X-Linked Inhibitor of Apoptosis Protein Is an Important Regulator of Vascular Endothelial Growth Factor–Dependent Bovine Aortic Endothelial Cell Survival

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Abstract—Vascular endothelial growth factor (VEGF) is a critical regulator of endothelial cell biology and vascular function. Chronic VEGF treatment has been shown to inhibit tumor necrosis factor–induced apoptosis in endothelial cells. However, the mechanism for this cell survival is unclear. Interestingly, VEGF also enhances the expression of X-linked inhibitor of apoptosis (XIAP), a well-established antiapoptotic factor. XIAP has been shown to suppress apoptosis by blocking caspase activity in cancer cells, but it remains under studied in the endothelium. Therefore, we hypothesized that VEGF affects important endothelial functions, such as apoptosis and cell migration, by regulating XIAP expression and downstream caspase activity. To test this hypothesis, we examined caspase activity, apoptosis, and cell migration were assessed following VEGF overexpression or depletion in bovine aortic endothelial cells. Much like VEGF treatment, ectopic expression of XIAP blocked tumor necrosis factor–induced apoptosis. Surprisingly, the mechanism was caspase-independent. In addition, XIAP-associated cell survival was the result of enhanced nitric oxide (NO) production, and XIAP was partially localized in caveolae. In these lipid rafts, XIAP interacted with a regulator of NO production, caveolin-1, via a binding motif (FtFgtwiY, where the bold letters represent aromatic amino acids) in the baculoviral IAP repeat-3 domain. Endothelial NO synthase binding to caveolin-1 was competitively inhibited by XIAP, suggesting that XIAP acts as a modulator of NO production by releasing endothelial NO synthase from caveolin-1. Further studies showed that endothelial cell migration was also controlled by XIAP-dependent NO. Taken together, these results suggest that XIAP plays a novel role in endothelial cells, interacting with caveolin-1 and acting as a regulator of vascular antiatherogenic function.

Key Words: VEGF ■ XIAP ■ caveolin-1 ■ nitric oxide ■ apoptosis

Extensive study of apoptosis has revealed a dichotomy of functions in the vascular system. In the endothelium, apoptosis contributes significantly to vascular pathology, as well as normal physiological function. In atherosclerotic plaques, apoptosis weakens the endothelial cell layer, promoting plaque rupture and atherothrombosis. In contrast, apoptosis also acts as an important physiological mediator, eliminating unbalanced, harmful cells and maintaining vascular homeostasis. Thus, the definition of apoptosis as an either physiological or pathological phenomenon requires careful dissection of distinct vascular apoptotic processes.

Vascular endothelial growth factor (VEGF) is an endothelial cytokine that acts through the activation of VEGF receptor tyrosine kinases, eg, flkA/KDR and flt-1. It is an important regulator of angiogenesis and cell survival, or antiapoptosis, and has been shown to act as a survival factor for newly formed blood vessels. Additional studies have found that VEGF also upregulates antiapoptotic proteins such as the inhibitors of apoptosis (IAPs) in human umbilical vein endothelial cells (HUVECs).

Cellular IAP homologs have been identified in many organisms, from yeasts to higher-order animals. An important family member of IAPs, X-linked inhibitor of apoptosis (XIAP), has been linked with biologically significant cellular activities including antiapoptosis. Unlike other IAPs, XIAP is not homogeneously distributed in different tissues and cells but is strongly expressed in epidermal keratinocytes and the esophageal epithelium. Subcellularly, XIAP localizes predominantly in the cytoplasm but has been identified in the membrane of endometrial gland cells and in a granular supranuclear position in acinar exocrine cells.

Previous work has shown that XIAP induces cell survival by inhibiting caspase-3,-7, and -9 through both molecular binding and ubiquitination. The XIAP protein is compr...
posed of 3 baculoviral IAP repeats (BIRs) and a carboxy-terminal RING finger domain. Caspase-3, -7, and -9 bind to XIAP via BIRs in the amino-terminal domains of XIAP. In addition, RING domains possessing E3 ubiquitin ligase activity initiate ubiquitination of caspases and synergistically promote cell survival.

Because of its potent antiapoptotic nature, XIAP is a target molecule in the development of therapeutic agents to treat malignant cancers.15,18–23 However, XIAP also modulates cell signaling molecules such as protein kinase B, nuclear factor-κB (NF-κB), transforming growth factor-β-activated kinase (TAK),25–28 and c-Jun N-terminal kinase (JNK),27 suggesting it is not limited to a cell survival role. In fact, XIAP domains for antiapoptotic activity are independent of the domains associated with other cell signaling pathways.27 Each functional domain possesses a distinct role, indicating that XIAP may act uniquely in a variety of cellular functions.

