PI3K/p110α inhibition selectively interferes with arterial thrombosis and neointima formation, but not re-endothelialization: potential implications for drug-eluting stent design

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**Background**
Impaired re-endothelialization and stent thrombosis are a safety concern associated with drug-eluting stents (DES). PI3K/p110α controls cellular wound healing pathways, thereby representing an emerging drug target to modulate vascular homoeostasis after injury.

**Methods and results**
PI3K/p110α was inhibited by treatment with the small molecule inhibitor PIK75 or a specific siRNA. Arterial thrombosis, neointima formation, and re-endothelialization were studied in a murine carotid artery injury model. Proliferation and migration of human vascular smooth muscle cell (VSMC) and endothelial cell (EC) were assessed by cell number and Boyden chamber, respectively. Endothelial senescence was evaluated by the β-galactosidase assay, endothelial dysfunction by organ chambers for isometric tension. Arterial thrombus formation was delayed in mice treated with PIK75 when compared with controls. PIK75 impaired arterial expression and activity of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1); in contrast, plasma clotting and platelet aggregation did not differ. In VSMC and EC, PIK75 inhibited expression and activity of TF and PAI-1. These effects occurred at the transcriptional level via the RhoA signalling cascade and the transcription factor NFκB. Furthermore, inhibition of PI3K/p110α with PIK75 or a specific siRNA selectively impaired proliferation and migration of VSMC while sparing EC completely. Treatment with PIK75 did not induce endothelial senescence nor inhibit endothelium-dependent relaxations. In line with this observation, treatment with PIK75 selectively inhibited neointima formation without affecting re-endothelialization following vascular injury.

**Conclusion**
Following vascular injury, PI3K/p110α inhibition selectively interferes with arterial thrombosis and neointima formation, but not re-endothelialization. Hence, PI3K/p110α represents an attractive new target in DES design.

**Keywords**
PI3K/p110α • Endothelium • Restenosis • Thrombosis • Re-endothelialization

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**Introduction**
Vascular injury may either occur in response to mechanical forces exerted by the circulation of blood or as a result of revascularization procedures, i.e. percutaneous transluminal coronary angioplasty (PTCA) or surgical endarterectomy and handling of bypass vessel. Under all circumstances, vascular injury may either heal with proper re-endothelialization or in certain cases lead to scar formation with luminal narrowing and/or thrombotic occlusion. With the increasing use of stents procedure-related vascular injury and...
restrictive remodelling leading to restenosis has become a significant problem. Thus, stents designed to release antiproliferative agents have been developed. Although the introduction of rapamycin- and paclitaxel-eluting stents has resulted in a significant reduction of restenosis and target-vessel revascularization compared with bare-metal stents (BMS), drug-eluting stents (DES) have failed to improve mortality and were associated with increased stent thrombosis rates. Impaired re-endothelialization as well as prothrombotic effects of the eluted drugs seems to play important roles under these conditions. Thus, ideally DES should release drugs that selectively inhibit vascular smooth muscle proliferation and thrombus formation, while allowing proper re-endothelialization still to occur.

By catalysing the synthesis of phosphatidylinositol second messengers (PIP2, PIP3, and PIP3), the phosphatidylinositol-3 kinase (PI3K) signalling pathway controls fundamental cellular processes such as cell survival, proliferation, and differentiation. The class I PI3Ks consist of a dimer with a regulatory (p85a) and a catalytic subunit (p110a, p110b, and p110c). Class I PI3Ks are activated by tyrosine kinases or G-protein coupled receptors and catalyse the generation of PIP3, which modulates downstream targets such as the Rho GTPases and the Akt/PDK1 pathway. The involvement of the PI3Ks in pathophysiological processes such as cancer, inflammation, or thrombosis has rendered these enzymes attractive therapeutic targets and recent efforts have been taken to synthesize cell-permeable small molecule PI3K inhibitors such as the PI3K/p110α inhibitor PIK75. However, because of their ubiquitous distribution, the understanding of how the several PI3K isoforms control pathophysiological responses and whether some provide selectivity for certain vascular processes remains an important issue in the development of PI3K-targeting drugs.

