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Ilk conditional deletion in adult animals increases cyclic GMP-dependent vasorelaxation

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Aims Integrin-linked kinase (ILK) regulates proliferation, differentiation, cell adhesion, and motility in many cell types and has been related to cancer progression, fibrosis, and vascular diseases. We designed the present study to directly explore the effect of ILK deletion on the regulation of vascular tone through the soluble guanylate cyclase (sGC) /protein kinase G (PKG) pathway in healthy adult mice.

Methods and results Experiments were carried out using a tamoxifen-inducible CRE-LOX system to conditionally delete the ILK gene in adult mice. Mice lacking ILK expression (cKO) presented increased vascular content and increased activity of sGC and PKG, resulting in a more intense vasodilatory response to a single dose of a nitric oxide (NO) donor [sodium nitroprusside (SNP)] or PKG agonist [8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt (8-Br)]. Five minutes after SNP or 8-Br administration the reduction in the systolic arterial pressure was enhanced in cKO mice (SNP WT: -7.4 ± 1.2 mmHG; SNP cKO: -14.0 ± 2.5; 8-Br WT: -2.9 ± 1.5 mmHG; 8-Br cKO: -10.0 ± 3.4 mmHG). ILK deletion restored the vascular response to SNP after chronic oral nitrite administration. In addition, ILK deletion also increased hypotensive SNP effect in angiotensin II-treated animals, suggesting a role for ILK in basal and pathological states.

Conclusion Deletion of ILK in adult animals increased the vascular response to NO. These findings show, for the first time, a requirement for ILK in regulating sGC–PKG expression *in vivo*.

Keywords Integrin-linked kinase • Nitric oxide • Vascular reactivity • Cyclic-GMP • Soluble guanylate cyclase

1. Introduction

Integrin-linked kinase (ILK) is a widely expressed serine/threonine kinase found as a multiprotein, transmembrane complex where initiation of the integrin-dependent signalling cascades occurs.[1,2] Through ILK, the extracellular matrix is linked with the actin cytoskeleton and other intracellular signalling pathways.[1,2] ILK regulates proliferation, differentiation, cell adhesion, and motility of many cell types, and has been associated with cancer progression,[3] fibrosis,[4] and vascular diseases.[5] Importantly, ILK deletion causes embryonic lethality in mice. For this reason, the use of CRE-LOX-driven recombination and RNA-interference technologies has been used to investigate the physiological roles of ILK in several organ systems.[3] These studies have revealed that, when the ILK gene is deleted or inactivated through RNA interference, significant developmental and tissue-homeostasis defects occur. In contrast, the expression of ILK is often elevated in human malignancies.[3] In the cardiovascular system, evidence from genetically modified mouse models has demonstrated important roles for ILK in embryonic vascular development,[6] VEGF-stimulated tumour angiogenesis[7] and cardiac hypertrophy.[8] In normal physiology, ILK contributes to maintaining cellular quiescence during the growth of arteries.[9] In vascular smooth muscle cells (VSMCs), ILK phosphorylates myosin light chain and myosin phosphatase and may therefore influence cytoskeletal function and cell contraction.[10,11]

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Nitric oxide (NO) plays a central role in regulating vascular tone in mammals.[12] Many of the physiological functions ascribed to NO are mediated through its primary receptor, soluble guanylate cyclase (sGC).[13] sGC is a heterodimer composed of α and β subunits, each of which exists in two isoforms, α1, α2, β1, and β2. The α_1 and β_1 isoforms predominate in VSMCs.[14] Different experimental approaches have demonstrated that both α and β subunits are required for sGC activity,[15] which can be altered by estrogen-, cytokine-, and NO donor-dependent changes in the expression of the subunits.[16,17] Activity can also be regulated by allosteric effectors.[16,17] By producing cyclic GMP (cGMP), sGC controls many physiological effects including the regulation of vascular tone and VSMC motility. To regulate vascular tone, cGMP interacts directly with downstream effectors such as cyclic nucleotide-gated channels, cGMP-regulated phosphodiesterases, and the family of cGMP-dependent protein kinases (PKG) that promote VSMC relaxation.[17] There are two different PKG genes, types I and II, encoding the synthesis of PKG protein. Type I is the gene product expressed in VSMCs, with two isoforms (PKG Iα and Iβ) generated by alternate mRNA splicing.[18] The expression of PKG I can be regulated by inflammatory cytokines, high glucose, and other stimuli at the transcriptional or post-transcriptional level.[19,20]

A link between ILK modulation and sGC has been proposed in contractile mesangial cells from kidney glomeruli. When cultured on collagen type I, these cells exhibit increased ILK activity and decreased sGC β_1 content.[21] In addition, a similar regulation has been reported in contractile vascular cells.[22] In contrast, endothelial cells cultured on collagen type I show a reduction of endothelial NO synthase (eNOS) protein content.[23] ILK deficiency causes an uncoupling of eNOS and impaired endothelium-derived vasodilation.[24] We hypothesized that ILK deletion could modulate vascular tone by modifying the intracellular content of sGC and/or PKG I, resulting in a subsequent increase in the vascular response to their agonists. The present study was designed to directly explore the effect of ILK deletion on the regulation of vascular tone through the sGC/PKG pathway in healthy adult mice.

2. Methods

2.1 Ethic statement

Animal were housed in a pathogen-free and temperature-controlled room (22 ± 2°C). Food and water were available *ad libitum*. All procedures involving animals were previously approved by the Institutional Animal Care and Use Committee from University of Alcala and were conform to the Directive 2010/63/EU of the European Parliament.

2.2 Conditional ILK knockout mice

Conditional inactivation of the ILK gene was accomplished by crossing C57Bl/6 mice homozygous for the floxed ILK allele, flanked by loxP sites ($ILK^{fl/fl}$)[25] with homozygous mice carrying a tamoxifen-inducible CreER (T) recombinase gene ($CRE^{+/+}$) which express Cre under the control of the cytomegalovirus promoter.[26] As previously described, 8-week-old male mice were injected intraperitoneally with 1.5 mg of tamoxifen (TX) once per day for 5 consecutive days to induce ILK deletion.[24,27] Three weeks following vehicle (VH, corn oil/methanol) or TX injections, routine genotyping of tail DNA samples was performed to monitor the Cre-driven ILK deletion.[24,27] TX-treated CRE-LOX mice displaying successful deletion of ILK are named hereafter conditional-KO ILK mice (cKO) and their control VH-treated CRE-LOX are named hereafter wild type (WT). For control experiments the parental $Cre^{+/+}$ (CRE) and $ILK^{fl/fl}$ (LOX) mice were also treated with TX or VH.

