Integrin-Linked Kinase Regulates Vasomotor Function by Preventing Endothelial Nitric Oxide Synthase Uncoupling

Role in Atherosclerosis


Rationale: Atherosclerotic lesions develop in regions of disturbed flow, whereas laminar flow protects from atherogenesis; however, the mechanisms involved are not completely elucidated. Integrins are mechanosensors of shear stress in endothelial cells, and integrin-linked kinase (ILK) is important for blood vessel integrity and cardiovascular development.

Objectives: To explore the role of ILK in vascular function by studying conditionally ILK-deficient (cKO) mice and human atherosclerotic arteries.

Results: ILK expression was detected in the endothelial cell layer of nonatherosclerotic vessels but was absent from the endothelium of atherosclerotic arteries. Live ultrasound imaging revealed that acetylcholine-mediated vasodilatation was impaired in cKO mice. These mice exhibited lowered agonist-induced nitric oxide synthase (NOS) activity and decreased cyclic guanosine monophosphate and nitrite production. ILK deletion caused endothelial NOS (eNOS) uncoupling, reflected in reduced tetrahydrobiopterin (BH4) levels, increased BH2 levels, decreased dihydrofolate reductase expression, and increased eNOS-dependent generation of superoxide accompanied by extensive vascular protein nitration. ILK reexpression prevented eNOS uncoupling in cKO cells, whereas superoxide formation was unaffected by ILK depletion in eNOS-KO cells, indicating eNOS as a primary source of superoxide anion. eNOS and ILK coimmunoprecipitated in aortic lysates from control animals, and eNOS-ILK–shock protein 90 interaction was detected in human normal mammary arteries but was absent from human atherosclerotic carotid arteries. eNOS-ILK interaction in endothelial cells was prevented by geldanamycin, suggesting heat shock protein 90 as a binding partner.

Conclusions: Our results identify ILK as a regulatory partner of eNOS in vivo that prevents eNOS uncoupling, and suggest ILK as a therapeutic target for prevention of endothelial dysfunction related to shear stress–induced vascular diseases. (Circ Res. 2012;110:00-00.)

Key Words: atherosclerosis • oxidative stress • uncoupling protein

Endothelial dysfunction is defined as impaired endothelium-dependent relaxation of blood vessels in response to the endogenous vasodilator nitric oxide (NO). Endothelial dysfunction is concomitant with changes in vascular structure associated with many forms of vascular disease, such as hypertension and atherosclerosis.1 Atherosclerotic lesions develop mostly in areas exposed to disturbed blood flow, whereas endothelial cells exposed to laminar flow are protected against inflammatory activation and show higher relative expression of endothelial NO synthase (eNOS) and superoxide dismutase.2,3

Most NO in the vasculature is produced by eNOS, with a minor contribution from neuronal-type nNOS expressed in vascular smooth muscle cells. Under inflammatory conditions, vascular cells can express iNOS, which produces large amounts of NO and contributes further to vascular damage.4 eNOS can be activated by hemodynamic forces, autacoids, hormones, and growth factors. NO relaxes vessels via activation of soluble guanylyl cyclase (sGC). The resulting elevated levels of cyclic guanosine monophosphate cause cGMP-dependent protein kinase type I (cGKI), which phosphorylates downstream targets that regulate the...
actin-myosin cytoskeleton and the calcium clearing mechanism, leading to vasorelaxation.  

Many endothelial cell molecules, including integrins, sense shear stress.  

Integrin-linked kinase (ILK), a key regulator of blood vessel integrity, is a phosphoinositide 3-kinase–dependent serine/threonine kinase that binds to the cytoplasmic domain of β-integrin and lies upstream of many intracellular signaling pathways. ILK plays pivotal roles in angiogenesis, matrix-endothelial cell interactions, endothelial and extracellular matrix–mediated signaling, cell survival, and the recruitment of endothelial progenitors. Transgenic mouse models demonstrate important roles for ILK in vascular development, tumor angiogenesis, and cardiac hypertrophy. Downregulation of ILK after arterial injury correlates with neointima formation and vascular repair and ILK is also implicated in the downregulation of eNOS in human endothelial cells after long-term culture in collagen type I. ILK-mediated regulation of vascular function in shear stress–related vascular diseases such as atherosclerosis has not been studied previously.

We investigated the vascular effects of ILK by analyzing vascular reactivity to eNOS agonists, NOcGMP pathway activity, vascular superoxide generation, and tetrahydrobiopterin (BH4)–mediated eNOS coupling in conditionally ILK-deficient (cKO-ILK) mice and in human and mouse atherosclerotic arteries. We show that ILK stabilizes association of eNOS with heat shock protein (Hsp)90 in vivo, thereby ensuring coupling of eNOS activity to physiological production of NO rather than oxidative derivatives. This association is disrupted in atherosclerosis, suggesting ILK as a therapeutic target.

Methods

An expanded Methods section is available in the Data Supplement at http://circres.ahajournals.org.

Human Arterial Specimens

Human carotid endarterectomy specimens were obtained from 25 patients (21 men: mean age, 75±5 years; 4 women: mean age, 71±8 years) with >70% carotid stenosis as demonstrated by digital subtraction angiography and Doppler ultrasonography. Nonatherosclerotic internal mammary artery specimens (controls) were obtained from 10 male patients (mean age, 65±5 years) undergoing coronary artery bypass surgery. All samples were obtained as surgical residues in accordance with local ethics committee regulations.

Conditional ILK Knockout Mice

Conditional inactivation of the ILK gene was accomplished by crossing mice carrying the floxed ILK allele (genotype: ILK<sup>KO</sup>/KO) with Cre<sup>ERT</sup> transgenic mice, which express Cre under the control of the cytomegalovirus promoter. Eight-week-old male mice were injected intraperitoneally with 1.5 mg tamoxifen (dissolved in a 10:1 volumetric mix of sunflower seed oil and ethanol) once a day for 5 consecutive days. Animals were killed 20 days after the last injection. Ultrasound images were captured 3–5 days before euthanasia. In selected experiments, L-NAME (intravenous; 10<sup>−5</sup> mol/L per day for 4 days) was administered before ultrasound recordings. A total of 50 animals were used. All animal procedures were approved by the institutional animal care and use committee.

