Transforming Growth Factor-β–Activated Kinase 1 Regulates Angiogenesis via AMP-Activated Protein Kinase-α1 and Redox Balance in Endothelial Cells

Nina Zippel, Randa Abdel Malik, Timo Frömel, Rüdiger Popp, Elke Bess, Boris Strilic, Nina Wettschureck, Ingrid Fleming,* Beate Fisslthaler*

**Objective**—Transforming growth factor-β–activated kinase 1 (TAK1) is a mitogen-activated protein 3-kinase and an AMP-activated protein kinase (AMPK) kinase in some cell types. Although TAK1−/− mice display defects in developmental vasculogenesis, the role of TAK1 in endothelial cells has not been investigated in detail.

**Approach and Results**—TAK1 downregulation (small interfering RNA) in human endothelial cells attenuated proliferation without inducing apoptosis and diminished endothelial cell migration, as well as tube formation. Cytokine- and vascular endothelial growth factor (VEGF)–induced endothelial cell sprouting in a modified spheroid assay were abrogated by TAK1 downregulation. Moreover, VEGF–induced endothelial sprouting was impaired in aortic rings from mice lacking TAK1 in endothelial cells (TAK1−/−). TAK1 inhibition and downregulation also inhibited VEGF–stimulated phosphorylation of several kinases, including AMPK. Proteomic analyses revealed that superoxide dismutase 2 (SOD2) expression was reduced in TAK1-deficient endothelial cells, resulting in attenuated hydrogen peroxide production but increased mitochondrial superoxide production. Endothelial cell SOD2 expression was also attenuated by AMPK inhibition and in endothelial cells from AMPKα1−/− mice but was unaffected by inhibitors of c-Jun N-terminal kinase, p38, extracellular signal–regulated kinase 1/2, or phosphatidylinositol 3-kinase/Akt. Moreover, the impaired endothelial sprouting from TAK1−/− aortic rings was abrogated in the presence of polyethylene glycol-SOD, and tube formation was normalized by the overexpression of SOD2. A similar rescue of angiogenesis was observed in polyethylene glycol-SOD–treated aortic rings from mice with endothelial cell–specific deletion of the AMPKα1.

**Conclusions**—These results establish TAK1 as an AMPKα1 kinase that regulates vascular endothelial growth factor–induced and cytokine-induced angiogenesis by modulating SOD2 expression and the superoxide anion:hydrogen peroxide balance. (Arterioscler Thromb Vasc Biol. 2013;33:2792–2799.)

**Key Words:** AMPK kinase • angiogenesis modulators • MAP kinase kinase kinase 7 • redox

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The transforming growth factor (TGF)–β–activated kinase 1 (TAK1) is a mitogen-activated kinase kinase kinase activated by TGF-β, tumor necrosis factor-α (TNF-α), and other cytokines including interleukin-1β (IL-1β),1,2 and thus it acts as a key player in proinflammatory cytokine signaling.3 TAK1 has also been proposed to act as an AMP-activated protein kinase (AMPK) kinase in some cell types4 as it is activated by well-established triggers of AMPK activity, such as 5-aminoimidazole-4-carboxamide riboside (AICAR), or ischemia.3 However, recent reports place the AMPK upstream of TAK1 in inflammatory signaling pathways, indicating a reciprocal interaction between both kinases.5,7

In endothelial cells, the AMPK can be activated by vascular endothelial growth factor (VEGF),3 adiponectin,8 as well as by hypoxia9 and other angiogenic factors.10 Moreover, dominant-negative AMPK or the inclusion of small interfering RNA (siRNA) directed against the AMPK inhibits angiogenesis in Matrigel plugs impregnated with basic fibroblast growth factor10 or VEGF.11 Although there is only limited information on the consequences of AMPK deletion on angiogenesis or vascular repair,12 the deletion of the Takt gene causes embryonic lethality, which is associated with vascular abnormalities including dilated blood vessels and the loss of smooth muscle differentiation.13 When targeted in endothelial cells, the loss of TAK1 impairs angiogenesis at the same time as increasing cell death and vessel regression at embryonic day 10.5.14 How TAK1 regulates vessel formation has yet to be elucidated.