Caveolae, or lipid rafts, affect diverse vascular functions by controlling endothelial cell signaling in a local environment.29 Caveolin-1 is a principal protein in caveolae and confers vascular regulation by binding to various signaling molecules containing the caveolin-1 binding motifs \( \phi XXXX\phi \) or \( \phi X\phi \) \( XXXX\phi \), where \( \phi \) represents an aromatic amino acid.32 Caveolin-1 has been shown to interact with heterotrimeric G proteins, Ras, Src, and endothelial nitric oxide synthase (eNOS).30,33–34

In this study, we present a unique endothelial apoptotic signaling pathway for the antiapoptotic molecule, XIAP. Our work shows that XIAP modulates endothelial apoptosis, interacting with caveolae/lipid rafts and caveolin-1 and enhancing nitric oxide (NO) production via eNOS. These novel findings clearly identify XIAP as a critical regulator of antitherogenic function in the vasculature.

**Materials and Methods**

**Cell Culture**

Bovine aortic endothelial cells (BAECs) obtained from descending thoracic aortas were cultured at 37°C and 5% CO\(_2\) in DMEM (1 g/L glucose; Life Technologies Inc) containing 20% FBS (Wel GENE Inc) with antibiotics.35–36 Cells from passages 3 to 10 were used in these studies.

HUVECs (Cambrex) were cultured at 37°C and 5% CO\(_2\) in EGM-2 Bullet Kit medium (Cambrex) containing 10% FBS (Wel GENE Inc). Cells from passage 4 to 7 were used for these studies.

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

Chronic VEGF Treatment Blocks Tumor Necrosis Factor-α-, Resveratrol-, and Etoposide-Induced Endothelial Cell Apoptosis in a Caspase-Independent Manner

It is well accepted that TNFα induces endothelial cell apoptosis.37 To investigate whether chronic VEGF treatment has an effect on physiological endothelial cell apoptosis, BAECs were incubated with VEGF for 48 hours and subsequently treated with tumor necrosis factor (TNF)α. As shown in Figure 1A and 1B, significantly fewer apoptotic cells were found in the VEGF-treated group compared with untreated control. However, there was no difference in caspase-3 activity between groups, indicating a caspase-3-independent pathway for VEGF-induced cell survival (Figure 1C and 1D).

Pharmaceutical drugs including resveratrol (Res) and etoposide (Eto) also induce endothelial cell apoptosis. In apoptosis studies, Res and Eto treatment induced 1.5- to 2.5-fold more apoptotic cells than TNFα, making such treatment ideal for subsequent cell survival assessments (Figure 1E). Despite this dramatic increase, VEGF pretreatment still significantly reduced apoptotic cell number, and this reduction did not correlate with caspase-3 activity (Figure 1F). In conjunction with TNFα studies, these findings further supported a VEGF-induced antiapoptotic pathway that does not involve caspase.

As such, the goal of further studies was to reveal this unique mechanism of VEGF-dependent cell survival.

VEGF Promotes Expression of XIAP in Endothelial Cells

Previous studies have shown that VEGF upregulates the expression of the antiapoptotic protein XIAP, suggesting a role for XIAP in VEGF-dependent cell survival.8 To investigate this mechanism of cell survival, XIAP expression was evaluated in response to growth factor treatment. As shown in Figure 2A, XIAP levels increased in a time-dependent manner following treatment of BAECs with a physiological concentration of VEGF. In contrast, 2 other growth factors (basic fibroblast growth factor and insulin-like growth factor), did not induce XIAP expression in either BAECs or HUVECs. This finding was consistent with previous reports and suggested that VEGF-specific induction of XIAP likely contributes to endothelial cell survival.

Figure 2. VEGF specifically upregulates the expression of an antiapoptotic protein, XIAP. A, BAECs were treated with 100 ng/mL VEGF as indicated. B, BAECs and HUVECs were treated with 100 ng/mL VEGF, basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF). After cell lysis, proteins in cell lysates were resolved by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with XIAP antibodies.

