iNOS-Derived Nitric Oxide Induces Integrin-Linked Kinase Endocytic Lysosome-Mediated Degradation in the Vascular Endothelium

Paula Reventun, Matilde Alique, Irene Cuadrado, Susana Márquez, Rocío Toro, Carlos Zaragoza, Marta Saura

Objective—ILK (integrin-linked kinase) plays a key role in controlling vasomotor tone and is decreased in atherosclerosis. The objective of this study is to find whether nitric oxide (NO) regulates ILK in vascular remodeling.

Approach and Results—We found a striking correlation between increased levels of inducible nitric oxide and decreased ILK levels in human atherosclerosis and in a mouse model of vascular remodeling (carotid artery ligation) comparing with iNOS (inducible NO synthase) knockout mice. iNOS induction produced the same result in mouse aortic endothelial cells, and these effects were mimicked by an NO donor in a time-dependent manner. We found that NO decreased ILK protein stability by promoting the dissociation of the complex ILK/Hsp90 (heat shock protein 90)/eNOS (endothelial NO synthase), leading to eNOS uncoupling. NO also destabilized ILK signaling platform and lead to decreased levels of paxillin and p-arvin. ILK phosphorylation of its downstream target GSK3-β (glycogen synthase kinase 3 beta) was decreased by NO. Mechanistically, NO increased ILK ubiquitination mediated by the E3 ubiquitin ligase CHIP (C terminus of HSC70-interacting protein), but ILK ubiquitination was not followed by proteasome degradation. Alternatively, NO drove ILK to degradation through the endocytic-lysosomal pathway. ILK colocalized with the lysosome marker LAMP-1 (lysosomal-associated membrane protein 1) in endothelial cells, and inhibition of lysosome activity with chloroquine reversed the effect of NO. Likewise, ILK colocalized with the early endosome marker EEA1 (early endosome antigen 1). ILK endocytosis proceeded via dynamin because a specific inhibitor of dynamin (Dyngo 4a) was able to reverse ILK endocytosis and its lysosome degradation.

Conclusions—Endocytosis regulates ILK signaling in vascular remodeling where there is an overload of inducible NO, and thus its inhibition may represent a novel target to fight atherosclerotic disease.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1272-1281. DOI: 10.1161/ATVBAHA.117.309560.)

Key Words: endocytosis ■ inflammation ■ mice ■ nitric oxide ■ nitric oxide synthase ■ vascular remodeling

Atherosclerosis is a chronic inflammatory disease mainly located in branches and bends of the vasculature exposed to disturbed blood flow. Disturbed and oscillatory flows harm the integrity of the vessel wall, resulting in endothelial dysfunction and leading to abnormal endothelial nitric oxide synthase (eNOS) enzymatic activity and vascular inflammation. Conversely, laminar shear stress plays the opposite effect, protecting the endothelial layer, and contributing to release endothelial-derived nitric oxide (NO), adjusting vascular tone, and thus playing protection against inflammation and atherosclerosis. The extensive inducible nitric oxide synthase (iNOS)–mediated NO formation associated with inflammatory cytokine milieu has been linked to the generation of harmful oxidative products and the progression of atherosclerosis. Endothelial cells respond to shear stress by activating cell membrane mechanoreceptors, such as integrins. On ligand binding, integrins cluster and recruit adaptor and signaling proteins to their cytoplasmic domains that mature into large signaling platforms named focal adhesions (FAs), linking extracellular matrix to the actin cytoskeleton. Integrin-linked kinase (ILK), a canonical component of the integrin cell adhesion machinery, binds to eNOS, protects eNOS from uncoupling, and prevents vascular oxidative stress by preventing reactive oxygen species production by uncoupled eNOS. In addition, ILK can regulate eNOS expression.