Given the limited information about the events linking TAK1 with angiogenesis, we set out to study TAK1 signaling...
in growth factor–stimulated and cytokine-stimulated endothelial cells and the potential role of AMPK activation.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**A**

**Antioxidant**

**AMPK**

**AMP-activated protein kinase**

**ERK1/2**

**extracellular signal–regulated kinase**

**FOXO**

**forkhead box class O**

**IL-1**

**interleukin-1**

**JNK**

**c-Jun N-terminal kinase**

**PEG-SOD**

**polyethylene-glycolyzed superoxide dismutase**

**ROS**

**reactive oxygen species**

**SOD**

**superoxide dismutase**

**TAK1**

**transforming growth factor-β–activated kinase 1**

**TAKΔEC**

**TAK1 in endothelial cells**

**TGF-β**

**transforming growth factor-β**

**TNF-α**

**tumor necrosis factor-α**

**VEGF**

**vascular endothelial growth factor**

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
<th>Description</th>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>(ERK)1/2</td>
<td>extracellular signal–regulated kinase</td>
</tr>
<tr>
<td>FOXO</td>
<td>forkhead box class O</td>
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<td>IL-1</td>
<td>interleukin-1</td>
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<td>JNK</td>
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<tr>
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**Results**

**Role of TAK1 in Endothelial Cell Proliferation, Migration, and Angiogenesis**

The siRNA-mediated downregulation of TAK1 in human endothelial cells (Figure 1A) significantly attenuated proliferation in the presence of 5% fetal calf serum (Figure 1B). Although the deletion of TAK1 sensitizes some cells to agonist-induced apoptosis,16 and increased TNF-α–induced apoptosis after TAK1 deletion has been linked with impaired vasculogenesis,14 there was no effect of TAK1 downregulation on either the expression of annexin V or the cleavage of caspase 3 under the conditions studied (Figure I in the online-only Data Supplement). TAK1 downregulation also attenuated endothelial cell migration in a scratch-wound assay (Figure 1C), as well as the ability to form tube-like structures on Matrigel. The defect in tube formation was evident in cells cultured in the absence and in the presence of VEGF (Figure 1D). The downregulation of TAK1 also attenuated basal and VEGF-induced sprouting in a modified spheroid assay (Figure 1E).

In mouse abdominal skeletal muscle, TAK1 was expressed clearly in small vessels where it colocalized with CD31 and smooth muscle actin (Figure 2A). Similarly, in the aorta, TAK1 was detected in endothelial cells and in vascular smooth muscle cells (Figure 2B). Thus, to determine the role of TAK1 in angiogenesis, endothelial cell sprouting (CD31-positive cells) was assessed in aortic rings from wild-type mice and animals lacking catalytically active TAK1 specifically in endothelial cells (TAKΔEC). Both the sprout number and the total sprout length were reduced markedly in aortic rings from TAKΔEC mice under basal conditions and after stimulation with VEGF (Figure 2C).

**VEGF Stimulates TAK1 and AMPK in Endothelial Cells**

Next, we determined the consequences of inhibiting TAK1 on VEGF signaling. In solvent-treated cells, VEGF elicited the phosphorylation of the c-Jun N-terminal kinase (JNK), p38 mitogen activated protein kinase, extracellular signal–regulated kinase (ERK1/2), and Akt, but responses were attenuated markedly in the presence of the TAK1 inhibitor oxozeaenol (Figure 3A). Similar effects were observed in cells treated with...
To identify TAK1-regulated proteins that are involved in cytokine-mediated angiogenesis, human endothelial cells treated with solvent or IL-1β were studied in the modified spheroid sprouting assay. Both cytokines stimulated endothelial cell sprouting in spheroids transfected with a control siRNA, but had little effect in endothelial cells treated with a TAK1 siRNA (Figure 4B).

