AMPK Alpha 1-Induced RhoA Phosphorylation Mediates Vasoprotective Effect of Estradiol

Marion Gayard, Christophe Guilluy, Anthony Rousselle, Benoit Viollet, Daniel Henrion, Pierre Pacaud, Gervaise Loirand, Malvyne Rolli-Derkinderen

Objective—Estradiol (E2) mediates numerous beneficial effects assigned to estrogens, but whereas mechanisms have been described at the endothelial level, direct effects on vascular smooth muscle cells (VSMC) are poorly documented. As evidence accumulates regarding the role of RhoA in vascular pathophysiology and the benefit of RhoA-Rho associated protein kinase (Rock) pathway inhibition, we analyzed if E2 could inhibit it in VSMC.

Methods and Results—We show that in VSMC, E2 inhibits the RhoA-Rock pathway in a time- and concentration-dependent manner. The inhibition of RhoA-Rock pathway results from E2-induced phosphorylation of the Ser188 of RhoA. Using pharmacological, transfection, and in vitro phosphorylation experiments, we demonstrate that AMP-activated protein kinase subunit alpha 1 (AMPKα1) is activated by estrogen receptor stimulation and catalyzes RhoA phosphorylation induced by E2. Ex vivo, ovariectomy leads to an increase in the amplitude of phenylephrine- or serotonin-induced contractions of aortic rings in wild-type mice but not in AMPKα1-knock-out mice or E2-supplemented animals. These functional effects were correlated with a reduced level of RhoA phosphorylation in the aorta of ovariectomized female, male, and AMPKα1 knock-out mice.

Conclusion—Our work thus defines AMPKα1 as (1) a new kinase for RhoA and (2) a new mediator of the vasoprotective effects of estrogen. (Arterioscler Thromb Vasc Biol. 2011;31:2634-2642.)

Key Words: cell physiology □ g proteins □ signal transduction □ vasoconstriction □ estrogen

In the vasculature, RhoA and its downstream effector Rho associated protein kinase (Rock) have been shown to regulate processes such as vascular smooth muscle cell (VSMC) contraction, proliferation and differentiations, endothelial permeability, platelet activation, and leukocyte migration. Accordingly, basal RhoA activity is required for homeostatic functions in physiological conditions, but its sustained overactivation has pathological consequences in the vascular system, particularly in VSMC. Activation of RhoA-dependent pathways is involved in excessive contraction, and thereby increases blood pressure, but also in excessive cell growth and migration that participate in pathological cardiovascular remodeling. Several regulatory proteins thus cooperate to provide a tight control of RhoA activity including RhoA exchange factors and RhoA GTPase activating proteins that control the GDP/GTP cycle, and Rho guanine dissociation inhibitor (RhoGDI) that sequesters RhoA in the cytosol. In addition to this regulation and independently of GDP-GTP cycling, recent reports have proposed that phosphorylation/dephosphorylation cycle also controls Rho protein activity. cAMP- and cyclic GMP-dependent protein kinases (PKA and PKG) phosphorylate RhoA on Ser188. Both in vitro and in vivo experiments indicated that Ser188 phosphorylation of RhoA induces increased association to GDI leading to cytosolic accumulation of RhoA, inhibition of RhoA-mediated target activation and functions and vasodilation. Recently, we showed that stimulation of angiostatin II type 2 receptor (AT2R) in VSMC induces RhoA phosphorylation by the Ste20-related kinase SLK, which contributes to the vasodilatory effect of AT2R. In fact, functional consequences of RhoA phosphorylation mimics RhoA-Rock inhibition that reverses or delays vascular pathology development. Conversely, vascular remodeling observed in pathological conditions in particular diabetes is associated with a loss of RhoA phosphorylation. RhoA phosphorylation thus appears as a simple physiological mechanism that could be used to control the dynamics of RhoA protein actions and to permit specific limitation or termination of RhoA protein signals.