2.3 Blood pressure measurements

Three weeks after the final injection of VH or TX, mice were injected intraperitoneally with the NO donor sodium nitroprusside (SNP, 2 μg/kg b.w.; Sigma-Aldrich) to specifically activate the soluble sGC, or 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt (8-Br, 5 μg/kg; Sigma-Aldrich) to activate PKG. In some animals, intraperitoneal injections with the sGC inhibitor, 1H [1, 2, 4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 mmol/kg b.w.; Sigma-Aldrich) or the synthetic peptide membrane-permeable PKG I inhibitor, RQIKIWFQNRRMKWKKLRKKKKKH (DT3, 0.5 g/kg b.w.; Calbiochem, Darmstadt, Germany),[28] were carried out 15 min before SNP or 8-Br administration, respectively. Alternatively, mice were pre-treated with the NO donor isosorbide dinitrate in the drinking water for 1 week (IDN, 300 mg/kg/day; Sigma-Aldrich) to induce NO tolerance,[29] or with intraperitoneal angiotensin II injection (AII, 0.8 μg/Kg b.w.; Sigma-Aldrich) to induce acute hypertension.[30] After treatments, arterial pressure was measured in conscious animals using a tail-cuff sphygmomanometer (LE 5001 Pressure Meter; Letica Scientific Instruments, Hospitalet, Spain).[29]

2.4 *Ex vivo* measurement of vascular reactivity

Animals were euthanized by cervical dislocation under anaesthesia with inhaled isofluorane 4%. The preparation of aortic rings and analysis of vascular reactivity were carried out similarly to that described previously.[31] Aortas were isolated, cleaned of loosely adhering fat and connective tissue under a dissecting microscope. In most cases, endothelium was removed by gentle rubbing with surgical thread. Endothelium-denuded or intact aortas were cut into 2 mm segments and incubated in an Automatic Organ Bath (Panlab, Spain). Aortic rings were suspended between two stirrups in a 5 mL organ bath filled with pre-warmed (37°C) and oxygenated (95% O_2/5% CO_2) modified Krebs-Ringer bicarbonate solution at pH 7.4 containing the following concentrations: 118 nM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 11.1 mM glucose, and 25 mM $NaHCO_3$. The rings were set at an initial resting tension of 0.5 g, and tension changes were recorded with an isometric force transducer (Biopac Systems, Inc., Sta Barbara, CA, USA). Tissues were allowed to equilibrate for 40 min prior to experimentation and the resting tension re-adjusted to 0.5 g if required. Vessels were exposed to 80 mM KCl to verify their functional integrity and, to confirm the lack of endothelium in the endothelium-denuded experiments, to 10 mM acetylcholine (ACH). Aortic rings were allowed to equilibrate for 30 additional minutes, and contraction was elicited with norepinephrine (NE, 300 nM; Sigma-Aldrich). Previously, an NE dose–response was performed. Endothelium-denuded aortic rings were treated with increasing doses of SNP (from 1 to 10 nM) or N2, 2′-*O*-dibutyrylguanosine 3′,5′-cyclic monophosphate sodium salt hydrate (DB, from 0.003 to 1 mM; Sigma-Aldrich) and tension was recorded. Some experiments were performed in the presence of 1 μM ODQ or with the membrane-permeable PKG I inhibitory peptide DT3 (1 μM). Intact aortic rings were treated with 0.01 μM ACH, 1 μM SNP, or both, and isometric tension was recorded. Contraction was calculated as the difference between measured and basal tension (0.5 g), and expressed in grams.

2.5 Measurement of cGMP levels in mouse aortic rings

Animals were euthanized as described above and aortic rings were harvested and immersed immediately in buffer (135 mM NaCl, 5 mM KCl, 16 mM sodium acetate, 5 mM Glucose, and 20 mM Trizma, pH 7.5) containing 3-isobutyl-L-methylxanthine (IBMX, 100 μM; Sigma-Aldrich), and incubated at 37°C for 15 min with 1 μM SNP. cGMP levels were determined by a radioimmunoassay (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) as described previously.[32] Some experiments were performed in the presence of 1 μM ODQ, for 30 min where noted.

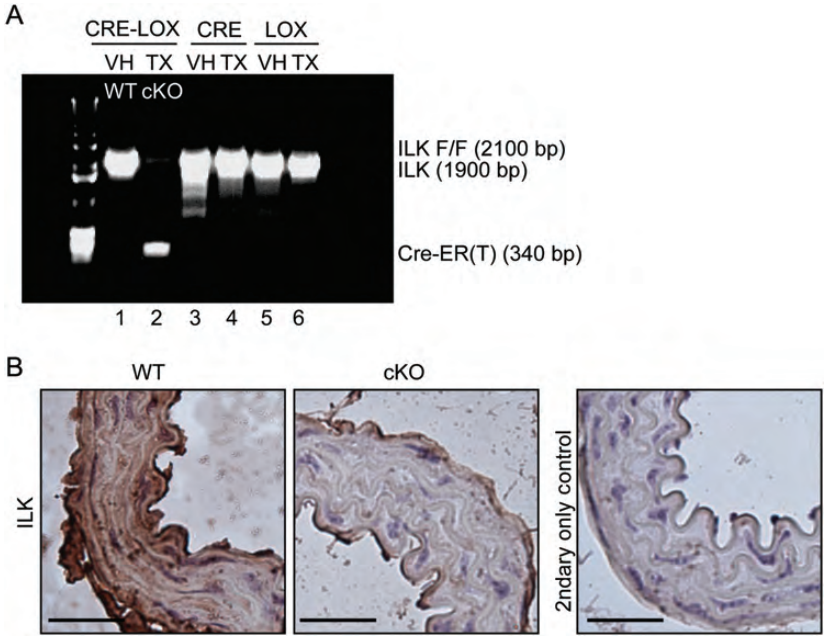

Figure 1 Conditional deletion of ILK in aorta. (*A*) PCR analysis of genomic DNA isolated from CRE-LOX mice aortas (lanes 1 and 2), LOX mice (lanes 3 and 4) and CRE mice (lanes 5 and 6) treated with corn oil (VH) or TX for 5 days. Amplification products, including the floxed ILK ($ILK^{F/F}$), the native ILK (ILK), and the CRE-excised DNA fragment (Cre-ER(T)) are shown. Lane 2 shows TX-treated CRE-LOX with ILK deletion (cKO) compared with the VH-treated control (WT). (*B*) Immunohistochemistry with indicated antibodies of aorta from the TX-treated CRE-LOX (ILK deleted, cKO) or VH-treated CRE-LOX (WT) mice. $n = 6$. Scale bar = 50 μM.

2.6 Immunohistochemistry analysis

After euthanasia, aortas were harvested from mice, fixed in 10% neutralized formaldehyde for 24 h, and embedded in paraffin. Sections were incubated with primary antibodies against ILK (R&D Systems, Minneapolis, MN, USA), sGC β1 (Sigma), and PKG 1β (Stressgen, Enzo Life Science Inc Farmingdale, NY, USA) in a humidified chamber, overnight at 4°C. Negative controls were carried out by omitting primary antibodies. Subsequently, sections were incubated with HRP-secondary antibody for 1 h, followed by incubation with the chromogen diaminobenzidine tetrahydrochloride (DAB; DAKO, Glostrup, Denmark). Sections were counterstained with Harris's haematoxylin.