Figure 3. XIAP plays an important role in VEGF-induced antiapoptosis. BAECs were transfected with vector alone, vector containing the xiap gene (wt-XIAP), scrambled siRNA, or xiap siRNAs. Then, confluent cells were treated with apoptosis-inducing agents (TNFα, etoposide, or resveratrol), as indicated. A, Apoptotic cells were detected as described in Figure 1A. B, Apoptotic cells were counted as described in Figure 1B and are plotted as bar graphs (means±SE, n=3 to 5). C, After cells were lysed, proteins in cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with caspase-3 antibodies. D, Caspase-3 activity was measured with substrate Ac-DEVD-pNA using the Caspase-3 Colorimetric Activity Assay Kit. The caspase-3 activity for various cell lysates are plotted as bar graphs (means±SE). *P<0.05. E, Apoptotic cells are plotted as line graphs (mean±SE). *P<0.05.
Ectopic Expression of XIAP Inhibits Res- and Eto-Induced Endothelial Cell Apoptosis in a Caspase-3-Independent Manner

To define the role of XIAP in the endothelium, apoptosis was assessed in response to ectopic XIAP expression. As illustrated in Figure 3A, treatment with the pharmaceutical drugs Res and Eto promoted endothelial cell apoptosis. As expected, overexpression of XIAP inhibited this increase in cell death, reducing the number of apoptotic cells by 20% to 25% (Figure 3B).

In various cancer cell lines, it has been shown that XIAP inhibits apoptosis by blocking caspase activity. Both Res and Eto increased caspase-3 activity in endothelial cells. However, unlike apoptosis, this caspase-3 activation was not affected by ectopic XIAP expression (Figure 3C). Moreover, the caspase-3 inhibitor Ac-DEVD-CHO blunted Res- and Eto-induced caspase-3 activity but had no effect on XIAP-dependent endothelial cell survival (Figure 3B and 3D). Taken together, these results indicated that caspase-3 is not involved in drug-stimulated endothelial cell apoptosis. Thus, XIAP expression likely reverses Res and Eto-induced apoptosis via a unique caspase-independent mechanism in the vasculature.

XIAP Knockdown Increases Apoptosis Induced by TNFα and Eto

The abovementioned studies, as shown in Figure 3, identified XIAP as an important antiapoptotic factor by an ectopic expression of XIAP. To investigate whether XIAP play this critical role endogenously, XIAP-depletion studies were performed in BAECs. XIAP-specific small interfering (si)RNA was used to transiently knockdown endogenous protein expression. Compared with control siRNA, XIAP siRNA considerably reduced XIAP levels (Figure 3E, bottom). In addition, functional studies showed that siRNA depletion of XIAP increased TNFα- and Eto-induced apoptosis (Figure 3E, top). These data further implicated XIAP in endothelial cell survival, but the mechanism behind this role remained unclear.

XIAP Increases Cell Survival in Endothelial Cells by Enhancing NO Production

Previous work by us has shown that NO, an important vasodilator, can significantly block Res-stimulated endothelial apoptosis. To confirm this finding, we pretreated BAECs with the NO-producing agent S-nitroso-N-acetyl penicillamine (SNAP) and, consecutively, with proapoptotic agents Res and Eto. Consistent with our previous report, Res-and Eto-induced apoptosis was inhibited by SNAP in a dose-dependent manner, indicating that NO has an inhibitory effect on endothelial cell apoptosis (Figure 4A).

To test whether NO participates in the unique mechanism for XIAP-induced cell survival, NO levels were evaluated in response to both ectopic XIAP expression and XIAP siRNA. Because of a limited sensitivity threshold, we were unable to detect differences between NO production in treated versus control cells (data not shown). To magnify the NO signal, cells were stimulated with A23187 (Ca2+ ionophore) before NO monitoring. As shown in Figure 4B, ectopic XIAP expression enhanced NO production, and XIAP-induced NO levels were comparable to those following VEGF treatment. In contrast, NO levels were reduced by XIAP knockdown via XIAP siRNA. Together, these data suggested that XIAP is able to regulate NO production in endothelial cells.

XIAP likely induces NO production via the enzyme endothelial NO synthase, which catalyzes the synthesis of NO. Increasing concentrations of XIAP caused concomitant phosphorylation of eNOS in a dose-dependent manner (Figure 4C). These results indicated that XIAP is an important vascular regulator, activating eNOS to promote NO production in endothelial cells.
To directly connect XIAP-dependent NO production to VEGF-dependent cell survival, we pretreated BAECs with the eNOS inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) before inducing apoptosis. As previously described, VEGF decreased the number of apoptotic cells present following Res and Eto treatment. In the presence of L-NAME, this effect was significantly reduced, indicating NO plays an important role in VEGF-dependent cell survival (Figure 4D). A similar effect was observed with ectopic XIAP expression. As shown in Figure 4E, XIAP reduced apoptosis, whereas L-NAME blunted this effect. Collectively, these results demonstrated a mechanism by which VEGF induces XIAP expression and, in turn, enhances NO production via eNOS to restrict endothelial cell apoptosis. Further studies focused on the mechanism of XIAP action of eNOS activity.

