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PI3Kγ inhibition reduces blood pressure by a vasorelaxant Akt/L-type calcium channel mechanism

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Aims The lipid and protein kinase phosphoinositide 3-kinase γ (PI3Kγ) is abundantly expressed in inflammatory cells and in the cardiovascular tissue. In recent years, its role in inflammation and in cardiac function and remodelling has been unravelled, highlighting the beneficial effects of its pharmacological inhibition. Furthermore, a role for PI3Kγ in the regulation of vascular tone has been emphasized. However, the impact of this signalling in the control of blood pressure is still poorly understood. Our study investigated the effect of a selective inhibition of PI3Kγ, obtained by using two independent small molecules, on blood pressure. Moreover, we dissected the molecular mechanisms involved in control of contraction of resistance arteries by PI3Kγ.

Methods and results We showed that inhibition of PI3Kγ reduced blood pressure in normotensive and hypertensive mice in a concentration-dependent fashion. This effect was dependent on enhanced vasodilatation, documented *in vivo* by decreased peripheral vascular resistance, and *ex vivo* by vasorelaxing effects on isolated resistance vessels. The vasorelaxation induced by PI3Kγ inhibition relied on blunted pressure-induced Akt phosphorylation and a myogenic contractile response. Molecular insights revealed that PI3Kγ inhibition affected smooth muscle L-type calcium channel current density and calcium influx by impairing plasma membrane translocation of the α1C L-type calcium channel subunit responsible for channel open-state probability.

Conclusion Overall our findings suggest that PI3Kγ inhibition could be a novel tool to modulate calcium influx in vascular smooth muscle cells, thus relaxing resistance arteries and lowering blood pressure.

Keywords Phosphoinositide 3-kinase γ • Blood pressure • Resistance artery • Myogenic tone • L-type calcium channel

1. Introduction

Phosphoinositide 3-kinase γ (PI3Kγ) is a lipid and protein kinase mainly expressed in bone marrow-derived cells and in the cardiovascular tissue[1] and, once activated, generates phosphatidylinositol-3,4,5-trisphosphate to recruit and activate downstream signalling molecules. In recent years, several studies have dissected the role played by PI3Kγ in different pathophysiological contexts involving inflammatory responses. In particular, it has been demonstrated that mice with PI3Kγ inactivated by genetic or pharmacological approaches are protected from disease development in a number of inflammation and autoimmune disease models.[2,3] This recent evidence has prompted

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the task of developing small molecule inhibitors of PI3Kγ, to attain the appropriate isoform selectivity profile.

So far, selective pharmacological inhibitors of PI3Kγ have been used to treat cardiovascular diseases with a substantial inflammatory component, such as myocardial ischaemia[4,5] and atherosclerosis.[6] Furthermore, we have recently demonstrated a beneficial role of PI3Kγ inhibition on cardiac remodelling in response to pressure overload, a stimulus in which challenges to cardiac and inflammatory cells are finely intertwined.[7] We have also described that mice lacking PI3Kγ are protected from hypertension induced by chronic administration of angiotensin II (AngII),[8] thus suggesting an additional role of PI3Kγ in the regulation of vascular tone.

Nevertheless, the impact of PI3Kγ inhibition on blood pressure (BP) is still poorly explored. The availability of transgenic mice and pharmacological approaches to inhibit PI3Kγ can help to disclose its role in BP regulation and then unveil mechanistic insights responsible for this effect. Here we investigated the effect of two independent small molecules inhibiting PI3Kγ, focusing on their impact on BP and the molecular mechanisms recruited. For these purposes, we evaluated concentration-dependent effects of PI3Kγ inhibitors on BP levels, in normotensive and hypertensive mice. Then, we explored how PI3Kγ inhibition exerted haemodynamic effects, by analysing cardiac output and total peripheral vascular resistance, the two components on which BP depends. Insights into the actions of PI3Kγ inhibition on vascular function, obtained in isolated vessels, finally prompted us to dissect molecular mechanisms residing in the vascular smooth muscle component, regulating calcium influx.

2. Methods

2.1 Animals

C57BL/6 mice (8–12 weeks old) were purchased from Charles River (Calco, Italy). Genetically engineered mice for PI3Kγ were generated as previously described.[9]

According to the New Directive 2010/63/EU, special attention was paid to animal welfare and to minimize animal suffering. Anaesthetized mice were closely monitored during the procedure to assure that they were maintained in the proper anaesthetic plane. In particular, the anaesthetic efficacy was assessed by pinching the tail of the animal as well as by checking the body temperature and the respiratory rate. Any reaction from the animal, hypothermia, or increase of the respiratory rate, indicated that the anaesthesia was too light and that additional anaesthetic should be given.

All experiments were conducted in conformity with European Communities Council Directive and approved by our Institution.

2.2 Synthesis of (*Z*)-5-(5-nitrofuryl-2-idene)-1, 3-thiazolidine-2,4-dione GE21

The detailed procedure for GE21 synthesis and for the assay of PI3K lipid kinase activity used to test GE21 selectivity is provided in the Supplementary material online, Methods.

2.3 GE21 plasma levels

The quantitative analysis in blood samples, collected by tail nicks at the indicated time points, was performed using the same chromatographic method coupled to ultraviolet detection, described for GE21 synthesis.

2.4 Administration of drugs

AS605240, from Merck Serono (Geneva, Switzerland)[2] was used as previously described.[3]

GE21 was dissolved in saline for i.p. injections and in water for oral administration, and used at the doses indicated in figures. Chronic administration was by daily i.p. injections or intragastrically by gavage, as indicated in figure legends. GE21 administration did not result in any sign of toxicity (see *Table 2*), and a long-lasting treatment of 3 months did not affect the viability of the mice (data not shown).

For Akt inhibition, we used 1L6-hydroxymethyl-chiro-inositol-2-(*R*)-2-*O*-methyl-3-*O*-octadecyl-sn-glycerocarbonate (Calbiochem #124005) at the doses indicated in figures.

2.5 Blood pressure measurements

Blood pressure was evaluated in conscious mice by tail-cuff plethysmography (Visitech Systems, Apex, NC, USA).

2.6 Cardiac output and total peripheral resistance

Cardiac catheterization was performed using a 1.4 French high-fidelity micromanometer catheter (Millar Instruments, Houston, TX, USA) in mice anaesthetized with a mixture of ketamine (110 mg/kg) and xylazine (20 mg/kg), before animals were killed with an overdose of sodium pentobarbital (250 mg/kg), as previously described.[10] Cardiac output was measured and total peripheral resistance calculated by dividing the mean arterial pressure by cardiac output.

2.7 Clinical chemistry analysis

GE21 was administered by a daily oral treatment at the maximal dose used in the *in vivo* studies (50 mg/kg/day) for 2 weeks. Mice were killed with an overdose of sodium pentobarbital (250 mg/kg) and serum was collected from the trunk. Clinical chemistry analyses were performed by Dimension EXL (Siemens Heathcare Diagnostic Inc., Deerfield, IL, USA) apparatus. Red (RBCs) and white blood cells (WBCs) were manually counted in plasma samples by May-Grünwald Giemsa staining and acquiring images using a DMI4000B Leica microscope (Leica Microsystems, Wetzlar, Germany).

2.8 Evaluation of locomotor activity

Locomotor activity was recorded by radiotelemetric transmitters (TA11PA-C20; Data Sciences International, St. Paul, MN, USA). Mice were anaesthetized with a mixture of ketamine (110 mg/kg) and xylazine (20 mg/kg), and radiotelemetric transmitters were inserted subcutaneously on the right flank. After 2 days recovery from surgery, continuous 24 h locomotor activity was monitored. Data were stored and analysed using DSI Ponemah 4.9 software (Data Sciences International, St. Paul, MN, USA).