2.7 Immunoblot analysis

Aortas were washed in PBS and solubilized (10 mmol/L Tris–HCl pH 7.4, 1 mmol/L EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 500 nmol/L sodium orthovanadate, 50 nmol/L NaF, 1 mmol/L pepstatin/leupeptin/aprotinin, and 1 mmol/L PMSF) for 30 min at 4°C. PVDF membranes were incubated with antibodies against sGC β1 (Sigma-Aldrich), PKG 1β (Stressgen), and Phospho-vasodilator stimulated phosphoprotein (VASP) Ser 239 (Cell Signaling Technology, Beverly, MA, USA). Specifically bound antibody was detected with enzymatic chemiluminiscence substrate (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA). Actin (Sigma-Aldrich) or VASP (Cell Signaling Technology) were used as loading controls. Densitometric analysis was carried out using Scion Image (National Institutes of Health, USA).

2.8 Statistics

Data are presented as the mean ± SEM of a variable number of experiments, detailed in the legends to the figures. Most experiments are presented as percentage of basal or control values. In these cases, absolute basal values are given in the legends to the figures. To compare the different experimental situations, two-way ANOVA or two-way ANOVA for repeated measurements (RMANOVA) were used, depending on the experiments. Pair-wise comparisons were performed with the Fisher's least significant difference method. The Dunnett test was used to analyse the changes with respect to basal values. A P-value of <0.05 was considered statistically significant.

3. Results

3.1 ILK deletion promotes sGCβ1 and PKG Iβ up-regulation in aorta

CRE-LOX mice were treated with TX or VH, and ILK deletion was verified using PCR analysis of aortic DNA (*Figure 1A*). Parental CRE and LOX mice were used as controls. Aortic ILK protein expression was analysed by immunohistochemistry, confirming a significant reduction in ILK expression in TX-treated CRE-LOX mice (referred to as cKO mice) compared with the VH-treated CRE-LOX mice (referred to as WT mice; *Figure 1B*).

To investigate the effect of ILK deletion on the sGC/PKG signalling pathway, the expression of sGC β1 and PKG Iβ in aortas isolated from cKO and WT mice was assessed by immunoblot. The expression of both proteins was significantly increased in cKO mice (*Figure 2A* and *B*). To exclude a possible effect of TX treatment on sGC β1 or PKG Iβ expression following ILK deletion, aortas from TX-treated CRE and LOX mice were also assessed. The TX treatment did not up-regulate protein expression in control CRE or LOX aortas (*Figure 2A* and *B*). The expression of sGC β1 and PKG Iβ was also assayed by immunohistochemistry. It showed a significant increase in the expression of both proteins in aortas from ILK-deleted mice compared with controls (*Figure 2C*).

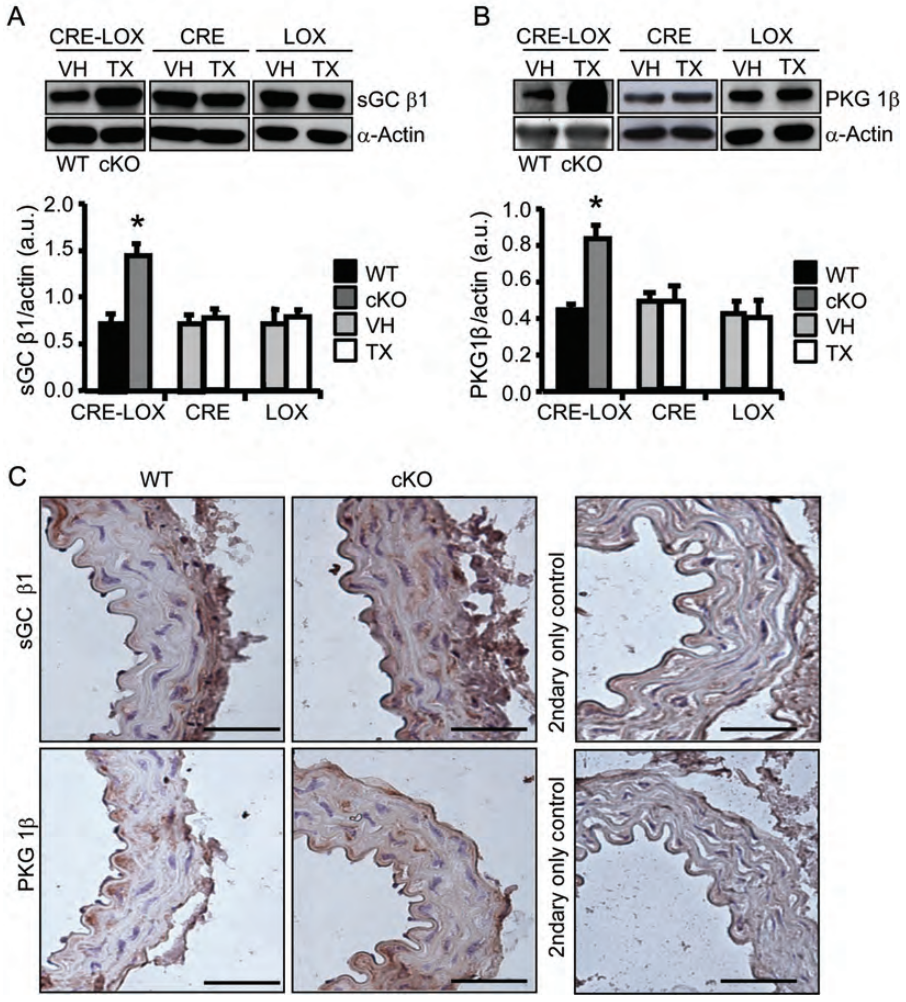

Figure 2 Loss of ILK results in increased sGC and PKG expression. (*A*) sGC β1 and (*B*) PKG 1β protein expression in aorta from VH or TX-treated CRE-LOX (WT or cKO), CRE and LOX mice, analysed by western blot. A representative immunoblot and the densitometric analysis of sGC β1 and PKG Iβ blots normalized against actin are shown as mean densitometric arbitrary units ± SEM from six independent experiments. *$P < 0.05$ vs. WT. (*C*) Representative immunohistochemistry of aortas from WT or cKO mice with indicated antibodies. $n = 6$. Scale bar = 50 μm.

No differences were observed in the basal systolic aortic pressure (SAP: WT: 115 ± 3 mmHg, cKO: 123 ± 7 mmHg) and mean arterial pressure (MAP: WT: 85 ± 7 mmHg, cKO: 95 ± 5 mmHg) of both groups of animals.

