Protection from angiotensin II–mediated vasculotoxic and hypertensive response in mice lacking PI3Kγ

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Hypertension affects nearly 20% of the population in Western countries and strongly increases the risk for cardiovascular diseases. In the pathogenesis of hypertension, the vasoactive peptide of the renin-angiotensin system, angiotensin II and its G protein–coupled receptors (GPCRs), play a crucial role by eliciting reactive oxygen species (ROS) and mediating vessel contractility. Here we show that mice lacking the GPCR-activated phosphoinositide 3-kinase (PI3K)γ are protected from hypertension that is induced by administration of angiotensin II in vivo. PI3Kγ was found to play a role in angiotensin II–evoked smooth muscle contraction in two crucial, distinct signaling pathways. In response to angiotensin II, PI3Kγ was required for the activation of Rac and the subsequent triggering of ROS production. Conversely, PI3Kγ was necessary to activate protein kinase B/Akt, which, in turn, enhanced L-type Ca^{2+} channel–mediated extracellular Ca^{2+} entry. These data indicate that PI3Kγ is a key transducer of the intracellular signals that are evoked by angiotensin II and suggest that blocking PI3Kγ function might be exploited to improve therapeutic intervention on hypertension.

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Abbreviations used: ANOVA, analysis of variance; DN-Akt, dominant-negative PB/Akt mutant; EGFR, epidermal growth factor receptor; ERK, extracellular signal–regulated kinase; GPCR, G protein–coupled receptor; GSK, glycogen synthase kinase; MCSA, media cross-sectional area; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); PI3K, phosphoinositide 3-kinase; PtdIns, phosphoinositides; PKB, protein kinase B; PTX, pertussis toxin; ROS, reactive oxygen species.

Angiotensin II is the primary effector peptide of the renin-angiotensin system and acts as a hormonal and local factor. It plays a key role in blood pressure homeostasis; high plasma levels of the peptide are a main trait of renovascular hypertension. In addition, angiotensin II overactivity has been involved in other widely diffused cardiovascular diseases, such as atherosclerosis and congestive heart failure.

The effects of angiotensin II are exerted on several target organs; however, especially the vascular action explains its impact on blood pressure. Angiotensin II increases vascular tone by activating calcium-flux, oxidative stress, and cell growth in vascular smooth muscle and, concomitantly, by promoting an inflammatory reaction in the vessel wall.

Several pharmacological interventions have been developed to attenuate angiotensin II vascular effects. In particular, inhibition of angiotensin II synthesis and, subsequently, blocking of its high affinity subtype-1 (AT_1) have allowed the targeting of angiotensin II-dependent negative effects.

Recent evidence suggests that the vasculotoxic effects of angiotensin II can be mediated via PI3K signaling pathways (1). PI3Ks are a family of lipid and protein kinases that are responsible for the phosphorylation of PtdIns at the position D3 of the inositol ring. These molecules act as secondary messengers and influence a variety of cellular responses, including proliferation, survival, and cytoskeletal remodeling (2). In vivo, PI3Ks of the class I subfamily produce PtdIns(3,4,5)P_3 that serves as a docking site for the pleckstrin homology domain that is present in numerous proteins that act as PI3K downstream effectors. Class I PI3Ks are divided in two subgroups depending on their biochemical properties. The class IA group

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consists of PI3Ks that—with the exception of PI3Kβ that also can respond to GPCRs—are activated mainly by tyrosine kinase receptors (3). Conversely, the unique member of class IB, PI3Kγ (p110γ), is activated exclusively by GPCRs; it binds directly to the βγ subunits of heterotrimeric G proteins (4) but its activity also can be modulated by interaction with an adaptor protein, p101 (5). Deletion of the *PI3Kγ* gene in mice is compatible with life and causes a protection from leukocyte recruitment by inflammatory stimuli (6, 7). A growing set of evidence indicates that PI3Kγ also is expressed in the cardiovascular system where it negatively controls cardiomyocyte contractility (8–10).

The specific PI3K isoform that is involved in angiotensin II signaling is still controversial. Using pharmacological inhibitors that block PI3K function without distinguishing between isoforms, it has been found that, in vascular smooth muscle cells, angiotensin II requires a PI3K activity to stimulate calcium channels and induce the calcium influx that governs the vascular contractile response (11). Although in porcine coronary artery smooth muscles, tyrosine phosphorylation and class IA PI3Ks may be involved (12), in rat portal vein myocytes, the free $\beta_1\gamma_3$ dimers that are generated by the activation of the G_{13}-coupled AT_{1A} receptor directly stimulate PI3K activity; this indicates a crucial role for the class IB enzyme, PI3Kγ (13). Recent evidence indicates that PI3Kγ and PI3Kα, but not PI3Kβ, are expressed by myocytes freshly isolated from rat portal veins (14). Although classes IA and B PI3K isoforms are present in rat portal vein myocytes, injection of antibodies that recognize different PI3K isoforms into these cells indicates that the angiotensin II-dependent activation of L-type Ca^{2+} current is inhibited by blocking PI3Kγ but not PI3Kα; this suggests a crucial role for PI3Kγ in angiotensin II signal transduction (15).

Despite the finding that smooth muscle cells require PI3Kγ for the angiotensin II–mediated intracellular Ca^{2+} concentration increase, in vivo studies that address the role of PI3Kγ in vascular responses to angiotensin II are missing. We examined the vascular responses to angiotensin II stimulation in mice lacking PI3Kγ and found that PI3K$\gamma^{-/-}$ vessels show reduced contractile responses to angiotensin II, a markedly decreased angiotensin II–mediated ROS production, and intracellular Ca^{2+} mobilization. As a consequence of these effects, mice lacking PI3Kγ are protected strongly from the hypertension that is induced by administration of angiotensin II in vivo.

RESULTS

PI3K$\gamma^{-/-}$ mice are protected from angiotensin II–induced hypertension

To investigate in an in vivo model whether PI3Kγ, the prototype GPCR-activated PI3K, plays a role in angiotensin II–

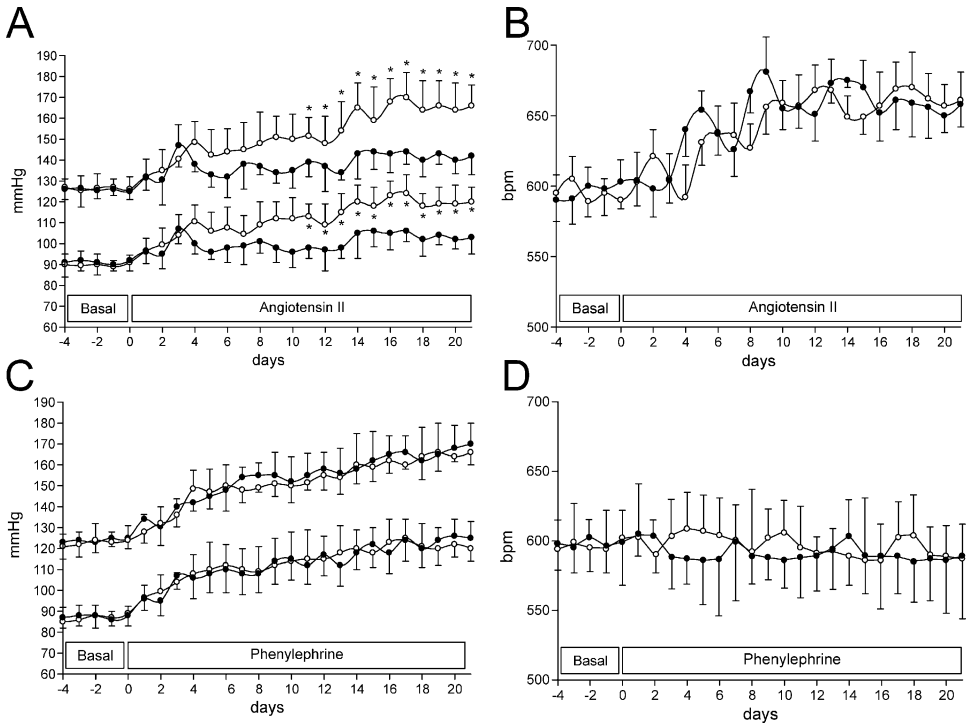

Figure 1. The lack of PI3Kγ protects in vivo from the hypertensive response evoked by angiotensin II. Daily systolic and diastolic blood pressure (A) and heart rate (B) profiles, recorded by radiotelemetry, in wild-type (empty circles; $n = 8$) and PI3K$\gamma^{-/-}$ (filled circles; $n = 8$) mice in response to 21 d of chronic infusion of angiotensin II. Angiotensin II is able to exert a significant hypertensive effect only in wild-type mice (PI3K$\gamma^{-/-}$ vs. wild-type controls *$P < 0.03$, two-way ANOVA analysis and Bonferroni post hoc test). Heart rate increase (10% over basal) is comparable in both mouse strains ($P = 0.90$). In contrast, the lack of PI3Kγ does not alter blood pressure (C) or heart rate (D) in response to phenylephrine.

induced hypertension, wild-type and PI3K$\gamma^{-/-}$ mice were challenged chronically with angiotensin II. As shown in Fig. 1 A, angiotensin II treatment induced a progressive increase in systolic and diastolic blood pressure in wild-type mice. In contrast, the hypertensive response seemed to be attenuated significantly in PI3K$\gamma^{-/-}$ animals; at the end of angiotensin II treatment, blood pressure increase of mutant mice was ~30% of that detected in wild-type controls (systolic blood pressure increase = 9 ± 4 mm Hg vs. 32 ± 5 mm Hg; % increase over basal diastolic blood pressure was 11% ± 4% vs. 29% ± 4%; n = 8). Nevertheless, chronic angiotensin II caused comparable changes of heart rate in both genotypes (Fig. 1 B). Echocardiographic analysis showed no signs of dilated cardiomyopathy or depressed systolic function in either mice strain (unpublished data); this excludes an involvement of cardiac effects on blood pressure. Infusion of phenylephrine influenced blood pressure (Fig. 1 C) and heart rate (Fig. 1 D) to a similar extent in both mouse strains.