ILK upregulation promotes a broadly cardioprotective role. In the vasculature, ILK regulates angiogenesis, endothelial survival, apoptosis, inflammation, vasomotor tone, vascular remodeling, and cardiac remodeling. In addition, ILK plays an important role during heart and vascular wall development. At the cellular level, ILK protein expression is known to be regulated by protein stabilization.
through Hsp90 (heat shock protein 90) binding. Inhibition of Hsp90 promotes polyubiquitination of ILK by the E3 ligase CHIP (C terminus of HSC70-interacting protein), leading to its proteasomal degradation.\textsuperscript{16}

We had previously reported the strike correlation between attenuation of ILK and atherosclerosis progression.\textsuperscript{6} We, therefore, questioned whether iNOS-derived NO could be involved in ILK-decreased expression during atherosclerosis and vascular remodeling. In the current work, we show that NO released by iNOS decreases ILK levels and induces ILK endocytosis and degradation in the lysosomal compartment ILK. This is a new unidentified pathway mediating endothelial dysfunction in atherosclerotic disease.

Materials and Methods

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

Results

ILK Expression Decreases in Human Atherosclerosis and in a Mouse Model of Vascular Remodeling

Because iNOS-mediated NO formation has been linked to the development of atherosclerosis,\textsuperscript{17,18} ILK and iNOS levels were studied in endarterectomy specimens of human carotid arteries (n=25) showing varying degrees of atherosclerosis (plaque) and in human arterial specimens free of atherosclerosis as controls (healthy, n=20). Confirming our earlier observations,\textsuperscript{6} Western blot analysis revealed a significant decrease in total ILK expression in atherosclerotic carotid arteries versus healthy vessels (Figure 1A). However, iNOS levels were increased in atherosclerotic vessels compared with healthy arteries, suggesting a link between ILK downregulation and iNOS expression. ILK is expressed at high levels in the endothelium of healthy arteries (Figure 1A in the online-only Data Supplement). However, its endothelial expression is reduced in atherosclerotic arteries (Figure 1B). The integrity of the endothelial layer was determined by CD31 staining. iNOS expression was patent in atherosclerotic arteries in disperse foci within the plaque and in the restenotic regions.

To further study the connection between ILK and iNOS protein expression, we examined ILK levels in a mouse model of vascular remodeling by carotid artery ligation (CAL) in wild-type (WT) and iNOS knockout mice (Figure 1C). First, ILK levels were studied by confocal microscopy in uninjured (control) group, finding a strong expression of ILK in the smooth muscle layer and a robust signal of ILK at the endothelial layer of normal arteries in both WT and iNOS knockout mice. Thus, indicating that ILK is normally expressed at the healthy endothelium. By contrast, 21-day permanent CAL resulted in a marked decrease of ILK expression in the endothelium in the WT mice group, whereas the iNOS knockout mice still exhibit a clear expression of endothelial ILK, indicating a role for iNOS-derived NO in the reduction of endothelial ILK during vascular remodeling. Confirming our human data, iNOS levels increased in the remodeled WT arteries correlating inversely with ILK levels (Figure 1D). To expand these findings, we also studied ILK expression at the protein and mRNA levels. Results in Figure 1B in the online-only Data Supplement show that ILK protein expression was reduced in CAL versus control groups in WT mice, whereas ILK did not decrease in iNOS knockout mice in CAL versus control animals. However, mRNA analysis showed no differences in ILK mRNA expression (Figure IC in the online-only Data Supplement).

iNOS-Derived NO Reduces ILK Content in Endothelial Cells by Decreasing Protein Stability

Intimal inflammation is an early step of atherosclerosis.\textsuperscript{19} To test whether inflammation may regulate endothelial ILK expression, we followed the levels of ILK in response to the proinflammatory cytokine TNF-\(\alpha\) (tumor necrosis factor \(\alpha\)), which induces several proinflammatory genes, including iNOS, in endothelial cells. In mouse aortic endothelial cell (MAEC) stimulated for 24 hours with 100 ng/mL TNF-\(\alpha\), a significant increase of iNOS was detected, together with reduced ILK levels (Figure 2A, top), whereas pharmacological inhibition of iNOS activity with 1400W prevented ILK decreased expression in TNF-\(\alpha\)-stimulated MAEC. Taken together, our data suggest that inflammation-induced downregulation of ILK is mediated by NO produced by iNOS. Supporting these findings, TNF-\(\alpha\) had no effect in iNOS knockout MAEC (Figure 2A, bottom).

