersham).
**Chromatin immunoprecipitation (ChIP) assay.**

A chromatin immunoprecipitation (ChIP) assay was performed with a commercially available kit (Upstate Biotechnology) following the manufacturer’s instruction. Antibodies for hemagglutinin and FOXO3a were used for the immunoprecipitation of the DNA fragments. The DNA fragments were analyzed by PCR with the primers specific for the TNF, TANK, TTRAP and κB-Ras1 promoters, respectively.

**Immunofluorescent staining**

To determine NF-κB localization, HUVECs were reacted with mouse monoclonal antibody against p65 (SantaCruz Biotechnology, INC) overnight, and with PE-conjugated goat anti-mouse IgG antibody for 1 hour, then incubated with 4,6-diamidino-2-phenylindole (DAPI) to visualize nuclei (Dako Corp.).

**Immunoprecipitation / In vitro JNK kinase assay**

Equal amounts of lysates were incubated with polyclonal anti-JNK1 antibody C-17 (Santa Cruz Biotechnology, Inc.) overnight and then with protein A/G-agarose (Calbiochem, San Diego, Calif.) for 3 hours. Solutions for the in vitro kinase assay were
made as previously described\textsuperscript{11}. Immunoprecipitated proteins separated by SDS-PAGE were incubated with anti-phospho-c-jun (Santa Cruz Biotechnology, INC) antibody to compare phosphorylating activity of JNK1, while JNK1 protein amount was identified with anti-JNK1 antibody C-17 (Santa Cruz Biotechnology, INC).

**Electrophoretic mobility shift assay (EMSA)**

The DNA binding activity of the nuclear protein was evaluated according to the established method with modifications\textsuperscript{12}. Briefly, after indicated treatments, the nuclear extracts from each group were prepared\textsuperscript{13} and protein concentrations were determined by a BCA\textsuperscript{TM} Protein assay kit (PIERCE). NF-κB oligonucleotides (forward primer: 5’-GTT AGT TGA GGG GAC TTT CCC AGG C-3’; reverse primer: 5’-CGT GCC TGG GAA AGT CCC CTC AAC-3’) were end-labeled with $[^{\alpha}-32P]\text{-ATP}$ by Klenow (Amersham). The competition assays included unlabeled AP-I probe. Equal amounts of nuclear protein (5µg) were incubated in the presence of 1µg poly(dI-dC), 1µg BSA, 0.1% NP-40 and 50% glycerol with or without 100-fold excess of unlabeled NF-κB or AP-I. Supershift reaction was performed through incubation of nuclear extracts (5µg) and p65 NF-κB antibody (2µg) in reaction buffer before mixed with labeled NF-κB oligonucleotides. After incubation, the DNA-protein complexes were electrophoresed,
and then exposed to X-ray film at -80°C overnight. The oligonucleotide sequences for TNF, TTRAP and TANK were indicated in supplemental figures 3-5.

**Evaluation of in vivo endothelial denudation and apoptosis after heat shock treatment on vessel**

Male Sprague-Dawley rats, 13 weeks old, weighing about 400 g (Daehan Biolink Co) were used for animal experiment as previously described\textsuperscript{14}. The experimental protocol was designed in accordance with the Guide for Experimental Animal Research issued by the Laboratory for Experimental Animal Research, Clinical Research Institute, Seoul National University Hospital. Animals were anesthetized with ketamine hydrochloride (50 mg/kg, Yuhan Corp, Bayer Korea) and xylazine (7 mg/kg, Yuhan Corp, Bayer Korea). 5x10\textsuperscript{8} pfu of the indicated adenovirus diluted in a total volume of 20 μL was delivered to injured segments, which were then incubated for 20 minutes with vascular clamp. Four hours after gene transfer, rats were euthanized by pentobarbital overdose. The carotid artery from the aortic arch to the bifurcation was dissected, incised longitudinally, pinned to a plastic board, and exposed to heat shock (42°C for 45 minutes suspended in a water-jacketed organ bath) to induce endogenous FOXO3a activation\textsuperscript{15}. Then the specimens were immunostained with antibodies against activated
caspase-3 and VE-cadherin to visualize apoptotic ECs, PECAM-1 to visualize ECs lining, and 4,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. After immunofluorescent staining, the luminal sides of the harvested vessels were observed en-face with the confocal microscope. In immuno-staining of PECAM-1 with DAPI staining, we quantitated the endothelial damage by counting the round nuclei with PECAM-1 positivity as viable ECs on the luminal surface of vessels. Apoptotic ECs on luminal surface were identified by double positive for the activated caspase-3 and VE-cadherin with typical morphological appearance: chromatin condensation, nuclear fragmentation, or apoptotic bodies\(^{16}\).

**FACS analysis of ECs apoptosis**

Apoptosis was quantified by measuring the hypodiploid DNA content using flow cytometry (FACS) analysis\(^{14}\). After transfection of the indicated adenoviral vectors for 24 hours, cells were harvested, fixed and analyzed by flow cytometry (Becton-Dickinson).

**Statistical analysis**
All data are expressed as mean ± SD. Comparisons between groups were performed using Mann-Whitney U test because of small sample numbers. A \( p \)-value of less than 0.05 was considered statistically significant.

References for the Methods

human endothelial cells stimulated with lipopolysaccharide or interleukin-1alpha. 