**XIAP Is Expressed in Caveolae or Lipid Rafts**
Interaction with caveolin-1, a principal protein of caveolae, is an important component of eNOS activity. Caveolin-1, the caveolar scaffolding protein, binds to and negatively regulates eNOS activity.\textsuperscript{29,39} Based on our finding that XIAP expression can induce eNOS activation, we hypothesized that ectopically expressed XIAP localizes in caveolae and interacts with caveolin-1 to activate eNOS. To test this hypothesis, lipid raft fractions were obtained and probed for XIAP with specific antibodies (Figure 5A). Fifteen to 20 percent of total cellular XIAP was found in the caveolar or lipid raft fraction. Immunostaining studies also supported this finding. Endogenous XIAP and V5-tagged XIAP colocalized with caveolin-1, indicating a portion of cellular XIAP is located in lipid rafts (Figure 5B and 5C). Although this data clearly supported our hypothesis, further
XIAP interacts with caveolin-1

Interestingly, the XIAP molecule contains a caveolin-1 binding motif (FtgwY, where the bold letters represent aromatic amino acids) (Figure 6A). To determine whether this binding motif plays an important role in molecular interaction between XIAP and caveolin-1, we constructed 2 XIAP mutants, △RING and BIR1-2, with and without the caveolin-1 binding domain, respectively. In vitro studies showed that wild-type (wt) XIAP was able to bind caveolin-1 ($K_d = 127 \pm 70$ nmol/L), whereas the BIR1-2 mutant was not (Figure 6B and 6C). Immunoprecipitation experiments supported this observation. As illustrated in Figure 6D, both wt XIAP and △RING coimmunoprecipitated with caveolin-1, whereas the BIR1-2 deletion mutant did not. Together, these data indicated that the BIR3 domain of XIAP is necessary for binding between caveolin-1 and XIAP.

XIAP Activates eNOS by Competitively Binding to Caveolin-1 in Caveolae or Lipid Rafts

The oligomerization domain (residues 61 to 101) of caveolin-1 is an essential binding domain for eNOS and other caveolin binding proteins, suggesting a potential competition between eNOS and XIAP for caveolin-1. In vitro binding assays, eNOS binding to caveolin-1 was inhibited as the concentration of wt XIAP was increased, whereas increasing the BIR1-2 mutant had no effect (Figure 7A). In addition, immunoprecipitation studies revealed that both ectopic XIAP and △RING expression inhibited eNOS binding to caveolin-1, whereas BIR1-2 did not (Figure 7B). In conjunction with colocalization studies, these results demonstrated that XIAP and eNOS compete for caveolin-1 in caveolae or lipid rafts, suggesting a critical role of caveolin-1 in the XIAP-dependent physiological effects.

This function for caveolin-1 was further explored using caveolin-1–specific siRNA. As shown in Figure 7C, caveolin-1 knockdown increased NO levels compared with control. However, this NO level was not different from that induced by exogenous XIAP expression or by a concomitant XIAP expression and caveolin-1 depletion. Additionally, only wt XIAP and △RING vectors were able to promote increased NO levels (Figure 7D). The BIR1-2 deletion mutant lacking the caveolin-1 binding domain did not. These findings suggested that the release of eNOS from caveolin-1, whether via competitive binding of XIAP or depletion of caveolin-1, promotes activation of eNOS. Furthermore, this mechanism also seemed to apply to XIAP-dependent cell survival. Caveolin-1 depletion blunted apoptosis in an eNOS-dependent manner, similar to wt XIAP expression or wt XIAP expression and caveolin-1 knockdown together (Figure 7E). Together, these studies showed that both XIAP and caveolin-1 act through NO to regulate endothelial cell functions.