We and other found that decreased ILK led to endothelial dysfunction by eNOS uncoupling.\textsuperscript{6,7,20} Thus, we tested superoxide levels in MAEC isolated from WT and iNOS knockout mice, finding an increase in superoxide production in TNF-\(\alpha\)-treated WT MAEC, which was not observed in iNOS knockout mice (Figure 2B). This increase could also be detected in WT MAEC treated with 2 different NO donors (Figure 2C), which differ in the timing of NO release. The NO donor–stimulated superoxide formation could be inhibited by the nitric oxide synthase inhibitor L-NAME (L-nitroarginine methyl ester). eNOS expression did not change in either condition, suggesting that exogenous NO and endogenous iNOS-derived NO reduce ILK levels facilitating eNOS uncoupling to produce superoxide anion in endothelial cells.

To further explore the contribution of iNOS-derived NO to eNOS uncoupling, we examined ILK binding to eNOS and Hsp90. Immunoprecipitation of ILK from WT MAEC lysates detected coprecipitation with eNOS and Hsp90 in control cells, whereas NO donor treatment or iNOS induction

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Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CAL</td>
<td>carotid artery ligation</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>FA</td>
<td>focal adhesion</td>
</tr>
<tr>
<td>Hsp90</td>
<td>heat shock protein 90</td>
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<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
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<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
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<tr>
<td>IPP</td>
<td>integrin-linked kinase, parvin, PINCH complex</td>
</tr>
<tr>
<td>MAEC</td>
<td>mouse aortic endothelial cell</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>SP-NO</td>
<td>spermidine-N0neate</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>tumor necrosis factor (\alpha)</td>
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Figure 1. Nitric oxide reduces ILK (integrin-linked kinase) content in atherosclerotic human arteries and mouse vascular remodeling.

A, Inducible nitric oxide synthase (iNOS) and ILK protein levels are detected by immunoblot in protein lysates from human endarterectomized carotid samples (plaque, P, n=25) or healthy arteries with no sign of atherosclerosis (H; n=20). GAPDH was used as loading control. Spear-whisker dot plot for ILK/GAPDH or iNOS/GAPDH expression, horizontal line means SD of 20 healthy and 25 atherosclerotic specimen analyzed. *P<0.05 vs healthy. A representative Western blot is shown.

B, Immunostaining for ILK, iNOS, and CD31 (endothelial cell marker, endothelial cells [EC]) in human atherosclerotic carotid sections and healthy arteries by confocal microscopy. ILK and iNOS were labeled in red, CD31 in green, and nuclei were labeled with Hoechst in blue. Squares indicate the regions magnified on the right, showing the decreased ILK staining in the CD31-positive endothelial cells within the plaque. Scale bar, 25 μm; 10 μm for enlarged images.

C, Immunostaining for ILK and CD31 in uninjured controls (CT) or carotid arteries 21 d after ligation (carotid artery ligation [CAL]) in wild-type (WT) mice and iNOS knockout (KO) mice (n=10). Arrowheads mark endothelial cells. Squares indicate the regions magnified on the right.

D, Immunostaining detection of iNOS and CD31 in the same animals as in C. Scale bar, 10 μm. L indicates lumen.
reduced the levels of both proteins in ILK immunoprecipitates (Figure 2D).

ILK forms a complex termed IPP (ILK, parvin, PINCH complex, which includes PINCH/α-parvin) and the FA protein paxillin as an essential element in integrin signaling. Thus, we explored the stability of the IPP complex in the presence of NO. Immunoprecipitation of ILK in protein lysates from MAEC treated with different NO donors revealed a significant reduction in the binding to α-parvin and paxillin. In contrast, PINCH association with ILK was slightly affected by NO (Figure 3A). In addition, immunoblot of protein lysates from 10^{-5} to 10^{-7} mol/L spermidine-NOnoate (SP-NO)–treated MAEC showed a marked decrease of paxillin and α-parvin levels in a dose-response manner while PINCH was reduced at the highest concentration of NO (Figure 3B). In addition, we analyzed ILK enzymatic activity detected as phosphorylation of its downstream mediator GSK3−β (glycogen synthase kinase 3 beta), finding that NO decreases GSK3−β phosphorylation by ILK (Figure 3C).