Endothelial ILK Is Downregulated in Atherosclerotic Human and Mouse Arteries

ILK expression levels and distribution were determined by immunohistochemistry in cross sections of endarterectomy specimens of human carotid arteries (n=25) showing varying degrees of atherosclerosis and in mammary arteries obtained from patients undergoing coronary bypass surgery (controls) (n=10).

Mammary arteries, with no sign of atherosclerosis by Masson trichrome staining, showed ILK expression in the adventitia, homogeneous staining for ILK in the media, and strong staining in the endothelial layer (Figure 1A, panel a). Intimal ILK expression was lower in endarterectomy specimens from carotid arteries showing moderate restenosis but no atherosclerotic plaque (Figure 1A, panel b). In atherosclerotic endarterectomy specimens, ILK staining was absent from the intima and was limited to dispersed foci in the primary plaque, whereas staining was dense and homogeneous in the restenotic regions (Figure 1A, panel c). Thus, ILK shows a radically different distribution in atherosclerotic arteries compared with nondiseased tissue, the most significant change being its disappearance from the endothelial cell layer as atherosclerosis progresses. The integrity of the endothelial layer was confirmed by eNOS staining. Despite these changes, Western blot analysis revealed only a small and nonsignificant decrease in total ILK expression in atherosclerotic carotid arteries (Figure 1B).

En face confocal immunostaining of mouse aorta revealed reduced ILK staining in the endothelium of atherosclerotic ApoE KO mice compared with wild-type (WT) mice, and ILK expression was also reduced in ApoE KO mouse aortic endothelial cells (MAEC) (Figure 1C and 1D).

ILK Regulates Vascular Reactivity In Vivo

To examine the role of ILK in endothelial function, we generated a tamoxifen-inducible ILK-deficient mouse. Cre recombinase was activated in Cre<sup>Lox</sup>/ILK<sup>KO</sup> (ILK Cre-Lox) mice by injection with 4-hydroxytamoxifen (TXF). Cre-recombinase-mediated excision of the Lox-P site from the floxed ILK gene in TXF-treated ILK Cre-Lox mice (cKO) was confirmed by PCR. Excision was not observed in vehicle-treated ILK Cre-Lox animals (CT) or TXF-treated Cre<sup>Lox</sup>/ILK<sup>KO</sup> (Lox) mice (Online Figure I). Efficient deletion of ILK after TXF administration was also observed by Western blot and immunohistochemical analysis of the aortic wall of cKO mice (Figure 2A and 2B), with ILK expression...
absent from vascular endothelium and reduced by 70% in smooth muscle cells. Intense ILK staining was maintained in the vascular wall of both CT and TXF-treated Lox mice (Online Figure I). Moreover, expression levels of the vascular smooth muscle marker \(\alpha\)-SMA and the endothelial cell marker PECAM-1 were similar in CT and cKO mice, indicating that ILK deletion had no significant effect on vascular wall structure (Online Figure I).

To test endothelium-dependent vasorelaxation, CT and cKO ILK Cre-Lox mice were sequentially injected with 10\(^{-10}\) and 10\(^{-8}\) mol/L acetylcholine (Ach), and vessel-wall diameter and Doppler blood flow in the left carotid artery were measured by high-resolution ultrasound imaging (35 MHz). Ach caused dose-dependent vasodilatation of the carotid artery in CT mice but had no significant vasodilator effect in cKO animals (Figure 2C). CT and cKO animals both
responded strongly to intravenous administration of $10^{-7}$ mol/L sodium nitroprusside, indicating that NO signaling remained functional and that the loss of the Ach-induced vasodilatory response in ILK-deleted animals might reflect impaired NO production. The vasodilatory action of Ach in CT mice was accompanied by increased mean carotid Doppler blood flow, whereas this increase was not seen in cKO mice, suggesting a direct effect of ILK on the vasodilatory action of Ach (Figure 2D).

We next analyzed the contribution of relaxing factors (NO, prostacyclin, and endothelium-derived hyperpolarizing factor [EDHF]) on Ach-mediated vascular responses in CT mice. Mice were pre-treated with L-NAME (10^{-5} mol/L) to inhibit NO synthesis or indomethacin (10^{-5} mol/L) to inhibit prostaglandin synthesis or both drugs 30 minutes before Ach (Figure 2E). L-NAME reduced vessel diameter compared with saline. Subsequent administration of $10^{-8}$ mol/L Ach did not produce an increase in diameter comparable with saline-injected mice (27±7% versus 81±10%). Indomethacin pretreatment had a limited inhibitory effect on Ach-induced dilation, whereas L-NAME and indomethacin coadministration almost completely blocked Ach effect. These findings show that the response of the mouse carotid artery to Ach depends largely on NO production. EDHF does not play a relevant role in Ach effects, since L-NAME plus indomethacin inhibited the Ach response by 85±4%.

Activity of the NO/cGMP Pathway Is Reduced in Vessels and Endothelial Cells of cKO Cre-Lox Mice

We first analyzed NOS isoform expression in ILK-CT and cKO mice, finding no significant differences for eNOS (Figure 3A and Online Figure II) or nNOS and iNOS (Online Figure II). However, isolated aortic rings from cKO animals failed to produce cGMP in response to Ach (Figure 3B), whereas treatment with the NO donor sodium nitroprusside increased cGMP production 11.8±1.2-fold, confirming that the sGC pathway is functional in cKO mice.

In further experiments, MAEC cultured from ILK Cre-Lox mice were treated with vehicle (CT MAEC, expressing ILK) or TXF to delete ILK (cKO MAEC). Treatment with the eNOS agonists calcium ionophore (A23187) and vascular endothelial growth factor (VEGF) induced accumulation of nitrite in the supernatants of CT MAEC, but this was
abolished in cKO MAEC (Figure 3C). To examine the effect of ILK deletion on eNOS activity, we compared conversion of L-arginine with L-citrulline in Ach-treated CT and cKO MAEC (Figure 3D). Although eNOS expression was similar in both cell populations, NO production was significantly lower in cKO cells. This lower NO production was paralleled by enhanced production of superoxide, detected by DHE oxidation (Figure 3D), suggesting that eNOS activity might be uncoupled in cKO cells.