3.2 Functional consequences of increased vascular sGC content

To analyse the functional consequences of the observed increase in aortic sGC expression, changes in blood pressure were recorded in cKO and WT mice in response to the NO donor SNP. All mice received a single dose of SNP, and the arterial pressure was recorded for the next 20 min. As expected, 5 min after SNP injection SAP clearly decrease in WT mice, with a further significant decrease in ILK cKO mice (*Figure 3A*). Similar results were found in MAP values (reduction in MAP after 5 min SNP injection: WT: 4.6 ± 0.5 mmHg; cKO: 9.5 ± 1.1 mmHg). There was a good correlation between SAP and MAP values ($r = 0.90$, $P < 0.001$). Mice pre-treated with ODQ, a specific inhibitor of sGC, did not respond to SNP injection, showing that the changes observed in the arterial pressure were specific to sGC (*Figure 3A*). No differences in the SNP response were observed in the VH- or TX-treated CRE or LOX control mice, confirming that the lack of ILK rather than TX treatment was responsible for the observed effects (*Figure 3B*).

To confirm this data, cGMP synthesis in response to SNP administration was measured in aortic rings isolated from WT and cKO mice. There were no significant differences in the cGMP basal synthesis (*Figure 3C*). However, SNP-stimulated cGMP synthesis was significantly increased in ILK-deficient mice compared with WT mice (*Figure 3C*). The absence of increased cGMP synthesis following SNP injection of mice pre-treated with ODQ, a sGC inhibitor, showed the specificity of the sGC response (*Figure 3C*). In addition, phosphorylation of VASP, a PKG substrate, was also analysed in aortic ring lysates. SNP induced VASP phosphorylation in WT mice, with a further significant increase in cKO mice. The increase in VASP phosphorylation was blocked with ODQ pre-treatment (*Figure 3D*).

3.3 Functional consequences of increased vascular PKG content

The functional consequences of PKG up-regulation were investigated using PKG agonists. A single dose of the specific PKG agonist 8-Br promoted a small drop in SAP in WT mice, whereas similar treatment of ILK-

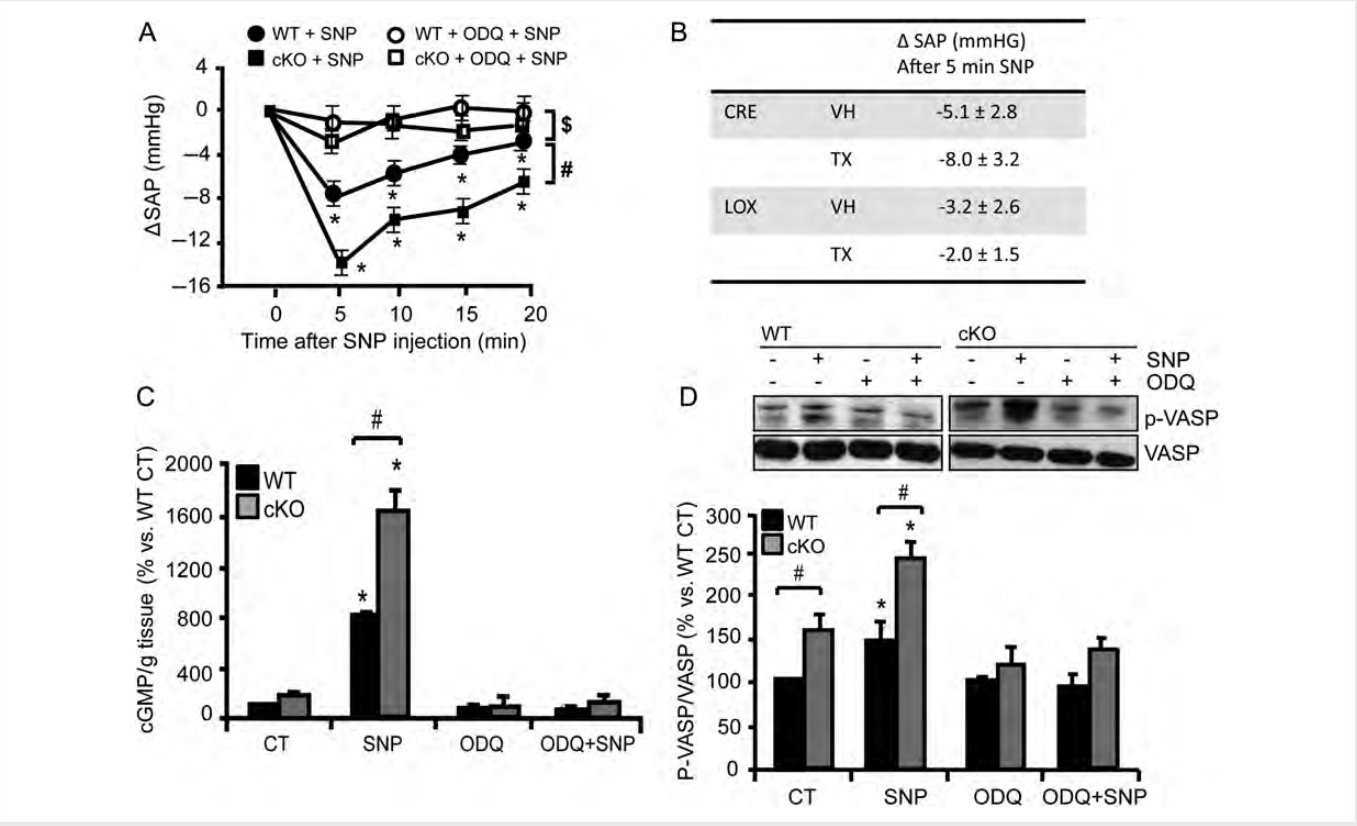

Figure 3 Loss of ILK increases sensibility to NO donors. (*A*) Systolic arterial pressure (SAP) was measured in WT or cKO mice after a single dose of the NO-donor SNP, with or without pre-treatment with a single dose of the sGC inhibitor ODQ. Graph shows the mean ± SEM of the changes in SAP (ΔSAP). The basal SAP values were: WT 115 ± 5, cKO 112 ± 3, ODQ + WT 110 ± 4, ODQ + cKO 116 ± 8 mmHg. $n = 10$. $^{*}P < 0.05$ vs. basal SAP (time 0), in the same group of mice. $^{\#}P < 0.05$ between WT + SNP vs. cKO + SNP groups. $^{\$}P < 0.05$ between the times 5, 10, and 15 min of the WT + SNP animals with respect to the same times of the groups treated with ODQ. (*B*) Table includes the mean reduction of SAP at 5 min after SNP administration in CRE and LOX control mice treated with VH or TX. The basal SAP values were: CRE-VH 106 ± 3, CRE-TX 109 ± 3, LOX-VH 105 ± 8, LOX-TX 102 ± 5 mmHg. Results are mean ± SEM from six independent experiments. TX did not induce any significant change in the SNP-dependent SAP reduction. (*C*) cGMP synthesis was measured after addition of SNP for 15 min in the presence or absence of ODQ in aortas isolated from WT or cKO mice. Results are mean ± SEM from six independent experiments, and they are expressed as percentage of the WT control. $^{*}P < 0.05$ vs. WT-CT. $^{\#}P < 0.05$ vs. WT-SNP. (*D*) Isolated aortas from WT or cKO mice were treated with the SNP in the presence or absence of ODQ. Lysates were subjected to western blot with indicates antibodies. Representative blots are shown and the graph represents the P-VASP normalized against total VASP densitometric values. Values are mean ± SEM from six independent experiments, and they are expressed as percentage of the WT control. $^{*}P < 0.05$ vs. WT-CT. $^{\#}P < 0.05$ between WT vs. cKO.