The incidence of cardiovascular diseases is lower among premenopausal women than in age-matched men or in postmenopausal women. Although the specific mechanisms by which female gender is vasoprotective are not fully understood, the vasoprotective effect of estradiol seems to be mediated in part by the inhibition of RhoA-Rock pathway by AMPKα1-mediated RhoA phosphorylation.
elucidated, the decreased protective effect against cardiovascular diseases, such as hypertension, in postmenopausal women is thought to be because of endogenous ovarian estrogen depletion. Estradiol (E2) predominant estrogen decreases arterial pressure by modulating autonomic function of the cardiovascular system and through direct effects on blood vessels, which have been essentially ascribed to an endothelial protective action. Genomic and nongenomic mechanisms have been described at the endothelial level to explain the protective effect of E2. However, estradiol-mediated vascular protection could also involve direct effect on vascular smooth muscle. In this context, inhibition of RhoA/Rho kinase by RhoA phosphorylation may constitute a plausible explanation for the effect ascribed to estradiol. Therefore, in the present study, we directly analyzed the effect of E2 on serine phosphorylation of RhoA both in vitro in VSMCs and in/ex vivo in artery samples. We describe a new signaling cascade activated following E2 stimulation that leads to AMP-activated protein kinase (AMPK) activation, RhoA phosphorylation, and RhoA/Rho kinase signaling inhibition. We thus identify AMPKα1 as a novel kinase for RhoA that may contribute to the vasoprotective effects of estrogen by limiting the contraction.

Methods

VSMC were obtained from explants of rat aorta and used at passage 2 to 4 for this study. Cells were placed in complete fresh Dulbecco’s modified minimal essential medium without phenol red 24 hours before the pharmacological treatments. Inhibitors were applied 45 minutes before other treatments. Mouse embryonic fibroblasts (MEFs) from wild-type and AMPKα1-deficient mice (described elsewhere) were cultured and treated as VSMC. Western Blot analysis was performed as previously described. Direct AMPK activity was measured using human recombinant AMPK (Merck) and γ[32P] incorporated into SAMS peptide substrate (HMRASMSGHLVKRR) or recombinant wild-type (WT) or phosphoresistant (S188A) RhoA. Rock-1 or Rock-2 were immunoprecipitated from lysates of VSMC or MEF treated or not with E2 for 6 hours and used for kinase assay on S6 substrate. After incubation for 30 minutes at 30°C, assays were terminated by phosphoric acid and revealed as phospho-cellulose binding assay. Wild-type or AMPKα1-deficient male mice (10 to 20 wk old) were treated daily with estrogen at a concentration of 10⁻⁹ mol/L for 0.5 to 20 hours, or with different concentrations during 1 hour before harvesting and analysis. Quantification of MYPT or RhoA phosphorylation level in VSMC expressing WT (WT-RhoA) or S188A RhoA, Rock-1 or Rock-2 was performed on total lysates (total lysate) or with antibodies on total lysate (total lysate).

Results

E2 Inhibits RhoA-Rock Pathway Through RhoA Phosphorylation in VSMC

To determine if estradiol can induce RhoA-Rock pathway inhibition and RhoA phosphorylation in smooth muscle, we analyzed the effect of E2 on VSMC. We measured the phosphorylation of the Rock target MYPT in VSMC stimulated with 10⁻⁹ mol/L E2 for different times, or with different E2 concentrations for 1 hour. E2 induced a time- and dose-dependent phosphorylation of MYPT (Figure 1A and 1C) and, in parallel, a time- and dose-dependent phosphorylation of RhoA (Figure 1B and 1D). Analysis of the RhoA-Rock pathway in VSMC expressing WT (WT-RhoA) or S188A RhoA, Rock-1 and Rock2 immunoprecipitated from lysates of VSMC treated with 10⁻⁹ mol/L of E2 for 6 hours. *P<0.05 and #P<0.01 treated vs nontreated. The data presented are representative of 4 to 5 independent experiments.
0.18±0.22 after 6 hours in WT-Rhoa expressing cells) (Figure 1E). Measurement of kinase activity of Rocks demonstrated that Rock1, but not Rock2, activity was significantly reduced in VSMC treated with E2 (Figure 1F). These results demonstrate that through direct action on VSMC, E2, at doses close to physiological concentrations (1 nmol/L), induced RhoA phosphorylation on Ser188 that is responsible for the RhoA-Rock pathway inhibition.