2.9 Evaluation of vascular function

The myogenic tone of second-order mesenteric arteries, isolated from mice killed with an overdose of sodium pentobarbital (250 mg/kg) was determined with a pressure arteriograph with stepwise perfusion pressure. Mesenteric arteries were subjected to a perfusion pressure of 100 mmHg for functional studies, and placed in a wire myograph for determination of structure, as previously described.[10] Where indicated, the endothelium was mechanically removed and endothelial function tested by evaluating the response to acetylcholine.

The percentage of myogenic tone was calculated with the following formula:

$$\left[\frac{(\text{internal diameter } Ca^{2+}\text{- free Krebs} - \text{internal active diameter})}{\text{internal diameter } Ca^{2+}\text{- free Krebs}}\right] \times 100.$$

For all studies vessels, were equilibrated and maintained in Krebs solution (mM: NaCl, 120; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 2.5; $NaHCO_3$, 25; KH_2PO_4, 1.1; glucose, 6; and $CaCl_2 \cdot 2H_2O$, 2.5).

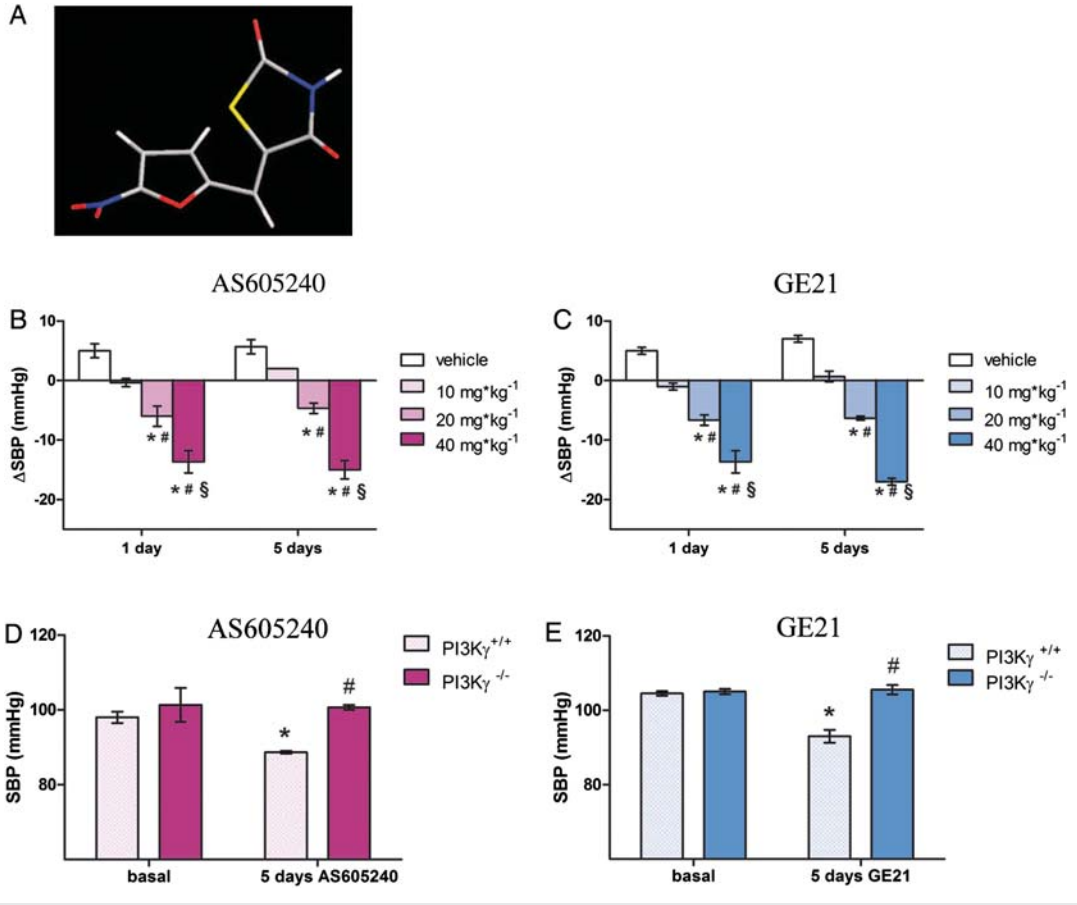

Figure 1 Two small molecules inhibiting PI3Kγ lower BP in mice. (*A*) Three-dimensional graphical representation of GE21, the (*Z*)-5-(5-nitrofuryl-2-idene)-1,3-thizoline-2,4-dione, a novel PI3Kγ inhibitor. GE21 structure obtained by a simulated annealing method. (*B* and *C*) Effects of AS605240 (*B*) and GE21 (*C*) in C57BL/6 mice ($n = 24$), injected i.p. daily for 5 days at different doses (10, 20 and 40 mg/kg for AS605240 and GE21). Changes in systolic blood pressure (ΔSBP) on days 1 and 5 are shown. $*P < 0.001$ vs. vehicle; $\#P < 0.001$ vs. medium dose; $\S P < 0.001$ vs. highest dose. (*D* and *E*) Effects of AS605240 (*D*) and GE21 (*E*) i.p. administration in PI3Kγ$^{+/+}$ and PI3Kγ$^{-/-}$ mice ($n = 20$) at the highest dose tested, given for 5 days. $*P < 0.001$ vs. basal and $\#P < 0.001$ vs. PI3Kγ$^{+/+}$.

2.10 Smooth muscle cells

Rat aortic smooth muscle cells (RASMCs), provided by ATTC (American Type Culture Collection, Sesto San Giovanni, Milan, Italy), clone A10, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (37°C; air supplemented with 5% CO_2) and harvested at confluence. Treatments were given in serum-free medium as indicated in the figures. GE21 (10^{-5} M) was incubated for 30 min before stimulation with AngII (1 μmol/L).

2.11 Evaluation of membrane current

Membrane current recordings were made on voltage-clamped RASMCs with a standard patch-clamp technique using a Multiclamp 700A patch-clamp amplifier (Molecular Devices, Sunnyvale, CA, USA) according to previously described methods.[11] Peak current density was expressed as the maximal amplitude of the Ba^{2+} current per capacitance unit (in picoamperes per picofarad). Current densities were recorded in the continuous presence of tetrodotoxin (10 μmol/L) to avoid activation of voltage-activated Na^+ channels, and with Cs^+ replacing K^+ in the intracellular solution. Currents were completely and reversibly inhibited by nifedipine (3 μmol/L).

2.12 Fluorescent measurement of Ca^{2+}

RASMCs were serum starved, mounted in chamber slides and loaded with fluo-4 AM (Invitrogen Molecular Probes, Monza, Italy; 1 μmol/L) for 1 h at 37°C and then stimulated as indicated above in section 2.10. Real-time movies were obtained with Leica DMI4000B (Leica Microsystems, CMS GmbH, Wetzlar, Germany) and relative fluorescence intensities at F_0 (basal fluorescence) and at F (peak fluorescence) were measured using LAS Application Suite and reported as $\Delta F = (F - F_0)/F_0$. AngII-induced fluorescence was completely inhibited by nifedipine (3 μmol/L).