The lack of PI3Kγ protects from angiotensin II–mediated vascular damage

To test whether the lack of PI3Kγ could protect vessels from the toxic effects of chronic angiotensin II stimulation, structural remodeling of the mesenteric wall and coincident inflammatory response were analyzed after 21 d of angiotensin II infusion. In wild-type mice, morphometric analysis of mesenteric arteries revealed a significant increase in MCSA and media/lumen ratio, but not in lumen diameter. This morphological pattern, typical of hypertrophic vascular remodeling, was blunted significantly in PI3K$\gamma^{-/-}$ animals (Fig. 2, A–C). Conversely, chronic infusion of phenylephrine induced eutrophic remodeling, with increased media/lumen ratio, but not MCSA (16), that was equally evident in mutant and control samples. The possible involvement of different recruitment of inflammatory cells in the vessel wall was evaluated next. Immunohistochemistry that used neutrophil-, macrophage-, and lymphocyte-specific markers showed equally low infiltrates in both genotypes (unpublished data). Nonetheless, PI3Kγ-deficient vessels expressed lower levels of vascular cell adhesion molecule-1, an adhesion receptor induced by angiotensin II, than wild-type controls (Fig. 2 D).

PI3K$\gamma^{-/-}$ mice show a blunted angiotensin II–dependent vasoconstriction

Angiotensin II can increase blood pressure after chronic treatment and in response to acute stimulation. PI3K$\gamma^{-/-}$ mice showed a reduced blood pressure increase in response to acute angiotensin II administration (Fig. 3 A); this indicates a specific involvement of PI3Kγ in the acute and chronic hypertensive effects of angiotensin II. As a consequence, the effects of the lack of PI3Kγ on angiotensin II–mediated vascular reactivity were studied next. In wild-type mesenteric arteries, angiotensin II induced a significantly blunted vasoconstriction in PI3Kγ-deficient vessels (Fig. 3 B). This effect also was observed in endothelium denuded

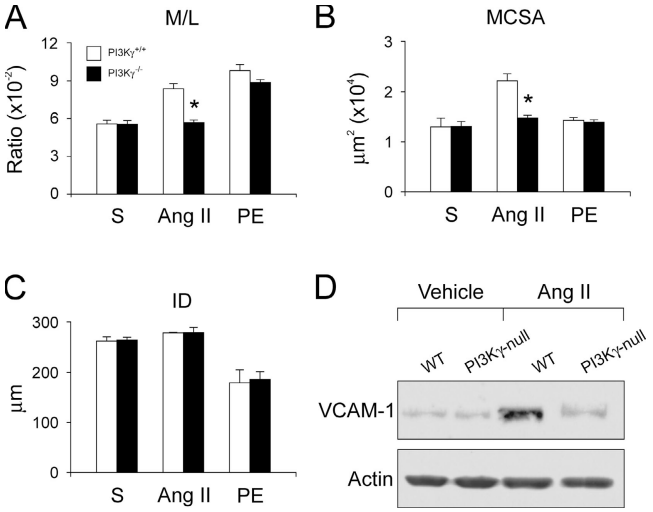

Figure 2. The lack of PI3Kγ protects from angiotensin II–induced vascular remodeling and activation. (A) Measurement of the media/lumen ratio (M/L) of aortas from wild-type (white bars) and PI3K$\gamma^{-/-}$ (black bars) mice before (S) and after 3 wk of chronic angiotensin II (Ang II) or phenylephrine (PE) infusion; n = 5 for both genotypes; *P < 0.05 by ANOVA. (B) Measurement of the MCSA; n = 5; *P < 0.05 by ANOVA. (C) Measurement of the internal diameter (ID); n = 5; P = 0.90 by ANOVA. (D) Western blot analysis of vascular cell adhesion molecule-1 (VCAM-1) expression in aortas of wild-type (WT) and PI3Kγ-deficient mice after chronic angiotensin II. Data are representative of three independent experiments.

vessels (unpublished data), which indicates that the lack of PI3Kγ affected smooth muscle cell–dependent responses. The maximal difference was obtained at 1 μM angiotensin II where contractility of PI3K$\gamma^{-/-}$ vessels was only 27% ± 5% of that of wild-type controls. The use of wortmannin reduced angiotensin II vasoconstriction in wild-type samples, and leveled vascular reaction to that observed in PI3K$\gamma^{-/-}$ vessels (Fig. 3 B). To prove further a specific involvement of PI3Kγ enzymatic activity in angiotensin II–mediated vascular contractility, vasoconstriction that was induced by angiotensin II was tested in vessels that were derived from PI3K$\gamma^{KD/KD}$ mice that expressed a catalytically inactive PI3Kγ (10). PI3K$\gamma^{KD/KD}$ mice showed an impaired angiotensin II–evoked increase in blood pressure (% mean arterial pressure increase: 25 ± 2% vs. 4 ± 1%, in wild-type and PI3K$\gamma^{KD/KD}$ mice, respectively; P < 0.01, one-way ANOVA; n = 4 for both genotypes) and vasoconstriction (max vasoconstriction: 620 ± 50 mg vs. 220 ± 35 mg in wild-type and PI3K$\gamma^{KD/KD}$ vessels, respectively; P < 0.01; n = 4 for both genotypes). This demonstrated that the enzymatic activity of PI3Kγ is required for angiotensin II–mediated vascular effects.

Furthermore, although pharmacological inhibition of AT_2 receptors did not affect the differences that were observed between the two genotypes, blockade of AT_1 receptors abolished vasoconstriction to angiotensin II in PI3K$\gamma^{+/+}$ and PI3K$\gamma^{-/-}$ mice (unpublished data). Other vasoactive

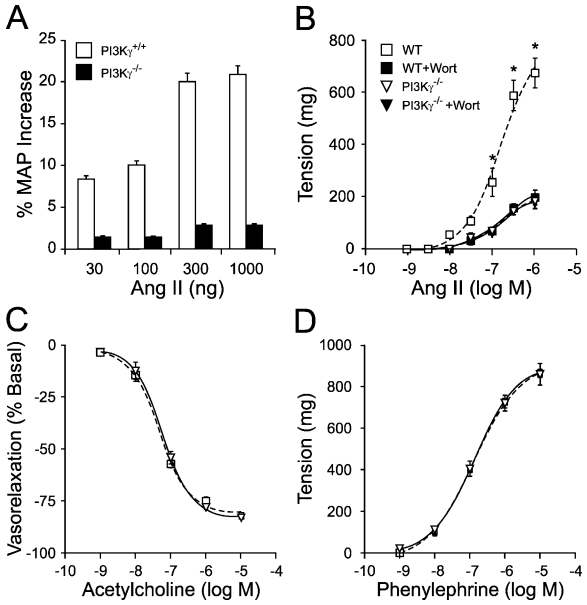

Figure 3. PI3Kγ modulates angiotensin II–dependent vasoconstriction. (A) Acute effect of angiotensin II on mean arterial pressure in wild-type (white bars; $n = 4$) and PI3K$\gamma^{-/-}$ (black bars; $n = 4$; wild-type vs. PI3K$\gamma^{-/-}$ $P < 0.01$ by ANOVA). Basal blood pressure was identical in the two genotypes (see Figure 1). (B) angiotensin II-dependent vasoconstriction in PI3K$\gamma^{-/-}$ (empty triangles, continuous line; $n = 12$) and wild-type (WT) vessels (empty squares, dotted line; $n = 12$; *$P < 0.01$, two-way ANOVA and Bonferroni post hoc test). In the presence of wortmannin (Wort, filled symbols), angiotensin II–mediated vasoconstriction is reduced only in wild-type arteries (filled squares, dotted line, $n = 12$; wild-type samples vs. wild-type samples in the presence of wortmannin *$P < 0.01$). (C, D) Vascular relaxation to acetylcholine or contraction to phenylephrine are not affected by the absence of PI3Kγ. Relaxation to acetylcholine is expressed as percentage of the tension attained with an initial stimulation with 1 μM phenylephrine.