**E2 Induces RhoA Phosphorylation and Rock1 Inhibition Through AMPK**

We then adopted a pharmacological approach to identify the kinase responsible for RhoA phosphorylation and RhoA-Rock pathway inhibition induced by E2 in VSMC. PKA is known to phosphorylate Rhoa on Ser188 and to be activated by E2.24 Pretreatment with the PKA inhibitor H89 affected neither RhoA phosphorylation nor RhoA-Rock pathway inhibition induced by E2 (Figure 2A) demonstrating no major involvement of PKA in this signaling. The AMP-activated protein kinase (AMPK) is another candidate serine-threonine kinase described to be activated by E2 in myotubes.25 The AMPK inhibitor compound C decreased the basal level of RhoA phosphorylation but also prevented the E2-induced RhoA phosphorylation (Figure 2A). This effect was associated with an increase in the basal MYPT phosphorylation and an inhibition of the dephosphorylation induced by E2 (Figure 2A). Consistently, analysis of the phosphorylation of AMPK itself and of its AMPK substrate acetyl-CoA carboxylase (ACC) showed that E2 activated AMPK in VSMC (Figure 2A and 2B). These results were confirmed by analysis in mouse embryonic fibroblasts (MEF) from AMPK α1-KO or -WT mice. E2-induced RhoA phosphorylation was almost lost in KO animals (Figure 2C). In the same manner, the decrease in the phosphorylation of 2 Thr residues of MYPT phosphorylated by Rock (Thr 697 and 855 in ratus sequences, and Thr 696 and 853 in human sequences) induced by E2 in VSMC and in WT MEFs is not observed in MEF from AMPK α1-KO mice (Figure 2A–C). Rock1 activity was also decreased in WT MEF treated with E2, whereas no significant changes were observed in AMPK α1-KO MEF (Figure 2D). These results demonstrate that E2 phosphorylates Rhoa and inhibits the RhoA-Rock pathway through AMPK activation in VSMC. This also shows that the AMPK is basally activated in VSMC.

**E2 Induces RhoA Phosphorylation Through Estrogen Receptor**

To analyze if E2 induces RhoA phosphorylation and RhoA-Rock pathway inhibition through estrogen receptor (ER) in VSMC, we pretreated the cells with the nonselective ER antagonist ICI182780. ICI182780 pretreatment prevented phosphorylation of Rhoa and ACC, and dephosphorylation of MYPT induced by E2 (Figure 2A). These results demonstrated that AMPK-induced RhoA phosphorylation and RhoA-Rock pathway inhibition are induced by binding of E2 to ER in VSMC.

**AMPK Directly Phosphorylates Rhoa on Ser188**

To assess the possibility that AMPK directly phosphorylates Rhoa on its serine residue 188, we first analyzed the affected sequence. Alignment of the C-terminal region of Rhoa sequences of different species to the consensus AMPK phosphorylation sequence showed that the K located 3 amino-acid before and the L located 5 amino-acid after the Ser188 corresponded to the consensus sequence (Figure 3A). Bioinformatic analysis (http://psps.biocuckoo.org/index.php) revealed that AMPK could phosphorylate Rhoa on Ser188 with a probability comparable to that obtained for PKG.
AMPK Activation Limits Vasoconstriction

Male or AMPK

RhoA Phosphorylation Is Reduced in Arteries of Male or AMPKα1 KO Female Mice

To analyze if RhoA phosphorylation induced by E2 through AMPKα1 could participate in gender differences at the level of the vascular system, we compared the activation of this pathway in aorta from female or male, WT, and AMPKα1-KO mice (Figure 4). The level of phosphorylated RhoA, phosphorylated AMPK and phosphorylated ACC was significantly lower in WT males than in WT females, suggesting a reduced activity of AMPKα1 pathway in males. Deletion of AMPKα1 induced a significant decrease in RhoA, AMPK and ACC phosphorylation in female that in male mice and could be responsible for vasoprotective effects of estrogens.