2.13 Protein analysis

Proteins were extracted as previously described.[12] In brief, vessels and cells were homogenized in a lysis buffer containing the following: Tris, 50 mmol/L (pH 7.4); NaCl, 150 mmol/L; EDTA, 1 mmol/L; sodium deoxycholate, 0.5%; NaF, 10 mmol/L; Na_3VO_4, 1 mmol/L; NP-40, 1%; and protease inhibitors. Following a centrifugation step, samples were separated by SDS–PAGE and blotted on a nitrocellulose membrane. Primary antibodies used were as follows: anti-phospho-(Ser^{473}) Akt (1:500 dilution) and anti-total-Akt (1:1000 dilution) from Cell Signaling Technology, Inc., Danvers, MA, USA; anti-CACNA1C (α1C subunit, L-Type Ca^{2+} channel; 1:300 dilution) from Novus Biologicals, Littleton, CO, USA;

and anti-p-Ser monoclonal antibody (4A3; 1:200 dilution), Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. The β2 subunit was immunoprecipitated with the previously characterized antibody.[12]

Protein detection was performed with Amersham™ ECL Plus Western Blotting Detection kit (GE Healthcare Europe GmbH, Milan, Italy) and densitometry was obtained with the NIH Image 1.61 software (developed at the U.S. National Institutes of Health).

2.14 Statistical analysis

Statistical significance was calculated with one-way ANOVA (for comparison of multiple groups) and two-way ANOVA (for multiple groups with factorial design), followed by Bonferroni *post hoc* test (data are presented as means ± SEM). For comparisons of two groups, Mann–Whitney *U* test was applied. The number of independent experiments and *P* value are indicated in each figure legend. Statistical analyses were performed with GraphPad software PRISM5 (GraphPad Software Inc., La Jolla, CA, USA).

Table 1 EC_{50} values of GE21 against PI3K isoforms, calculated from enzyme inhibition assay

	PI3Kα	PI3Kβ	PI3Kγ	PI3Kδ
EC_{50} (μmol/L)	18.2 ± 1.3	18.1 ± 1.4	5.3 ± 1.4	84.2 ± 1.3
E_{max} (% of inhibition)	108.4 ± 7.4	98.3 ± 7.4	102.7 ± 7.1	114.6 ± 7.6
n	24	24	24	24

3. Results

3.1 Pharmacological inhibition of PI3Kγ lowers blood pressure in normotensive and hypertensive mice

We investigated BP effects of a pharmacological inhibition of PI3Kγ obtained by using two independent small molecules: AS605240, already commercially available, and GE21, a novel one. We developed this latter analogue inhibitor by designing molecules that should fit into the ATP-binding pocket of the enzyme.[13] GE21 (*Figure 1A*) was the most selective molecule synthesized, with an EC_{50} for PI3Kγ lower than that needed to inhibit other PI3K isoforms (*Table 1*).

The effects of PI3Kγ inhibition on BP were analysed in normotensive C57BL/6 mice. PI3Kγ inhibitors (AS605240 in *Figure 1B* and GE21 in *Figure 1C*) showed a similar concentration-dependent hypotensive effect, compared with vehicle alone, after an i.p. treatment over 5 days. Then, to demonstrate specificity of this pharmacological strategy, we treated PI3Kγ$^{+/+}$ and PI3Kγ$^{-/-}$ mice chronically, at the

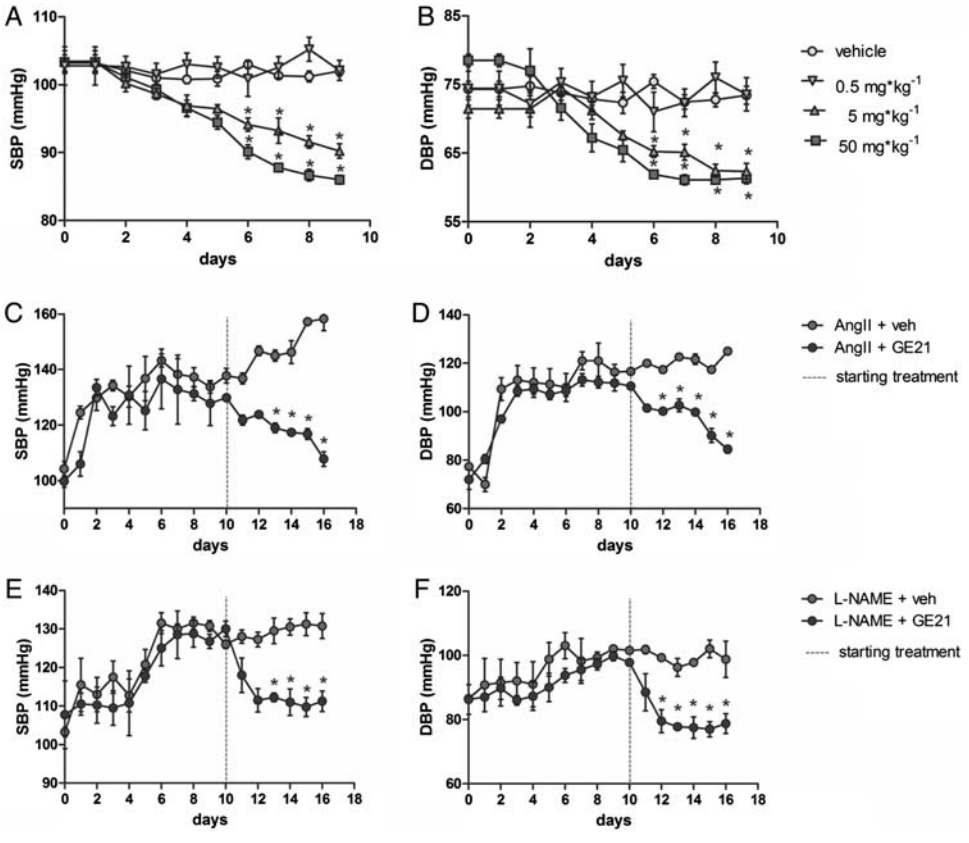

Figure 2 A daily oral treatment of GE21 lowers BP in normotensive and hypertensive mice. (*A* and *B*) Dose–response of a daily oral treatment of GE21, given over a wide range of concentrations (0.5–50 mg/kg/day) in C57BL/6 mice ($n = 32$). *$P < 0.001$ vs. vehicle. (*C–F*) Antihypertensive effects of daily oral treatment with GE21 (5 mg/kg/day) in two murine models of hypertension obtained by chronic administration through osmotic minipumps of AngII (*C* and *D*; $n = 10$; 0.5 mg/kg/day) or L-NAME (*E* and *F*; $n = 10$; 10 mg/kg/day). *$P < 0.001$ vs. vehicle.

maximal effective dose and time, determined in the dose–response experiment. We confirmed that basal BP was comparable in both mouse strains and, as expected, both AS605240 (*Figure 1D*) and GE21 (*Figure 1E*) reduced BP levels in PI3Kγ$^{+/+}$ but not in PI3Kγ$^{-/-}$ mice.

Table 2 Peripheral haematological and clinical chemistry values in vehicle- or GE21 (10^{-2} M)-treated mice, and animal weights before and after treatment

	Units	Vehicle	GE21
Total bilirubin	mg/dL	0.21 ± 0.1	0.22 ± 0.1
Creatinine	mEq/L	0.26 ± 0.1	0.24 ± 0.1
Aspartate aminotransferase	IU/L	64 ± 11	72 ± 18
Alanine aminotransferase	IU/L	37 ± 7	44 ± 7
Creatine kinase	IU/L	871 ± 166	1060 ± 409
Alkaline phosphatase	IU/L	68 ± 28	60 ± 23
Insulin	μIU/mL	1.8 ± 0.3	2.4 ± 0.6
RBCs	$\times 10^{12}$/L	8.6 ± 0.6	8.9 ± 0.5
WBCs	$\times 10^{9}$/L	5.2 ± 1	4.7 ± 1
Weight gain	g	+0.37 ± 0.5	+0.58 ± 0.6

Data are expressed as means ± SEM of $n = 5$–10 mice for each group.