**NO Induces ILK Ubiquitination and Leads to ILK Degradation in the Lysosome**

Next, to investigate the molecular mechanism of NO-mediated modulation of ILK, mRNA and protein levels of ILK were analyzed in MAEC incubated with 10^{-5} mol/L SP-NO for 0 to 24 hours, finding that rather than a transcriptional effect (Figure IIA in the online-only Data Supplement) SP-NO induced a time-dependent decrease of ILK protein, starting 6 hours after incubation with NO (Figure IIB in the online-only Data Supplement).
Data Supplement), indicating that NO promotes a post-transcriptional modification of ILK.

To further assess the role of NO on ILK-decreased protein levels, cycloheximide was used to block de novo protein synthesis in MAEC. As shown in Figure IIC in the online-only Data Supplement, ILK decay rate was slow in cycloheximide-treated cells because a 20±0.5% of ILK was reduced 24 hours after treatment with 50 μmol/L cycloheximide while in MAEC coincubated with 50 μmol/L cycloheximide+10−5mol/L SP-NO, ILK half-life was shortened by a 70±1%.

Because NO disrupts ILK–Hsp90 interaction (Figure 2D) and Hsp90 prevents ILK degradation by the proteasome,23 we addressed whether NO induces ILK ubiquitination. Protein lysates from NO-treated MAEC were immunoprecipitated with antiubiquitin and anti-ILK specific antibodies. ILK immunoprecipitation revealed a significant increase of ubiquitinated ILK in SP-NO–treated MAEC (Figure 4A, top), and conversely ubiquitinated extracts contained significant levels of ILK in the same cells (Figure 4A, bottom). In human atherosclerotic protein lysates, in which we previously found a significant increase of iNOS (Figure 1A), ILK immunoprecipitates also revealed extensive ubiquitination (Figure IIIA in the online-only Data Supplement), suggesting that NO may play a role on ILK ubiquitination.

ILK ubiquitination can be accomplished by the E3 ubiquitin ligase CHIP, driving its degradation by the proteasome.19 We could detect a significant increase of the complex CHIP/ILK in SP-NO–treated MAEC (Figure IIIB in the online-only Data Supplement). Next, we measured ILK levels in MAEC transfected with CHIP small interfering RNA or scrambled small interfering RNA as control. In small interfering RNA control, but not in CHIP-silenced cells, NO reduced ILK levels (Figure IIIC in the online-only Data Supplement), indicating that CHIP-mediated ubiquitination of ILK is an important step in NO effects. Then, the role of proteasomal activation was studied. Pre-treatment of MAEC with 10 μmol/L MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, Z-LLL-CHO) or lactacystin (reversible and irreversible respectively proteasome inhibitors)
did not block the NO-induced degradation of ILK. Interestingly, they potentiated the inhibitory effect of NO (Figure 4C). NO did not increase proteasome activity and MG-132, accurately inhibited proteasome activity as expected (Figure III D in the online-only Data Supplement), suggesting that the proteasome pathway may not contribute to ILK degradation in response to NO.

Ubiquitinated proteins can be cleared from the cells by the proteasome and alternatively by lysosome-mediated degradation or by the autophagy pathway. Pre-treatment of MAEC with the lysosomal inhibitor chloroquine (10 µmol/L) reversed the effect of NO on ILK levels (Figure 4C). To go deeply into the mechanism, we found that ILK was mainly located at the cell surface in resting MAEC (Figure 4D, control). In contrast, ILK was internalized into discrete vesicles and colocalized with the lysosomal marker Lamp-1 in response to 10−5 mol/L SP-NO (Figure 4E; SP-NO). MAEC treatment with 10 µmol/L chloroquine reversed the NO-mediated internalization of ILK (Figure 4D; SP-NO + chloroquine), whereas 10 µmol/L MG-132 did not show any change in ILK localization and even potentiated the response to NO (Figure 4D; NO+MG-132), suggesting that NO targets ILK into the degradative lysosome pathway. Besides, 10−5 mol/L SP-NO also stimulated internalization of paxillin (Figure IVA in the online-only Data Supplement). Because NO can initiate the formation of the autophagosome during autophagy, SP-NO–treated MAEC extracts were immunoblotted at different times with the autophagic markers Beclin-1, p62, and LC3, showing that NO did not induce any significant differences in the protein content (Figure IVB in the online-only Data Supplement).