**TAK1 Regulates Reactive Oxygen Species Production in Endothelial Cells**

To assess the consequences of TAK1 deletion on the endothelial production of ROS, H$_2$O$_2$ production was assessed in Amplex Red–loaded cells. H$_2$O$_2$ production was lower in endothelial cells from TAK$^{ΔEC}$ mice than wild-type mice (Figure 5E). SOD2 mRNA levels (reduction of 43±3%; $P<$0.05; n=4 different cell batches) and protein expression (Figure 5B) were also decreased in human endothelial cells after TAK1 downregulation using siRNA. The TAK1 siRNA-mediated downregulation of SOD2 could not be mimicked by the JNK inhibitor SP600125, the p38 inhibitor SB203580, the ERK1/2 inhibitor U0126, or the phosphatidylinositol 3-kinase-Akt inhibitor compound C, however, compound C, however, decreased SOD2 expression, an effect that was not further affected by TAK1 downregulation using siRNA. The TAK1 siRNA-mediated downregulation of SOD2 could not be mimicked by the JNK inhibitor SP600125, the p38 inhibitor SB203580, the ERK1/2 inhibitor U0126, or the phosphatidylinositol 3-kinase-Akt inhibitor wortmannin. The AMPK inhibitor, compound C, however, decreased SOD2 expression, an effect that was not further affected by TAK1 downregulation (Figure 5C). To determine which AMPK subunit was involved in regulating SOD2 expression, endothelial cells were isolated from AMPKα1−/− and AMPKα2−/− mice and their respective wild-type littermates. SOD2 levels were comparable in wild-type and AMPKα2−/− mice but significantly attenuated in endothelial cells from AMPKα1−/− mice (Figure 5D).

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TAK1 Regulates SOD2 Expression and Angiogenesis

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Discussion

The results of the present study indicate that TAK1 acts as an AMPKα1 kinase in endothelial cells and positively regulates VEGF-induced, TNF-α-induced, and IL-1β-induced angiogenesis by increasing the expression of the antioxidative mitochondrial enzyme, SOD2. Indeed, superoxide production and the mitochondrial membrane potential were reduced in the absence of TAK1, and the impaired endothelial cell sprouting from aortic rings in TAK1-deleted endothelial cells in the presence or absence of polyethylene-glycolated (PEG)-SOD, which increases cellular SOD activity and protects against the damaging effects of ROS. As before, endothelial sprouting from aortic rings from TAK1Δ mice was attenuated under basal conditions, as well as in the presence of VEGF. Remarkably, the inclusion of PEG-SOD normalized basal and VEGF-induced sprouting (Figure 6A). Similar effects were observed in human endothelial cell spheroids after the downregulation of TAK1 (Figure II in the online-only Data Supplement). In line with the finding that only the mitochondrial SOD2 isosform is affected by TAK1, the overexpression of SOD1 was attenuated in TAK1-deleted endothelial cells (Figure III in the online-only Data Supplement). The overexpression of SOD1 was without effect.

Basal and VEGF-induced endothelial cell sprouting was attenuated in aortic rings from animals lacking the AMPKα1 subunit specifically in endothelial cells versus their wild-type littermates. The defective endothelial cell sprouting in the rings lacking endothelial AMPKα1 was also rescued by PEG-SOD (Figure 6B).