In the vasculature, the PI3K/p110α isoform is abundantly expressed in endothelial cell (EC) and vascular smooth muscle cell (VSMC). This study was designed to address the role of p110α in injury-induced arterial thrombus formation. Since VSMC and EC proliferation as well as migration are critically involved in vascular healing following coronary intervention, we also investigated the contribution of PI3K/p110α in those responses.

## Methods

Expanded methods are provided in Supplementary material online.

### Carotid artery thrombosis model

All animal procedures were approved by the local ethical committee (Kantonales Veterinäramt Zürich, Switzerland) and performed in accordance with institutional guidelines. Ten-week-old male C57Bl6 mice weighing on average 27 g received a daily i.p. injection of PIK75 (10 mg/kg body weight, Axon Medchem; Groningen, The Netherlands) or vehicle (0.5% methylcellulose plus 15% dimethylsulfoxide) for 7 days. Thrombus formation was induced by photochemical injury 24 h after the last injection. Mice were anaesthetized by i.p. injection of sodium pentobarbital (87 mg/kg; Butler, Columbus, OH, USA). Rose bengal (Fisher Scientific, Fair Lawn, NJ, USA) was diluted in phosphate-buffered saline (PBS) and then injected into the tail vein at a final concentration of 50 mg/kg. The right common carotid artery was exposed following a midline cervical incision and the blood flow monitored using a Doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY, USA) connected to a flowmeter (Model T106, Transonic Systems). Photochemical injury was induced by a 1.5 mW green light laser (540 nm; Melles Griot, Carlsbad, CA, USA) 6 min after i.v. rose bengal injection. From the onset of injury, blood flow was monitored until occlusion occurred, which was defined as flow < 0.1 mL/min for at least 1 min.

### Cell culture

Human aortic endothelial cell (HAEC), human aortic smooth muscle cell (AoSMC), and human umbilical vein endothelial cell (HUVEC; all from Lonza, Basel, Switzerland) were cultured as described. Saphenous vein smooth muscle cells (SVSMC) were isolated from human saphenous veins, characterized, and cultured as described. Briefly, AoSMC and SVSMC were grown on flasks (BD Biosciences, Bedford, MA, USA) in Dulbecco’s modified Eagle’s medium (Invitrogen, Basel, Switzerland) plus 10% foetal calf serum (FCS) using passages 5 to 9. Human aortic endothelial cell and HUVEC were grown on fibronectin-coated flasks in endothelial cell growth medium-2 (EGM-2) (Lonza) containing 10% FCS.

### Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donors by density centrifugation in BD Vacutainer cell preparation tubes with sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA) and purified by magnetic cell sorting using anti-human CD14 antibody (Miltenyi Biotec, Cologne, Germany) conjugated with magnetic beads. Monocyte purity was assessed by flow cytometry (FACS Canto, BD, Heidelberg, Germany). Monocytes were cultured in RPMI-1640 medium (Invitrogen) containing 25 mM HEPES supplemented with 10% FCS. PBMCs were incubated with PIK75 (0.1–10 nM) for 24 h following starvation.

### Generation of recombinant adenoviruses and adenoviral infection

Recombinant adenoviruses were generously provided by Prof. Zihong Yang (Department of Physiology, University of Fribourg, Fribourg, Switzerland). Cells were transduced with the respective adenoviral vector at moi 800. After a 24-h growth period, the cells were rendered quiescent for 24 h before harvesting.

### Neointima formation

To investigate the impact of PIK75 treatment on neointima formation in vivo, 10–14-week-old male C57Bl6 mice weighing on average 22–25 g were anaesthetized by inhalation of 3% isoflurane. The left common carotid artery was dissected and ligated near the carotid bifurcation as previously described. Importantly, all animals recovered and showed no symptoms of a stroke. Animals received a daily i.p. injection of PIK75 (10 mg/kg body weight, Axon Medchem; Groningen, The Netherlands) or vehicle (0.5% methylcellulose plus 15% dimethylsulfoxide) for 14 days. After 2 weeks, animals were euthanized and the isolated arteries were fixed with 4% paraformaldehyde in PBS. The common carotid arteries were 8–10 mm long. Proximal and distal segments (2 mm) were discarded and the remaining portion was embedded in paraffin. Serial sections (5 μm thick) were cut for haematoxylin–eosin staining. Neointima formation was quantified by determining the mean neointima/media ratio in triplicates of each sample.