**ILK Deletion Leads to eNOS Uncoupling and Protein Nitration**

Pretreatment of cells with L-NAME prevented Ach-induced DHE oxidation in both cell populations, indicating that superoxide formation was due to eNOS activity (Figure 4A). Neither 7-nitroindazole (inhibitor of nNOS) nor 1400W (inhibitor of iNOS) prevented superoxide formation, indicating that nNOS and iNOS are not involved in superoxide production. Inactive BH2 is reduced back to inactive BH4 by regeneration enzymes such as dihydrofolate reductase (DHFR) to replenish cellular BH4, which is also synthesized de novo from GTP by GTP-cyclohydrolase I (GTP-CHI). GTP-CHI expression was similar in CT and cKO mice, whereas the steady-state levels of DHFR decreased in cKO MAEC, indicating that ILK deficiency decreases the rate of BH4 regeneration, thus disrupting the BH4/BH2 balance (Figure 4D).

Factors that contribute to eNOS uncoupling include oxidation of the critical NOS cofactor tetrahydrobiopterin (BH4) to 7,8-dihydrobiopterin (BH2), depletion of L-arginine, reduced BH4 regeneration rate and reduction in BH4 levels due to defective synthesis.28,27 Both the BH4/BH2 ratio and BH4 levels determine the outcome of NOS activity and are under stringent control. HPLC analysis of aortic extracts revealed that BH4 levels are decreased in ILK-cKO mice, accompanied by an increase in BH2 (Figure 4C), whereas the total pteridine concentration remained unchanged. WT ILK overexpression reversed this effect and restored the BH4/BH2 ratio (Online Figure III). Inactive BH2 is reduced back to BH4 by regeneration enzymes such as dihydrofolate reductase (DHFR) to replenish cellular BH4, which is also synthesized de novo from GTP by GTP-cyclohydrolase I (GTP-CHI). GTP-CHI expression was similar in CT and cKO mice, whereas the steady-state levels of DHFR decreased in cKO MAEC, indicating that ILK deficiency decreases the rate of BH4 regeneration, thus disrupting the BH4/BH2 balance (Figure 4D).

To identify the source of the superoxide responsible for BH4 deficiency in ILK-deficient mice, we measured superoxide production in ILK-cKO MAEC in the presence of specific inhibitors of candidate generating systems: NADPH oxidase, a major endothelial source of superoxide (Apocynin, 10−4 mol/L), NOS (L-NAME, 10−4 mol/L), xanthine oxidase (Allopurinol, 10−4 mol/L), and the mitochondrial respiratory chain (Rotenone, 10−5 mol/L). Allopurinol and rotenone had no effect on superoxide production, whereas apocynin reduced superoxide but did not reverse the effect of ILK deficiency. In contrast, L-NAME blocked the increase in superoxide in cKO MAEC (Figure 5A). To confirm eNOS as the source of the increased superoxide, we siRNA-silenced ILK expression in MAEC from eNOS-deficient mice. ILK
Figure 4. ILK deletion increases superoxide production and protein nitration in intact vessels and endothelial cells. A, Superoxide produced by CT and cKO MAEC untreated (NT), treated with L-NAME (10^{-4} mol/L, 18 hours), or with Ach (10^{-7} mol/L, 30 minutes), or with Ach + L-NAME (n=5, mean±SD; *P<0.05 versus NT CT, **P<0.05 versus CT). B, Confocal detection of 3-nitrotyrosine in aortic sections from CT mice, cKO mice, and cKO mice treated with L-NAME (n=3, bars=75 μm). C, HPLC analysis of the content of total biopterins, BH4 and BH2, in aortic extracts from CT and cKO mice (n=10, mean±SD; *P<0.05 versus CT BH4; **P<0.05 versus CT BH2). D, Immunoblot analysis of GTP-CH I, DHFR, ILK, and GADPH in extracts from CT and cKO MAEC. Two independent samples of each cell type are shown (n=5, mean±SD; *P<0.05 versus CT).

silencing increased superoxide anion production in WT but not eNOS-KO MAEC, demonstrating a primary role for eNOS in superoxide production (Figure 5B). Furthermore, exposure of ILK Cre-Lox MAEC to apocynin from 24 hours before the initiation of tamoxifen-induced ILK depletion did not prevent the increase in superoxide production (Figure 5C), whereas treatment with L-NAME under identical conditions did so.

The increased superoxide production by cKO MAEC was reversed by overexpression of WT ILK, which also led to a small but nonsignificant reduction in superoxide production in CT MAEC (Figure 5D). Accordingly, ILK overexpression reversed the effects of ILK depletion on NO production, as measured by DAF-2 oxidation, and restored NO release to CT levels (Online Figure III). In addition, eNOS-KO MAEC show a significant decrease in DHFR expression, compared with WT cells. ILK silencing decreased DHFR levels in WT but not in eNOS KO MAEC, demonstrating a novel role for endothelial NO availability in regulating BH4:BH2 ratio by the salvage pathway (Figure 5E).

ILK Interacts With eNOS in Endothelial Cells Through Hsp90

ILK was originally identified as a Ser/Thr kinase that also acts as a scaffold protein that sustains multiprotein complexes. To investigate whether ILK-mediated coupling of eNOS to NO production depends on ILK kinase activity, cKO MAEC were transfected with V5-tagged WT ILK (ILK-WT), kinase-dead ILK (ILK-kDa), or empty vector, and eNOS activity was analyzed after stimulation with calcium ionophore. ILK-WT and ILK-kDa were equally effective at restoring eNOS activity in cKO MAEC (Online Figure IV). ILK can activate Akt, which activates eNOS via phosphorylation on Ser1177. Treatment with the Akt activators VEGF or calcium ionophore increased eNOS phosphorylation in CT MAEC but not in cKO MAEC, although Akt phosphorylation is increased in cKO MAEC (Online Figure IV). Taken together, ILK Ser/Thr kinase activity does not contribute to the effects observed on ILK depletion.