TAK1-Dependent Angiogenesis Requires SOD2 Activity

To demonstrate a causal link among TAK1, AMPKα1, and SOD2 in angiogenesis, we reassessed endothelial cell sprouting in the presence or absence of polyethylene-glycolated (PEG)-SOD, which increases cellular SOD activity and protects against the damaging effects of ROS. As before, endothelial sprouting from aortic rings from TAK1Δ mice was attenuated under basal conditions, as well as in the presence of VEGF. Remarkably, the inclusion of PEG-SOD normalized basal and VEGF-induced sprouting (Figure 6A). Similar effects were observed in human endothelial cell spheroids after the downregulation of TAK1 (Figure II in the online-only Data Supplement). In line with the finding that only the mitochondrial SOD2 isosform is affected by TAK1, the overexpression of SOD1 was attenuated in TAK1-deleted endothelial cells (Figure III in the online-only Data Supplement). The overexpression of SOD1 was without effect.

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The global deletion of TAK1 impairs embryonic development in C. elegans, zebrafish, and mice. Both the global and endothelial-specific deletions of TAK1 result in vascular malformation, dilated capillaries, and defects in vascular stabilization, and correlate well with the phenotype of endothelial cell–specific TGF-β type II receptor conditional mutants. In addition to its role in vascular development, TAK1 has also been implicated in the TGF-β–induced regulation of matrix metalloproteinase-9 and tumor vascularization as the suppression of TAK1 signaling by a dominant-negative TAK1 mutant reduced tumor growth and formation of lung metastases in severe combined immunodeficiency mice. The results of the present investigation confirm that the inhibition/downregulation of TAK1 in endothelial cells inhibits endothelial cell proliferation, migration, and sprouting. These effects could not be linked to the induction of apoptosis, assessed as changes in annexin V levels and caspase 3 cleavage, although TAK1 is known to prevent TNF-α–induced death in other cell types.

Endothelial cell function and angiogenesis are tightly regulated by growth factors, particularly by VEGF. The VEGF signaling pathway in endothelial cells is complex but involves the activation of Akt, ERK1/2, JNK, p38, and AMPK. Determining whether TAK1 is part of a signaling cascade is complicated by the fact that the activity of the kinase is regulated by its association with regulator/activator proteins and phosphorylation, as well as by ubiquitination and methylation. Therefore, we used a combination of a pharmacological TAK1 inhibitor, that is, (5Z)-7-oxozaeanol, and siRNA-mediated TAK1 downregulation to study the role of the kinase in endothelial cell function. Using these tools it was possible to demonstrate that TAK1 activity is required for the VEGF-induced phosphorylation of JNK, p38, ERK1/2, and Akt. These effects could not be attributed to an indirect effect on the VEGF receptor as VEGF-R2 expression and its phosphorylation were unaffected by TAK1 inhibition.

The AMPK is activated by phosphorylation on Thr172, generally as a result of the inhibition of the protein phosphatases PP1 and PP2A or the direct phosphorylation of the enzyme by an upstream kinase (for review see Carling et al). The best studied AMPK kinases are the Ca2+/calmodulin-dependent protein kinase kinase in the immune system and regulator/activator proteins and phosphorylation, as well as by ubiquitination and methylation. Therefore, we used a combination of a pharmacological TAK1 inhibitor, that is, (5Z)-7-oxozaeanol, and siRNA-mediated TAK1 downregulation to study the role of the kinase in endothelial cell function. Using these tools it was possible to demonstrate that TAK1 activity is required for the VEGF-induced phosphorylation of JNK, p38, ERK1/2, and Akt. These effects could not be attributed to an indirect effect on the VEGF receptor as VEGF-R2 expression and its phosphorylation were unaffected by TAK1 inhibition.

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TAK1 Regulates SOD2 Expression and Angiogenesis

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Figure 6. Rescue of angiogenesis in aortic rings from transforming growth factor-β-activated kinase 1 (TAK1ΔEc) and AMPKα1ΔEc mice by polyethylene-glycolated superoxide dismutase 2 (PEG-SOD). Endothelial cell sprouting from aortic rings from (A) wild-type (WT) and TAKΔEc littermates and (B) WT and AMPKα1ΔEc littermates in the presence of solvent (Sol) or vascular endothelial growth factor (VEGF; 10 ng/mL) and the absence and presence of PEG-SOD (100 U/mL); green=CD31, bar=1 mm. The graphs summarize data from 4 to 5 different animals; *P<0.05, **P<0.01, and ***P<0.001 as indicated. AMPK indicates AMP-activated protein kinase.