Estradiol Induces RhoA Phosphorylation and Limits Vasoconstriction Through AMPKα1

To analyze the vascular consequences of the newly described E2-AMPK-RhoA pathway in arteries, we measured the vasoreactivity of aorta rings from WT or AMPKα1-KO female mice, ovariectomized or not, and daily supplemented with E2 or not. As previously described, all experiments were performed in presence of l-NAME (2 × 10−4 mol/L). The amplitude of the contraction induced by 60 mmol/L of KCl was similar in WT and AMPKα1-KO aortic rings (Figure 5B). Ovariectomy significantly increased the contraction of WT aorta in response to phenylephrine. This increase was prevented by the daily supplementation with E2 (Figure 5C). In contrast ovariectomy or E2 supplementation had no effect on AMPKα1-KO aortic ring responses to phenylephrine, that, in control condition were already higher than those obtained in WT mice and comparable to the responses of ovariectomized WT aortic rings (Figure 5C). The same results were observed for the contractile response to serotonin (5HT) (Figure 5D). Measurement of RhoA phosphorylation in aorta form WT or AMPKα1-KO mice showed that ovariectomy decreased RhoA phosphorylation and that E2 supplementation prevented this effect in aortas from WT mice. No change was observed in aorta of AMPKα1-KO mice in which RhoA phosphorylation remained low in the 3 conditions (Figure 6). AMPK phosphorylation was also greatly reduced in aortas from WT ovariectomized mice and this effect was reversed by E2 supplementation (Figure 6). On the contrary, MYPT phosphorylation was increased in ovariectomized WT mice and in AMPKα1-KO mice. Ovariectomy or E2 supplementation did not induce any effect on MYPT phosphorylation in AMPKα1-KO mice (Figure 6). All together, these results suggest that RhoA phosphorylation and RhoA-Rock pathway

AMPK Activation Limits Vasoconstriction Independently of NO

To evaluate the effect of AMPK on VSMC function, we first have used a pharmacological approach consisting in analyzing the effect of AMPK activators, AICAR and metformin on...
inhibition induced by E2 through AMPKα1 could limit vasoconstriction. To analyze in vivo if the differences in the level of RhoA phosphorylation observed in arteries of WT females and males and in AMPKα1 KO mice were associated with change in blood pressure regulation, we have measured systolic blood pressure in female and male AMPKα1-KO mice (Supplemental Figure II). Under basal condition, systolic blood pressure in male and female AMPKα1-KO mice was similar to that of WT mice. Under these conditions, the level of RhoA phosphorylation observed in arteries of male or AMP-activated protein kinase (AMPK) alpha 1 knock-out (KO) female mice. RhoA phosphorylation was measured by Western blot analysis using (p)Ser188 antibodies on total lysates. AMPK pathway activation was measured by using (p)AMPK and (p)ACC antibodies. Quantification of AMPK, ACC, and RhoA levels of phosphorylation are represented relative to the level measured in wild-type (WT) females taken as 1. §P<0.05 KO vs WT; *P<0.05 treated by E2 vs non-treated. The data presented are representative of 3 to 7 independent experiments (5 to 10 mice per group).
of RhoA/Rho kinase activation and P-MYPT phosphorylation is low. It is therefore logical that the inhibitory action of AMPKα1-mediated RhoA phosphorylation on this pathway had no effect on basal blood pressure. This is in agreement with the absence of effect of Rho kinase inhibitors on blood pressure in normotensive animals.26 1-NAME treatment revealed differences between the AMPKα1-KO and WT mice. In WT mice, 1-NAME treatment induced hypertension earlier and stronger in male than in female (Supplemental Figure II). After 2 days of 1-NAME treatment, maximal increase in blood pressure was already observed in males (around 35 mm Hg) whereas there was no change in blood pressure in females, and the maximal rise observed after 14 days of 1-NAME in females was smaller than in males (around 20 mm Hg). In AMPKα1-KO mice, there was no difference in males and females: 1-NAME induced a rise (around 35 mm Hg) in blood pressure that was already maximal after 2 days of treatment. These data thus suggest that AMPKα1 partially protects females from 1-NAME induced-hypertension. All together, our data support the idea that estrogen-dependent, AMPKα1-mediated phosphorylation of RhoA observed in arteries of female mice participates to the vasoprotective effects of estrogens.

**Discussion**

Our work identifies AMPKα1 as a new kinase that phosphorylates Ser188 of RhoA and establishes a novel signaling cascade induced by estradiol. In VSMC, ER stimulation by E2 activates AMPK that phosphorylates RhoA thereby reducing Rho-Rock signaling pathway activity and limiting vasoconstriction. Our results also demonstrate that AMPKα1-RhoA pathway is constitutively active in female mice and could thus participate to the vasoprotective effect of estrogens.