To test whether ILK interacts with eNOS by acting as a chaperone protein we used a yeast 2-hybrid strategy. Neither full-length ILK nor fragments corresponding to residues 1–150 or 155–452 showed a positive interaction with either the heme oxygenase or the reductase domain of eNOS (Online Table I). Control experiments showed strong interaction between eNOS heme oxygenase domain monomers and between the heme oxygenase and reductase domains.

The most likely explanation of these results is that ILK associates with eNOS through a linker protein, a likely candidate being Hsp90, a binding partner of both eNOS and ILK. Immunoprecipitation of eNOS from aortic lysates detected coprecipitation of ILK and Hsp90 in CT mice, and the levels of both proteins in eNOS immunoprecipitates were significantly lower in cKO extracts (Figure 6A). Association between ILK, eNOS, and Hsp90 was confirmed by cross coimmunoprecipitation with anti-ILK and anti-eNOS antibodies in CT and cKO MAEC lysates (Figure 6B). Immunoblotting confirmed similar Hsp90 levels in CT and cKO mice (Figure 6B: input). These results suggest that ILK may serve as a scaffold protein in a complex that regulates eNOS activity in endothelial cells.

To confirm the clinical significance of these results, we examined ILK interactions in human tissue extracts. Immuno precipitation of ILK and eNOS from extracts of human mammary and carotid artery detected interaction between ILK and eNOS in nonatherosclerotic tissue, but this interaction was absent in atherosclerotic samples (Figure 6C). Moreover, Hsp90 was coprecipitated with ILK or eNOS in protein extracts of healthy mammary arteries and this interaction was reduced in atherosclerotic carotid arteries.

To isolate complexes containing eNOS-interacting proteins, we passed cell lysates through Ni-NTA agarose columns loaded with 6xHis-tagged recombinant eNOS. Lysates of bovine aortic endothelial cells (BAEC), which express high levels of ILK, were added to the column and eluted proteins were detected by immunoblot. ILK and Hsp90 were eluted from the column together with eNOS in lysates of...
untreated BAEC, but pretreatment of cells with the Hsp90 inhibitor geldanamycin significantly reduced the levels of coeluting ILK and eNOS (Figure 7A). Similar results were obtained when MAEC were treated with GA and subjected to ILK immunoprecipitation (Figure 7B).

The role of ILK in regulating eNOS-Hsp90 interaction was confirmed by overexpression of WT ILK in cKO cells, which reverted the effects of ILK deletion on the eNOS:Hsp90 ratio (Figure 7C). These results indicate that ILK regulates eNOS activity by stabilizing a multiprotein complex of which Hsp90 is an essential component.

Discussion

Our results identify an important role for ILK as a mediator of endothelial function. By stabilizing the association of eNOS with Hsp90 in a signaling complex, ILK protects eNOS from enzymatic uncoupling and promotes the physiological production of NO rather than oxidative derivatives, thus preventing endothelial dysfunction.

We have established a link between atherosclerosis and decreased expression of ILK in the endothelium in human atherosclerotic blood vessels and in atherosclerotic mice. Proatherogenic stimuli such as oxidized LDL and angiotensin II regulate ILK activity and expression in cultured endothelial and vascular smooth muscle cells. ILK expression is also reduced in several models of arterial injury characterized by intimal thickening. These findings suggest a role for ILK in the progression of pathological vascular remodeling such as atherosclerosis.

In the cardiovascular system, ILK plays a role in blood vessel integrity and participates in angiogenesis and endothelial cell migration. ILK also has a critical role in cardiac development and function, particularly as a component of the cardiac stretch sensor. However, little is known about the postnatal physiological role of ILK in relation to NO synthesis and vascular function. Our use of a conditional ILK knockout (KO) model circumvents the embryonic lethality of conventional ILK KO. Conditional deletion of ILK in adult mice resulted in vasomotor dysfunction as a consequence of impaired endothelial production of nitric oxide. Individual vascular beds exhibit differences in vascular reactivity. NO is generally regarded as a major contributor to endothelium-dependent relaxation in large conduit arteries, whereas EDHF may play an important role in small arteries. However, our
results demonstrate that the response to Ach was largely
dependent on NO production and EDHF had only a small
contribution.

eNOS uncoupling, characterized by increased production of
superoxide by eNOS and reduction of NO production, is a
common feature of hypertension and atherosclerosis. Our
data show that ILK deficiency leads to a shift in eNOS
activity from NO production to superoxide production, sug-
gesting eNOS uncoupling as the underlying mechanism for
the observed effects. Indeed, ILK overexpression reversed
eNOS uncoupling and restored eNOS enzyme activity.

PKG-1, the target of cGMP, was nitrated in the absence of

Figure 6. ILK immunoprecipitates with
eNOS. A, Aortic protein lysates from CT
and cKO mice were immunoprecipitated with anti-eNOS antibody, and ILK. Hsp90,
and eNOS were analyzed by immunoblot. Representative immunoblots for each
group are shown (n=4, mean±SD; *P<0.05 versus CT mice). B, Protein lysates from CT and cKO MAEC were
immunoprecipitated with anti-eNOS or anti-ILK antibodies, and ILK. Hsp90, and
eNOS content was analyzed by immuno-
blot (n=4, mean±SD; *P<0.05 versus CT
MAEC). C, Protein extracts from human
mammary artery (M) or atherosclerotic
carotid artery (C) were immunoprecipi-
tated with anti-eNOS or anti-ILK antibod-
ies, and ILK, eNOS, and Hsp90 levels
were analyzed by immunoblot (white
bars: mammary artery; black bars:
carotid artery; n=6, mean±SD; *P<0.05
versus M).