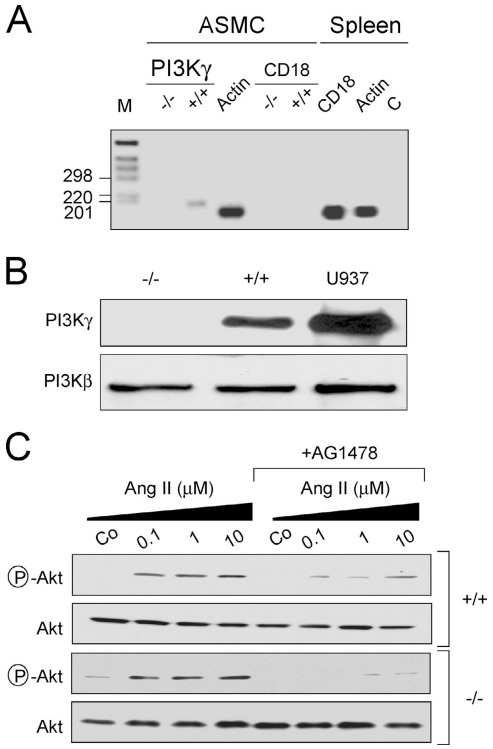

Figure 4. PI3Kγ expression in murine smooth muscle cells. (A) RT-PCR analysis of PI3Kγ expression in wild-type and PI3K$\gamma^{-/-}$ primary ASMCs. PI3Kγ expression can be detected only in wild-type samples. The leftmost Actin lane corresponds to a control amplification of the PI3K$\gamma^{-/-}$ sample. The absence of CD18 expression excludes a possible contribution of contaminating leukocytes to the PI3Kγ signal. C, control amplification with water only. M, molecular weight markers. (B) Detection of PI3Kγ (p110γ) expression by immunoprecipitation of 1 mg of proteins extracted from primary wild-type and PI3K$\gamma^{-/-}$ ASMCs. Detection of PI3Kβ (p110β) expression by Western blot analysis of supernatants. (C) Angiotensin II-induced PKB/Akt phosphorylation is mediated by EGFR transactivation in wild-type (+/+) and PI3K$\gamma^{-/-}$ (−/−) in vitro cultured ASMCs. Encircled "P" denotes phosphorylated. Cells were stimulated for 5 min with saline (Co) angiotensin II. EGFR transactivation was inhibited by AG1478 (250 nmol/L) before angiotensin II stimulation. Panels are representative of five independent experiments.

agonists, such as acetylcholine and phenylephrine, evoked a similar response in the two mouse strains (Fig. 3 C and D) that was unaffected by pretreatment with wortmannin (not depicted). Altogether, these results indicate that the catalytic activity of PI3Kγ plays a specific key role in the AT_1-mediated signal transduction that leads to vascular contraction.

Impaired angiotensin II–dependent Akt phosphorylation in PI3Kγ-deficient aortas

To test a possible involvement of PI3Kγ in angiotensin II–mediated signal transduction and in the vascular responses that lead to hypertension, primary smooth muscle cells were isolated from aortas of wild-type and PI3Kγ null mice. The cells were stained positive for smooth muscle actin and, when derived from wild-type animals, presented the mRNA for PI3Kγ (Fig. 4 A). Similarly, only wild-type cells showed the expression of the PI3Kγ protein, albeit at very low levels (Fig. 4 B, top). In PI3K$\gamma^{-/-}$ samples, no changes in expression of other PI3Ks (e.g., PI3Kβ) were detected in mutant samples (Fig. 4 B, bottom). Because PI3Kγ could play a role in GPCR-mediated signaling, cultures were expanded for no more than five passages and cells were stimulated with angiotensin II. In apparent contrast to a putative role of PI3Kγ in angiotensin II signaling, analysis of the PI3K-dependent phosphorylation of protein kinase B (PKB)/Akt at Ser473 did not show any difference between wild-type and mutant cells (Fig. 4 C). However, in aortic smooth muscle cells (ASMCs) of both genotypes, the angiotensin II activation of PKB/Akt was blocked by the EGFR kinase blocker, AG1478 (Fig. 4 C). These results are in agreement with the previous finding that in vascular smooth muscle cells, angiotensin II–mediated signaling triggers EGFR transactivation (17), and thus, bypasses PI3Kγ function by an EGFR-dependent activation of class IA PI3Ks.

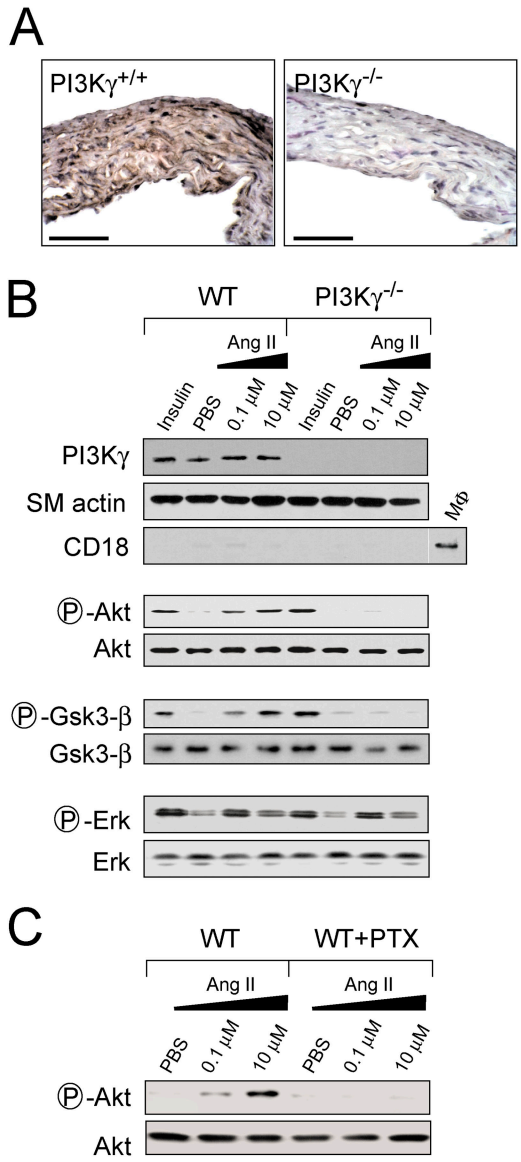

Figure 5. Aortic smooth muscle PI3Kγ modulates angiotensin II–dependent activation of PKB/Akt. (A) Immunohistochemical localization of PI3Kγ expression in a section of a mouse aorta. Note brown reactivity only in the smooth muscle cell layer of wild-type ($PI3K\gamma^{+/+}$). Bars, 100 μm. (B) PI3Kγ expression in aortic tissue extracts. Phosphorylation of PKB/Akt and GSK3-β shows a concentration-dependent increase in angiotensin II–stimulated wild-type aortas, which is impaired in PI3Kγ-deficient vessels. Conversely, angiotensin II-mediated ERK1 and 2 phosphorylation are not affected. Stimulation of PKB/Akt and GSK3-β phosphorylation is identical in aortas of the two genotypes treated with insulin. Equal loading is evidenced by the detection of the total nonphosphorylated proteins. Panels are representative of three independent experiments. PBS, saline control; SM, smooth muscle. (C) angiotensin II–dependent PKB/Akt is mediated by a $G_{\alpha i}$-coupled receptor. angiotensin II-dependent phosphorylation of PKB/Akt is lost equally in PTX-treated and PI3Kγ-deficient aortas. Panels are representative of three independent experiments.

To test whether PI3Kγ function was required for angiotensin II–dependent signal transduction in vivo, studies were conducted on intact aortas. First, PI3Kγ expression was investigated by immunohistochemistry and the smooth muscle cell layer was stained positive for the enzyme (Fig. 5 A). Next, expression was validated in aortic tissue preparations that expressed PI3Kγ and smooth muscle actin but little CD18; this indicated a relatively small contamination of leukocytes and a major presence of smooth muscle cells (Fig. 5 B). After i.p. stimulation with different doses of angiotensin II, only wild-type samples showed PKB/Akt phosphorylation (1.8 ± 0.6 and 3.5 ± 0.3 fold induction vs. unstimulated control for 0.1 and 10 μM angiotensin II, respectively; $n = 7$; $P < 0.05$ and $P < 0.01$, respectively; one-way ANOVA). This process was impaired strongly in PI3Kγ-deficient aortas (1 ± 0.1 and 1.3 ± 0.3 fold induction over the unstimulated control for 0.1 and 10 μM angiotensin II, respectively; $n = 7$, not significant). i.p. administration of 0.5 mg/kg AG1478 (suspended in 0.2% carboxymethylcellulose and sonicated), known to abrogate EGFR activation in vivo (18), in wild-type mice did not affect PKB/Akt activation; this indicates that, in vivo, EGFR transactivation is not involved (unpublished data). To prove further the specificity of this defect, PKB/Akt activation was tested after stimulation with insulin, an agonist that is known to signal through PI3K isoforms different from PI3Kγ. As shown in Fig. 5 B, tissue samples from mutant and control mice showed an identical ability to phosphorylate PKB/Akt. As expected from a reduced PKB/Akt activation, phosphorylation of glycogen synthase kinase (GSK)3-β, a known substrate of PKB/Akt, was decreased in PI3Kγ-deficient aortas after angiotensin II, but not insulin, stimulation. Conversely, no difference in extracellular signal–regulated kinase (ERK)1/2 activation was detected in mice of the two genotypes.