Taken together, our results indicate that NO induces CHIP-mediated ILK ubiquitination, but neither the proteasome nor the autophagy plays a role in the regulation of ILK by NO. NO, on the contrary, induces the lysosomal-dependent degradation of ILK.

NO Triggers ILK Endocytosis Through a Dynamin-Dependent Pathway

A key step in protein trafficking from the plasma membrane to specific intracellular compartments is internalization in endocytic vesicles. Thus, we sought to investigate whether NO
may promote ILK endocytosis. MAEC were treated for 30 minutes to 2 hours with $10^{-5}$ mol/L SP-NO, and localization of ILK in early endosomes was visualized by confocal microscopy. ILK was detected as early as 30 minutes in NO-treated MAEC, colocalizing with the early endosome marker EEA1 (early endosome antigen 1; Figure IVC in the online-only Data Supplement) indicating that NO promotes ILK endocytosis.

Endocytosis of integrins and associated proteins can be accomplished by chladrin-dependent, caveolae-dependent, and noncaveolae-mediated pathways.27–29 The first 2 processes rely on the GTPase dynamin for the scission of endocytic intermediates to generate early endosomes.30 Previous studies have demonstrated that NO can stimulate endocytosis by S-nitrosilation of dynamin.31,32 Therefore, we tested whether dynamin-mediated endocytosis was involved in NO-induced ILK degradation by using a specific inhibitor of dynamin activity, Dyno 4a. Confocal microscopy showed that dynamin was mediating the NO-dependent internalization of ILK into endosomes. Dyno 4a prevented ILK localization in vesicles containing the early endosome marker EEA1 observed after 30-minute treatment with the NO donor (Figure 5A). Conversely, ILK localization in lysosomes after 6-hour treatment with the NO donor was prevented by Dyno 4a. Accordingly, Dyno 4a reversed the increased degradation of ILK induced by NO (Figure 5B). In fact, Dyno 4a restored NO inhibitory effect on ILK enzymatic activity (Figure 5C). These results demonstrate that NO-induced ILK internalization and degradation in lysosomes are mediated by a dynamin-dependent mechanism.

Discussion
The data presented in this article show that iNOS-derived NO plays a crucial role during atherosclerosis by regulating the endocytic-lysosomal degradation of ILK in endothelial cells. NO-mediated ILK degradation is described here for the first time.
time, through a dynamin-depend endocytosis that drives ILK to a lysosome degradative pathway. Our results shed light about the molecular mechanisms by which inflammatory NO contributes to endothelial dysfunction and provide with a new target to fight against atherosclerosis.

We previously found an inverse correlation between progression of atherosclerosis and the levels of ILK in the vessel wall. Here, we also found an association between ILK decrease and iNOS induction in atherosclerotic arteries. A key early step in the progression of atherosclerosis involves endothelial damage, adhesion of oxidized low-density lipoprotein to the vascular endothelium, and infiltration of inflammatory leukocytes. Endothelial dysfunction perpetuates impaired NO production and initiate an inflammatory reaction in the vessel wall which lead to extensive NO/iNOS production, proinflammatory cytokine activation, extracellular matrix degradation, smooth muscle cell migration, increased reactive oxygen and nitrogen species, endothelial and VSMC (vascular smooth muscle cells) necrosis, apoptosis, and accumulation of cellular debris, leading to plaque formation. We provide molecular evidence about the mechanisms that lead to iNOS-induced endothelial cell dysfunction, by promoting endocytosis and lysosomal degradation of ILK. We previously demonstrated that eNOS form a complex with Hsp90/ILK to regulate vascular tone. In the absence of ILK, uncoupled eNOS switch to produce reactive oxygen species, leading to cellular damage. Our data show that iNOS-NO lead to eNOS uncoupling by decreasing ILK and disrupting eNOS–Hsp90–ILK interaction. We propose that preventing ILK-mediated degradation could represent a new target against atherosclerosis. In support of this hypothesis, ILK prevents endothelial cells apoptosis induced by oxidized low-density lipoprotein, and restoring the levels of ILK can rescue the repairing activities of endothelial progenitor cells.