A daily oral treatment of GE21 demonstrated, even with this route of administration, a concentration-dependent lowering effect on both systolic BP (SBP; *Figure 2A*) and diastolic BP (DBP; *Figure 2B*), which became statistically significant 5 days after the start of the treatment and was sustained until the end of our analysis.

When we evaluated the efficacy of PI3Kγ inhibition as an antihypertensive strategy, we found that the daily oral treatment completely normalized BP levels in two murine models in which hypertension was induced by chronic administration of AngII (SBP, *Figure 2C*; and DBP, *Figure 2D*) or L-NAME, a nitric oxide synthase inhibitor (SBP, *Figure 2E*; and DBP, *Figure 2F*).

Overall, this first piece of evidence demonstrated that PI3Kγ inhibition had a clear antihypertensive effect, and the fact that this was accomplished using two independent molecules, AS605240 and GE21, further strengthens our findings.

3.2 PI3Kγ inhibition does not influence health of mice

Chronic oral treatment with GE21, at the maximal dose used in our experiments (50 mg/kg/day) and for a maximal observation period of 2 weeks, showed no relevant sign of drug toxicity on clinical chemistry analysis (*Table 2*). The locomotor activity of GE21- and vehicle-treated mice was comparable (*Figure 3A*).

Next, we measured the GE21 plasma concentrations reached after a daily oral treatment given at the dose of 5 mg/kg/day (the dosage used for antihypertensive purposes), and found that it peaked 1 h

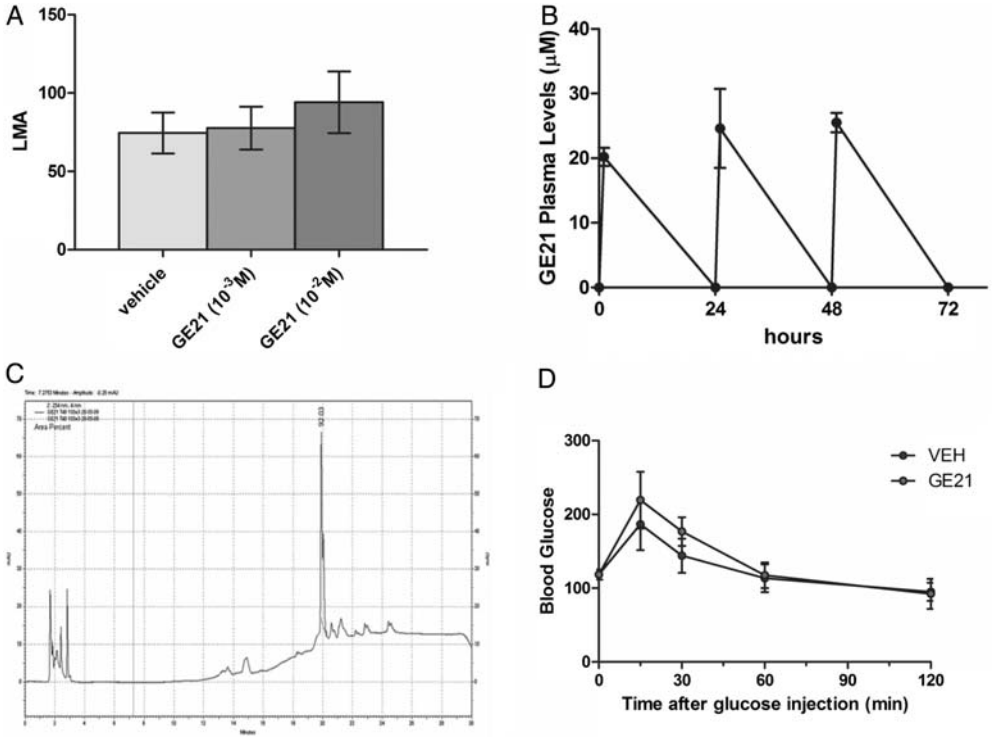

Figure 3 Chronic treatment with the maximal dose of GE21 does not affect general health of the mice. (*A*) Effects of 2 weeks treatment with 10^{-2} M GE21 on locomotor activity (LMA), evaluated by radiotelemetry ($n = 8$). (*B*) Concentration of GE21 in the plasma after daily oral treatment (5 mg/kg/day; $n = 5$). (*C*) An example of chromatographic analysis for GE21 levels in blood (peak corresponding to 49 h, i.e. 1 h from intake). (*D*) Glucose tolerance test on mice orally administered with GE21 (5 mg/kg/day) or vehicle alone after a 6 h fasting by i.p. injection of glucose (2 g/kg) at time zero ($n = 10$).