depleted mice induced a further significant decrease in SAP (*Figure 4A*). In both cases, the observed decrease in SAP was abolished by the administration of the PKG inhibitor DT3 prior to 8-Br injection (*Figure 4A*). No differences in the 8-Br response were observed between the VH- and TX-treated CRE or LOX control mice, confirming that the lack of ILK rather than TX treatment was responsible for the observed effects (*Figure 4B*). VASP phosphorylation was assessed in aortic ring lysates isolated from WT and cKO mice. Treatment with the PKG-agonist DB induced VASP phosphorylation in WT aortic ring lysates, with a further significant increase in cKO aortic ring lysates, even in basal conditions (*Figure 4C*). The increase in phosphorylation of VASP was blocked by pre-treatment with DT3 inhibitor (*Figure 4C*).

3.4 Increased vascular sGC and PKG content regulates vascular reactivity

The effect of ILK deficiency on vascular reactivity was assessed in both denuded endothelium rings and intact rings (*Figure 5*) isolated from the thoracic aorta of WT and cKO mice. First, denuded aortic rings were incubated with increasing concentrations of NE (from 0.1 to 2 μM) to promote vasoconstriction (*Figure 5A*). At 300 nM NE, vasoconstriction was at 92% of the maximum for aortic ring from both WT and cKO mice. For subsequent experiments, this level of contraction was designated as 100%. After contraction of endothelium-denuded rings, we recorded isometric tension to analyse the vasorelaxation effect of increasing concentrations of SNP (*Figure 5B*) or DB (*Figure 5C*). The relaxing effect of both sGC and PKG agonists was more marked in denuded aortic rings from ILK-deficient cKO mice compared with WT mice. Both ODQ and DT3 inhibited the vasodilatory effect of SNP and DB, respectively (*Figure 5B* and *C*).

Additionally, vascular reactivity was also assessed in intact aortic rings from WT and cKO mice (*Figure 5D*). The contraction elicited by 300 nM NE was comparable in aortic rings from both groups of mice. Compared with WT aortic rings, rings from cKO mice exhibited a relative lack of response to 0.01 μM ACH and an increased relaxation when treated with

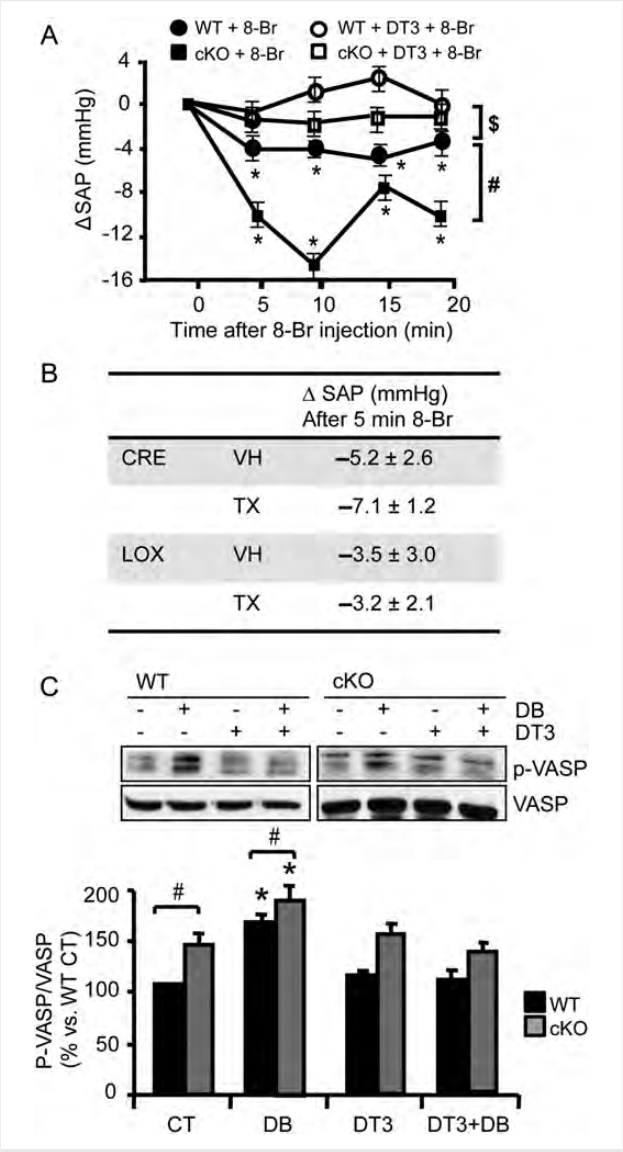

Figure 4 Loss of ILK increases sensibility to PKG agonists. (*A*) SAP was measured in WT or cKO mice after a single dose of cGMP analogue 8-Bromo-GMP (8-Br), with or without pre-treatment with a single dose of the PKG I inhibitor DT3. The graph shows the mean ± SEM of the changes in SAP (ΔSAP). The basal SAP values were WT 114.5 ± 2, cKO 118 ± 3, DT3 + WT 112 ± 3, and DT3 + cKO 113 ± 6 mmHg. $n = 10$. $^{*}P < 0.05$ vs. basal SAP (time 0), in the same group of mice. $^{\#}P < 0.05$ between cKO + 8-Br vs. WT + 8-Br groups at all times. $^{\$}P < 0.05$ between WT + 8-Br vs. WT-8-Br-DT3 groups at all times. (*B*) The table includes the mean reduction of SAP at 5 min after 8-Br administration in CRE and LOX control mice treated with VH or TX. The basal SAP values were CRE-VH 106 ± 3, CRE-TX 109 ± 3, LOX-VH 105 ± 6, and LOX-TX 102 ± 5 mmHg. Results are the mean ± SEM from six independent experiments. (*C*) Isolated aortas from WT or cKO mice were treated with the cGMP analogue dibutiryl-GMP (DB) in the presence or absence of DT3. Lysates were subjected to western blot with indicated antibodies. Representative blots are shown and the graph represents the P-VASP normalized against the total VASP densitometric values. Values are the mean ± SEM from six independent experiments and they are expressed as percentage of the WT control. $^{*}P < 0.05$ vs. WT-CT. $^{\#}P < 0.05$ between WT vs. cKO.