In a model of vascular remodeling by CAL, we observed that ILK was decreased in the vascular wall in WT mice after carotid ligation compared with the levels found in iNOS knockout animals, indicating that inducible NO regulates the levels of ILK during the process of remodeling. In agreement with these findings, downregulation of ILK after myocardial infarction caused impairment of angiogenesis, angiogenic dysfunction, and myocyte cell loss. By contrast, sustained expression of ILK by adenoviral delivery could effectively promote angiogenesis and preserve heart function. Other models of neointimal formation have reported similar results, including balloon catheter carotid injury, where a decrease in ILK has been reported at the media layer, and re-expression at the luminal surface may attenuate this process during later stages of the injury response. Additional examples include ablation of ILK in cultured endothelial cells, resulting in defective cell spreading, or progenitor endothelial cells lacking ILK which lead to defective vasculogenesis and embryonic lethality between E8.5 and E12.5, whereas, in the adult, the absence of ILK produced endothelial dysfunction.

Despite the relevant role of ILK in the cardiovascular system, the mechanism of ILK stability has not been studied in detail. It is being reported that Hsp90 binds to ILK at the kinase homology domain, preventing ILK proteasomal degradation, thus we hypothesized that NO would increase ILK ubiquitination and proteasomal degradation, by promoting the disruption of the complex Hsp90/ILK. However, our results showed that even when ILK was effectively ubiquitinated in presence of NO and during atherosclerosis, proteasome inhibitors did not reverse such effect, rather potentiated the effect of NO. We might argue that NO could modulate the ubiquitin/proteasome system, as previously reported, but NO had no effect in this work, concluding that the decrease in ILK stability induced by NO was not dependent on the proteasome pathway. Others found that NO regulates the expression of ILK in kidney cells, but as today, our work provides the first molecular evidence about the post-transcriptional effect of NO on ILK protein stability.

What is the mechanism responsible of ILK ubiquitination? It is known that inhibition of Hsp90 promotes ILK ubiquitination by CHIP, which drives ILK to proteasome degradation. CHIP acts not only as a E3 ubiquitin ligase but also as a protein chaperone linking Hsp90 to ILK. CHIP seems to be a key step in the degradative road, as demonstrated in CHIP-silenced cell, in which ILK degradation induced by NO was partially reversed. One explanation is that NO targets Hsp90 to disrupt CHIP–ILK interaction. Hsp90 plays a critical role in ILK stability and signaling, it protects ILK from protein degradation and promotes ILK complex with α-parvin and the cytoskeleton, and we found that Hsp90–ILK interaction is necessary to prevent endothelial nitric oxide synthase enzymatic uncoupling that leads to endothelial dysfunction. It is accepted that S-nitrosylation of Hsp90 inhibits Hsp90 chaperone and ATPase activities required to bind ILK to α-parvin and paxillin. So, S-nitrosylation of Hps90 could also support the loss of α-parvin and paxillin from the complex in NO-treated MAEC, which could explain how increasing amounts of NO contribute to vascular remodeling and also inhibit vascular relaxation. Alternatively, NO may modify ILK by tyrosine nitration to promote protein destabilization. ILK protein contains several tyrosine residues in its pseudokinase domain, which is involved in ILK interaction with Hsp90 and α-parvin. Thus, we cannot exclude that NO effects may be mediated at least, in part, by protein nitration of ILK.