### Senescence-associated β-galactosidase assay

Human aortic endothelial cells were plated on fibronectin-coated six-well dishes and grown for 24 h to 80% confluence in EGM-2 plus 10% FCS. After replacing the medium (EGM-2 plus 10% FCS), cells were treated with vehicle (0.1% dimethylsulfoxide), PIK75 (10 nM), rapamycin (10 nM), or paclitaxel (10 nM) for another 24 h. Human aortic
endothelial cells were washed three times with PBS and fixed with 3% paraformaldehyde for 3 min, incubated with a senescence-associated β-galactosidase (SA-β-gal) stain solution for 14 h, and the proportion of SA-β-gal positive cells determined as previously described.19

Endothelial function studies
Aortas obtained from male C57Bl6 mice were cut into rings (2–3 mm long). Each ring was connected to an isometric force transducer (Multi-Myograph 610M; Danish Myo Technology, Aarhus, Denmark), suspended in an organ chamber filled with 5 mL of control solution (37°C, pH 7.4), and bubbled with 95% O2 and 5% CO2. Isometric tension was recorded continuously. After a 30-min equilibration period, rings were gradually stretched to the optimal point of their length–tension curve as determined by the contraction to 60 mM KCl. Concentration-dependent contractions were established by using norepinephrine (NE) (1 nM–10 μM; Sigma Aldrich). Concentration–response curves were obtained in a cumulative fashion. Several rings cut from the same artery were studied in parallel. Responses to acetylcholine (1 nM–1 μM) were obtained in a cumulative fashion. Several rings cut from the same weight, liver enzyme activities, and lipid profiles were observed.

Results
PI3K/p110α promotes TF and PAI-1 expression in vascular cells and monocytes
PIK75 (0.1–10 nM) inhibited TNF-α-induced TF expression in HAEC as well as basal TF expression in AoSMC in a concentration-dependent manner (n = 4; P < 0.05; Figure 2A and B). In PBMC treatment with PIK75 resulted in a similar concentration-dependent decrease in TF expression (n = 4; P < 0.05; Supplementary material online, Figure S2). PIK75 also strongly impaired PAI-1 mRNA expression in HAEC and AoSMC (n = 4; P < 0.05; Figure 2C and D). No cytotoxic effect of PIK75 was observed in any of these cell types at any of the concentrations used (n = 4; data not shown).

To investigate the contribution of the other PI3K isoforms in TF and PAI-1 expression, HAEC and AoSMC were incubated with corresponding specific small molecule inhibitors. In contrast to the inhibitory effect of PIK75, pharmacological inhibition of PI3K/p110β, γ, and δ with TGX221, A5605240, and IC87114, respectively, resulted in a further stimulation of TNF-α-induced TF expression in HAEC (n = 4; P < 0.05; Figure 2E). In AoSMC, PIK75, decreased basal TF expression, while inhibitors of PI3K/p110β, γ, and δ had no effect (n = 4; P < 0.05; Figure 2F).

PI3K/p110α silencing mimics the effect of PIK75 on TF and PAI-1
Treatment of AoSMC and HAEC with siRNA targeting p110α markedly decreased p110α protein expression in both cell types (n = 4; P < 0.01; Supplementary material online, Figure S3A and B). Silencing of PI3K/p110α was paralleled by an inhibition of TF expression in HAEC as well as in AoSMC (n = 4; P < 0.05; Figure 3A and B). Inhibition of PI3K/p110α with siRNA also decreased PAI-1 expression in both cell types (n = 4; P < 0.05; Figure 3C and D).