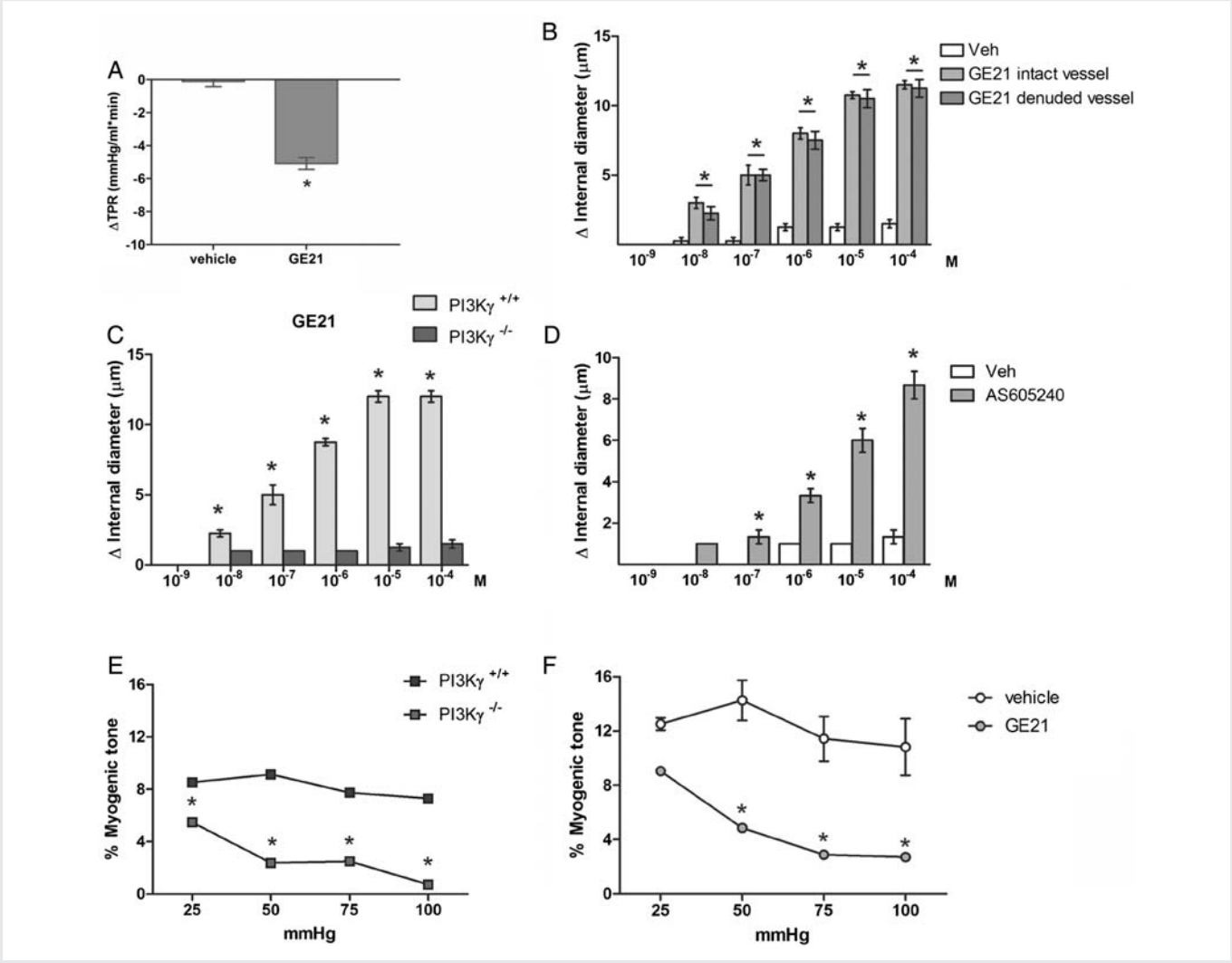

Figure 4 PI3Kγ inhibition evokes vasorelaxant effects through modulation of calcium influx. (*A*) Changes in total peripheral resistance (ΔTPR) evoked by GE21 (4 mg/kg) or vehicle in C57BL/6 mice ($n = 10$). *$P < 0.01$ vs. vehicle. (*B*) Effect of GE21 on intact or endothelium-denuded mesenteric arteries from C57BL/6 mice ($n = 10$), compared with vehicle alone ($n = 5$). *$P < 0.01$ vs. vehicle. (*C*) Changes in the internal diameter of mesenteric arteries ($n = 12$) from PI3Kγ$^{+/+}$ and PI3Kγ$^{-/-}$ mice with increasing doses of GE21. *$P < 0.01$ vs. PI3Kγ$^{-/-}$. (*D*) Changes in the internal diameter of mesenteric arteries ($n = 10$) from C57Bl/6 mice with increasing doses of AS605240, compared with vehicle. *$P < 0.01$ vs. vehicle. (*E* and *F*) Myogenic response obtained in mesenteric arteries from PI3Kγ$^{+/+}$ and PI3Kγ$^{-/-}$ mice (*E*) and from C57BL/6 mice (*F*) treated with GE21 (10^{-4} M) or vehicle alone. *$P < 0.01$ vs. PI3Kγ$^{+/+}$ or vs. vehicle.

after drug administration (*Figure 3B*), as evaluated by chromatographic analysis (*Figure 3C*), at levels compatible with the reported EC_{50} for PI3Kγ.

As other isoforms of the PI3K family have been demonstrated to be crucial in insulin signalling, we investigated whether GE21 treatment may interfere with glucose metabolism. We performed a glucose tolerance test in mice given GE21 orally at the maximal dose (50 mg/kg/day) or given vehicle alone. As shown in *Figure 3D* and in *Table 2*, we found no difference in fasting glucose and insulin levels between the two groups and, more importantly, i.p. glucose injection in the GE21-treated mice resulted in an increase in glycaemia at 15–30 min, comparable to that of vehicle-treated mice. This result indicated that chronic administration of GE21, even at a dose higher than that used to produce a hypotensive effect, does not interfere with other PI3K isoforms controlling glucose metabolism.

3.3 PI3Kγ inhibitors exert smooth muscle vasorelaxant effects by inhibiting pressure-mediated Akt activation

Our next aim was to characterize the mechanisms through which PI3Kγ inhibitors exert antihypertensive effects. In this regard, we should mention that PI3Kγ is expressed in cardiac as well as in vascular cells, making it conceivable that its inhibition may affect one or both of them. To verify whether the decrease in BP induced by PI3Kγ inhibition was dependent on a negative effect on cardiac function or on decreased vascular resistance, we measured cardiac output and calculated total peripheral resistance, before and after drug injection. PI3Kγ inhibition significantly reduced vascular resistance (*Figure 4A*) without modifying cardiac output (5588 ± 329 vs. 5307 ± 215 μL/min; $n = 5$ mice per group; not significant).

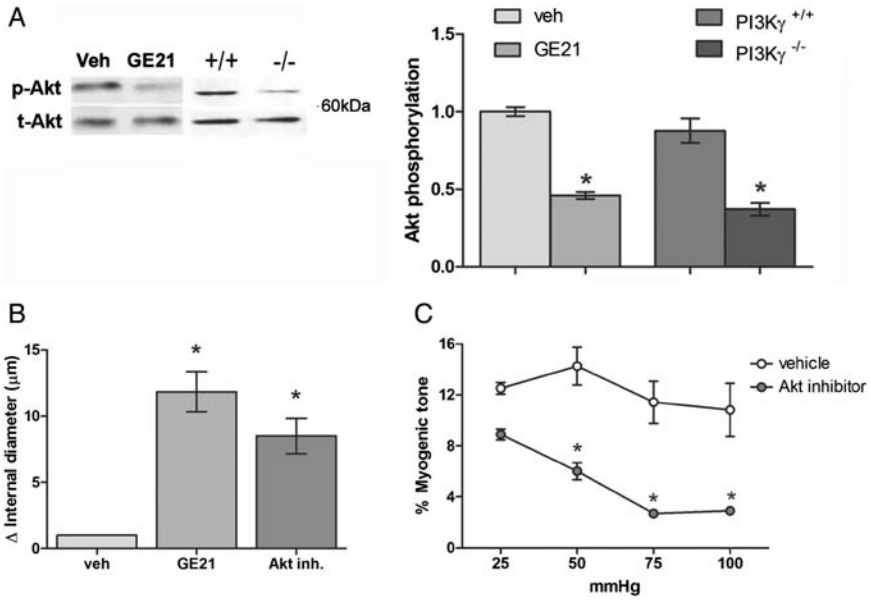

Figure 5 PI3Kγ inhibition counteracts pressure-induced Akt activation in resistance vessels. (*A*) Effect of GE21 (10^{-4} M) or vehicle on Akt phosphorylation in mesenteric arteries from C57BL/6 mice, perfused at 100 mmHg. Representative western blot (left) and quantification of phospho/total ratio (mean ± SEM; right) of $n = 5$ independent experiments. *$P < 0.01$ vs. vehicle and PI3Kγ$^{+/+}$, respectively. (*B*) Effect of GE21 (10^{-4} M) or Akt inhibitor (10^{-5} M) on changes in the internal diameter of mesenteric arteries from C57BL/6 mice ($n = 4$), perfused at 100 mmHg, compared with vehicle alone. Data are means ± SEM. *$P < 0.01$ vs. vehicle. (*C*) Myogenic response obtained in mesenteric arteries from from C57BL/6 mice treated with Akt inhibitor (10^{-5} M) or vehicle alone. *$P < 0.01$ vs. vehicle.

This finding prompted us to explore actions of PI3Kγ on vascular tissue. In particular, we evaluated the effects of PI3Kγ inhibition on isolated resistance vessels, i.e. mesenteric arteries. As shown in *Figure 4B*, GE21 exerted a concentration-dependent increase in the internal diameter of perfused mesenteric arteries, indicating a clear vasorelaxing effect, which remained completely unaffected by removal of the endothelium. This latter finding was confirmed by the lack of concentration-dependent vasorelaxation to acetylcholine (data not shown). Thus, as GE21 was still able to induce vasorelaxant effects in the absence of endothelium, PI3Kγ inhibition should recruit a mechanism of action in smooth muscle cells to provoke vasodilatation.