ILK form a ternary complex known as the IPP. The formation of the IPP complex is crucial for the stability of the 3 proteins and is a pre-requisite for them to locate to the cell-extracellular matrix adhesion sites. Mutations in PINCH, ILK, or parvin that disrupt the formation of the complex will prevent the other members from locating to FAs and promote their internalization or eventually degradation. Accordingly, our results show that NO was able to destabilize the IPP complex and drive ILK and parvin to lysosomes while it only had a slight effect over PINCH. Paxillin, another FA protein that also interacts with ILK to localize at the FAs, was also detected in lysosomes after NO stimulation. In addition, phosphorylation of ILK target GSK3β was reduced in the presence of NO. Thus, NO acts as a molecular disruptor of endothelial ILK signaling platform during atherosclerosis and vascular remodeling.

Mechanistically, our results show that lysosomal inhibition reversed the effect of NO, describing a colocalization of ILK with the lysosome marker LAMP-1 in cells coincubated with NO and potentiated by the proteasome inhibitor...
MG-132, proving that ILK is degraded in the lysosome compartment. Autophagy is a process that involves the packaging of cytoplasmic proteins or organelles into vesicles and fused to the lysosome to form an autophagosome. Autophagy plays an important role during atherosclerosis progression. NO can induce autophagy lysosome-mediated claudin-5 degradation in oxygen-glucose deprivation–treated endothelial cells. However, we found here that NO does not induce autophagy at the time and dose used in our study.

Endocytic trafficking has emerged as a critical mechanism for controlling the length and strength of signals via sorting of internalized receptors and their associated signaling complexes for recycling or lysosomal degradation. Several reports demonstrate a role for NO in clathrin- and dynamin-mediated endocytosis and also integrins can be endocytosed by these pathways. NO S-nitrosylates dynamic increasing dynamin oligomerization and GTPase activity, thereby increasing endocytosis. Our results support a role for dynamin in ILK internalization because a specific inhibitor of dynamin activation reverses ILK degradation and localization in endosomes and later in lysosomes, thus proving that NO drives ILK endocytosis to direct ILK to lysosome degradation.

In summary, ILK is a key mechanosensitive protein that regulates heart contractility, vascular remodeling, and endothelial cell homeostasis regulating endothelial NO production. We provide for the first time molecular evidence about the negative effect of NO on ILK stability by inducing internalization and molecular degradation of ILK in lysosomes (summarized in Figure V in the online-only Data Supplement). This work opens a new target for intervention reversing or stabilizing arterial remodeling by preventing ILK degradation by NO and represents a promising approach for both prevention and treatment of cardiovascular complications caused by hypertension and atherosclerosis in which uncoupling of eNOS leads to impaired NO-dependent vessel relaxation.

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Disclosures
None.

References
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**Highlights**

- Inducible nitric oxide (NO) synthase—NO expression correlates inversely with ILK (integrin-linked kinase) presence in human atherosclerosis, pathological arterial remodeling in mice and in models of cardiovascular disease.

- NO does not inhibit transcriptional expression of ILK but induces ILK protein destabilization.

- NO induces ubiquitination of ILK. However, ILK does not undergo proteasome degradation. NO destabilization of ILK begins with disruption of Hsp90 (heat shock protein 90)—ILK complex, which is required for endothelial NO synthase to produce NO in vascular endothelial cells leading to endothelial dysfunction. Hsp90 binding to ILK is also required to transmit mechanical forces to actin cytoskeleton through ILK-binding proteins; thus NO decreases ILK signaling.