XIAP Promotes the Endothelial Cell Migration via NO

It has been well established that VEGF is a proangiogenic factor and that NO induces endothelial cell migration, a proangiogenic property. Combined with the present finding that XIAP is a functional mediator for VEGF-induced cell survival, these studies imply that XIAP expression may affect endothelial migration. To test this hypothesis, we evaluated endothelial cell migration in response to XIAP expression. Overexpression of XIAP, generated by either chronic VEGF treatment or ectopic expression, significantly enhanced endothelial cell migration, whereas XIAP siRNA reduced migration (Figure 8A and 8B). As shown in Figure 8B, increased migration was inhibited by the L-NAME pretreatment, implicating NO in XIAP-promoted endothelial cell migration. Overall, this work showed that XIAP acts as an important...
and is initiated by TNF and TNF receptor binding. Subsequently, XIAP blocks TNF-, and Res-, and Eto-induced apoptosis in a caspase-independent manner. XIAP plays an important role in the endothelial cell migration. Confluent endothelial cells were treated with 2 mmol/L thymidine twice for 18 and 17 hours and then scraped with a razor blade. The cells were subsequently incubated for an additional 16 hours in starvation media containing various agents (100 ng/mL VEGF, 1 mmol/L L-NAME) as indicated. Migrated cells were observed with microscopy. A, Images are representative of at least 3 different observations. The solid line indicates the boundary line immediately after scraping. B, Quantification was performed by counting the number of cells migrated in the same field. Bar graphs represent means±SE (n=3). *P<0.05, ** P<0.003.

Discussion

Through exogenous XIAP expression and siRNA depletion studies, we have shown that XIAP regulates important vascular functions in endothelial cells. Our work provides evidence for the following mechanism: (1) VEGF upregulates XIAP in both BAECs and HUVECs; (2) XIAP competitively binds to caveolin-1, releasing and activating eNOS; and (3) eNOS activation results in NO production which promotes both cell survival and cell migration. Previous reports have stated that XIAP cannot be detected in the endothelium when monitored by immunohistochemistry. Here, we were able to identify XIAP in both HUVECs and BAECs by Western blots. This is consistent with previous reports. These conflicting results are likely attributable to a low expression level in non–VEGF-treated cells.

In other studies, XIAP has been shown to block apoptosis in cancer cells by inhibiting caspase activity. Although these previous reports indicated that VEGF upregulates XIAP in endothelial cells, until now, the physiological effect of XIAP in endothelial cells has not been well understood. Through a number of functional studies, we have shown that VEGF-induced XIAP blunts apoptosis and acts as a potent anti-atherogenic molecule. In addition, our work has shown that XIAP blocks TNF-, and Res-, and Eto-induced apoptosis in a caspase-independent manner.

The pathway for TNF-induced apoptosis is well described and is initiated by TNF and TNF receptor binding. Subsequent recruitment of serine adaptor proteins, including TNF receptor–associated death domain protein (TRADD) and Fas-associated death domain protein (FADD), results in caspase-8 activation. Caspase-8 then activates other caspases, including caspase-3, to induce apoptotic cell processes. Based on this established signaling pathway, a caspase-3–independent mechanism for VEGF/XIAP-induced cell survival is not easily understood. A model for the caveolin-1–dependent regulation of TNF-induced apoptosis is more consistent with our findings. Previously, an essential role of caveolin-1 in the regulation of TNF signaling has been documented. In addition, caveolin-1 was suggested to play an important role in the regulation of apoptosis via a phosphatidylinositol 3-kinase/Akt signaling pathway. Thus, our findings support this caveolin-associated mechanism and further suggest an important role of XIAP interacting with caveolin-1 in the regulation of endothelial apoptosis or TNF signaling.

Via 2 distinct fractionation assays, we found that ~20% of endothelial XIAP was localized to detergent-insoluble membrane raft fractions. Our unpublished observations using β-methyl cyclodextrin indicate that caveolar or lipid raft fraction of XIAP can be dynamically changed by certain stimuli, such as shear stress, to affect target molecules, such as ERK (unpublished data, 2008). Together, these data imply that XIAP is compartmentalized in endothelial cells to define function according to location. Like XIAP, caveolin-1 is not ubiquitously expressed in all tissues and cells. These observations suggest that in caveolin-1–expressing cells, like endothelial cells, XIAP behaves differently based on its location, with caveolin-associated functions or caspase-dependent functions occurring independently. The finding that XIAP promotes NO production further supports this concept.