Interestingly, PI3Kγ$^{-/-}$ mice had a different structure of the mesenteric arteries. In particular, they had greater media thickness (see Supplementary material online, *Figure S1A*) and media cross-sectional area (see Supplementary material online, *Figure S1B*), accompanied by an increased internal diameter (see Supplementary material online, *Figure S1C*). However, the internal diameter of PI3Kγ$^{-/-}$ mesenteric arteries was not further increased by GE21 compared with PI3Kγ$^{+/+}$ (*Figure 4C*), thus supporting a selective action of GE21 on the γ isoform. Like our analogue inhibitor GE21, AS605240 induced a comparable concentration-dependent vasorelaxation in mesenteric arteries (*Figure 4D*).

The maximal dose of PI3Kγ inhibitor that induced a vasorelaxing effect showed kinetics of vasodilatation that reached a plateau in 15–20 min, as shown for GE21 in Supplementary material online, *Figure S2A*.

A more detailed characterization of the influence of PI3Kγ signalling in the smooth muscle was obtained by studying the regulation of the vascular myogenic tone developed in response to stepwise increases in perfusion pressure.[14] We found that absence of PI3Kγ or its pharmacological inhibition by GE21 counteracted the pressure-induced contractile myogenic response starting at 25 mmHg (*Figure 4E* and *F*; see Supplementary material online, *Figure S2B*), thus demonstrating that PI3Kγ signalling is crucial to establish a vascular myogenic response to pressure.

A downstream target of PI3Kγ signalling, Akt, has been also proposed to be among the main pathways involved in vascular mechanotransduction. In this regard, we detected that, at the maximal inhibition of perfusion pressure-induced vascular myogenic tone (100 mmHg), Akt phosphorylation was higher than that observed in vessels treated with GE21 and in mice lacking PI3Kγ (*Figure 5A*). Strikingly, when we inhibited Akt phosphorylation, we evoked a significant vasorelaxant effect, comparable to that induced by GE21 (*Figure 5B*). Interestingly, we found that Akt inhibition impaired the myogenic response to increasing steps in perfusion pressure (*Figure 5C*). Finally, when we tested both GE21 and Akt inhibitor on the myogenic response of PI3Kγ$^{-/-}$ arteries, they failed to exert any effect (see Supplementary material online, *Figure S2C*). These findings suggested that PI3Kγ inhibition influences vascular tone by blunting its downstream Akt signalling.

3.4 PI3Kγ inhibitors reduce nifedipine-sensitive whole-cell currents and cytosolic Ca^{2+} in vascular smooth muscle cells

It is known that the PI3Kγ/Akt pathway could drive calcium influx in smooth muscle cells through increased L-type calcium channel (LTCC) open probability;[8,15–18] thus, we reasoned that

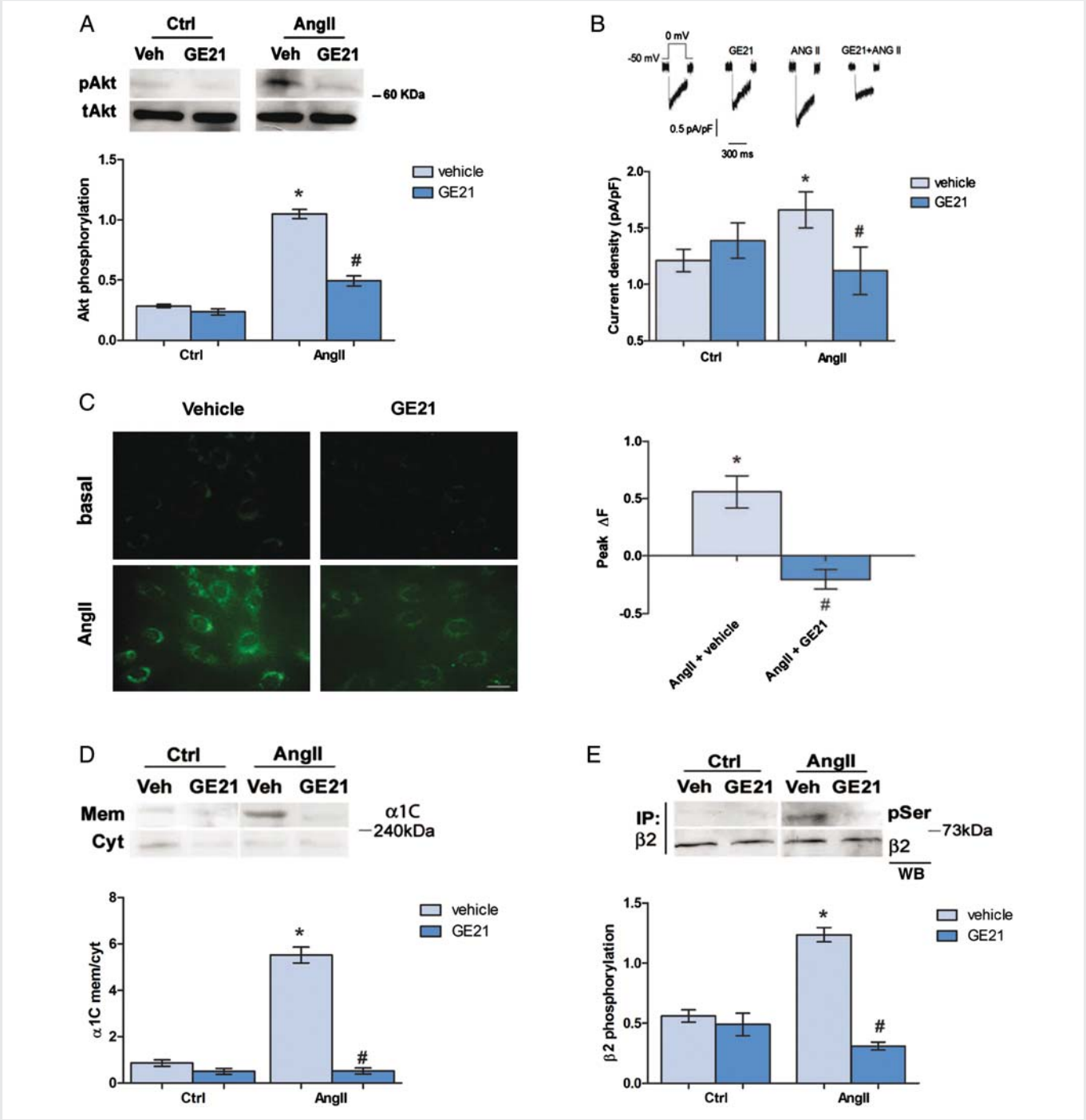

Figure 6 Molecular mechanism involved in vasorelaxation evoked by PI3Kγ inhibition. (*A*) Effect of GE21 (10^{-4} M) or vehicle on Akt phosphorylation in vascular smooth muscle cells in control conditions and stimulated with AngII (1 μM). Representative western blot (top) and quantification (bottom) of $n = 5$ independent experiments. *$P < 0.01$ vs. control–vehicle and #$P < 0.01$ vs. AngII–vehicle. (*B*) Current densities in RASMCs evoked by depolarization (from −50 to 0 mV, 300 ms) in control conditions and with AngII (1 μM), after 30 min pre-treatment with GE21 (10^{-4} M) or vehicle. Representative traces (top) and quantification (bottom) of $n = 8$ independent experiments. *$P < 0.01$ vs. control–vehicle and #$P < 0.01$ vs. AngII–vehicle. (*C*) Changes in peak fluorescence intensity (F/F_0) in vascular smooth muscle cells, loaded with fluo-4 AM, evoked by AngII (1 μM), after 30 min pre-treatment with GE21 (10^{-5} M) or vehicle. Representative images of peak fluorescence (left panel) and quantification (right panel) of $n = 4$ independent experiments. *$P < 0.01$ vs. AngII–vehicle. (*D*) Effect of GE21 (10^{-4} M) or vehicle on α1C membrane/cytoplasm ratio in RASMCs in control conditions and stimulated with AngII (1 μM). Representative western blot (top) and quantification (bottom) of $n = 3$ independent experiments. *$P < 0.01$ vs. control–vehicle and #$P < 0.01$ vs. AngII–vehicle. (*E*) Effect of GE21 (10^{-4} M) or vehicle on immunoprecipitated β2 serine phosphorylation, in control conditions and stimulated with AngII (1 μM). Representative western blot (top) and quantification of phosphorylated/total ratio (bottom) of $n = 5$ independent experiments. *$P < 0.01$ vs. control–vehicle and #$P < 0.01$ vs. AngII–vehicle.

pharmacological inhibition of this target could affect such a mechanism. The best way to study whether PI3Kγ inhibition affects LTCC regulation in smooth muscle cells, thus controlling muscle contraction, was to dissect the molecular mechanisms in isolated smooth muscle cells. In this experimental setting, AngII was able to activate Akt phosphorylation in a PI3Kγ-dependent manner, as pre-treatment of cells with GE21 blunted AngII-recruited Akt phosphorylation (*Figure 6A*).