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PI3K/p110α promotes TF and PAI-1 transcription via RhoA-GTPase and NFkB

In AoSMC and HAEC, treatment with PIK75 or siRNA targeting p110α decreased TF and PAI-1 mRNA expression \((n = 4; \ P < 0.05; \text{Figures } 4A \text{ and } D)\). Pull-down assays demonstrated that pharmacological inhibition of PI3K/p110α reduced activity of the GTPase RhoA and the transcription factor NFkB in both cell types \((n = 4; \ P < 0.05; \text{Figures } 4B \text{ and } E)\). Activation of the mitogen-activated protein (MAP)-kinases was not affected by PI3k/p110α inhibition in none of the cell types \((n = 4; \text{data not shown})\).

A role of RhoA in mediating the induction of TF and PAI-1 was confirmed by adenoviral overexpression of the constitutively active RhoA mutant Rho63 which enhanced both basal TF and PAI-1 expression in AoSMC and TNF-α-induced TF and PAI-1 expression in HAEC, while overexpression of the dominant negative mutant Rho19 inhibited TF and PAI-1 expression in both cell types \((n = 4; \ P < 0.05; \text{Figures } 4C \text{ and } F)\).

PI3K/p110α regulates vascular smooth muscle cell proliferation, chemotaxis, and neointima formation in vivo

Since the RhoA-GTPase has been shown to play important roles in the proliferative potential of vascular SMC, the impact of PI3K/p110α inhibition on the proliferative properties of vascular SMC was further investigated. Treatment with PIK75 \((0.1–10 \text{ nM})\) inhibited PDGF-BB-induced proliferation of AoSMC in a concentration-dependent manner as well as migration of AoSMC \((n = 4; \ P < 0.05; \text{Figure } 5A)\). Experiments performed in venous...
Figure 2  PI3K/p110α regulates tissue factor and plasminogen activator inhibitor-1 expression in vascular cells. (A) PIK75 inhibits TNF-α-induced TF expression in human aortic endothelial cell in a concentration-dependent manner \((n = 4; \ast P < 0.05)\). (B) In aortic smooth muscle cell PIK75 (0.1–10 nM) abrogates basal TF expression \((n = 4; \ast P < 0.05)\). (C and D) PIK75 inhibits PAI-1 expression in human aortic endothelial cell (C) and aortic smooth muscle cell (D) \((n = 4; \ast P < 0.05)\). (E) In contrast to PIK75, pharmacological inhibition of PI3K/p110β, γ, and δ with TGX 221, AS602240, and IC87114, respectively, enhances TNF-α-induced TF expression in human aortic endothelial cell \((n = 4; \ast P < 0.05)\). (F) In aortic smooth muscle cell, solely PIK75 decreased basal TF expression \((n = 4; \ast P < 0.05)\).
SMC (SVSMC) obtained from the saphenous vein confirmed the results obtained in AoSMC (n = 4, Supplementary material online, Figure S4A and C). In line with this observation, silencing of PI3K/p110α by siRNA decreased proliferation and impaired chemotaxis of AoSMC in response to PDGF-BB (n = 4; P < 0.01 vs. control; Figure 5B). Treatment with PIK75 (10 nM), rapamycin (10 nM), or paclitaxel (10 nM) resulted in a comparable inhibition of PDGF-BB-induced proliferation and migration of AoSMC (n = 4; P < 0.005; Figure 5C).

To confirm the relevance of the antiproliferative effects of PIK75 observed at the cellular level in vivo, injury-induced neointima formation was studied in a murine carotid artery ligation model. Two weeks after ligation of the carotid artery neointimal hyperplasia was observed in the carotid arteries of control animals (n = 6; Figure 5D). Treatment with PIK75 or rapamycin inhibited neointima formation following vascular ligation as determined by the assessment of the intima/media ratio by (n = 6; P < 0.01 vs. control; Figure 5D and E).