In wild-type vessels, PKB/Akt phosphorylation after angiotensin II stimulation was reduced by a pretreatment with PTX to the level that was detected in mutant samples (Fig. 5 C). In contrast, no further decrease in PKB/Akt phosphorylation could be detected in PTX-treated mutant vessels; this indicates the involvement of a PTX-sensitive $G_{\alpha i}$-coupled angiotensin II receptor in PI3Kγ activation.

$PI3K\gamma^{-/-}$ mice are protected from angiotensin II–evoked vascular ROS generation

In hypertension, the increase of vascular ROS contributes to endothelial dysfunction and vessel contraction. Because angiotensin II induces ROS production in vascular smooth muscle cells, the involvement of PI3Kγ in this process was evaluated next. To test the effects of angiotensin II–mediated ROS production, vascular contractility was measured after the combined administration of angiotensin II and tiron, a potent ROS scavenger. As expected, in wild-type vessels, tiron significantly reduced the angiotensin II–dependent enhancement of vascular wall tension. In contrast, in mutant vessels, the addition of tiron did not modify further the

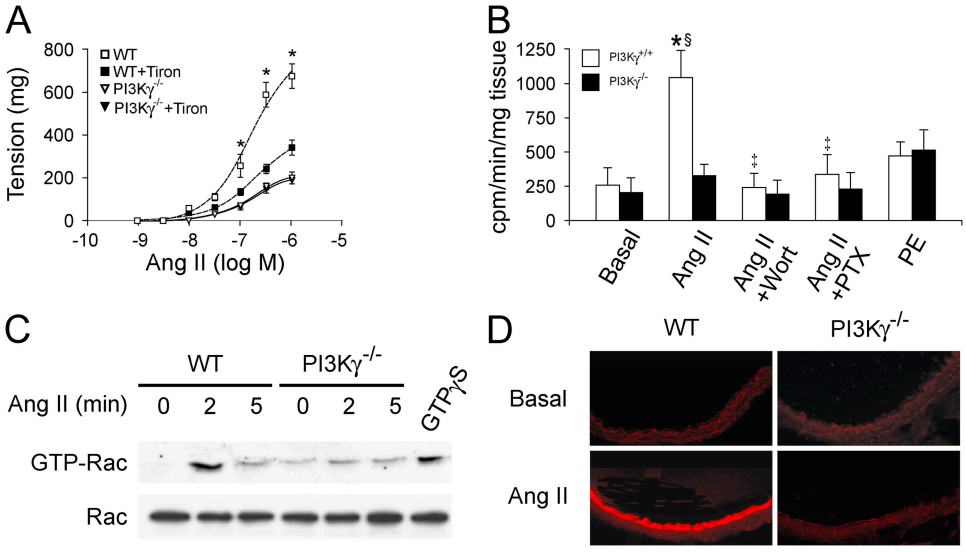

Figure 6. PI3Kγ is involved in the vascular ROS production induced by angiotensin II. (A) Tiron reduced angiotensin II–mediated vasoconstriction of only wild-type mesenteric arteries ($n = 6$; *$P < 0.01$, two-way ANOVA and Bonferroni post hoc test). (B) Measurement of ROS by chemiluminescence before and after stimulation of vessels from wild-type ($n = 4$) and PI3K$\gamma^{-/-}$ mice ($n = 4$) with angiotensin II (1 μM) alone and after PTX (250 ng/ml), wortmannin (100 nM), or phenylephrine (1 μM). Angiotensin II–evoked ROS production is blunted significantly in PI3K$\gamma^{-/-}$ mice ($^{\S}P < 0.01$ basal vs. stimulated, *$P < 0.01$ wild-type vs. PI3K$\gamma^{-/-}$ stimulated vessels, one-way ANOVA). Similarly, PTX treatment reduces the ROS production only in the wild-type samples ($^{\dagger}P < 0.01$ stimulated vs. PTX-treated stimulated wild-type vessels). (C) Representative immunoblot of GTP-bound Rac. At 2 min after angiotensin II stimulation, Rac activation is reduced markedly in PI3K$\gamma^{-/-}$ samples. Mutant vessels, however, show normal Rac activation after treatment with the unspecific Rac activator, GTPγS. (D) Immunohistochemical detection of nitrotyrosines in aortas from wild-type and PI3K$\gamma^{-/-}$ mice stimulated with 1 μM angiotensin II. Angiotensin II–induced nitration of vascular proteins is blunted in PI3K$\gamma^{-/-}$ mice. Panels are representative of three independent experiments.

blunted response to angiotensin II; this suggests that angiotensin II-evoked ROS generation requires the activation of PI3Kγ (Fig. 6 A). To prove further that PI3Kγ was involved in the angiotensin II–mediated ROS production, the generation of ROS was measured directly in wild-type and mutant vessels after angiotensin II stimulation. The levels of ROS production in tissues of the two genotypes were comparable in basal conditions (Fig. 6 B). After administration of angiotensin II, wild-type vessels responded with a significant increase of ROS generation over the basal level (370% ± 70% over control, $P < 0.01$); this effect was blocked by preincubation with wortmannin (Fig. 6 B). In contrast, stimulated PI3Kγ-deficient aortas showed a response that was reduced by 70% over that of similarly treated wild-type samples ($P < 0.05$); wortmannin did not affect this response further. PI3K$\gamma^{KD/KD}$ mice showed a similarly impaired response (unpublished data); this confirms that the catalytic activity of PI3Kγ is a key event that is necessary for the angiotensin II–dependent generation of ROS. This effect was mediated by AT_1 receptors because it was inhibited by candesartan, but not by PD123319 (unpublished data). Angiotensin II-mediated ROS production was inhibited significantly by pretreatment with PTX; this further suggested an involvement of a $G_{\alpha i}$-coupled angiotensin II receptor in vessel (Fig. 6 B). In addition, vascular ROS production that was evoked by phenylephrine, an agonist of a $G_{\alpha q}$-coupled receptor, was very weak and seemed to be similar in both mouse strains (Fig. 6 B).

The signaling pathway that links PI3Ks to ROS production involves the PI3K-dependent activation of Rac, which, in turn, triggers nicotinamide adenine dinucleotide (phosphate) (NAD[P]H)-oxidase–dependent ROS generation (17, 19). Although Rac activation increased in stimulated wild-type samples, this effect was reduced markedly in mutant vessels (Fig. 6 C). These data suggest that the lack of PI3Kγ impairs ROS production because of a reduced activation of Rac in response to angiotensin II.

In the vasculature, superoxide can react with other free radicals, such as nitric oxide, and generate peroxynitrites that lead to nitrotyrosine production. In wild-type samples, tyrosine nitration was undetectable in basal conditions but became apparent after stimulation with angiotensin II (Fig. 6 D, left). In contrast, the level of nitrotyrosines in aortas that were derived from PI3K$\gamma^{-/-}$ mice was undetectable in basal conditions and after angiotensin II stimulation (Fig. 6 D, right).

PI3Kγ is required for angiotensin II–mediated extracellular Ca^{2+} entry through activation of PKB/Akt

Although in wild-type vessels, contractility in response to angiotensin II was reduced by tiron, the impact of the antioxidant was smaller than that caused by the lack of PI3Kγ (Fig. 6 A). This observation suggested that in the absence of