We have established that XIAP blunts cell death in an NO-dependent manner. Although it is not clear how NO functions as an inhibitor for TNF-dependent apoptosis, several studies support such a protective role. For example, Hida et al reported that NO plays a role in mitochondria and acts as an antiapoptotic factor. Thus, it is possible that NO promotes cell survival by inhibiting signaling molecules downstream of caspase-3 in the TNF-induced pathway. Beyond cell survival, association of XIAP with other important molecules suggests a broader role in vascular function. It has been established that XIAP is related to endothelial cell survival and activation of cell signaling molecules including NF-κB. Because NF-κB regulates inflammatory responses, cell proliferation, and cell survival, correspondingly, XIAP likely participates in several physiological functions in the vascular system. Via in vitro and the in vivo assays, we showed that XIAP not only colocalizes with caveolin-1 but also interacts with it. Caveolin-1 is involved in modulation of vascular tone, angiogenesis, and atherosclerosis. The molecular interaction between caveolin-1 and XIAP provides insight into a wider spectrum of vascular functions exerted by XIAP. In this study, we have shown that XIAP expression is associated with important vascular functions such as NO production, apoptosis, and migratory responses. These vascular functions are essential to maintain homeostasis of blood vessels and to prevent disease. For instance, NO modulates basal vascular tone, endothelial cell proliferation and survival, and angiogenesis. Reduction in NO bioavailability induces a number of major cardiovascular diseases such as arterial hypertension and dyslipid-
emia\(^{47}\) and inhibition of angiogenesis.\(^{46}\) However, overexpression of NO has also been related to infectious disease and immune disease mediation.\(^{48}\) These different physiological or pathophysiological roles for NO are dependent on NO concentration and cell type. Given that immune cells stimulated by infection produce 10-fold more NO than quiescent cells\(^{49}\) and XIAP induces less than a 2-fold increase in NO, XIAP is likely involved in maintaining vascular physiology rather than vascular disease. We were able to verify that XIAP does indeed participate in vascular functions other than cell survival. Scratch assay results showed that ectopically expressed XIAP modulated endothelial cell migration as well.

Endothelial cell migration is critical to vascular remodeling, inflammation, wound healing, and angiogenesis. The finding that XIAP participates not only in VEGF-induced cell survival but also in cell migration suggests that XIAP is an important vascular factor. Although these studies were limited to bovine aortic endothelial cells and investigation of other cell types is warranted, this work clearly identifies a novel role for XIAP in vessel physiology. In conclusion, XIAP can now be studied as a significant functional regulator in the cardiovascular system.

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**Disclosures**

None.

**References**

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Detailed Materials and Methods

Transfection of BAEC with XIAP (wt-XIAP) Gene

Ninety % confluent BAEC were transfected with pcDNA3.1 vector carrying either the xiap gene or an empty control vector (1.5µg/well each). Transfections were performed in the presence of 5µL/well LipofectAMINE 2000 Transfection Reagent (Invitrogen Life Technologies) according to supplier recommendation. After 4h incubation, medium was replaced with fresh medium containing antibiotics, and cells were maintained for additional 24h before confirmation of transgenic gene expression.

Transfection of BAEC with Stealth siRNA against the XIAP Gene

Duplex oligoribonucleotide Stealth siRNAs (25 nucleotides) were obtained from Invitrogen Life Technologies. 30-50% confluent BAEC were transfected with 300 pmol/mL of either scrambled control siRNA or xiap-specific siRNA (5’-GCAGATTATGAAGCACGGATCTTTA-3’) according to the manufacturer’s protocol (Invitrogen Life Technologies). Transfection efficiency was assessed by Western Blot.

Co-transfection of BAEC with XIAP (wt-XIAP) Gene and oligonucleotide siRNA against the caveolin-1 Gene

Duplex oligonucleotide siRNAs (21 nucleotides) were obtained from Dhharmacon RNA Technologies. Ninety % confluent BAEC were transfected with either pcDNA3.1 vector carrying the xiap gene or an empty control vector (1µg/well each) and 200 pmol/mL of either scrambled control siRNA or caveolin-1-specific siRNA (5’-CCAGAAGGGACACACAGUdTdT-3’) using LipofectAMINE 2000 Transfection
Reagent (Invitrogen Life Technologies) according to manufacturer’s protocol. Transfection efficiency was assessed by Western Blot.

**Preparation of Cell Lysate**

Cells were washed in ice-cold phosphate buffered saline (PBS), scraped in 250 μL RIPA buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS and 1mmol/L phenylmethysulfonyl fluoride) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), and solubilized for 15 minutes at 4°C, as previously described [1]. To obtain the Triton-soluble fraction, cells were lysed with 250 μL of lysis buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 1 mmol/L vanadate, 1 mmol/L dithiothreitol, 1% Triton X-100 and 1 mmol/L phenylmethysulfonyl fluoride) containing protease inhibitor cocktail, solubilized for 15 minutes at 4°C and centrifugated for fractionation. Total protein content of soluble cell lysate was measured using a Bio-Rad DC assay kit (Bio-Rad).