Figure 3 PI3K/p110α silencing inhibits tissue factor and plasminogen activator inhibitor-1. (A and B) Silencing of PI3K/p110α with siRNA inhibits TF expression in human aortic endothelial cell as well as in aortic smooth muscle cell (n = 4; *P < 0.05). (C and D) Inhibition of PI3K/p110α with siRNA also decreased PAI-1 expression in both cell types (n = 4; *P < 0.05).
PI3K/p110α regulates tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) transcription via RhoA-GTPase and NFκB. Treatment with PIK75 (10 nM) or siRNA targeting p110α inhibits TF and PAI-1 mRNA expression in aortic smooth muscle cell (A; n = 4; *P < 0.05) and human aortic endothelial cell (D; n = 4; *P < 0.05). Inhibition of PI3K/p110α with PIK75 reduces activity of the RhoA-GTPase and the transcription factor NFκB under basal conditions in aortic smooth muscle cell (B; n = 4; *P < 0.05) and in TNF-α stimulated human aortic endothelial cell (E; n = 4; *P < 0.05). (C and F) Adenoviral overexpression of the constitutively active RhoA mutant Rho63 enhances TF and PAI-1 expression in aortic smooth muscle cell (n = 4; *P < 0.05 vs. control) and human aortic endothelial cell (n = 4; *P < 0.05 vs. TNF-α), while overexpression of the dominant negative mutant Rho19 blunts TF and PAI-1 expression in aortic smooth muscle cell (n = 4; *P < 0.05 vs. control) and human aortic endothelial cell (n = 4; **P < 0.01 vs. TNF-α).
PI3K/p110α inhibition does not affect endothelial proliferation, chemotaxis, and nitric oxide synthesis

Treatment with PIK75 (0.1–10 nM) did not affect serum-induced proliferation and migration of HAEC (n = 4; Figure 6A). Consistent with this observation, silencing of PI3K/p110α by siRNA inhibited the proliferation and chemotaxis of aortic smooth muscle cell in response to PDGF-BB in a similar manner (n = 4; *P < 0.01 vs. PDGF-BB). (C) Treatment with PIK75, rapamycin, or paclitaxel results in a similar inhibition of PDGF-BB-induced proliferation and migration (n = 4; *P < 0.005 vs. PDGF-BB). (D) Treatment with PIK75 or rapamycin for 14 days inhibits neointima formation following vascular ligation when compared with untreated controls (representative photograph of carotid arteries stained with haematoxylin and eosin; M, media; NI, neointima; black arrow indicates internal elastic membrane). (E) Antiproliferative effects of PIK75 and rapamycin were quantified by determining the intima/media ratio (n = 6; *P < 0.05 vs. control).

In contrast to PIK75, the mTOR inhibitors rapamycin and paclitaxel exerted anti-proliferative and anti-migratory effects in ECs (n = 4; P < 0.005; Figure 6C).

Since down-regulation of endothelial NO expression (eNOS) expression is critically involved in both endothelial proliferation and migration, eNOS expression was assessed in HAEC by western blot. Rapamycin and paclitaxel, but not PIK75, decreased total eNOS expression in HAEC within 24 h (n = 4; P < 0.005; Figure 6D), resulting in impaired endogenous NO production in HAEC (n = 4; P < 0.05, Figure 6E).
PIK75 does not affect endothelial senescence, function, and repair capacity

The decrease in eNOS expression observed in HAEC treated with rapamycin and paclitaxel for 24 h was paralleled by the induction of a senescent phenotype characterized by an enlarged and elongated cellular morphology (Figure 7A). These morphological changes were not observed with PIK75 (n = 4; Figure 7A). The SA-β-gal assay confirmed senescence in HAEC treated with rapamycin or paclitaxel, but not in PIK75-treated cells (n = 4; P < 0.05; Figure 7B).

Isometric tension studies in organ chambers demonstrated no difference in endothelium-dependent relaxations to acetylcholine between aortas treated with PIK75 and controls (n = 8; P = 0.45, Figure 7C). Endothelium-independent relaxations to the NO donor sodium nitroprusside were identical in both groups, denoting no difference in the response of VSMCs to NO (n = 8; P = 0.56; Figure 7D).

Consistent with those findings, endothelial healing in the carotid artery 3 days after electric injury was not affected by pharmacological inhibition of PI3K/p110α with PIK75 (n = 6; P = 0.38 vs. control; Figure 7E and F). In contrast, treatment with rapamycin significantly delayed endothelial repair following vascular injury (n = 6; P < 0.01 vs. control and PIK75; Figure 7E and F).