AMPK is an ubiquitous heterotrimeric serine/threonine protein kinase activated by pathological stimuli, such as oxidative damage, osmotic shock, hypoxia, and glucose deprivation, as well as by physiological stimuli such as exercise and muscle contraction, and by hormones including leptin and adiponectin.27 In general, AMPK is activated in response to decreased cellular energy charge (increased in AMP/ATP ratio) and regulates carbohydrate and lipid metabolism.28–30 Although there is a robust correlation between the activity of the AMPK and the metabolic control in skeletal muscle or heart, the role of the AMPK activity in other organs is less studied. In the vasculature, AMPK can be regulated by hypoxia as well as fluid shear stress and has been implicated in the regulation of fatty acid oxidation and nitric oxide production as well as inflammation in endothelial cells.31 More recently, AMPK has been shown to preserve endothe-

[knock-out (KO) female mice. RhoA phosphorylation was measured by Western blot analysis using (p)Ser188 antibodies on total lysates. RhoA-Rock pathway and AMPK pathway activation were measured by using (p)AMPK and (p)MYPT antibodies. Quantification of AMPK, MYPT, and RhoA phosphorylation levels are represented relative to the level measured in WT females taken as 1. $P<0.05$ OVX vs control and $^*P<0.05$ OVX+E2 vs OVX. §$P<0.05$ KO vs WT. The data presented are representative of 3 independent experiments (3 to 4 mice per group).
As predominant estrogen, E2 mediates numerous beneficial effects assigned to estrogens, such as blood pressure lowering,\textsuperscript{12,14} angiogenesis promotion, or early atheroma prevention.\textsuperscript{13} E2 actions are mediated by genomic and nongenomic mechanisms. The classical genomic mechanism of E2 action involves activation of its nuclear receptor (ER-\textgreek{a} or -\textgreek{b}), receptor dimerization, and subsequent binding to ER response elements located in the promoter of target genes. More recently, E2 has been shown to have rapid, nongenomic biological effects, believed to be mediated through membrane-bound subpopulation of ER-\textgreek{a} and ER-\textgreek{b} and/or the newly described G protein--coupled receptor 30.\textsuperscript{3,4} The results obtained using the ICI182780 inhibitor, argue against a role for the G protein--coupled receptor 30 but for the involvement of ER-\textgreek{a} or ER-\textgreek{b} in the vasoprotective effects of E2 observed. Concerning the vasculature, ER-\textgreek{a} and ER-\textgreek{b} are present both on endothelial and smooth muscle cells,\textsuperscript{11} but most of these studies concern the endothelium, and a few consider a possible smooth muscle implication. Indeed, endothelial ER stimulation induces vascular wall dilatation\textsuperscript{16,18} mainly through an increased nitric oxide production\textsuperscript{48} due to rapid eNOS activation\textsuperscript{18,46,47} or induction of eNOS expression.\textsuperscript{17} E2 has also been shown to stimulate endothelial cell survival and formation of primary capillary tube.\textsuperscript{48} At the media level, E2 regulates smooth muscle cell proliferation,\textsuperscript{12} differentiation, apoptosis,\textsuperscript{49} and even contraction.\textsuperscript{17} The mechanisms underlying these effects are poorly described, implicating ERK/MAPKs and p38/SAPKs balance or PI3K-Akt and Src signaling, and cannot explain all the E2 effects.\textsuperscript{47,49,50} Recently, although the mechanism involved has not been identified, it has been suggested that inhibition of RhoA-Rock pathway in the central nervous system or in the vasculature might participate to the limitation of arterial contraction induced by E2.\textsuperscript{51}

The observation that E2-induced AMPK-mediated RhoA phosphorylation on Ser188 provides the molecular mechanism of these observed effects. Both in vitro and in vivo experiments indicated that Ser188 phosphorylation of only a fraction of total RhoA efficiently inactivated RhoA signaling through an increased association to GDI, leading to cytosolic sequestration of RhoA,\textsuperscript{6} inhibition of RhoA-mediated target activation and functions\textsuperscript{7} and vasodilation.\textsuperscript{5} In agreement with these reports, here we show that expression of a phosphoresistant form of RhoA blocked MYPT dephosphorylation induced by E2 thus suggesting that E2-induced inhibition of RhoA-Rho kinase signaling resulted from phosphorylation of RhoA on Ser188.