Figure 7. ILK and eNOS interact
through Hsp90. A, BAEC were treated
with geldanamycin (10⁻⁶ mol/L, 18 hours)
and protein lysates were passed through
an Ni-NTA agarose column loaded with
6xHis-tagged full-length recombinant
eNOS. Bound proteins were eluted and
ILK, Hsp90, and eNOS content was ana-
lyzed by immunoblot (NT: nontreated; GA:
geldanamycin; n=3). "Input" shows
eNOS, Hsp90, and ILK levels in input
samples. Blots are from a representative
experiment. B, Protein extracts from
MAEC, treated as in
A, were immunopre-
cipitated with anti-ILK antibody, and ILK,
eNOS, and Hsp90 levels were analyzed
by immunoblot. A representative experi-
ment of 3 is shown
C, Effect of ILK over-
expression on eNOS complex formation.
CT (white bars) and cKO MAEC (gray
bars) were untransfected or transfected
with V5-tagged WT ILK (black bars) and
then immunoprecipitated with anti-ILK.
Data are mean±SD (n=3; *P<0.05 versus
untransfected CT MAEC; **P<0.05 versus
untransfected cKO MAEC).
ILK silencing in eNOS null MAEC did not downregulate and subsequent decrease in NO production by eNOS, since downregulation is a direct consequence of ILK deletion in the absence of ILK. Our data strongly suggest that DHFR downregulation could contribute secondarily or in parallel to BH4/BH2 imbalance. ILK reexpression in cKO MAEC reduced superoxide production, increased NO production, and restored the BH4:BH2 ratio, supporting the idea that ILK deficiency is upstream of BH4/BH2 imbalance in eNOS uncoupling.

In search of a mechanism that explained how ILK regulated eNOS and its production of NO and superoxide, we discovered that ILK interacts with eNOS in endothelial cells, in CT mice, and in human healthy mammary arteries. This interaction is not present in human atherosclerotic arteries or ILK-deficient vessels. However, this interaction is indirect and is prevented by inhibition of Hsp90, strongly suggesting that eNOS and ILK form part of a multiprotein complex containing Hsp90. Accordingly, ILK overexpression in cKO MAEC reversed the observed defect in eNOS-Hsp90 association. Hsp90 binds to and stabilizes eNOS, promotes NO generation, and inhibits eNOS-mediated superoxide anion generation. ILK also interacts with Hsp90, preventing ILK degradation by the proteasome. It may be that Hsp90 regulates interaction between eNOS and ILK, because Hsp90 can interact with other client proteins that could compete with ILK or even recruit ubiquitin ligases that could regulate protein stability of either eNOS or ILK. Our data thus suggest a role for ILK-eNOS-Hsp90 interaction in eNOS activity and indicate that disruption of this interaction might underlie the endothelial dysfunction associated with vascular diseases such as atherosclerosis.

Considering that ILK forms part of a signaling platform that transduces mechanical forces (such as shear stress) to the cell interior, the interaction of ILK with Hsp90 and eNOS provides an additional control on eNOS enzymatic activity.

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Disclosures

None.

References

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Novelty and Significance

What Is Known?

- Integrin-linked kinase (ILK) is a serine threonine kinase that binds to the cytoplasmic domain of integrins.
- ILK forms part of a multiprotein signaling platform that transduces mechanical forces (such as shear stress) activating multiple intracellular signaling pathways.
- ILK is critical for cardiovascular development and regulates vascular repair processes.
- Endothelial nitric oxide synthase (eNOS) uncoupling, characterized by increased production of superoxide by eNOS and reduction of NO production, is a common feature of hypertension and atherosclerosis.
- Atherosclerosis develops in areas of disturbed blood flow whereas laminar flow protects from atherogenesis.

What New Information Does This Article Contribute?

- ILK participates in the regulation of vasomotor tone.
- ILK regulates endothelial nitric oxide production.
- ILK is decreased in endothelium from atherosclerotic human and mouse blood vessels.
- ILK interacts eNOS and protects eNOS from enzymatic uncoupling.

The role of ILK-mediated regulation of vascular function in shear stress–related vascular diseases such as atherosclerosis has not been studied previously. Our results show a reduction in endothelial ILK expression in human and mouse atherosclerotic blood vessels compared with healthy blood vessels. Conditional deletion of ILK in adult mice resulted in vasomotor dysfunction as a consequence of impaired endothelial production of nitric oxide. ILK deficiency led to eNOS uncoupling, reflected by increased eNOS-dependent superoxide anion production and extensive vascular protein nitration. ILK deletion decreased BH4 levels, a cofactor for NOS enzymes required for efficient NO production, accompanied by an increase of its oxidation product BH2. The decrease in BH4 levels in KO MAEC could be attributable to defective BH4 regeneration by an ILK-dependent downregulation of DHFR (dihydrofolate reductase), the enzyme that reconverts BH2 to BH4. We show that ILK stabilizes association of eNOS with Hsp90 in vivo, thereby ensuring coupling of eNOS activity to physiological production of NO rather than oxidative derivatives. We found that this association was disrupted in atherosclerosis. The interaction of ILK with Hsp90 and eNOS provides an additional level of control of eNOS enzymatic activity, suggesting ILK as target for therapeutic intervention.
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SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

MATERIALS

Recombinant human VEGF and ILK antibody were from R&D Systems. eNOS antibody was from BD Biosciences. Anti-rabbit secondary antibody (Hilyte Fluor 647 conjugate) was from AnaSpec. Protein A/G PLUS- Agarose were from Santa Cruz Biotechnology. V5 antibody, dihydroethidium (DHE) and Lipofectamine were from Invitrogen. HRP-conjugated anti-mouse secondary antibody and liquid DAB substrate were from Dako (Carpinteria, CA). Bovine recombinant eNOS was from Cayman Chemical (Ann Arbor, MI). Human recombinant 6xHis tagged ILK was from AbCam Laboratories (Cambridge, MA). Tissue culture media, ECB, matrigel and growing supplements were from BioWhittaker. Geldanamycin was from Stressgen Bioreagents. Collagenase type IA, A23187, DAN, IBMX, Ach, SNP, L-NNAME, indomethacin, apocynin, allopurinol, rotenone, tamoxifen, HRP-conjugated anti-rabbit secondary antibody and GADPH antibody were from Sigma-Aldrich. All other antibodies were from Cell Signaling Technology.