Rapamycin and paclitaxel, but not PIK75, induce endothelial TF and PAI-1 expression

Treatment of HAEC with rapamycin (10 nM) or paclitaxel (10 nM) induced basal TF expression (n = 4; P < 0.05 vs. control; Figure 8A) as well as TNF-α-induced TF expression (n = 4; P < 0.05 vs. TNF-α alone; Figure 8B). In contrast, PIK75 inhibited basal- and TNF-α-induced TF expression (n = 4; P < 0.01; Figure 8A and B). In a similar manner, rapamycin and paclitaxel, but not PIK75, enhanced basal PAI-1 expression in HAEC (n = 4; P < 0.05; Figure 8C). Only treatment with PIK75 abrogated TNF-α-induced PAI-1 expression (n = 4; P < 0.01 vs. TNF-α alone; Figure 8D).

Discussion

This study provides evidence for a functional diversity of PI3K/p110α isoforms. Specifically, we demonstrate that PI3K/p110α plays a crucial role in the induction of pro-thrombotic proteins such as TF and PAI-1 via RhoA-GTPase and NFκB in vitro and in arterial thrombus formation in vivo. Moreover, PI3K/p110α regulates proliferation and migration of VSMCs via RhoA while sparing ECs.

The small molecule inhibitor PIK75 is an imidazopyridine developed in the context of a PI3-kinase drug discovery programme. PIK75 selectively inhibits the catalytic subunit p110α encoded by the PIK3CA gene. The dose of PIK75 used for our in vitro studies is within the range of that used in previously published studies that demonstrated a potent inhibition of PI3K/p110α in several PIK75-treated tissues. Measurement of plasma levels in our study also provides evidence that the concentrations employed for the experiments are in a pharmacologically relevant range. Several studies have confirmed that at these in vitro concentration ranges, PIK75 is a highly selective inhibitor of PI3K/p110α compared with other class I PI3Kinas (p110β and p110δ). Inhibition of the three other PI3K isoforms resulted in induction rather than inhibition of TF and PAI-1 expression in cultured vascular cells, which provides evidence for the biological selectivity of different PI3K isoforms and the pharmacological selectivity of different PI3K isoform inhibitors. Based on results from previous in vivo studies and the plasma levels measured in the present study, it is reasonable to assume that the potent antithrombotic and antiproliferative effects observed after treatment with PIK75 are mediated by inhibition of PI3K/p110α activity in the vessel wall even though PI3K/p110α activity was not directly measured in carotid artery lyses.

A limitation of the present study is that only pharmacological inhibition of PI3K/p110α was applied to investigate the role of PI3K/p110α in thrombus formation in vivo. However, PIK75 is a highly specific inhibitor of PI3K/p110α and, to our knowledge, an appropriate genetic model is not available. For instance, endothelial-specific homozygous inactivation of PI3K/p110α causes embryonic lethality, while mice heterozygous for the D910A knock-in mutation (p110αD933A/WT) are viable and fertile, but display a strong metabolic phenotype which would probably interfere with the study endpoint.

Another potential limitation of the study is related to the observation that performing experimental studies with a small number of animals and a single strain carries the risk of over- or underestimating the biological effect observed.

Suppression of basal- and cytokine-induced TF and PAI-1 expression through PI3K/p110α inhibition is of particular relevance, since both proteins are critically involved in the pathogenesis of stent thrombosis. During acute coronary syndromes and PTCA, periprocedural cytokine plasma levels are increased to concentrations high enough to induced TF and PAI-1 expression in surrounding vascular cells. While rapamycin and paclitaxel enhance both basal- and TNF-α-induced TF and PAI-1 expression, PI3K/p110α inhibition with PIK75 exerts potent antithrombotic effects. In vascular cells, inhibition of PI3K/p110α resulted in decreased expression of TF and PAI-1 mediated by reduced activation of RhoA GTPase and the transcription factor NFκB. The role of RhoA activation and NFκB in regulating TF and PAI-1 has previously been described.