- NO directly destabilizes ILK by inducing dynamin-dependent ILK endocytosis and degradation in lysosomes.
iNOS-Derived Nitric Oxide Induces Integrin-Linked Kinase Endocytic Lysosome-Mediated Degradation in the Vascular Endothelium
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Supplementary Figure I. ILK and iNOS expression during atherosclerosis and vascular remodeling. A. Immunostaining for ILK and CD31 (endothelial cell marker) in human healthy arteries sections by confocal microscopy. ILK was labeled in red, CD31 in green and nuclei were labeled with Hoechst in blue. Regions magnified on the right showing the clear ILK staining in the CD31 positive endothelial cells. Scale bar = 50 μm; 10 μm for enlarged images. A representative western blot of some of the samples analyzed for ILK protein expression is shown on the right. GAPDH was used as loading control. B. Immunoblot detection of ILK in protein lysates from WT or iNOS KO mice who underwent carotid artery ligation (CAL) or uninjured controls (n=10). GAPDH was used as loading control (n=10). Graph bar represent mean ± SD *p<0.05 vs WT CT. C. RT-PCR detection of ILK mRNA in WT and iNOS KO mice who underwent carotid artery ligation (CAL) or sham controls (n=10). β-actin mRNA expression was used as control. Graph bar represent mean ± SD *p<0.05 vs WT.
Supplementary Figure II. NO effect on ILK occur at a posttranscriptional level. A. RT-PCR detection of ILK mRNA in MAEC treated with Spermidine NO noate (SP-NO) 10^{-5} mol/L at 0, 2, 4, 8 and 24 h (n=5). GADPH mRNA expression was used as control. Graph bar represent mean ± SD *p<0.05 vs WT. B. Immunoblot detection of ILK in MAEC treated as in A. β-actin was used as loading control. Data are mean ± SD (n=3; *p<0.05 vs CT). A representative western blot is shown. C. Immunoblot detection of ILK in protein lysates from MAEC treated with 50 μmol/L Cycloheximide (CHX) or 50 μmol/L CHX/ 10^{-5} mol/L SP-NO, and isolated at the times indicated β-actin was used as loading control. (n=5 with duplicates in every condition. Mean ± SD *p<0.05 vs CHX+NO).
**Supplementary Figure III. Nitric oxide induces ILK ubiquitination.**

A. Immunoblot detection of Ub and ILK from mammary (M) or carotid (C) arteries, immunoprecipitated with anti-ILK antibody. (n=6). Mean ± SD *p<0.05 vs M). B. Immunoblot detection of CHIP and ILK from MAEC WT treated with or without Sp-NO, immunoprecipitated with anti-ILK antibody. (n=6). Mean ± SD *p<0.05 vs M). C. Immunoblot detection of ILK and CHIP from control MAEC (siRNA CT) or MAEC in which CHIP was silenced by RNA interference in presence and absence of Sp-NO treatment (NO) (n=3). Mean ± SD *p<0.05 vs CT in siRNA CT). D. Proteasome activity from MAEC treated with 10-5 mol/L SP-NO, 10-5 mol/L MG132, or a combination of both. (n=3) . Mean ± SD *p<0.05 vs CT
Supplementary Figure IV. NO effect on ILK is independent of the autophagy lysosome pathway.

A. Confocal microscopy detection of Paxilllin and the lysosome marker LAMP1 in MAEC treated with SP-NO 10^{-5} mol/L (n=3).

B. Immunoblot detection of autophagic markers Beclin-1, p62, and LC3I/II in MAEC incubated with 10^{-5} mol/L SP-NO for the times indicated. GAPDH was used as a loading control (n=3, Mean ± SD).

C. Confocal microscopy detection of ILK (Alexa Fluor 647, red) and EEA1 (FICT, green) from MAEC treated with or without SP-NO for 30 min. Nuclei were stained with Hoechst (blue). Co-localization was visualized in yellow (n=3). Scale bar= 10 μm.
Supplementary Figure V. Proposed mechanisms by which inflammatory NO regulates ILK levels and signaling. First, inflammation of the vascular wall during atherosclerosis and vascular remodeling induces iNOS which releases vast amounts of NO. NO will disrupt ILK interactions with Hsp90 and eNOS leading to eNOS uncoupling and therefore, to oxidative and nitrosative damage that will further dissociate ILK signaling complex. Mechanistically, NO increases ILK ubiquitination activating ILK endocytosis through a dynamin dependent mechanism. NO induced ILK endocytosis leads to ILK degradation in lysosomes. Thus, NO-induced ILK decreased content in endothelial cells leads to endothelial dysfunction and correlates with atherosclerosis progression.