**Measurement of Caspase-3 Activity**

Caspase-3 activity was measured via Caspase-3 Colorimetric Activity Assay Kit with Ac-DEVD-pNA as the substrate (Chemicon). Briefly, confluent BAEC were starved for 8 hours prior to treatment with 100 ng/mL VEGF (R&D systems) or transfection with empty control vector, vector containing the xiap gene, scrambled control siRNA or xiap-specific siRNA. After 48 hours, cells were treated with various apoptotic agents (50 ng/mL TNFα (sigma), 100 μmol/L etoposide (Sigma) or 100 μmol/L resveratrol (Sigma)) and then lysed. In 96-well plates, cell lysates were incubated with reaction
buffer containing 236 μmol/L Ac-DEVD-pNA with or without caspase-3-specific inhibitor (0.1 μmol/L Ac-DEVD-CHO) for 2 hours at 37°C. Caspase-3 activity was determined by absorbance (405 nm) via microplate reader (Bio-Rad, Model 550).

**Measurement of Apoptosis**

Apoptotic cells were identified via visual assessment of cell morphology (cell rounding and shrinking). Following pretreatment with 100 ng/mL VEGF for 48 h, BAEC apoptosis was induced via TNFα (50 ng/mL), etoposide (100 μmol/L) or resveratrol (100 μmol/L). Cells were exposed to apoptotic agents in the presence of caspase-3 specific inhibitor (0.1 μmol/L Ac-DEVD-CHO), nitric oxide synthase inhibitor L-NAME (1 mmol/L obtained from BIOMOL Research Labs) or vehicle control. After 18 hours, the number of apoptotic cells was assessed (Zeiss Autoplan 2). In additional experiments, 90-95% confluent BAEC were starved for 12 hours, then treated with apoptotic agents (100 μmol/L etoposide or 100 μmol/L resveratrol) and varying concentrations of SNAP (Calbiochem, 0 μmol/L to 200 μmol/L) for an additional 18 hours. After incubation, apoptotic cells were observed as above. For quantification, apoptotic cells were counted in a uniform visual field [2].

**Hoechst 33258 Staining of Nuclei**

Morphological changes in nuclear chromatin were detected by staining with the DNA-binding fluorescent dye, Hoechst 33258 (Sigma). BAEC grown on glass coverslips were pretreated with VEGF and transfected with empty vector or xiap vector. As described above, cells were subsequently treated with apoptosis-inducing agents. In preparation for staining, cells were washed twice with PBS, fixed with Carnoy’s fixative.
for 10 minutes, and air-dried for an additional 10 minutes. After drying, cells were stained with Hoechst 33258 solution (12.5 μg/mL, Sigma) for 30 min at room temperature, then were thoroughly washed with PBS. Nuclei were observed fluorescence microscopy (Zeiss Autoplan 2) [2].

Cell Migration

For the in vitro cell migration assays, transfected BAEC were treated with 2mM thymidine for 18 h (first block), then washed with PBS and released for 9h. Released cells were treated with an additional 2mM thymidine for 17 h (second block), and scraped with a razor blade. Cells were thoroughly washed with PBS, incubated in starvation media with or without 100 ng/mL VEGF or 1 mmol/L L-NAME, and then allowed to migrate for 12 hours. Migrating cells were then observed via microscopy. The number of migrating cells was quantified by counting the number of cells within the defined wound area in a uniform visual field [3,4].

Western Blots

Proteins (30 μg) in soluble lysate were resolved by 10% SDS-PAGE, then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked in 5% non-fat milk in PBS containing 0.1% Tween-20 (PBST) for 1 hour at room temperature and probed with antibodies specific to XIAP (BD Biosciences), caveolin-1 (BD Biosciences), caspase-3 (Cell signaling), V5 (Invitrogen Life Technologies), or endothelial nitric oxide synthase (Cell signaling or Upstate). Subsequently, the membrane was thoroughly washed in PBST, and incubated with
HRP-conjugated secondary antibody for 1 hour at room temperature. Finally, the membrane was developed by enhanced chemiluminescence detection method (Amersham) [1].

**Lipid Raft Isolation**

Lipid rafts were isolated using Triton X-100 as previously described [5]. 90% confluent endothelial cells from four 150-mm dishes were lysed with 1 mL of lysis buffer (25 mmol/L HEPES, pH 6.5, 150 mmol/L NaCl, 2% TritonX-100, 1 mmol/L EDTA, and 1 mmol/L PMSF) containing a protease inhibitor cocktail, homogenized 20 times with a tight Dounce homogenizer (Kontes, Vineland, NJ), and then incubated for 20 min at 4°C. The lysate was mixed with 1 mL of 80% sucrose, transferred to a SW41Ti centrifuge tube, and overlaid with 6.5 mL of 30% sucrose solution and 3.5 mL of 5% sucrose solution (25 mmol/L HEPES and 150 mmol/L NaCl, pH 6.5). The discontinuous sucrose gradients were ultracentrifuged for 18 hours at 4°C in a SW41Ti rotor (Beckman Instruments, Palo Alto, CA) at 200,000g. The gradient was then fractionated into 12 fractions from bottom to top. Fractions 7-9 were pooled and assayed for lipid raft caveolae.