Pharmacological inhibition of PI3K/p110α decreased expression and activity of both TF and PAI-1 in the arterial wall without affecting plasma clotting times and platelet aggregation. This indicates that PI3K/p110α inhibition impairs local injury-induced arterial thrombosis by regulating the expression and activity of prothrombotic mediators within the artery wall, rather than inducing any functionally relevant alterations in the activity of plasmatic coagulation factors or circulating platelets.

While a trend towards inhibition of platelet aggregation could be observed in the treated group, this effect did not reach statistical significance which may be due to the use of small sample sizes in the in vivo studies potentially resulting in an underestimation of the effect. Interestingly, a previous study described a role of PI3K/p110α in insulin-like growth factor-1-mediated propagation of platelet activation after stimulation with the PAR-1 agonist SFLLRNR. It is important to note that in contrast to the current study the direct effect of PIK75 on ADP or collagen was not investigated in the cited study, which renders side-by-side comparisons of the results difficult. In addition, concentrations of PIK75 used in that study were significantly higher than the one in our study, where concentrations in vitro as well as in vivo peaked in the nanomolar range.
Vascular smooth muscle cell proliferation and migration are crucial cellular mechanisms triggering a response to injury and in turn restenosis in coronary arteries following PTCA and intimal hyperplasia and occlusion of venous grafts after coronary artery bypass operations. In particular, the RhoA-GTPase has been shown to play an important role in the proliferative response to mechanical stress in VSMC. To confirm the in vivo relevance of the antiproliferative response to PI3K/p110α inhibition, neointima formation was studied in an established animal model. Murine models of restenosis based on arterial injury carry the distinctive advantage of inducing smooth muscle-rich neointimal hyperplasia while thrombus formation remains limited. Complete ligation of the carotid artery near the carotid bifurcation has been shown to induce proliferation of smooth muscle cells leading to significant neointima formation. Hence, this model appears particularly suitable to investigate the antiproliferative effects of PIK75 on VSMCs in vivo.

The PI3K family consists of several isoforms with regard to the regulatory p85 and the catalytic p110 subunits. Studies on the p85α/p110α dimer showed that the p85α regulatory subunit inhibits the catalytic activity of p110α and that oncogenic p85α mutations act by disrupting the inhibitory contact with p110α. A more recent study demonstrated that phosphorylation of p85α at serine 83 (pSer83) resulted in inhibition of VSMC and reduced neointima formation after balloon injury without affecting endothelial regeneration. The present study demonstrates for the first time that inhibition of p110α is very likely to mediate this selective antiproliferative effect in VSMC. Since p85α can interact with different p110 isoforms and the expression of the p110 isoforms varies greatly between tissues, modulation of p85α activity and consecutive regulation of downstream cellular responses is likely complex and currently not well understood. Inhibition of p110α instead offers the advantage of targeting a single PI3K pathway and may be a safer option.

Delayed arterial healing manifested by impaired re-endothelialization and enhanced thrombogenicity remains a concern associated with the use of DES, but is relevant for vascular injury and healing at large. Morphological studies have indeed demonstrated a
marked delay of re-endothelialization with the use of both rapamycin- and paclitaxel-eluting stents when compared with BMS. Indeed, rapamycin and paclitaxel suppress EC proliferation and migration as demonstrated in several studies. In addition, by inducing a senescent phenotype and a decrease in eNOS expression, recent data suggest that those drugs impair endothelial senescence, eNOS expression and re-endothelialization capacity. (A) Rapamycin and paclitaxel, but not PIK75 induce a senescent phenotype in human aortic endothelial cell (representative figure of four independent experiments). (B) Senescence-associated β-galactosidase staining demonstrates senescence in human aortic endothelial cell treated with rapamycin or paclitaxel, but not in PIK75-treated cells (n = 4; *P < 0.05). (C) Isometric tension studies demonstrate no difference in endothelium-dependent relaxations to acetylcholine between aortas treated with PIK75 and controls (n = 8; P = 0.45). (D) PIK75 had no effect on endothelium-independent relaxations to the nitric oxide donor sodium nitroprusside (n = 8; P = 0.56). (E) Representative photographs of carotid arteries from mice. Endothelial injury was confirmed by Evans blue dye staining (representative picture of six independent experiments. (F) In contrast to rapamycin, PIK75 did not impair arterial re-endothelialization after electrical injury (n = 6; *P < 0.05).
Regrowth, Paclitaxel and rapamycin treated control groups were not included in the organ chamber experiments in the context of the current study since previous studies already convincingly demonstrated that both compounds also affect endothelial function. \(^{36–38}\) In line with this observation, rapamycin as well as paclitaxel exerts direct prothrombotic effects by enhancing both basal- and cytokine-induced endothelial expression of TF and PAI-1.\(^ {39,40}\) Most of these biological effects have also been observed with...
compounds used for second generation DES and suggest that the thrombotic risk of DES is a function of both impaired endothelial coverage and function.41–43