SNP. No differences were observed in the isometric tension generated in WT and cKO aortic rings treated simultaneously with Ach and SNP (*Figure 5D*).

3.5 Relevance of ILK deficiency in pathophysiological conditions

To induce NO tolerance, WT and cKO mice received the NO donor IDN for 1 week. IDN did not modify ILK expression in aortas from WT mice (*Figure 6A*). SAP was measured before and after IDN administration. IDN did not modify SAP in WT animals, but it decreased SAP in cKO mice (*Figure 6B*). Immediately after the last SAP measurement, a single injection of SNP was administered to all animals, and SAP was recorded for 10 min. SNP did not modify SAP in WT mice, whereas an additional significant 20% reduction in SAP occurred in the ILK-deficient mice (*Figure 6B*). In these experimental conditions, the aortic content of sGC was determined by western blot (*Figure 6C*). As expected, the deletion of ILK in cKO mice induced a significant increase of sGC, whereas chronic IDN administration reduced the expression of sGC in only the WT group. The IDN-treated cKO mice showed an sGC protein content similar to that observed in WT mice not treated with IDN (*Figure 6C*).

To assess the role of ILK in an acute hypertension model, WT and cKO mice were treated with a single injection of AII. AII treatment induced a rapid increase in SAP in both groups of mice. Immediately following the AII treatment, mice were treated with SNP, and the SAP was recorded for 20 min. SNP reduced SAP in both groups of animals, but this reduction was significant only in the cKO mice (*Figure 6D*).

4. Discussion

The conditional deletion of ILK in adult mice results in healthy mice without any obvious pathology and provides a very efficient model to study the role of ILK in aortic rings as previously shown.[24] The main finding of the present work is that depletion of ILK in aortic vessels leads to increased vascular content and activity of the NO receptor sGC and its downstream target PKG, both of which promote vasorelaxation.[15,17] These results must be interpreted with previous reports from our group that demonstrated that ILK-deficient mice show an uncoupling of eNOS and diminished production of NO.[24] The net effect on the arterial pressure of these opposite changes in ILK-deficient animals would be a combination of decreased production of NO by the endothelium and increased vascular content of sGC and PKG. In fact, mice lacking ILK do not show changes in the arterial blood pressure.

We did not investigate the mechanisms underlying the ILK-dependent changes in sGC and PKG expression. One hypothesis for the sGC changes could be that the decreased NO synthesis characteristic of the cKO animals is involved in the up-regulation. This is consistent with the well-established NO-induced decrease in sGC content.[33,34] However, this does not seem to be an adequate explanation. Previous studies demonstrated that the activation of ILK in mesangial and smooth muscle vascular cells decreases the sGC β1 cellular content,[21,22] a finding that supports our *in vivo* findings, and these two type of cells do not synthesize NO in the absence of significant stimulation. Concerning the PKG over-expression, it could be suggested a link between the changes in cGMP and the synthesis of PKG. However, in basal conditions, no changes were observed in the cGMP content in arterial walls of ILK-deficient mice, and the best described effect for cGMP in the synthesis of PKG is a reduction of the protein.[35] Consequently,

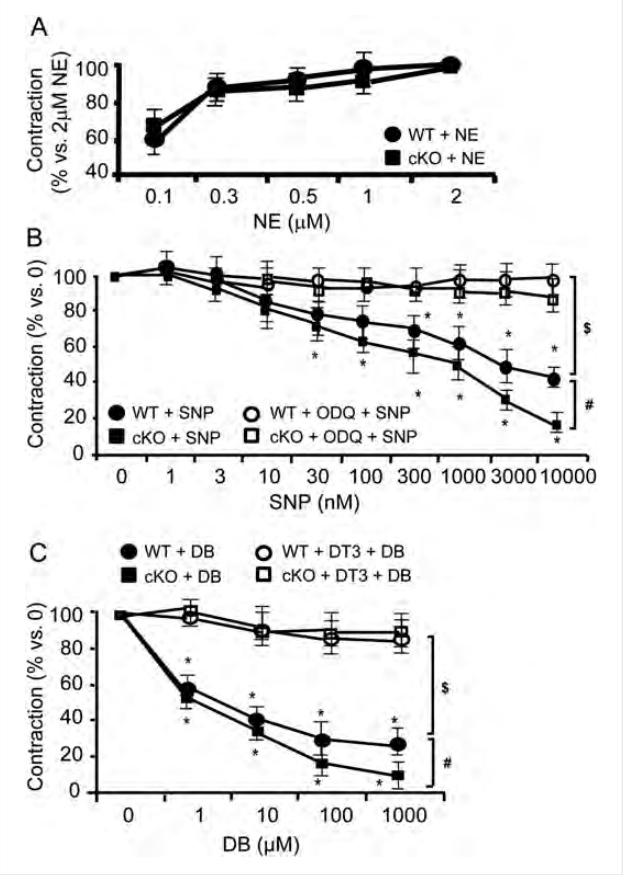

Figure 5 ILK deficiency increases vascular reactivity to SNP and dibutiryl-cGMP (DB). Aortic rings isolated from WT or cKO mice were suspended in an organ bath, basal tension was settled at 0.5 g in every experiment, and vascular contraction was studied. (*A*) Endothelium-denuded rings from WT and cKO mice were treated with increasing additive NE concentrations and contraction was determined by the changes in the isometric tension. Data show the NE concentration–response curve compared with the maximal contraction obtained (1.07 ± 0.01 g at 2 μM NE). Results are the mean ± SEM from three independent experiments. (*B* and *C*) Endothelium-denuded rings were treated with 0.3 μM NE, and incremental doses of the NO-donor SNP with or without the sGC inhibitor ODQ (*B*) or incremental doses of the cGMP analogue DB with or without the PKG I inhibitor DT3 (*C*) were added. Changes in isometric tension were recorded. The contraction induced by 0.3 μM NE (WT 0.65 ± 007 and cKO 0.68 ± 0.04 g, in the (*B*) and *C*) was taken as 100%, and every isometric tension recorded was expressed as percentage of this value. The results are shown as the mean ± SEM from 10 independent experiments. $^{*}P < 0.05$ vs. 100% NE contraction (concentration 0). $^{\#}P < 0.05$ between the concentrations 3000 and 10 000 in the cKO + SNP aortic rings (*B*) or 100 and 1000 in the cKO + 8-Br aortic rings (*C*) with respect to the same concentrations in the WT + SNP (*B*) or WT + 8-Br (*C*) aortic rings. $^{\$}P < 0.05$ between the concentrations 100–10 000 in the WT + SNP aortic rings (*B*) or 1–1000 in the WT + 8-Br aortic rings (*C*) with respect to the same concentrations in the aortic rings of animals treated with ODQ (*B*) or DT3 (*C*). (*D*) Intact, not-endothelium-denuded rings were treated with 0.3 μM NE plus ACH (0.01 μM), SNP (1 μM), or both together. Changes in isometric tension were recorded. The contraction induced by NE (WT 0.88 ± 0.04, cKO 0.84 ± 0.06 g) was taken as 100%, and every tension recorded was expressed as percentage of this value. Values are shown as the mean ± SEM from four independent experiments. $^{*}P < 0.05$ vs. WT-CT. $^{**}P < 0.05$ vs. rest of WT groups. $^{\#}P < 0.05$ vs. cKO mice with the same treatment.