Likewise, GE21 significantly blunted smooth muscle nifedipine-sensitive voltage-dependent current density and consequently $[Ca^{2+}]_i$ transients induced by AngII (*Figure 6B* and *C*; see Supplementary material online, *Figure S3* and *Movies S1* and *S2*). Overall, the results obtained in the isolated whole vessel and in isolated smooth muscle cells suggested that GE21-induced impairment in vascular myogenic contraction could be ascribed to an impairment of Ca^{2+} influx through LTCCs.

The LTCC open probability is finely tuned by the regulation of subunit assembly in the plasma membrane. In particular, the translocation in the plasma membrane of $Ca_v\alpha1C$, the pore-forming subunit, is necessary for increased open probability, whereas the $Ca_v\beta2$ adaptive subunit, when phosphorylated, antagonizes $Ca_v\alpha1C$ degradation, allowing its translocation.[17,18] Interestingly, in smooth muscle cells we found that AngII stimulation induced translocation in the plasma membrane of $Ca_v\alpha1C$ (*Figure 6D*) and that this process was impaired by PI3Kγ inhibition with GE21 (*Figure 6D*). In addition, GE21 blunted AngII-induced phosphorylation of $Ca_v\beta2$ (*Figure 6E*).

Overall, our data reveal that pharmacological inhibition of PI3Kγ induces vasorelaxation through an action on an intracellular pathway finely modulating LTCC functional regulation and calcium influx, by regulating subunit assembly in the plasma membrane (see model in Supplementary material online, *Figure S4*).

4. Discussion

In this study, we provide evidence that pharmacological inhibition of PI3Kγ reduces arterial BP, by exerting a significant relaxant action on the smooth muscle component of resistance vessels. This effect is mediated through a fine regulation of the LTCC, a pivotal molecular switch for calcium influx in smooth muscle cells, by PI3Kγ inhibition.

These results uncover novel potential applications for PI3Kγ inhibitors and also better delineate the haemodynamic contribution of this strategy to the beneficial effects already observed on overloaded hearts.[7] Indeed, until now, PI3Kγ has gained increasing attention only as a promising pharmacological target for the treatment of inflammation,[2–6] and successively, for protection from maladaptive cardiac remodelling.[7] Here we present new insights suggesting that PI3Kγ inhibition could be exploited to develop novel antihypertensive treatments.

The antihypertensive effect of PI3Kγ inhibition was obtained with two independent molecules, AS605240 and GE21, thus clearly proving that this molecular target is of outmost importance in BP control. Moreover, the fact that these molecules failed to lower BP in mice with genetic ablation of PI3Kγ demonstrates their selectivity of action.

The effect of PI3Kγ inhibitors on BP is realized by reducing total peripheral resistance, thus depicting the involvement of a vascular mechanism. Indeed, PI3Kγ inhibitors evoked a concentration-dependent relaxant effect in resistance arteries, a mechanism demonstrated to be relevant for efficacy of antihypertensive treatments. On this issue, it should be considered that the relaxant effect of PI3kγ inhibition could be not only limited to the mesenteric compartment, but also extended to other resistance beds, such as the renal and skeletal muscle ones.

Our results also clarify that the vasorelaxation induced by PI3Kγ inhibition is independent from effects on endothelium and has to be ascribed to an impact on smooth muscle function. Our data reveal, for the first time, that inhibition of PI3Kγ, as well as of its downstream signalling, Akt, is able to impair the vascular myogenic contractile response markedly, in an experimental setting for isolated vessels where the myogenic response to perfusion pressure represents the main component of vascular tone, in the absence of any neurohumoral influence. In this regard, it is tempting to speculate that PI3Kγ/Akt signalling could be one of the main pathways recruited by pressure-induced mechanical stress. Our novel data on PI3Kγ add a further piece of knowledge on how the mechanical stimulus residing in pressure itself could be sensed by the vessel. The signalling pathway activated by the vessel will in turn allow the myogenic contractile response counteracting the increasing BP. In contrast, it is well known that the PI3Kγ isoform is activated by the βγ subunit of G-protein coupled receptor (GPCR). Among these, Angiotensin II receptor type 1 (AT1R) in vascular myocytes has been demonstrated to be responsible for PI3Kγ activation that, in turn, regulates LTCCs.[19] This result is in agreement with our data obtained in isolated vascular smooth muscle cells, in which the proposed mechanism of LTCCs through PI3Kγ inhibition, has been recruited by AngII. Accordingly, our results obtained in isolated resistance arteries, through perfusion pressure, recapitulate the same effects. Cellular mechanotransduction of pressure could be mediated by a membrane receptor and associated second messengers. In particular, AT1Rs are required for mechanosensitivity, even in the absence of the ligand AngII (the condition reproduced in the isolated vessel, without neurohumoral influences). These receptors, when mechanically activated, adopt an active conformation, allowing G-protein coupling and thus βγ activation.[20] Taken together, these mechanisms through AT1R/βγ could activate PI3Kγ signalling, thus regulating LTCCs and the myogenic response in both a ligand-dependent and −independent manner.

Although PI3Kγ inhibitors lower BP, genetic ablation of PI3Kγ counteracts only agonist-induced hypertension, without affecting basal BP. This may seem counterintuitive, but a perusal of the data indicates that $PI3K\gamma^{-/-}$ mice have a larger lumen of isolated resistance vessels accompanied by a marked enhancement in cardiac muscle contractility, as documented by our previous observations.[21] Thus, the reduced peripheral resistance, coupled to increased myocardial contractility, could explain the development of normal basal BP in $PI3K\gamma^{-/-}$ mice.

A main role in myogenic vascular contraction is played by calcium influx through LTCCs,[14] and we have previously reported that PI3Kγ represents an upstream signalling in response to AngII, modulating Akt-dependent calcium influx through LTCCs.[15] Interestingly, here we extend those observations, demonstrating that PI3Kγ signalling, converging on Akt phosphorylation, is also recruited by pressure. Finally, the fact that an Akt inhibitor induces a vasorelaxant effect comparable to that observed with a PI3Kγ inhibitor suggests that the PI3Kγ/Akt pathway has a crucial role in regulating vascular contraction.

Akt is of key importance for the structural organization and functionality of the LTCC complex at the plasma membrane.[18] In particular, regulation of LTCC activity is directly related to the Akt-mediated

phosphorylation of the accessory/chaperone subunit $Ca_v\beta2$, which in turn, protects the pore-forming $Ca_v\alpha1C$ subunit from the proteolytic degradation system and results in increased density of $Ca_v\alpha1C$ in plasma membrane and consequent LTCC open probability.[18] Here we show that PI3Kγ inhibitors interfere with this signalling pathway by impairing both $Ca_v\beta2$ phosphorylation and $Ca_v\alpha1C$ translocation in the plasma membrane of smooth muscle cells, thus explaining their vasorelaxant effects, which are responsible for their antihypertensive properties.