In contrast to rapamycin or paclitaxel, inhibition of p110α with PIK75 or the corresponding siRNA did neither affect proliferation nor migration of arterial or venous EC. While the current study demonstrates that TF and PAI-1 expression are regulated by RhoA-GEF and Rac1 in both cell types, proliferation of EC does not depend on RhoA and is rather regulated by the small GTPase Rac-1.29 Hence, the decrease in RhoA activity observed in both cell types is consistent with the selective interference of PI3K/p110α inhibition with VSMC proliferation. Mechanistically, Rac1 triggers proliferative signalling through the p21-activated protein kinase and microinjection of Rac1 has been shown to stimulate DNA synthesis.34 Interestingly, PI3K/p110α-dependent activation of Rac-1 regulates EC migration in response to hydrogen sulfide.45 Another study suggested a regulatory role for PI3K/p110α in endothelial barrier function via Rac-1 activation.46 While the same authors demonstrated a crucial role for PI3K/p110α in developmental angiogenesis, tube formation, and EC migration through the small GTPase RhoA, they did not observe any inhibitory effect on EC proliferation, which is in line with our observation as well as that of another group demonstrating that PI3K/p110α also regulates Rac-1 in endothelial cells.46,47

Inhibition of PI3K/p110α did neither affect endothelial NO generation nor endothelium-dependent vascular relaxation, which is of particular interest since eNOS activation and endothelial NO generation are critically involved in endothelial repair in vivo.48 Nitric oxide is a major regulator of EC migration and proliferation which is supported by the finding that NO is generated by EC upon stimulation with VEGF and that depletion of the eNOS gene in knockout mice results in impaired angiogenesis.49,50 Interestingly, the RhoA/Rho-kinase pathway plays an important role in eNOS suppression in EC leading to reduced NO synthesis. Transfection of EC with a dominant negative RhoA mutant increases eNOS expression.51 In the current study, we observed that inhibition of PI3K/p110α suppressed TNF-α-induced RhoA activation in EC. Hence, it is conceivable that PI3K/p110α may contribute to re-endothelialization by an increased, or at least stable, NO production.

Finally, it is important to mention that the use of different chemotactants (i.e. VEGF, TNF-α, and serum) makes it difficult to compare the results between the different in vitro studies. Re-endothelialization of injured vessels in vivo involves several cellular mechanisms including chemotactic, mechanotactic, and haptotactic migration as well as proliferation of EC. In the current study, we demonstrate for the first time that pharmacological inhibition of PI3K/p110α does not affect the complex process of re-endothelialization following vascular injury, which is a particularly interesting observation in the context of DES design. However, further molecular studies will be required to decrypt the cellular pathways underlying the cell-specific anti-proliferative effects of PI3K/p110α inhibition in different cell types.

In summary, the present study provides evidence that inhibition of PI3K/p110α impairs arterial thrombus formation and differentially regulates smooth muscle and endothelial cell activation. Since specific targeting of p110 isoforms is currently evaluated in different areas of medicine, local inhibition of p110α might represent a novel strategy in the context of DES design, in order to prevent restenosis without adding the risk of impaired re-endothelialization and enhanced thrombogenicity.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

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**Conflict of interest:** Drs E.W.H., T.F.L., and F.C.T. filed a patent on the results reported in the present study and their potential clinical applications. The remaining authors report no conflicts of interests related to this study.

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