The identification of AMPK activation and RhoA-Rock inhibition can explain E2 effects, and it would be interesting to consider AMPK activation and RhoA phosphorylation as inducers of estrogen vasoprotection, and even as mediators of estrogen signaling in the whole organism.

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References


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AMPKalpha1-induced RhoA phosphorylation mediates vasoprotective effect of estradiol

Marion Gayard, Christophe Guilluy, Anthony Rousselle, Benoit Viollet, Daniel Henrion, Pierre Pacaud, Gervaise Loirand and Malvyne Rolli-Derinderen
INSERM, UMR915, l’institut du thorax, Nantes, F-44000 France

Supplementary Methods

Cell Culture, transfection and treatments. VSMC were obtained from explants of rat aorta. VSMC at passage 2 to 4 were used for this study. Cells were cultured in DMEM containing 10% of fetal calf serum, 100U/ml penicillin and 100U/ml streptomycin and placed in complete fresh DMEM without phenol red 24h before the treatment with AICAR (5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, 3 mmol/L for the indicated time), metformin (1,1-Dimethylbiguanide, Hydrochloride, 4 mmol/L) or 17-β-estradiol (E2; 10⁻⁹ mol/L) for the indicated times. Estrogen receptor antagonist (ICI 182, 780 1 µmol/L), protein kinase A inhibitor H89 (N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 10 µmol/L) or AMPK inhibitor (compound C, 10 µmol/L)) were performed 45 min before other treatments. For transfection, VSMC were plated at 70-80% confluence for cDNA transfection using the Nucleofector (LONZA/Amaxa) according to the manufacturer’s instructions. Twenty four hour post-transfection, culture medium is replaced and VSMC are treated or not with E2 (10⁻⁹ mol/L) for one hour. cDNA coding for the constitutively active (CA) or the dominant inactive (DN) forms of AMPK are gifts from Benoit Viollet. cDNA encoding for RhoA mutants were previously described. They are fused to an HA-tag thereby expression of RhoA-mutants were analysed using anti-HA antibody (HA). Mouse embryonic fibroblasts (MEFs) from wild-type and AMPKalpha1 deficient mice (described elsewhere)
were cultured in DMEM containing 10% of fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin and treated as VSMC.

**Western Blot Analysis.** As previously described. Male or female mice used for these analyses were 14 to 22 week old.

**AMPK kinase assay.** Direct AMPK activity was measured using human recombinant AMPK (MERCK) and γ[32P] incorporated into SAMS peptide substrate (HMRSAMSGLHLVKRR) (MERCK) or recombinant Wild Type- (WT-) or phosphoresistant- (S188A-) RhoA for 30 min at 30°C using a final concentration of 50 μM ATP according to the manufacturer instructions (MERCK). Assays were terminated by phosphoric acid and revealed as phospho-cellulose binding assay. 4

**Rock kinase assays.** Rock-1 or Rock-2 were immunoprecipitated (with anti-rock1 H85 sc5560 1/200 or anti-rock2 C20 sc1851 1/200, Santa Cruz Biotechnology) from lysates (NETF lysis buffer) of VSMC or MEF treated or not with E2 for 6h. Immunoprecipitates were resuspended in 50 μl 1× kinase buffer (25 mmol/L Tris, pH 7.5; 10 mmol/L MgCl₂; 5 mmol/L β-glycerol phosphate; 0.1 mmol/L Na₃VO₄; and 2 mmol/L DTT) containing 1 μmol/L cold ATP, 10 μCi of [γ-³²P]ATP and S6 substrate. After incubation for 30 minutes at 30°C, assays were terminated by phosphoric acid and revealed as phospho-cellulose binding assay. 4

**Animals.** Wild type or AMPKalpha1 deficient mice have been described elsewhere. After bilateral ovariectomy (OVX) under isoflurane anesthesia, wild type or AMPKalpha1 deficient female mice (10 to 20 weeks old) were daily treated with E2 (subcutaneous, 0.1 mg/kg/day; OXV+E2) or placebo and buprenorphine and carprofen analgesia for the following procedure. After 7 to 10 days, they were killed by cervical dislocation. Body weight did not
vary significantly between the groups, but uterus weight was already diminished in the OVX group (0.113±0.016g for OVX versus 0.152±0.018 for OVX+E2). Mice were killed by cervical dislocation. Their spleens were weighted to control the mice genotype (WT spleen 103±45 versus alpha1 KO spleen 252±60mg) and their aorta used for Western Blot or tension analysis.