**Immunofluorescence**

Subcellular localization of caveolin-1 and XIAP was determined via immunofluorescence microscopy. Cells were fixed in 2% paraformaldehyde for 15 minutes, permeabilized in 0.1% Triton X-100 and 50 mmol/L lysine for 30 minutes, and blocked in 2% BSA, 10% goat serum, and 0.1% Triton X-100 for 1 hour. Fixed cells were incubated with anti-caveolin-1 (BD Biosciences), anti-XIAP (BD Biosciences), or
anti-V5 (Invitrogen Life Technologies) primary antibodies overnight at 4°C, followed by TRITC-conjugated goat anti-rabbit (Zymed) or FITC-conjugated goat anti-mouse (Zymed) antibodies for 30 minutes. Cells were mounted with Slow-Fade mounting medium, and fluorescence intensity was quantified via fluorescence microscope and an image analysis program (Zeiss Autoplan 2) [6].

Purification and In Vitro Binding Assay of GST-fusion Protein

GST-fusion proteins (the full-length caveolin-1, XIAP, or fragments of XIAP) expressed in *Escherichia coli* were purified by Glutathione-Sepharose affinity chromatography as previously described [7]. A 1:1 slurry (40μL) of glutathione-sepharose beads combined with GST-caveolin-1 was resuspended in 500 μL PBS buffer and incubated with varying concentrations of (0-265 nmol/L) purified recombinant proteins (XIAP or BIR 1-2) overnight at 4°C. Bound complexes were washed at least seven times with the lysis buffer, incubated with Laemmli sample buffer on heating blocks for 5 minutes, resolved by SDS-PAGE, and immunoblotted with monoclonal XIAP antibodies.

Immunoprecipitation

BAEC (90% confluent) were transfected with the empty, wt *xiap* or *xiap* deletion mutant (*ΔRING, BIR1-2*) vector. 24 hours after transfection, cells were washed with ice-cold PBS and scraped in 600 μL of a lysis buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 80 mmol/L β-octyl glucopyranoside, 0.5% sodium deoxycholate, 10 mg/mL leupeptin, 10 mg/mL aprotinin, 100 mg/mL iodoacetamide, and 1 mmol/L PMSF). Cell lysates were prepared as previously described [7]. Lysates were homogenized by passing 10 times through a 22-gauge needle and were then centrifuged
for 10 minutes at 15,000 X g at 4°C. Total protein concentration was determined by a protein assay kit (Bio-Rad), and protein concentration was adjusted to 1 mg/mL. Equal volumes of lysate were incubated with goat anti-mouse V5 (4 µg/mL) or goat anti-rabbit polyclonal caveolin-1 (4 µg/mL) antibodies at 4°C overnight, followed by incubation with 50µL Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) at 4°C with gently rocking for 2 hours. The immunoprecipitated complexes were washed at least seven times with lysis buffer, incubated with Laemmli sample buffer on heating blocks for 5 minutes, resolved by SDS-PAGE, and immunoblotted.

**Competition Assay**

20 µg of the glutathione-sepharose beads combined with GST-caveolin-1 were resuspended in 600 µL of the lysis buffer and incubated with varying concentrations (0-600 nmol/L) of purified XIAP or BIR1-2, as well as cell lysate (600µg of total proteins) overnight at 4°C. Binding complexes were thoroughly washed and desolved in Laemmli sample buffer. Proteins in the complexes were resolved by 10% SDS-PAGE and immunoblotted using a polyclonal anti-eNOS antibody (Cell Signaling).

**NO Measurement**

NO was monitored via the fluorescence spectrum of DAF-2 (Calbiochem). Cells were pre-incubated with a HEPES buffer (5 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5 mmol/L Glucose, pH7.4) containing 1 µmol/L Ca²⁺ ionophore, A23187 (Sigma) for 20 minutes. Subsequently, cells were incubated at 37°C and 5% CO₂with 0.1 µmol/L DAF-2 for 15 minutes. Harvested
cells were lysed by sonication. Supernatants were collected following centrifugation and scanned by a spectrofluorophotometer (RF 5301PC Shimadzu) at excitation and emission of 495 and 515 nm (slit 10nm), respectively. NO was calculated via DAF-2 fluorescence intensity [8].
References