Mice

Conditional KO model

Cre-Lox mice were genotyped by tail DNA analysis. Cre primers were 5'-CATTTCGTGGGATGTGCTTATAACAC-3' and 5'-TATTGAAACTCCAGCGGGCC-3', yielding a 391-bp fragment. ILK genotype primers were 5'CCAGGTGGCAGAGGTAAGTA-3' and 5'CAA GGAATAAGGTGAGCTTCAGAA-3', yielding amplimers of 2.1-kb and 230-bp for the floxed and Cre-excised alleles, respectively.

ApoE knock-out mice and diet

Wild-type C57BL/6 mice, and apoE-KO mice were purchased from The Jackson Laboratory. Atherosclerosis was induced in mice fed with Western diet for 16 weeks (42% fat, Harland Teklad, TD88137). 8 weeks ApoE KO mice used as control were fed regular diet. All animals were housed in isolated rooms.

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cell culture and transient transfection.

Mouse aortic endothelial cells (MAEC) were cultured from aortas extracted from anesthetized Cre-lox mice or ApoE-KO mice as described previously. Cells were purified by flow cytometry (Dako Cytometrix). Purification was verified by confocal immunofluorescence with mouse monoclonal anti-ICAM-2 and anti-PCAM-1 antibodies (BD Biosciences). The purity of MAEC cultures used for experiments was greater than
99%. To delete ILK, MAEC cultures were treated with 1μmol/L tamoxifen or vehicle for 96 h.

MAEC were transfected with empty vector (pcDNA) or plasmids encoding wild-type ILK (ILK-WT) or kinase-dead ILK (ILK-KD) using Lipofectamine.

Bovine aortic endothelial cells (BAEC) were isolated as previously described. Studies were performed on confluent monolayers at passages 2-7, made quiescent by overnight serum deprivation.

Immunohistochemistry.

For human samples, specimens were fixed in 10% neutral buffered formalin within 2 min of surgical removal. Serial 3 µm thick sections were cut from paraffin blocks and mounted on poly-L-lysine (Sigma) coated slides. Serial sections were stained with hematoxylin & eosin and Masson trichrome to evaluate morphological damage by light microscopy (Eclipse 50i, Nikon, Tokyo, Japan), according to American Heart Association recommendations. All carotid specimens contained ruptured lipid-rich plaques, and thin fibrous cap atheromata occurred together with other stages of atherosclerosis in the same specimen. All mammary artery segments showed adaptive intimal thickening and duplication of the internal elastic membrane.

Aortas harvested from control ILK Cre-Lox mice (CT) and tamoxifen-treated ILK Cre-Lox mice (cKO) were paraffin embedded, cut into 3-5 µm serial sections and incubated with anti-ILK antibody (Millipore). Aortic sections from human and mice specimens were then incubated with biotin-conjugated goat anti-mouse antibody (Dako) followed by exposure to avidin–peroxidase complex (Dako). Stain was developed by addition of diaminobenzidine substrate (DAB; Dako). Samples were counterstained with Mayer’s hematoxylin and mounted with DPX. Sections from cKO and WT mice were reacted with antibodies to ILK, eNOS and nitrotyrosine, and staining was visualized by confocal microscopy after incubation with anti-rabbit secondary antibody conjugated to HiLyte Fluor 647 (excitation wavelength 653 nm; emission wave length 671 nm). Nuclei were stained with Hoechst. Negative controls were obtained by omitting primary antibodies.

En face immunohistochemical measurement of endothelial ILK expression

To measure ILK in endothelium ex vivo, aortas were extracted from 8 week-old C57/BL6 mice, 8 week-old old ApoE-KO mice fed a regular diet and ApoE-KO mice fed a high-fat diet for 16 weeks. Aortic rings were cut into 30-μm-thick segments and incubated with antibodies to ILK (red) and ICAM-1 (endothelial cell marker) green. The rings were then washed three times, and the endothelial layer was visualized in en face preparations with a Zeiss LSM 510 Meta confocal microscope (Zeiss, Göttingen, Germany). Fluorescent images were captured at ×10 magnification. Hoechst 33258 (Sigma Aldrich) was used to visualize nuclei. Intensity of endothelial ILK and ICAM-1 staining was analyzed using Imaris x64 7.3.1 (Bitplane, Zurich, Switzerland) imaging software. Ten full fields were analyzed per vessel, with one image per field. The mean fluorescence intensities of stained cells in the endothelium detected as ICAM-1 positive cells were calculated for each ring, and averaged for each animal in the group. Unstained aortas and vessels preincubated with polyethylene glycol (PEG)-SOD were used for background correction and negative control, respectively.

Micro-ultrasound imaging.

Ultrasound imaging parameters of the left carotid artery were measured with the Vevo2100 system (Visualsonics, Toronto, Canada). Images were captured with a 55-
MHz scan head with a 4.5-mm focus and an axial resolution of 30 \( \mu \text{m} \). Mice were anesthetized with 2% isoflurane gas, yielding a heart rate of \(~500\) beats/min. A long axis view in B mode was used to identify the carotid artery by its characteristic flow pattern. Probe position was optimized to show clear vessel wall/lumen interfaces, allowing automated recognition by the analysis software. Experiments were started after a 15-min equilibration period to allow achievement of a stable core body temperature. Flow dilatation was measured before and after infusion of acetylcholine (Ach) or sodium nitroprusside (SNP). After obtaining baseline readings for diameter and heart rate, control injections (saline) were administered via tail-vein catheter (30G; BD Biosciences). Readings were taken immediately after injection (0 min; t 0) and after 1, 2, 4 and 10 min. 15 min after the last measurement, two increasing bolus doses of Ach (\(10^{-10}\) and \(10^{-8}\) mol/L) were administered at 15 min intervals, and recordings taken 0, 1, 2, 4, and 10 min after each injection. To test endothelium-independent vasorelaxation, SNP was infused at \(10^{-7}\) mol/L, (30\( \mu \text{L}\)) and recordings taken as above. In another experimental group, mice were pretreated with saline, L-NAME (\(10^{-5}\) mol/L), indomethacin (INDO) (\(10^{-5}\) mol/L) or both drugs 30 min before infusion with Ach and SNP. Every treatment was injected in a 30 \( \mu \text{L} \) volume. Image and blood-flow analyses were performed off-line from recorded loops using an automated system (Brachial Analyzer 5, Medical Imaging Applications, Coralville, IA). Baseline and pharmacologically-induced flows are expressed as mean flow (mL/min) calculated from flow velocity and vessel cross-sectional area. Dilatation was expressed as the percentage diameter change from baseline (time 0). Diameter readings represent the difference between systolic and diastolic diameters, and flow velocity represents the mean-angle-corrected doppler-flow velocity.