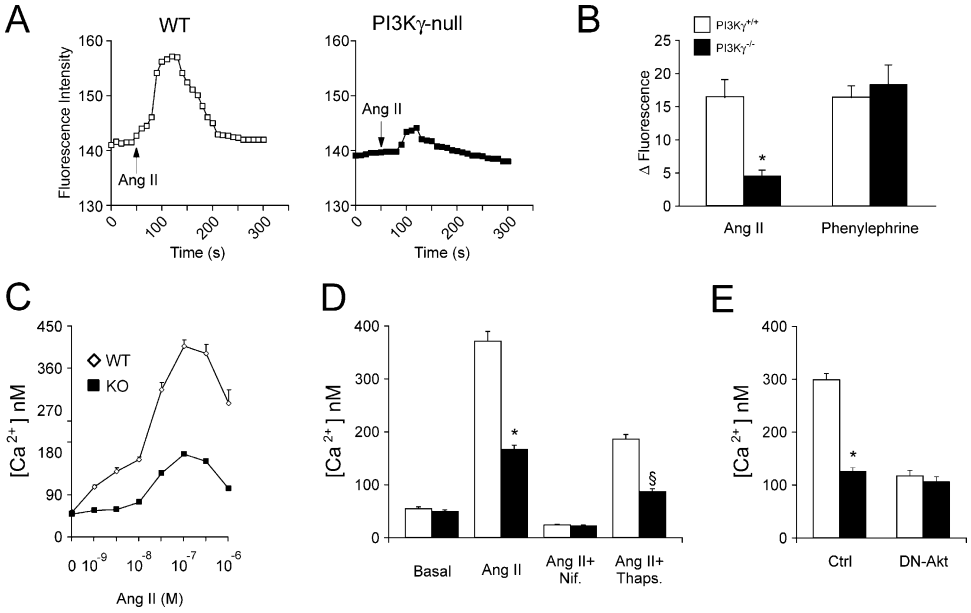

Figure 7. The PI3Kγ/Akt pathway is involved in the extracellular calcium entry induced by angiotensin II. (A) Time-dependent changes in vascular Ca^{2+} release in the vessels expressed in arbitrary units. (B) Average of peak-to-basal change in Fluo4-AM fluorescence intensity in mesenteric arteries from wild-type ($n = 7$) and PI3K$\gamma^{-/-}$ ($n = 8$) mice in response to angiotensin II and phenylephrine. Angiotensin II–evoked calcium flux is impaired significantly in vessels from PI3K$\gamma^{-/-}$ mice (*$P < 0.005$, Student's t test), whereas the effect of phenylephrine is comparable in both mouse strains ($P = 0.95$). (C) Dose-response quantification of Ca^{2+} flux induced by angiotensin II. PI3K$\gamma^{-/-}$ vessels show a significantly reduced response at all concentrations ($n = 5$ for each genotype, $P < 0.001$ as assessed by two-way ANOVA). (D) PI3K$\gamma^{-/-}$ vessels show reduced extracellular Ca^{2+} entry upon angiotensin II (0.1 μM) stimulation. Vessels were treated with vehicle (basal) or angiotensin II before and after 10 min pretreatment with 2 μM thapsigargin (Thaps.) or 1 μM nifedipine (Nif.). Vessels were exposed to vehicle (basal) or 0.1 μM angiotensin II. Data represent the mean peak Ca^{2+} mobilization from five independent experiments (*$P < 0.02$ PI3K$\gamma^{-/-}$ vs. wild-type; §$P < 0.01$ PI3K$\gamma^{-/-}$ vs. wild-type as assessed by ANOVA). (E) Transfection of wild-type aortas with a DN-Akt reduces angiotensin II–mediated $[Ca^{2+}]_i$ increase to the level detected in PI3K$\gamma^{-/-}$ samples. The empty plasmid (Ctrl) does not exert any effect.

PI3Kγ, the impaired angiotensin II–mediated vasoconstriction was due not only to reduced ROS production. During vessel contraction, a crucial role is played by the increase of intracellular Ca^{2+}, which stimulates the contractile machinery. To test whether the reduced contractile response to angiotensin II in PI3K$\gamma^{-/-}$ vessels was due to a defective induction of intracellular calcium increase, the ability of angiotensin II to enhance $[Ca^{2+}]_i$ was evaluated in wild-type and mutant mesenteric artery preparations. In resting conditions, vessels of wild-type and mutant mice showed similarly low fluorescence levels (Fig. 7 A). The addition of angiotensin II caused a time-dependent increase in fluorescence intensity with a maximal peak at 70 s after agonist exposure (Fig. 7 A). However, the peak fluorescence that was detected in PI3Kγ-deficient vessels was 73% weaker than that measured in wild-type controls (Fig. 7 B, $n = 7$). In contrast, phenylephrine elicited a comparable fluorescence response in wild-type and mutant samples (Fig. 7 B).

To define better the role of PI3Kγ in angiotensin II–evoked Ca^{2+} mobilization, a concentration response curve for angiotensin II was determined at 70 s after stimulation and $[Ca^{2+}]_i$ was quantitated from the fluorescence measurements (Fig. 7 C). Vessels of both genotypes showed a maximal Ca^{2+} mobilization with 0.1 μM angiotensin II; however, the $[Ca^{2+}]_i$ induced in mutant samples always was weaker than in wild-type controls. In particular, the maximal response of mutant samples was reduced by 60% ($P < 0.05$, Bonferroni post hoc test); this confirms further that the lack of PI3Kγ impairs vascular calcium mobilization in response to angiotensin II. Because this difference may be due to abnormal intracellular calcium release or extracellular calcium entry, Ca^{2+} flux was analyzed after depletion of intracellular Ca^{2+} stores by preincubation with thapsigargin, a specific inhibitor of Ca^{2+}-ATPase pumps (20). After this treatment, the residual angiotensin II–induced increase in $[Ca^{2+}]_i$ (Fig. 7 D) and vasoconstriction (unpublished data) remained higher in wild-type samples than in PI3K$\gamma^{-/-}$ vessels; this excludes an involvement of PI3Kγ in angiotensin II–induced intracellular Ca^{2+} fluxes. Next, calcium entry was analyzed by incubation of vessels in a Ca^{2+}-free medium that contained 0.1 mM EGTA, or in the presence of the L-type Ca^{2+} channel antagonist, nifedipine (1 μM). Consistent with angiotensin II causing Ca^{2+} entry mainly through L-type channels, both treatments blocked the angiotensin II–mediated Ca^{2+} influx in wild-type and PI3K$\gamma^{-/-}$ samples (Fig. 7 D). In addition, inhibition of T-type Ca^{2+} channels

with flunarizine dihydrochloride (10 μM) did not modify the angiotensin II–dependent Ca^{2+} response (unpublished data). These findings demonstrate that PI3Kγ modulates angiotensin II–dependent extracellular calcium entry via L-type Ca^{2+}channels.

L-type Ca^{2+} channels can be regulated by the levels of the second messenger, cAMP. Our previous results indicated that in the heart, PI3Kγ helps to reduce cAMP concentration independently of its kinase activity (10). PI3Kγ$^{-/-}$ mice, where PI3Kγ is absent, show increased cAMP in basal conditions but no effect can be detected in PI3Kγ$^{KD/KD}$ mice that express a catalytically inactive PI3Kγ. As increased cAMP levels could influence L-type Ca^{2+} channels of smooth muscle negatively, cAMP concentration was determined in wild-type and PI3Kγ$^{-/-}$ aortas. In agreement with the finding that in PI3Kγ$^{-/-}$ and in PI3Kγ$^{KD/KD}$ vessels angiotensin II–dependent vasoconstriction is affected equally, measurements in resting conditions and after stimulation with the β-adrenergic agonist, isoproterenol (1 μM), never revealed differences between wild-type and PI3Kγ-deficient aortas (basal [cAMP]: 0.081 ± 0.019 nmol/g and 0.062 ± 0.022 nmol/g, respectively; isoproterenol-induced [cAMP]: 0.391 ± 0.140 nmol/g and 0.280 ± 0.174 nmol/g, respectively; $n = 6$ for each genotype). The finding that angiotensin II-dependent vasoconstriction requires the kinase activity of PI3Kγ demonstrates the involvement of signaling pathways that are linked to PtdIns(3,4,5)P_3 production. Because the enzymatic activity of PI3Kγ is required for PKB/Akt phosphorylation, PKB/Akt activation might mechanistically couple PI3Kγ to extracellular Ca^{2+} entry. To explore this hypothesis, PKB/Akt was blocked by transfecting a DN-Akt in isolated vessels. A reduction of angiotensin II–evoked vasoconstriction was detected only in DN-Akt–transfected wild-type samples that were leveled to the response of PI3Kγ$^{-/-}$ vessels (24 ± 6% and 19 ± 8% increase over basal in wild-type and PI3Kγ$^{-/-}$ samples, respectively; not significant; $n = 4$ for each genotype). This result was supported fully by data on calcium fluctuations which showed that DN-Akt expression blunted angiotensin II effects only in wild-type vessels (Fig. 7 E). In contrast, expression of DN-Akt did not affect the PKB/Akt-independent vascular response and calcium mobilization that are induced by potassium (unpublished data). These data indicate that angiotensin II acts on L-type Ca^{2+} channels through a PI3Kγ/Akt pathway.

DISCUSSION

In this study, we demonstrated for the first time that PI3Kγ is required for angiotensin II–evoked vasoconstriction and that the inactivation of this enzyme protects from the hypertensive response that is elicited by angiotensin II administration. We also provide evidence that PI3Kγ is a crucial mediator of angiotensin II–dependent signaling in vascular smooth muscle, where PI3Kγ controls vasoconstriction through ROS production and PKB/Akt activation that lead to extracellular Ca^{2+} entry from L-type Ca^{2+} channels.

It is well known that angiotensin II represents one of the major mediators that is involved in the development of hypertension. The protection from blood pressure increase after angiotensin II administration that was seen in PI3Kγ$^{-/-}$ mice has to be ascribed mainly to the vascular phenotype and not to the previously reported enhancement of cardiac contractility (8, 10), that should, in principle, increase cardiac output and blood pressure. In agreement with this view, several studies demonstrated that in the pathogenesis of angiotensin II–mediated hypertension, a critical role is played by ROS production (21) as well as imbalanced homeostasis of calcium (22, 23). Therefore, it is possible that the protection from angiotensin II–induced hypertension that is observed in the absence of PI3Kγ could be due to the beneficial reduction of oxidative stress and intracellular calcium concentration. However, PI3Kγ-deficient vessels also were protected from the chronic vascular remodeling that occurred as a consequence of hypertensive vascular insult. Although a minor involvement of inflammatory response, evoked particularly by vascular ROS production, could not be excluded, our data point to a crucial role of PI3Kγ in vascular smooth muscle, rather than in the onset of vascular inflammation.