Figure 6. Rescue of angiogenesis in aortic rings from transforming growth factor-β-activated kinase 1 (TAK1ΔEc) and AMPKα1ΔEc mice by polyethylene-glycolated superoxide dismutase 2 (PEG-SOD). Endothelial cell sprouting from aortic rings from (A) wild-type (WT) and TAKΔEc littermates and (B) WT and AMPKα1ΔEc littermates in the presence of solvent (Sol) or vascular endothelial growth factor (VEGF; 10 ng/mL) and the absence and presence of PEG-SOD (100 U/mL); green=CD31, bar=1 mm. The graphs summarize data from 4 to 5 different animals; *P<0.05, **P<0.01, and ***P<0.001 as indicated. AMPK indicates AMP-activated protein kinase.

The expression of SOD2 is regulated by a series of transcription factors, including nuclear factor κB and Forkhead box class O transcription factors. The deletion of both TAK1 and AMPKα1 attenuated SOD2 expression in endothelial cells, and both kinases have been reported previously to target nuclear factor κB and Forkhead box class O. There are strong links between SOD2 activity and angiogenesis as the mitochondrial generation of H2O2 by SOD2 has been reported previously to promote endothelial cell sprouting. Moreover, SOD2 overexpression increases H2O2-dependent blood vessel formation in the chicken chorioallantoic membrane. It was possible to make a direct link between the endothelial deletion of TAK1 or AMPKα1 and the downregulation of SOD2 as the defective angiogenesis observed in aortic rings and endothelial cells from TAKΔEc and AMPKα1ΔEc mice could be rescued by both PEG-SOD and the overexpression of SOD2.

Taken together, the results of this study indicate that TAK1 is a signaling intermediate in the VEGF- and cytokine-signaling cascades upstream of p38, JNK, ERK1/2, Akt, and AMPK activation. The downregulation or inactivation of TAK1 attenuated endothelial cell migration and angiogenesis, phenomena that could be attributed to the downregulation of SOD2, and the subsequent inadequate conversion of O2− to
Disclosures

None.

References


**Significance**

The results of this study demonstrate that the transforming growth factor-β–activated kinase 1 (TAK1) is part of the proangiogenic signaling cascade activated in endothelial cells by vascular endothelial growth factor and the cytokines interleukin-1β and tumor necrosis factor-α. The downregulation or inactivation of TAK1 attenuated endothelial cell migration and angiogenesis, phenomena that could be attributed to the downregulation of superoxide dismutase 2, inadequate conversion of $O_2^-$ to $H_2O_2$, and could be rescued by polyethylene-glycolated superoxide dismutase 2. TAK1 is suggested to be an AMP-activated protein kinase (AMPK) kinase in some cells, and TAK1 inhibition prevented the vascular endothelial growth factor–induced phosphorylation of AMPK and the inhibition or deletion of AMPKα1 abrogated the stimulation of angiogenesis by vascular endothelial growth factor and attenuated SOD expression. Angiogenesis in aortic rings lacking AMPKα1 in endothelial cells was disturbed and rescued by polyethylene-glycolated superoxide dismutase 2. These findings show that TAK1 and AMPKα1 play critical roles in vascular endothelial growth factor–stimulated and cytokine-stimulated angiogenesis.
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Online Figure I. Effect on TAK1 on endothelial cell apoptosis. (A) Effect of solvent (Sol), TNF-α (10 ng/mL) or Camptothecin (Cam, 5 µmol/L) for 8 hours on the binding of Annexin V to cells treated with control siRNA (siCTL) or TAK1 siRNA (siTAK). (B) Effect of control siRNA and TAK1 on caspase 3 cleavage in cells stimulated with solvent (Sol) or VEGF (10 ng/mL, 24 hours). Staurosporine (1 µmol/L, 3 hours) was used as a positive control (pc). The graphs summarise data from 4 batches of human umbilical vein endothelial cells.
Online Figure II. Rescue of defective angiogenesis in TAK1-deficient endothelial cells by PEG-SOD. Human endothelial cells were treated with either control siRNA (siCTL) or TAK1 siRNA (siTAK). Sprouting was assessed in a modified spheroid assay after incubation with solvent (Sol), VEGF (10 ng/mL) and/or PEG-SOD (100 U/mL) for 24 hours. The graphs summarise data from 4 independent cell batches; *P<0.05, **P<0.01 ***P<0.001 as indicated.
Online Figure III. Rescue of endothelial tube formation by SOD2 overexpression.