Overall, we clarify that PI3Kγ inhibition has important haemodynamic effects that need to be considered when this molecular target is considered for a therapeutic strategy. Moreover, our findings strongly indicate that PI3Kγ inhibitors could be further studied as a potential tool to treat hypertension. It is noteworthy to emphasize that very recently PI3Kγ emerged as a new locus influencing pulse pressure and mean arterial pressure in a human genome-wide study on a very large population (over ∼120 000 individuals).[22] Thus, this recent discovery of genome-wide association of PI3Kγ and BP encourages the possibility of translating to humans our previous[8] and present findings on the pathophysiological role of PI3Kγ in BP regulation.

Moreover, PI3Kγ signalling is also a promising pharmacological target for fighting inflammation,[2–4] even in atherosclerosis,[6] and this represents a consistent pathophysiological trait of hypertension-induced organ damage;[23] therefore, the use of PI3Kγ inhibitors could be an appealing strategy with combined beneficial effects for limiting cardiovascular risk. On this particular issue, the possibility of having a common therapeutic target for both inflammation and hypertension is particularly intriguing, because the use of classical non-steroidal anti-inflammatory drugs is frequently reported to dampen the BP-lowering actions of various antihypertensive medications,[24,25] in contrast to that observed with PI3Kγ inhibitors.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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References

1. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* 2010;**11**:329–411.
2. Camps M, Rückle T, Ji H, Ardissone V, Rintelen F, Shaw J *et al.* Blockade of PI3Kγ suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat Med* 2005;**11**:936–943.
3. Barber DF, Bartolomé A, Hernandez C, Flores JM, Redondo C, Fernandez-Arias C *et al.* PI3Kγ inhibition blocks glomerulonephritis and extends lifespan in a mouse model of systemic lupus. *Nat Med* 2005; **11**:933–935.
4. Doukas J, Wrasidlo W, Noronha G, Dneprovskaia E, Fine R, Weis S *et al.* Phosphoinositide 3-kinase γ/δ inhibition limits infarct size after myocardial ischemia/reperfusion injury. *Proc Natl Acad Sci USA* 2006;**103**:19866–19871.
5. Siragusa M, Katare R, Meloni M, Damilano F, Hirsch E, Emanueli C *et al.* Involvement of phosphoinositide 3-kinase γ in angiogenesis and healing of experimental myocardial infarction in mice. *Circ Res* 2010;**106**:757–768.
6. Fougerat A, Gayral S, Gourdy P, Schambourg A, Rückle T, Schwarz MK *et al.* Genetic and pharmacological targeting of phosphoinositide 3-kinase-γ reduces atherosclerosis and favors plaque stability by modulating inflammatory processes. *Circulation* 2008; **117**:1310–1317.
7. Damilano F, Franco I, Perrino C, Schaefer K, Azzolino O, Carnevale D *et al.* Distinct effects of leukocyte and cardiac phosphoinositide 3-kinase γ activity in pressure overload-induced cardiac failure. *Circulation* 2011;**123**:391–399.
8. Vecchione C, Patrucco E, Marino G, Barberis L, Poulet R, Aretini A *et al.* Protection from angiotensin II-mediated vasculotoxic and hypertensive response in mice lacking PI3Kγ. *J Exp Med* 2005;**201**:1217–1228.
9. Costa C, Barberis L, Ambrogio C, Manazza AD, Patrucco E, Azzolino O *et al.* Negative feedback regulation of Rac in leukocytes from mice expressing a constitutively active phosphatidylinositol 3-kinase γ. *Proc Natl Acad Sci USA* 2007;**104**:14354–14359.
10. Zacchigna L, Vecchione C, Notte A, Cordenonsi M, Dupont S, Maretto S *et al.* Emilin1 links TGF-β maturation to blood pressure homeostasis. *Cell* 2006;**124**:929–942.
11. Macrez N, Mironneau C, Carricaburu V, Quignard JF, Babich A, Czupalla C *et al.* Phosphoinositide 3-kinase isoforms selectively couple receptors to vascular L-type Ca^{2+} channels. *Circ Res* 2001;**89**:692–699.
12. Haase H, Alvarez J, Petzhold D, Doller A, Behlke J, Erdmann J *et al.* Ahnak is critical for cardiac $Ca_V1.2$ calcium channel function and its β-adrenergic regulation. *FASEB J* 2005;**19**:1969–1977.
13. Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP *et al.* Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Mol Cell* 2000;**6**:909–919.
14. Hill MA, Meininger GA, Davis MJ, Laher I. Therapeutic potential of pharmacologically targeting arteriolar myogenic tone. *Trends Pharmacol Sci* 2009;**30**:363–374.
15. Vecchione C, Carnevale D, Di Pardo A, Gentile MT, Damato A, Cocozza G *et al.* Pressure-induced vascular oxidative stress is mediated through activation of integrin-linked kinase 1/βPIX/Rac-1 pathway. *Hypertension* 2009;**54**:1028–1034.
16. Le Blanc C, Mironneau C, Barbot C, Henaff M, Bondeva T, Wetzker R *et al.* Regulation of vascular L-type Ca^{2+} channels by phosphatidylinositol 3,4,5-trisphosphate. *Circ Res* 2004;**95**:300–307.
17. Viard P, Butcher AJ, Halet G, Davies A, Nürnberg B, Heblich F *et al.* PI3K promotes voltage-dependent calcium channel trafficking to the plasma membrane. *Nat Neurosci* 2004;**7**:939–946.
18. Catalucci D, Zhang DH, DeSantiago J, Aimond F, Barbara G, Chemin J *et al.* Akt regulates L-type Ca^{2+} channel activity by modulating $Ca_v\alpha1$ protein stability. *J Cell Biol* 2009;**184**:923–933.
19. Quignard JF, Mironneau J, Carricaburu V, Fournier B, Babich A, Nurnberg B *et al.* Phosphoinositide 3-kinase γ mediates angiotensin II-induced stimulation of L-type calcium channels in vascular myocytes. *J Biol Chem* 2001;**276**:32545–32551.
20. Patel A, Sharif-Naeini R, Folgering JR, Bichet D, Duprat F, Honoré E. Canonical TRP channels and mechanotransduction: from physiology to disease states. *Pflugers Arch* 2010;**460**:571–581.
21. Crackower MA, Oudit GY, Kozieradzki I, Sarao R, Sun H, Sasaki T *et al.* Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. *Cell* 2002;**110**:737–749.
22. Wain LV, Verwoert GC, O'Reilly PF, Shi G, Johnson T, Johnson AD *et al.* Genome-wide association study identifies six new loci influencing pulse pressure and mean arterial pressure. *Nat Genet* 2011;**43**:1005–1011. doi: 10.1038/ng.922.
23. Cohuet G, Struijker-Boudier H. Mechanisms of target organ damage caused by hypertension: therapeutic potential. *Pharmacol Ther* 2006;**111**:81–98.
24. Chan AT, Manson JE, Albert CM, Chae CU, Rexrode KM, Curhan GC *et al.* Nonsteroidal antiinflammatory drugs, acetaminophen, and the risk of cardiovascular events. *Circulation* 2006;**113**:1578–1587.
25. Brophy JM. Cardiovascular risk associated with celecoxib. *N Engl J Med* 2005;**352**:2648–2650.