Although a previous report suggests that PI3Kγ is not present in rat aortas (24), our finding of PI3Kγ expression in aortic smooth muscle is in agreement with the established notion of its existence in rat portal vein myocytes (14, 15). Nevertheless, PI3Kγ was expressed at low levels and it is possible that the different nature of the antibodies that were used in the two studies might account for this discrepancy. Our data further located p110γ expression in cultured ASMCs; however, analysis of angiotensin II stimulation revealed that mutant cultured cells responded equally as well as wild-type controls. In contrast, PI3Kγ$^{-/-}$ intact vessels showed a clearly impaired response to angiotensin II. As an explanation for these divergent results, and in agreement with previous studies (17) in ASMCs cultured in vitro, our results suggest that the angiotensin II–dependent transactivation of the EGFR induces class IA PI3K-dependent activation and bypasses the requirement for PI3Kγ function. Our data suggest that in intact aortas, the pathway that is dependent on EGFR transactivation is less critical, and that, in vivo, PI3Kγ is used preferentially for angiotensin II–mediated PKB/Akt activation. Thus, our apparently contrasting findings indicate that for the analysis of vascular signal transduction events, in vivo experiments in genetically altered organisms are required; in vitro studies with primary ASMCs might be limited by the adaptation of cells to culture conditions. A similar situation was described in rat portal vein myocytes that, when freshly isolated, express only p110α, -γ, and -δ, but start to express p110β after a few days of culture (14). Because angiotensin II receptors can relay equally to PI3Kβ and -γ (14), the presence of PI3Kβ in cultured cells might compensate for the absence of PI3Kγ. The limited amount of low-passage primary ASMCs that is obtainable from mice and the lack of specific antibodies for immunohis-

tochemistry prevented us from investigating whether PI3Kβ is absent from ASMCs.

Nonetheless, our analysis of in vivo stimulated aortas clearly indicated that multiple angiotensin II–mediated vascular responses depend on PI3Kγ. So far, several mechanisms have been proposed to explain the activation of PI3K and the subsequent phosphorylation of PKB/Akt that is induced by angiotensin II. Although some studies include a role for tyrosine kinase–activated class IA PI3Ks (1, 12, 17), others reported that activation of G_q-coupled angiotensin II receptors can mediate PKB/Akt phosphorylation (25). Despite these indications, we showed by genetic means that angiotensin II signals through PI3Kγ in intact aortas and that this process is mediated by the activation of a G_i-coupled receptor. In general, signaling by the angiotensin II AT_1 receptors mainly is dependent on G_q-containing heterotrimeric G proteins (26); however, other studies also point to an involvement of G_i–coupled angiotensin II AT_1 receptors, particularly in the angiotensin II–dependent inhibition of adenylate cyclase (27). In addition, an involvement of G_i and PI3K is crucial for the signal transduction events that lead to Raf-1 activation in response to angiotensin II (28). In further agreement with a role of G_i in the angiotensin II–mediated signal transduction in smooth muscle cells, we and others (29) found that treatment with PTX inhibits the angiotensin II–dependent ROS generation. Our data indicate that the G_i-dependent branch of angiotensin II signaling might have considerable importance in the vascular responses that lead to vasoconstriction and hypertension.

The finding that the addition of wortmannin, a PI3K inhibitor with no isoform selectivity, in wild-type tissues blunted the angiotensin II–dependent vascular response to the level that was observed in mutant arteries, indicated that in intact vessels, PI3Kγ probably is the unique PI3K isoform that is involved in the process. In vivo blockade of EGFR-dependent signaling did not exert significant effects on angiotensin II–mediated PKB/Akt phosphorylation. Because other agonists, such as phenylephrine or acetylcholine, exerted identical effects in the two genotypes, the absence of PI3Kγ did not induce a generalized impairment of vascular function; this demonstrates a specific involvement of PI3Kγ in the angiotensin II–dependent vascular contractile response. In agreement with previous reports that suggested a role of ROS in vasoconstriction (30), scavenging of angiotensin II–mediated ROS production reduced vasoconstriction in wild-type preparations but was unable to exert a further inhibitory effect in PI3Kγ$^{-/-}$ samples. Consistently, we found a marked reduction of ROS production in response to angiotensin II in PI3Kγ-null vessels. As an explanation for this defective ROS generation, PI3Kγ seemed to be crucial for angiotensin II activation of Rac, a key event that is required for NAD(P)H oxidase assembly and ROS generation (17, 31). Our finding is in agreement with the PtdIns(3,4,5)P_3-dependent activation of GTP exchange factors triggering Rac. The involvement of a similar mechanism in the activation of NAD(P)H oxidase recently was outlined by the cloning of P-Rex, a GTP exchange factor for Rac, that, in neutrophils, mediates the respiratory burst response in a GPCR- and PI3K-dependent way (32).

The mechanism by which free radicals increase vascular tone has been attributed to a direct smooth muscle effect and a reduced nitric oxide bioavailability (33). The finding of increased nitration of tyrosines in wild-type, but not mutant, vessels that were stimulated with angiotensin II, further supports this view and suggests that PI3Kγ contributes to the angiotensin II–dependent depletion of vascular nitric oxide. Although antioxidant agents reduced the angiotensin II–dependent vasoconstriction in wild-type control samples, the overall effect that was caused by the lack of PI3Kγ was significantly stronger. This fact suggested that mechanisms other than decreased ROS production could be triggered concomitantly by PI3Kγ signaling and contribute to the impact on angiotensin II vascular response. In addition to through oxidative stress, angiotensin II can control vascular tone by increasing intracellular calcium concentration in smooth muscle cells (34). It is known that voltage-gated L-type Ca^{2+} channels represent the major pathway for calcium entry and play an important role in excitation-contraction coupling (35). Previous reports indicated that PI3Kγ may act as a key mediator of angiotensin II–dependent voltage gated L-type Ca^{2+} channel activation in rat portal vein myocytes (13, 14). In these cells, stimulation of angiotensin II receptors frees the βγ dimer of the G_{13} protein and activates PI3Kγ, which, in turn, causes an increase in intracellular Ca^{2+} concentration. Furthermore, intracellular infusion of an anti-PI3Kγ antibody causes reduced PtdIns(3,4,5)P_3 generation, and the inhibition of angiotensin II elicited stimulation of Ca^{2+} current (15). In agreement with these data, our results clearly showed that the angiotensin II–mediated elevation of intracellular Ca^{2+} concentration was defective in PI3Kγ$^{-/-}$ vessels. Our results with pharmacological inhibitors further clarify that PI3Kγ is involved in the mobilization of extracellular Ca^{2+} through L-type channels, and does not influence Ca^{2+} release from intracellular stores. Thus, the PI3K emerging signaling that was described to be involved in the regulation of Ca^{2+} release from intracellular stores in cardiac cells through membrane anchoring of Tec and subsequent phospholipase C activation seems not to be recruited by angiotensin II at the vascular level (36). Recently, PI3Kγ was implicated, in this case in the heart, in a kinase-independent activation of PDE3B, an enzyme that hydrolyzes cAMP, a secondary messenger that controls L-type Ca^{2+} channels function (10). Despite this fact, cAMP concentration in aortas was not affected by the absence of PI3Kγ; in addition, although cardiac contractility was different in mice lacking PI3Kγ (PI3Kγ$^{-/-}$) or expressing a kinase-dead mutant (PI3Kγ$^{KD/KD}$) (10), the angiotensin II–mediated vasoconstriction was equally blunted in the two PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$ genotypes. This clearly excludes a cAMP-related kinase-independent function of PI3Kγ in the angio-

tensin II–mediated modulation of L-type Ca^{2+} channel activity at the vascular level. Conversely, in agreement with an involvement of the catalytic activity of PI3Kγ, PI3Ks were found to enhance native voltage-dependent L-type Ca^{2+} currents through the activation of PKB/Akt, which causes rapid plasma membrane relocalization of channel subunits (37). Similarly, the expression of a constitutively active PKB/Akt mutant in the murine heart leads to increased L-type Ca^{2+} channel activation and enhanced contractility (38). ROS production can sustain PKB/Akt phosphorylation by the oxidative stress–dependent inactivation of the PtdIns(3,4,5)P_3 3-phosphatase, phosphatase with tensin homology (39). In this way, it could be hypothesized that the concurrent production of PtdIns(3,4,5)P_3 and ROS, induced by angiotensin II, cooperate to keep PKB/Akt in its phosphorylated state, and consequently, increase L-type Ca^{2+} channel activity. Our results, which were obtained with transfection of vessels with a dominant-negative form of PKB/Akt blocked angiotensin II–evoked $[Ca^{2+}]_i$ increase, clearly indicate that a PI3Kγ/Akt signaling pathway crucially regulates angiotensin II–mediated vascular contractility.