(A&B) Tube formation by human endothelial cells transfected with a control plasmid (YFP) or overexpression plasmids encoding SOD1 or SOD2 as well as either a control (CTL) siRNA or siRNA directed against TAK1. The graph summarizes data from 4 different cell batches. (C) Western blot showing the successful overexpression of SOD1 and SOD2. Similar results were obtained in 3 additional experiments.
### Online Table I. Consequences of TAK1 downregulation on the endothelial proteome following IL-1β stimulation.

Human endothelial cells were treated with either a control siRNA (siCTL) or a TAK1 siRNA (siTAK) 48 hours prior to stimulation with IL-1β (10 ng/mL) for 16 hours. Protein expression was assessed by isotope coded protein labeling combined with mass spectrometry. (A) Proteins regulated more than 30% in control siRNA treated endothelial cells following IL-1β stimulation compared to solvent. (B) Regulation of the proteins listed in (A) in cells treated with TAK1 siRNA versus to control siRNA, both following IL-1β stimulation. The results represent the data from 2 independent cell preparations.

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<td>DNA-binding protein A</td>
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<tr>
<td>SODM</td>
<td>Superoxide dismutase [Mn], mitochondrial</td>
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<tr>
<td>HNRL2</td>
<td>Heterogeneous nuclear ribonucleoprotein U-like protein 2</td>
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</tr>
<tr>
<td>VDAC2</td>
<td>Voltage-dependent anion-selective channel protein 2</td>
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<tr>
<td>PRDX1</td>
<td>Peroxiredoxin-1</td>
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<td>SAP</td>
<td>Proactivator polypeptide</td>
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<td>TBA1B</td>
<td>Tubulin alpha-1B chain</td>
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<td>PRDX4</td>
<td>Peroxiredoxin-4</td>
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<td>TPIS</td>
<td>Triosephosphate isomerase</td>
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<tr>
<td>ACTN4</td>
<td>Alpha-actinin-4</td>
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SUPPLEMENTAL MATERIAL

Transforming growth factor-β-activated kinase 1 regulates angiogenesis by modulating the AMP-activated protein kinase α1 and redox balance in endothelial cells

Expanded Methods Section

Materials

The antibodies against p-p38, p38, phospho-Thr172AMPK, AMPKα2, phospho—Ser473 Akt, Akt, phospho-ACC, ACC, phospho ERK1/2, total ERK, phospho-tyrosine and caspase 3 were from Cell Signaling (New England Biolabs, Frankfurt, Germany). The anti-p-JNK, and VEGFR2 antibodies were from Invitrogen (Karlsruhe, Germany), the CD31 antibody was from BD Biosciences (Heidelberg, Germany), the antibodies against JNK and peroxiredoxin 1 were from Santa Cruz (Heidelberg, Germany). The TAK1, SOD1, SOD2, SOD3 and catalase antibodies as well as the TAK1 inhibitor (5Z)-7-oxozeaenol were from Merck (Darmstadt, Germany). VEGF and IL-1β were from PeproTech (Hamburg, Germany). All other substances are from Sigma Aldrich (Munich, Germany).