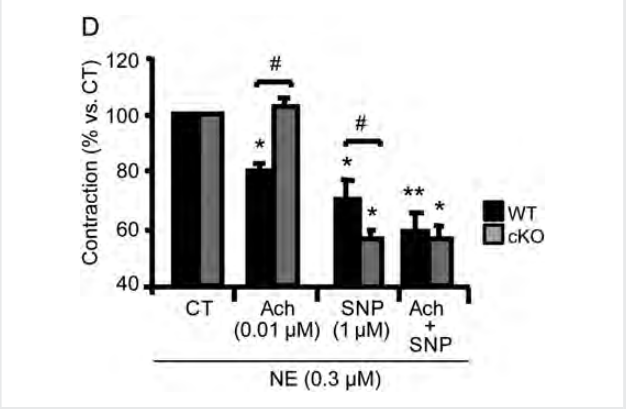

Figure 5 *Continued*

alternative mechanisms must be involved. It has been widely demonstrated the ability of ILK to modulate the activity of different transcription factors and genes. For instance, we previously demonstrated that the arginine–glycine–aspartic acid stimulation of TGFß1 synthesis is dependent on ILK, through AP-1 activation and increased activity of the cytokine promoter.[36] The deletion of ILK could determine the changes in the activity of different kinases and transcription factors, and these changes would determine the over-expression of certain proteins, such as sGC or PKG isoforms. Forthcoming studies should be devoted to explore the pathways linking ILK to these proteins.

The demonstration of an increased amount of protein in a particular tissue does not necessarily imply an increased activity of this protein. This is especially important in the case of sGC, as this enzyme is formed by two subunits, and we measured only the β1 isoform. Previous studies have shown that an increase in the sGC β1 isoform can be used to indicate the global sGC activity, which in turn regulates blood pressure and vascular tone.[21,22,28] Thus, to confirm the functional relevance of changes observed in the sGC β1 isoform and PKG Iβ, we quantified the differences in vascular tone in adult mice. We selected three functional parameters: SAP as a measurement of the effect on the arterial pressure; GMP synthesis, as the direct consequence of sGC activation[33,34]; and P-VASP, as the direct product of activated PKG.[37] The use of SAP values as the index of vasodilation could be problematic, since tail-cuff measurement of SAP is very sensitive to arterial properties other than vasodilation. However, the measurement of diastolic arterial pressure in our animals provided multiple technical problems and MAP measurement quality depend on this. Thus, we preferred to use SAP. Nevertheless, in a wide group of animals, particularly those used in the SNP response analysis, we observed similar changes in SAP and MAP, as well as a good correlation between both values.

In the cKO animals, the changes induced by SNP treatment in SAP, cGMP synthesis, and VASP phosphorylation were significantly more marked than those observed in WT mice. Similar experiments were performed with a PKG agonist, 8-Br,[38] and SAP and VASP phosphorylation were evaluated. Again, the increased amount of PGK Iβ content in the ILK-depleted mice determined a more marked hypotensive effect and VASP phosphorylation in these animals. Taken together, these data suggest that the increased expression of guanylate cyclases and PKG are likely responsible for the blood pressure and biochemical changes observed.

ILK-deficient mice had a higher P-VASP expression under basal conditions, even without significant changes in cGMP synthesis; however,

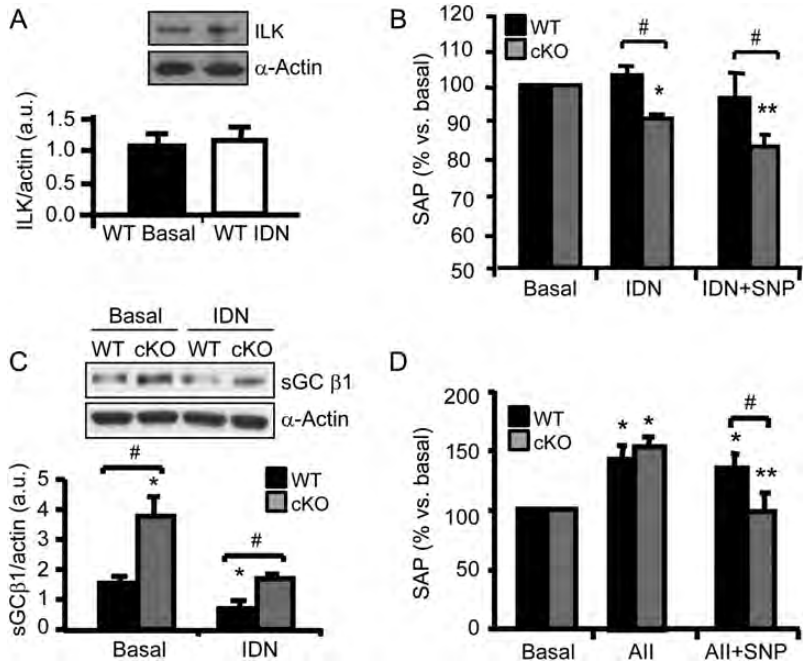

Figure 6 ILK deficiency protects from NO tolerance and improves the hypotensive response to NO donors. (*A*) WT mice received IDN in their drinking water for 1 week. At the end of the treatment period, aortas were lysed and ILK and actin protein content assessed by western blot. A representative blot is shown, and the graph represents the ILK normalized against actin densitometric values, from three different experiments. (*B*) SAP was measured in WT or cKO mice treated with IDN and after a single dose of SNP was administered intraperitoneally. The results are expressed as % of basal SAP values (previous to IDN treatment) and are the mean ± SEM from six independent experiments. $^{*}P < 0.05$ vs. basal WT, $^{**}P < 0.05$ vs. basal and IDN WT. $^{\#}P < 0.05$ vs. WT mice under the same treatments. Basal SAP values were WT 110 ± 2 and cKO 108 ± 4 mm Hg. (*C*) At the end of the IDN treatment period, WT and cKO aortas were lysed and sGC β1 and actin protein content assessed by western blot. A representative blot is shown, and the graph represents the mean ± SEM of the sGC β1 normalized against actin densitometric values, from six different experiments. $^{*}P < 0.05$ vs. basal WT. $^{\#}P < 0.05$ vs. WT under the same treatments. (*D*) WT or cKO mice received a single intraperitoneal injection of AII, immediately after a single intraperitoneal injection of SNP. SAP was recorded for 20 min. The results are expressed as % of basal SAP values (previous to AII treatment) and are the mean ± SEM from six independent experiments. $^{*}P < 0.05$ vs. basal WT, $^{**}P < 0.05$ vs. basal and AII WT. $^{\#}P < 0.05$ vs. WT under the same treatments. Basal SAP values were WT 114 ± 3 and cKO 116 ± 4 mmHg.