In light of these results, PI3Kγ represents a crucial intracellular signaling molecule which drives multiple mechanisms that are responsible for the angiotensin II–dependent vasculotoxic and hypertensive effects; thus, targeting this enzyme with specific inhibitors could be exploited to expand the therapeutic strategy that is aimed at treating hypertension.

MATERIALS AND METHODS

Mice. PI3Kγ$^{-/-}$ (6) and PI3Kγ$^{KD/KD}$ (10) mice were generated as previously described. For this study, 2-mo-old male 129sv inbred mice were used. Mice were kept in standard cages under a 12-h light/dark cycle and fed *ad libitum* and cared for according to guidelines of our institution. Experiments conformed with institutional and national guidelines, and were approved by the Italian Ministry of Health.

Evaluation of blood pressure in conscious mice. Radio-telemetric analysis of blood pressure and echocardiography were assessed as described (40). Angiotensin II (0.5 mg/kg/d in 0.9% NaCl), phenylephrine (0.15 mg/kg/d in 0.2% ascorbic acid), or vehicle were infused for 21 d through osmotic minipumps. Because blood pressure response in the first 3 d of chronic infusion was masked by the stress response to surgical procedures, acute blood pressure response to angiotensin II was evaluated by arterial catheterization as described (41).

Evaluation of vascular reactivity. Vascular reactivity and structure were assessed in mesenteric arteries as described previously (42). Increasing doses of angiotensin II (10^{-9} to 10^{-6} M) were tested alone and in the presence of the PI3K inhibitor, wortmannin (10^{-7} M, 30 min); the antioxidant agent, tiron (10^{-3} M, 10 min); the AT_1 antagonist, candesartan (10^{-6} M, 15 min); or the AT_2 antagonist, PD123319 (10^{-6} M, 15 min). Moreover, phenylephrine (10^{-9} to 10^{-5} M) and acetylcholine (10^{-9} to 10^{-5} M) vascular responses were tested in all vessels. For selected experiments, mechanical removal of the endothelial layer was demonstrated by the absence of acetylcholine-mediated vasorelaxation.

Isolation of ASMCs. ASMCs were isolated from male wild-type and PI3Kγ$^{-/-}$ mouse aortas using published procedures (43). All experiments were performed using cells at the fourth/fifth passage. Cell lysates were prepared from ASMCs at 80% confluence, starved for 24 h, and then stimulated with the agonists. For RT-PCR analysis, total RNA was extracted using RNAeasy columns (QIAGEN). Primers used were described previously (6).

In vivo aortic tissue stimulation. For in vivo angiotensin II stimulation, mice were anesthetized with i.p. thiopental (50 mg/kg). Angiotensin II was infused by i.p. injection. For PTX inhibition experiments, mice were injected i.p. with PTX (150 mg/kg) 24 h before angiotensin II stimulation. Effectiveness of the PTX treatment was assayed by the detection of full inhibition of muscarinic chronotropic response. At the end of angiotensin II stimulation, thoracic aortas were removed and proteins were extracted.

Three distinct methods were used to evaluate oxidative stress: chemiluminescence with lucigenin (16), histochemistry with dihydroethidium, and immunohistochemistry with anti-nitrotyrosine antibodies (44). Vascular Rac activity was measured using a commercially available kit (Upstate Biotechnology).

Antibodies. Mouse monoclonal and rabbit polyclonal antibodies against PI3Kγ were provided by R. Wetzker (University Hospital, Jena, Germany). Immunohistochemistry was performed on paraffin sections of mouse aorta by the ABC peroxidase method (Strepta ABComplex/HRP; Dako-Cytomation). Antibodies against murine F4/80 antigen (CI:A3-1) and CD18 (YTS 213.1) were from BMA Biomedicals. Rabbit polyclonal antibodies against phospho-Ser473-Akt, Akt, and phospho-ERK1/2 were obtained from New England BioLabs, Inc., antibodies against ERK1 (C16) were obtained from Santa Cruz Biotechnology, Inc.

Evaluation of vascular angiotensin II–dependent Ca^{2+} flux. Mesenteric arteries were placed at 37°C, in Krebs' buffer (mM: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4 \times 7\ H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 5.6 glucose), in a Mulvany micromyograph (Danish Myo Technology), and stretched to the appropriate tension. The myograph was placed on the stage of an inverted confocal microscope (Nikon). After the equilibration period, the vascular responsiveness was tested with 80 mM KCl three times. To view intracellular Ca^{2+} oscillations, vessels were incubated for 2.5 h with Fluo4-AM (60 μM) plus 0.2% pluronic acid. Vessels were washed three times with Krebs' buffer and stimulated with angiotensin II (1 μM) or phenylephrine (1 μM). Confocal images and $[Ca^{2+}]_i$ measurements were acquired as described previously (45).

Mesenteric artery transfection. Vessels of PI3Kγ$^{-/-}$ and wild-type mice were transfected as described previously (46). Vessels were placed in a Mulvany pressure system with DMEM/F12 medium, containing pCMV6 Vector (Origene Technologies) carrying a HA-tagged dominant negative mutation of PKB/Akt (K179 ≥ M179) at the concentration of 3 μg/ml. An empty plasmid was used as a negative control. The vessels were perfused at 100 mm Hg of pressure for 1 h, and, subsequently, at 60 mm Hg for 5 h. Transfection efficacy was tested by immunofluorescence with anti-HA monoclonal antibodies (BabCo) in transfected and control sections of mesenteric artery. The vascular contractility of transfected vessels, perfused at constant flow, was assessed by pressure changes that were induced by angiotensin II (1 μM) or KCl (80 mM).

Statistical analysis. Data are expressed as the mean ± SEM. Comparisons used were unpaired Student's *t* test for differences between wild-type and PI3Kγ-deficient mice, or repeated measures two-way ANOVA followed by Bonferroni post hoc test. p-value of <0.05 was assigned statistical significance.