Animals

Mice (C57BL/6x, LDL receptor-/-) harboring endothelial cell specific TAK1 deletion (TAK1ΔEC) were generated by breeding TAK1loxPloxP mice1 (kindly provided by Shizuo Akira, Osaka, Japan) with LDLR-/- mice. The endothelial TAK1 was deleted after breeding this strain with transgenic mice expressing the Cre recombinase under the control of the VE-cadherin promoter. Endothelial specific AMPKα1ΔEC mice were generated by breeding “floxed” AMPKα1 mice (provided by B. Viollet, Paris, France) with VE-cadherin-Cre mice both strains are in the C57BL/6 background. Animals were housed in conditions that conform to the guidelines for the housing and care of animals in directive 2007/526/EC (EU 2007). For the isolation of organs, mice were sacrificed using 4% isoflurane in air and subsequent exsanguination.

Cell Culture

Murine lung endothelial cells were isolated and cultured as described,2 from TAK1ΔEC, AMPKα1ΔEC mice or their wild-type littermates and used up to passage nine. Human umbilical vein endothelial cells were isolated and cultured as described3 and used up to passage 2. The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki4 and the isolation of endothelial cells was approved in written form by the ethics committee of the Goethe-University.

TAK1 Downregulation

To silence TAK1 gene expression, endothelial cells were transiently transfected with TAK1 siRNA (Santa Cruz, Heidelberg, Germany) or with control oligonucleotides (Eurogentec (Seraing, Belgium) of medium GC content using the Gene Trans II transfection kit (MoBiTec, Göttingen, Germany)

SOD Overexpression

For the overexpression of SOD, human endothelial cells were transfected by electroporation with pcDNA3.1 plasmids for SOD1 (E. Fisher, University College London, England; provided by Addgene) and SOD2, subcloned from pBI-EGFP2SOD2 (Bert Vogelstein, John Hopkins University, USA, provided by Addgene) or as a control YFP (Takara-Europe, Saint-Germain-en-Laye France) using the NEON transfection kit (Invitrogen). Trypsinised cells were resuspended (6x10^5 cells, 105 µL in buffer R provided with the kit) and incubated with 6 µg plasmid in a
cuvette. Cells were electroporated twice with 1050 V for 30 ms. Transfected cells were directly transferred to 3.5 cm plates and cultured in EBM medium with 8% FCS.

Proteomics

Human endothelial cells were treated with a control siRNA or siRNA directed against TAK1. After 24 hours, cells were treated with either solvent or IL1-β (10 ng/mL, 16 hours) and lysed with a sample grinding kit (GE Healthcare, No. 80-6483-37). The identification and quantification of differentially expressed proteins was achieved by isotope coded protein labeling (ICPL) in combination mass spectrometry by TOPLAB (Martinsried, Germany).

Cell Proliferation

Endothelial cells were seeded on 24-well plates (50,000 cells/well) coated with fibronectin in EBM medium (Invitrogen, Karlsruhe, Germany) containing 5% FCS. Endothelial cell proliferation was monitored using live cell imaging (IncuCyte, Essen Bioscience, Ann Arbor, MI, USA).

Cell Migration

Scratch wound-healing assays were performed in 96-well plates. Cells were cultured in EBM containing FCS (5%) and VEGF (10 ng/mL). Endothelial cell migration was monitored until confluency by live cell imaging (IncuCyte, Essen Bioscience, Ann Arbor, MI, USA). The distance migrated was calculated using ImageJ software.