**Nitrite assay and NO production**

The diaminonaphthalene (DAN) assay was performed in MAEC supernatants as described in Misko et al with minor modifications. The fluorescent signal was read on a Perkin Elmer LS50B luminescence spectrometer (excitation 330 nm, emission 426 nm).

NO production was measured in cells loaded with DAF-DA diacetate (5mM), and propidium iodide (1 \( \mu \text{g/mL} \)) was used to determine cell viability. After exposure to different experimental conditions, cells were trypsin-dispersed and labeled with the fluorochromes at 37°C, followed by cytofluorometric analysis with a Fluorescence Activated Cell Sorted scanner (Becton Dickinson, New York, NY). A total of 10,000 events were analyzed for each condition.

**Measurement of cGMP in mouse aortic rings.**

Mouse aortas were harvested after tamoxifen treatment and cut into 15 \( \mu \text{m} \) rings. Rings were immersed immediately in a solution of 3-isobutyl-L-methylxanthine (IBMX; 0.1 mmol/L) and incubated at 37°C for 5 min with Ach (\(10^{-6}\) mol/L) or SNP (\(10^{-7}\) mol/L). cGMP was measured with a RIA kit (GE Healthcare) as described previously. Four aortas from four mice were examined per experimental group.

**Immunoblotting.**

Immunoblotting was performed as described. Immunoreactive bands were visualized with the SuperSignal detection system (Pierce).

**eNOS catalytic activity.**

eNOS catalytic activity in intact MAEC was determined by measuring the conversion of \([^{3}\text{H}]\) L-arginine to \([^{3}\text{H}]\) L-citrulline with a NOS Activity Assay Kit (Cayman Chemical).
RNA interference

ILK expression was silenced by transfecting MAEC with siRNA targeting human ILK (Cell signaling). Transfection was performed with Lipofectamine 2000 according to the manufacturer’s instructions. Gene silencing was monitored by immunoblot of cell extracts isolated 96 h post transfection. Controls were performed with scrambled siRNA (Ambion).

Immunoprecipitation.

Extracts from aortas or MAEC were pre-cleared with protein A-sepharose and control IgG corresponding to the host of the primary antibody. After centrifugation the cell lysate was incubated overnight with the appropriate antibody and protein A-sepharose. The pellets were collected by centrifugation, washed 3 times with RIPA buffer, and resuspended in electrophoresis sample buffer.

Superoxide anion production.

MAEC were incubated with vehicle or 0.1 mmol/L L-NAME for 18 h, harvested, and treated with 10^−5 mol/L diphenyleneiodonium chloride (DPI) for 30 min to inhibit NADPH oxidase. Cells were then treated with 10^−7 M Ach for 30 min followed by 1 μmol/L DHE for 15 min, and superoxide was determined by FACS (FASCalibur, Becton Dickinson).

Pteridine measurements

For oxidation under acidic or alkaline conditions, frozen isolated mouse aortas were part-thawed and homogenized in 0.5 vol of acid-iodine solution (2% I₂, 3% KI in 0.1N HCl) or alkaline-iodine solution (2% I₂, 3% KI in 0.2N NaOH). Biopterin and pterin were determined by fluorescence HPLC (excitation, 350; emission, 450 nm). The solid phase was Fine-SIL C18T-5 and the mobile phase was 7% methanol. I₂-oxidation under acidic conditions stoichiometrically converts biopterins of various redox states—such as tetrahydro-, quinonoid dihydro- and 7,8-dihydrobiopterin—to fully-oxidized biopterin; therefore biopterin measured after acid-I₂ oxidation was defined as total biopterin. Tetrahydrobiopterin converts to pterin in a selective and stoichiometric manner. We thus defined BH4 as the difference between total biopterin after acid oxidation and biopterin after alkaline-oxidation. Since the amount of fully-oxidized biopterin detected in fresh tissue extracts was insignificant, the difference between “total biopterin” and “BH4” was defined as “BH2”. BH4, BH2 and total biopterin content were quantified by comparison with external standards and normalized to sample protein content.

RNA interference

We performed gene silencing of ILK, by transfecting MAECs with human ILK (Cell signaling). Transfection was performed with Lipofectamine 2000 according to the manufacturer instructions. Gene silencing was monitored by immunoblot of cell extracts isolated 96 hours after transfection. Controls were performed by with a scrambled siRNA (Ambion).

Yeast two hybrid analysis of ILK–eNOS interaction.

The eNOS heme domain (residues 1-527) was amplified by PCR and cloned into the yeast two-hybrid vectors pGBT9 and pGAD (Clontech). The bait plasmid pGBT9 expresses the binding domain of GAL4 in frame with the desired protein, whereas the prey plasmid pGAD expresses the activator domain of GAL4. In parallel, the PCR-amplified reductase domain of eNOS (residues 489-1205) was cloned into pGAD, while human ILK (a generous gift of Dr. Dedhar, British Columbia Cancer Research Center) was PCR amplified and was cloned into pGBT9. Three ILK constructs were generated:
full-length (residues 1-452), the ankyrin repeat domain (residues 1-150) and the catalytic domain (residues 155-452). These three ILK constructs were confronted in a yeast two-hybrid screen with the heme oxygenase and reductase domains of eNOS. As positive controls the heme oxygenase domain was confronted with itself, since this domain is homodimeric, and with the reductase domain, since these domains are known to interact efficiently. Double transformants were plated in Leu-/Trp- plates whereas positive interactions were selected in Leu-/Trp-/His- plates. Interacting proteins were confirmed using the X-Gal assay\textsuperscript{12}.