**Tension Measurement.** Isometric tension of arterial rings of thoracic aorta was measured as previously described.6

**Chemicals and Drugs.** Mouse monoclonal anti-RhoA antibody (26C4 ; sc-418), mouse anti-phospho Ser 188 of RhoA (sc32954, (p)Ser188), rabbit anti-phospho-MYPT (rat Thr697/human Thr696 sc-17556, (p)MYPT) and goat anti-phospho-MYPT (rat Thr855/human Thr853 sc 17432, (p)MYPT) antibodies were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Rabbit anti-phospho-Acetyl-CoA Carboxylase (Ser79) ((p)-ACC), rabbit anti-phospho-AMPK (Thr172, (p)AMPK) and pan-AMPK antibody were purchased from Cell Signaling (TEBU Bio). Mouse monoclonal anti-phosphoserine was purchased from (ZYMED). Mouse monoclonal anti-HA (HA.11) was purchased from Berkely Antibody Co. (BabCO, Richmond, CA, USA). 8-pCPT-cGMP was purchased from Biolog Life Science Institute (Bremen, Germany). 17-β-estradiol (E2) and ICI 182, 780 were purchased from TOCRIS (Bioscience, Bristol, UK). For *in vitro* VSMC stimulation, 17-β-estradiol powder was resuspended in DMSO to constitute a 1 mol/L stock solution that was then diluted (10⁻⁹ mol/L final concentration) in the culture medium. For *in vivo* injection 17-β-estradiol powder was resuspended in DMSO then diluted 1000 times in NaCl 0.9% before being injected. Controls were done with same dilutions of DMSO alone (1/10⁹ and 1/1000 for *in vitro* and *in vivo* experiments, respectively). Compound C, AICAR, Metformin and H89
were purchased from Calbiochem (MERCK biochemicals). All other reagents were purchased from Sigma (Saint-Quentin Fallavier, France).

**Statistics.** All results are expressed as the mean±SD of sample size n. Statistical analysis were performed with 2-ways ANOVA. A value of p <0.05 or less was considered to be statistically significant and is specified for each experiment in the figure legend.

**References**


Figure 1: AMPK activation induces RhoA phosphorylation and RhoA-Rock pathway inhibition.

To consider a more general effect and confirm that, in cells, AMPK is able to induce RhoA phosphorylation and RhoA-Rock pathway inhibition, we next analyzed the effects of AMPK activators AICAR and metformin on VSMC. Both induced the phosphorylation of RhoA (A) in concomitance with dephosphorylation of MYPT (B). Similar effects were obtained by expressing constitutively active mutant of AMPK (CA) in VSMC (C and D). In contrast, expression of a dominant negative form of AMPK (DN) increased MYPT phosphorylation and decreased the RhoA phosphorylation (C and D). These results show that independently of the tested activator, AMPK activation induces RhoA phosphorylation and RhoA-Rock pathway inhibition.

(A) and (B), Western blot analyzes of RhoA phosphorylation and RhoA-Rock pathway inhibition using (p)SER antibodies on RhoA IP or (p)MYPT on total lysates (tot lysate). VSMC were treated with AICAR (3 mmol/L) or metformin (Met, 4 mmol/L) activators of AMPK for the indicated time. (C) and (D), Same analyzes were performed on VSMC expressing the constitutively active (CA) or the dominant inactive (DN) forms of AMPK. Quantification of RhoA or MYPT phosphorylation level is represented relative to the non stimulated condition taken as 1. *p<0.05 and #p<0.01 treated versus non-treated or VSMC expressing a mutant versus mock at the same time. The data presented are representative of three to five independent experiments.
Figure II: Delayed hypertension development in female is due to AMPKa1.

Mean systolic blood pressure was measured by tail-cuff in male or female wild type mice (WT) and AMPKalpha1 knock-out (KO) mice during 14 days. # p<0.05 L-NAME-treated mice (100mg/kg per day in drinking water) versus control; ! p<0.05 WT male or KO female or male versus WT female at the same time. The data presented are representative of two independent experiments (five to eight mice per group).