Tube Formation

For the investigation of endothelial cells to form capillary-like structures, a tube formation assay was performed as previously described. Endothelial cell tube formation was assessed using 3*10^5 HUVECs seeded on angiogenesis microscope slides (Ibidi, Martinsried, Germany) coated with Matrigel. Cells were cultured in EBM supplemented with 5% FCS and either solvent or VEGF (10 ng/mL). The plates were incubated at 37°C, 5% CO₂ for 8 hours, and the tubes were imaged by phase contrast microscopy using an Axioscope.A1 Mikroskop with an Axio MRc5 camera (Zeiss, Jena, Germany). After 48 hours, angiogenesis was quantified in five randomly chosen fields of view by measuring tube length with a computer-assisted microscope. The total length of the tubes of five independent pictures per well out of three wells per experimental group was measured using the AxioVision 4.8 software (Zeiss).

Spheroid Sprouting Assay

Spheroids containing 800 cells were generated as described in EBM supplemented with 5% FCS and either solvent or VEGF (10 ng/mL). After 24 hours in a collagen gel, angiogenesis was quantified by measuring the cumulative length of all capillary like sprouts originating from an individual spheroid using a computer assisted microscope. At least five spheroids per gel out of 3 gels per experimental group and experiment were analyzed. Experiments were repeated at least three times.

Aortic Ring Assay

Aortae from wild-type, TAKΔEC or AMPKα1ΔEC mice were removed, cleaned, and embedded in a collagen type I gel (BD Biosciences, Heidelberg, Germany) in a 48 well plate containing EBM medium supplemented with murine serum (2.5%). After 24 hours, murine VEGF (10 ng/mL) was added and the tube-like structures were allowed to develop over 7 days. Thereafter, the samples were fixed (4% paraformaldehyde) and endothelial cells were visualized using antibodies against CD31.

Determination of Hydrogen Peroxide

Mouse lung endothelial cells were grown on 12-well plates. Cells were washed once with phosphate-buffered saline and subsequently incubated in cell culture medium containing Amplex
Red (50 μmol/L; Invitrogen, Karlsruhe, Germany) and horseradish peroxidase (2 units/mL). After 30 minutes, the supernatant was transferred to a 96-well plate, and the H₂O₂-dependent oxidation of Amplex Red was measured using a microplate reader (excitation λ540 nm, emission λ580 nm).

**Flow Cytometry**

Adherent cell cultures of mouse lung endothelial cells were detached with trypsin and transferred to culture tubes as a cell suspension. Cells were incubated for 20 minutes with the fluorescent MitoSOX dye (5 μmol/L; Invitrogen, Karlsruhe, Germany) to measure mitochondrial specific superoxide production. To measure the mitochondrial membrane potential, cells were incubated with Mitotracker JC-1 (5 μmol/L; Invitrogen, Karlsruhe, Germany) for 20 minutes. In order to measure the total mitochondrial number, cells were incubated with 100 nmol/L Mitotracker FM (Invitrogen, Karlsruhe, Germany) for 20 minutes. Absorbance was measured using a flow cytometer (BD FACS Calibur or BD FACSVerse).

**Immunoblotting**

Cells were lysed in Triton X-100 buffer. Detergent-soluble proteins were heated with SDS-PAGE sample buffer and separated by SDS-PAGE, and specific proteins were detected by immunoblotting. To assess the phosphorylation of proteins, either equal amounts of protein from each sample were loaded twice and one membrane incubated with the phospho-specific antibody and the other with an antibody recognizing total protein, or blots were reprobed with the appropriate antibody.

**Immunoprecipitation**

Porcine endothelial cells expressing flk-1 were treated with VEGF (60 ng/mL) for 10 minutes with and without oxozeaenol. For immunoprecipitation cells were lysed in Triton X-100 lysis buffer. After being precleared with protein A/G sepharose, proteins were immunoprecipitated from the cell supernatant with a custom phospho-tyrosine antibody and immunoblotted with a flk-1 antibody.

**Statistics**

Values are expressed as the mean ± SEM, and statistical evaluation was performed using Student t-test for unpaired data, 1-way ANOVA or ANOVA for repeated measures followed by a Newman-Keuls t-test, where appropriate. Values of P<0.05 were considered statistically significant.

**References**