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REFERENCES

1. Steinberg, S.F. 2001. PI3King the L-type calcium channel activation mechanism. *Circ. Res.* 89:641–644.
2. Wymann, M.P., M. Zvelebil, and M. Laffargue. 2003. Phosphoinositide 3-kinase signalling—which way to target? *Trends Pharmacol. Sci.* 24: 366–376.
3. Vanhaesebroeck, B., S.J. Leevers, K. Ahmadi, J. Timms, R. Katso, P.C. Driscoll, R. Woscholski, P.J. Parker, and M.D. Waterfield. 2001. Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* 70:535–602.
4. Stoyanov, B., S. Volinia, T. Hanck, I. Rubio, M. Loubtchenkov, D. Malek, S. Stoyanova, B. Vanhaesebroeck, R. Dhand, B. Nurnberg, et al. 1995. Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science.* 269:690–693.
5. Stephens, L.R., A. Eguinoa, H. Erdjument-Bromage, M. Lui, F. Cooke, J. Coadwell, A.S. Smrcka, M. Thelen, K. Cadwallader, P. Tempst, and P.T. Hawkins. 1997. The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell.* 89: 105–114.
6. Hirsch, E., V.L. Katanaev, C. Garlanda, O. Azzolino, L. Pirola, L. Silengo, S. Sozzani, A. Mantovani, F. Altruda, and M.P. Wymann. 2000. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science.* 287:1049–1053.
7. Wymann, M.P., S. Sozzani, F. Altruda, A. Mantovani, and E. Hirsch. 2000. Lipids on the move: phosphoinositide 3-kinases in leukocyte function. *Immunol. Today.* 21:260–264.
8. Crackower, M.A., G.Y. Oudit, I. Kozieradzki, R. Sarao, H. Sun, T. Sasaki, E. Hirsch, A. Suzuki, T. Shioi, J. Irie-Sasaki, et al. 2002. Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. *Cell.* 110:737–749.
9. Alloatti, G., R. Levi, D. Malan, L. Del Sorbo, O. Bosco, L. Barberis, A. Marcantoni, I. Bedendi, C. Penna, O. Azzolino, et al. 2003. Phosphoinositide 3-kinase gamma-deficient hearts are protected from the PAF-dependent depression of cardiac contractility. *Cardiovasc. Res.* 60: 242–249.
10. Patrucco, E., A. Notte, L. Barberis, G. Selvetella, A. Maffei, M. Brancaccio, S. Marengo, G. Russo, O. Azzolino, S.D. Rybalkin, et al. 2004. PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. *Cell.* 118:375–387.
11. Seki, T., H. Yokoshiki, M. Sunagawa, M. Nakamura, and N. Sperelakis. 1999. Angiotensin II stimulation of Ca2+-channel current in vascular smooth muscle cells is inhibited by lavendustin-A and LY-294002. *Pflugers Arch.* 437:317–323.
12. Saward, L., and P. Zahradka. 1997. Angiotensin II activates phosphatidylinositol 3-kinase in vascular smooth muscle cells. *Circ. Res.* 81:249–257.
13. Viard, P., T. Exner, U. Maier, J. Mironneau, B. Nurnberg, and N. Macrez. 1999. Gbetagamma dimers stimulate vascular L-type Ca2+ channels via phosphoinositide 3-kinase. *FASEB J.* 13:685–694.
14. Macrez, N., C. Mironneau, V. Carricaburu, J.F. Quignard, A. Babich, C. Czupalla, B. Nurnberg, and J. Mironneau. 2001. Phosphoinositide 3-kinase isoforms selectively couple receptors to vascular L-type Ca(2+) channels. *Circ. Res.* 89:692–699.
15. Quignard, J.F., J. Mironneau, V. Carricaburu, B. Fournier, A. Babich, B. Nurnberg, C. Mironneau, and N. Macrez. 2001. Phosphoinositide 3-kinase gamma mediates angiotensin II–induced stimulation of L-type calcium channels in vascular myocytes. *J. Biol. Chem.* 276:32545–32551.
16. Vecchione, C., and R.P. Brandes. 2002. Withdrawal of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors elicits oxidative stress and induces endothelial dysfunction in mice. *Circ. Res.* 91:173–179.
17. Seshiah, P.N., D.S. Weber, P. Rocic, L. Valppu, Y. Taniyama, and K.K. Griendling. 2002. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ. Res.* 91:406–413.
18. Voisin, L., S. Foisy, E. Giasson, C. Lambert, P. Moreau, and S. Meloche. 2002. EGF receptor transactivation is obligatory for protein synthesis stimulation by G protein-coupled receptors. *Am. J. Physiol. Cell Physiol.* 283:C446–C455.
19. Welch, H.C., W.J. Coadwell, L.R. Stephens, and P.T. Hawkins. 2003. Phosphoinositide 3-kinase-dependent activation of Rac. *FEBS Lett.* 546:93–97.
20. Thastrup, O., P.J. Cullen, B.K. Drobak, M.R. Hanley, and A.P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. *Proc. Natl. Acad. Sci. USA.* 87:2466–2470.
21. Laursen, J.B., S. Rajagopalan, Z. Galis, M. Tarpey, B.A. Freeman, and D.G. Harrison. 1997. Role of superoxide in angiotensin II–induced but not catecholamine-induced hypertension. *Circulation.* 95:588–593.
22. Ohya, Y., I. Abe, K. Fujii, Y. Takata, and M. Fujishima. 1993. Voltage-dependent Ca2+ channels in resistance arteries from spontaneously hypertensive rats. *Circ. Res.* 73:1090–1099.
23. Wilde, D.W., P.B. Furspan, and J.F. Szocik. 1994. Calcium current in smooth muscle cells from normotensive and genetically hypertensive rats. *Hypertension.* 24:739–746.
24. Northcott, C.A., M.N. Poy, S.M. Najjar, and S.W. Watts. 2002. Phosphoinositide 3-kinase mediates enhanced spontaneous and agonist-induced contraction in aorta of deoxycorticosterone acetate-salt hypertensive rats. *Circ. Res.* 91:360–369.
25. Takahashi, T., T. Taniguchi, H. Konishi, U. Kikkawa, Y. Ishikawa, and M. Yokoyama. 1999. Activation of Akt/protein kinase B after stimulation with angiotensin II in vascular smooth muscle cells. *Am. J. Physiol.* 276:H1927–H1934.
26. Touyz, R.M., and E.L. Schiffrin. 2000. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol. Rev.* 52:639–672.
27. Dinh, D.T., A.G. Frauman, M. Sourial, D.J. Casley, C.I. Johnston, and M.E. Fabiani. 2001. Identification, distribution, and expression of angiotensin II receptors in the normal human prostate and benign prostatic hyperplasia. *Endocrinology.* 142:1349–1356.
28. Smith, R.D., A.J. Baukal, P. Dent, and K.J. Catt. 1999. Raf-1 kinase activation by angiotensin II in adrenal glomerulosa cells: roles of Gi, phosphatidylinositol 3-kinase, and Ca2+ influx. *Endocrinology.* 140: 1385–1391.
29. Rodriguez-Puyol, M., M. Griera-Merino, G. Perez-Rivero, M.L. Diez-Marques, M.P. Ruiz-Torres, and D. Rodriguez-Puyol. 2002. Angiotensin II induces a rapid and transient increase of reactive oxygen species. *Antioxid. Redox Signal.* 4:869–875.
30. Rajagopalan, S., S. Kurz, T. Munzel, M. Tarpey, B.A. Freeman, K.K. Griendling, and D.G. Harrison. 1996. Angiotensin II–mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J. Clin. Invest.* 97:1916–1923.
31. Griendling, K.K., D. Sorescu, and M. Ushio-Fukai. 2000. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ. Res.* 86:494–501.
32. Welch, H.C., W.J. Coadwell, C.D. Ellson, G.J. Ferguson, S.R. Andrews, H. Erdjument-Bromage, P. Tempst, P.T. Hawkins, and L.R. Stephens. 2002. P-Rex1, a PtdIns(3,4,5)P(3)- and gbetagamma-regulated guanine-nucleotide exchange factor for Rac. *Cell.* 108:809–821.
33. Pelaez, N.J., T.R. Braun, R.J. Paul, R.A. Meiss, and C.S. Packer. 2000. H(2)O(2) mediates Ca(2+)- and MLC(20) phosphorylation-independent contraction in intact and permeabilized vascular muscle. *Am. J. Physiol. Heart Circ. Physiol.* 279:H1185–H1193.
34. Kim, S., and H. Iwao. 2000. Molecular and cellular mechanisms of angiotensin II–mediated cardiovascular and renal diseases. *Pharmacol. Rev.* 52:11–34.
35. Benham, C.D., P. Hess, and R.W. Tsien. 1987. Two types of calcium channels in single smooth muscle cells from rabbit ear artery studied with whole-cell and single-channel recordings. *Circ. Res.* 61:I10–I16.
36. Bony, C., S. Roche, U. Shuichi, T. Sasaki, M.A. Crackower, J. Penninger, H. Mano, and M. Puceat. 2001. A specific role of phosphatidylinositol 3-kinase gamma. A regulation of autonomic Ca(2)+ oscillations in cardiac cells. *J. Cell Biol.* 152:717–728.
37. Viard, P., A.J. Butcher, G. Halet, A. Davies, B. Nurnberg, F. Heblich, and A.C. Dolphin. 2004. PI3K promotes voltage-dependent calcium

channel trafficking to the plasma membrane. *Nat. Neurosci.* 7:939–946.
38. Kim, Y.K., S.J. Kim, A. Yatani, Y. Huang, G. Castelli, D.E. Vatner, J. Liu, Q. Zhang, G. Diaz, R. Zieba, et al. 2003. Mechanism of enhanced cardiac function in mice with hypertrophy induced by overexpressed Akt. *J. Biol. Chem.* 278:47622–47628.
39. Leslie, N.R., D. Bennett, Y.E. Lindsay, H. Stewart, A. Gray, and C.P. Downes. 2003. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *EMBO J.* 22:5501–5510.
40. Vecchione, C., L. Fratta, D. Rizzoni, A. Notte, R. Poulet, E. Porteri, G. Frati, D. Guelfi, V. Trimarco, M.J. Mulvany, et al. 2002. Cardiovascular influences of alpha1b-adrenergic receptor defect in mice. *Circulation.* 105:1700–1707.
41. Lembo, G., C. Vecchione, L. Fratta, G. Marino, V. Trimarco, G. d'Amati, and B. Trimarco. 2000. Leptin induces direct vasodilation through distinct endothelial mechanisms. *Diabetes.* 49:293–297.
42. Vecchione, C., A. Aretini, A. Maffei, G. Marino, G. Selvetella, R. Poulet, V. Trimarco, G. Frati, and G. Lembo. 2003. Cooperation between insulin and leptin in the modulation of vascular tone. *Hypertension.* 42:166–170.
43. Lepretre, N., J. Mironneau, and J.L. Morel. 1994. Both alpha 1A- and alpha 2A-adrenoreceptor subtypes stimulate voltage-operated L-type calcium channels in rat portal vein myocytes. Evidence for two distinct transduction pathways. *J. Biol. Chem.* 269:29546–29552.
44. Zhang, D.X., A.P. Zou, and P.L. Li. 2003. Ceramide-induced activation of NADPH oxidase and endothelial dysfunction in small coronary arteries. *Am. J. Physiol. Heart Circ. Physiol.* 284:H605–H612.
45. Gentile, M.T., C. Vecchione, A. Maffei, A. Aretini, G. Marino, R. Poulet, L. Capobianco, G. Selvetella, and G. Lembo. 2004. Mechanisms of soluble beta-amyloid impairment of endothelial function. *J. Biol. Chem.* 279:48135–48142.
46. Mann, M.J., G.H. Gibbons, H. Hutchinson, R.S. Poston, E.G. Hoyt, R.C. Robbins, and V.J. Dzau. 1999. Pressure-mediated oligonucleotide transfection of rat and human cardiovascular tissues. *Proc. Natl. Acad. Sci. USA.* 96:6411–6416.