**eNOS pull-down assay.**

Approximately 300 µL of a Ni-NTA agarose resin were loaded in a pasteur pipette and the resins allowed to equilibrate in PBS containing 100 µM CaCl\(_2\) and protease inhibitors (buffer A). Full-length recombinant eNOS (250 µg) was loaded twice, to bind it to the resin through the hexa-His tag at the N-terminus\textsuperscript{13}. Columns turned slightly brown upon loading with full-length eNOS. Columns were washed with 3 mL buffer A and lysates from control or geldanamycin-treated (1µmol/L, 18 h) BAEC were loaded. The columns were subsequently washed with 5 mL buffer A and the recombinant protein and bound proteins were eluted in 200 mmol/L imidazole in buffer A. The eluted fractions were collected and ILK, Hsp90 and eNOS were detected by western blot.

**Statistical Analysis.**

Every experimental condition was duplicated within each experiment and each experiment was repeated at least three times. For animal studies, n values refer to the number of individual animals used. Comparisons were made by analysis of variance, followed by Dunnett’s modification of the \(t\)-test when comparisons were made with a common control, and the unpaired two-tailed Student’s \(t\)-test for other comparisons. Results are expressed as mean±SD, and differences were considered statistically significant at \(p<0.05\).

**REFERENCES**


Online Figure I. A. PCR analysis of blood tail DNA from eight ILK tamoxifen-conditional ILK Cre-Lox knockout mice (Cre+/ILK\(^{fl/fl}\)) treated with vehicle (CT) and tamoxifen (cKO). In CT mice, a single band corresponding to the floxed alleles (2.1kb) is detected. In cKO mice, the excised allele (230bp) is detected. A representative example of 20 analyzed is shown. B. PCR analysis of blood tail DNA from ILK\(^{fl/fl}\) mice treated with vehicle (VH) or tamoxifen (TXF). In both groups only the 2.1kb band corresponding to the floxed alleles is seen; tamoxifen did not excise ILK. A representative example of 20 analyzed is shown. D. Detection of ILK in aortic rings from ILK\(^{fl/fl}\) mice treated with vehicle (VH) (a) or tamoxifen (TXF) (b). Panels (c) and (d) show secondary antibody controls. Magnification 100X. E. Immunostaining of endothelium (PECAM1) and smooth muscle (\(\alpha\)-SMA) in aortic rings from CT or cKO mice. Magnification 100X.
Online Figure II. A. Confocal detection of total eNOS (red) in aortic rings from CT and cKO mice. (n=8, Bar=50 μm, nuclei Hoechst blue). B. Immunoblot of nNOS and iNOS protein in aortic extracts from CT and cKO mice. Lower. The bar chart shows relative NOS bands intensities from 10 mice per group. Data are means ±SD; *p<0.05 vs CT. C. Immunohistochemical detection of nNOS and iNOS in aortic rings from CT and cKO ILK mice. D. Flow cytometry measurement of superoxide anion production in CT and cKO MAEC non-treated (NT), MAEC incubated with L-NAME (10^{-4} mol/L, 18 h), treated with 7-nitroindazole to inhibit nNOS activity (10^{-4} mol/L, 18 h) and treated with 1400W to inhibit iNOS (10^{-4} mol/L, 18h). Data are means ±SD, and are expressed as the fold change with respect to non-treated CT MAEC (n=5; *p > 0.05 vs NT CT, **p> 0.05 vs NT cKO).
Online Figure III. A. Protein extracts from CT and cKO MAEC were immunoprecipitated (IP) with anti-3-nitrotyrosine antibody, and PKG-1 levels were analyzed by immunoblot. 'Input' shows PKG-1 and ILK levels in the input samples. Blots are from a representative experiment (n=3). B. HPLC analysis of bipterin content in CT and cKO MAEC transfected or not with V5-tagged wild-type ILK (ILK-WT). Data are means ± SD, n=6; *p < 0.05 vs. BH4 CT; **p<0.05 vs. BH2 CT ). C. MAEC were transfected with V5-tagged wild-type ILK (ILK-WT). After 24 h, cells were left unstimulated (NS) or treated with calcium ionophore A23187 (10^{-6} mol/L, 15 min). NO production was measured by flow citometry using the NO probe DAF-2DA . Data are means ±SD of 3 independent experiments. *p < 0.05 vs. non stimulated MAEC in each condition.
Online Figure IV. A. MAEC were transfected with V5-tagged wild-type ILK (ILK-WT) or kinase dead ILK (ILK-KD). After 24 h, cells were left untreated (NT, white bars) or treated with calcium ionophore A23187 (10^{-6} mol/L, 15 min). eNOS activity was measured as L-arginine to L-citrulline conversion. Data are means ± SEM of 8 independent experiments and are expressed relative to unstimulated CT MAEC transfected with empty vector (pcDNA). The representative immunoblot shows V5 epitope and eNOS expression. *p < 0.05 vs. non stimulated MAEC in each condition. B. Immunoblot detection of phospho-Ser1177 eNOS and total eNOS from CT and cKO MAEC, non stimulated (NT, white bars) or treated with either VEGF (50 ng/ml, 2.5 min, grey bars) or A23187 (A23) (10^{-6} mol/L, 15 min, black bars). (n=3). *p<0.05 vs NT CT; **< 0.05 vs VEGF or A23 in CT MAEC. C. Western blot analysis of aortic protein extracts from CT and cKO MAEC showing Akt phosphorylation (Ser473 and Thr308 residues) (n=6). *p<0.05 vs S473 in CT.
Online table I. Yeast two hybrid analysis of ILK and eNOS interactions.

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