this was not enough to decrease SAP in these animals. The lack of changes in basal cGMP synthesis was likely to have been due to the opposite changes in eNOS activity and sGC content that took place in the cKO mice. The increased phosphorylation of VASP could be explained by the increased PKG content or by c-GMP-independent mechanisms of PKG activation.[39] The absence of SAP changes with increased VASP phosphorylation in cKO animals could be attributed to the fact that basal blood pressure is regulated by different compensatory mechanisms besides the vascular wall contractility. In fact, *in vivo* blood pressure is finely maintained by the modulations of a wide variety of effectors, including neural, endocrine, and local factors. Thus, the changes from just one effector mechanism may not be sufficient to modify blood pressure.

Although the experimental data strongly supported the critical functional role of increased sGC and PKG protein content in the vascular tissue of cKO mice, we performed additional experiments with specific inhibitors of both sGC (ODQ) and PKG (DT3).[28,40] The observed changes in SAP, cGMP, and P-VASP were abolished by these inhibitors, thus confirming the functional relevance of the changes in sGC and PKG. DT3, a selective PKG I pharmacological inhibitor, prevented the changes induced by cGMP analogues but did not modify the basal phosphorylation of VASP. Perhaps, the chemical nature of the inhibitor determines this kind of response, as other authors have shown the same effect.[28,41]

To exclude a possible direct role of the drug TX that could interfere with the results, even though the experiments were performed 3 weeks after the TX administration, we treated parental CRE and LOX mice with TX, as we did with CRE-LOX. We found no differences in either the vascular content of sGC and PKG or in SAP in CRE or LOX mice treated with VH or TX. This confirmed that TX treatment alone had no effects on these parameters.

The interpretation of data obtained from haemodynamic studies in the animals can be difficult. The regulation of blood flow is very complex, involving sGC and PKG along with neurohumoral mechanisms, including the synthesis of endothelial vasoactive factors. Using these same cKO animals, Herranz *et al.*[24] demonstrated that ILK deletion is responsible for a marked NOS dysfunction, resulting in decreased vascular response to ACH treatment, measured as changes in arterial blood flow. In contrast, they also observed, as we did, an increased vascular response to SNP, pointing to the possibility that changes in the vascular wall independent from the endothelium could be present in these mice. To better analyse the whole animal results, we evaluated the contractility of aortic rings from WT and cKO mice. In basal conditions, we found no differences in the vascular response to NE between the strains of mice or between intact or denuded aortic rings. ILK deficiency did increase the sensitivity to SNP and DB vasorelaxants, an effect that was

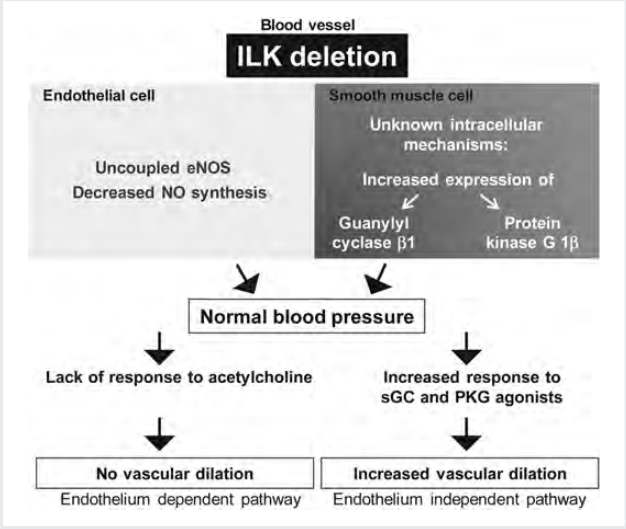

Figure 7 Proposed integrative mechanisms involved in vascular reactivity in the ILK-deleted mice. The deletion of ILK in the vasculature induces eNOS uncoupling with reduced NO synthesis in the endothelium[24] and increased expression of sGC and PKG in the smooth muscle (present results). In basal conditions, ILK-deficient mice do not show changes in blood pressure. The treatment with exogenous ACH did not affect endothelium-dependent vascular dilation. However, the use of sGC or PKG agonist increased the smooth muscle-dependent vasodilation pathway.

abolished by ODQ and DT3 pre-treatment, respectively. When incubated with ACH and SNP, the vasodilation was similar in intact aortic rings from WT and cKO animals, probably because of compensation due to the involvement of different effector mechanisms. The results in aortic rings confirm our *in vivo* findings concerning the changes in blood pressure and vascular sGC and PKG protein content, as well as the description by Herranz *et al*.[24] An integrated view of these mechanisms is shown in *Figure 7*. In this figure, we propose that after the deletion of ILK, the administration of exogenous NO donors or cGMP agonists increases vascular blood flow, even in the presence of decreased endogenous NO synthesis.

To further investigate the clinical implications of our findings, we assessed the role of ILK in tachyphylaxis and acute hypertension, two pathological conditions characterized by altered vascular response. Nitrates are powerful vasodilators and NO donors are widely used for the treatment of acute hypertensive emergencies. However, they have no role in the treatment of chronic hypertension because of the common development of tachyphylaxis, a rapid tolerance to drug treatment following its repeated administration. In our current study, IDN treatment induced tolerance in WT mice, whereas ILK-deficient mice showed a decreased SAP just after the long-term treatment excluding the induction of tolerance. Importantly, we found that ILK-deficient mice responded to SNP treatment after the IDN administration whereas WT mice did not. Moreover, ILK deletion increased the hypotensive effects of the NO donor SNP in the AII-induced acute hypertension. AII induced a rapid and transient increase in SAP in both WT and cKO animals, but the decrease in SAP after SNP administration was significantly higher in cKO than in WT mice. Interestingly, the percentage of SAP reduction after SNP was higher in cKO animals with AII treatment than after SNP treatment in basal conditions (without AII administration). These findings suggest that ILK might be a viable target in the field of acute hypertension treatment.

In summary, this study describes, for the first time, a role for ILK in the regulation of the sGC–PKG system that contributes to vasomotor control *in vivo*. Our data suggest that ILK inhibitors should be investigated as possible tools, in combination with other vasoactive agonists, in the management of diseases that require increased vascular relaxation.

Conflict of interest: none declared.

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