Inactivation of PI3Kδ Induces Vascular Injury and Promotes Aneurysm Development by Upregulating the AP-1/MMP-12 Pathway in Macrophages

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Objective—An aneurysm is an inflammatory vascular condition. Phosphatidylinositol 3-kinases δ is highly expressed in leukocytes, and play a key role in innate immunity. However, the link between phosphatidylinositol 3-kinases δ and aneurysm development has not yet been elucidated.

Approach and Results—Carotid ligation unexpectedly induced characteristic aneurysm formation beneath the ligation point in p110δD910A/D910A mice (n=25; P<0.001 versus wild-type). Besides, p110δ inactivation exacerbated CaCl2-induced abdominal aortic aneurysms development. A reverse transcription polymerase chain reaction microarray revealed significant extracellular matrix components degradation and matrix metalloproteinases (MMPs) upregulation in the abdominal aorta of p110δD910A/D910A mice. Similarly, the expression of both collagen I and IV was significantly decreased (n=10; P<0.05 versus wild-type) in carotid artery. Western blot assay confirmed that MMP-12 was significantly upregulated in arteries of p110δD910A/D910A mice (n=10; P<0.01 versus wild-type). In vitro, p110δ inactivation marked increase peritoneal macrophages recruitment and synergistically enhance tumor necrosis factor-α-induced recruitment. A specific phosphatidylinositol 3-kinases δ inhibitor (IC87114) or genetic p110δ inactivation upregulated MMP-12 expression and c-Jun phosphorylation (n=6; P<0.05 versus wild-type macrophages). IC87114 also increased activator protein-1 DNA-binding activity (n=6; P<0.001 versus control) and enhanced the effect of tumor necrosis factor-α on activator protein-1 binding activity (n=5; P<0.01 versus tumor necrosis factor-α treatment groups). Knockdown of c-Jun suppressed the effect of the IC87114 and tumor necrosis factor-α on MMP-12 mRNA expression (n=5 in each group; P<0.01 versus scrRNA treatment groups).

Conclusions—Our findings demonstrate that p110δ inactivation leads to extracellular matrix degradation in vessels and promotes aneurysm development by inducing macrophages migration and upregulating the activator protein-1/MMP-12 pathway in macrophages. (Arterioscler Thromb Vasc Biol. 2015;35:368-377. DOI: 10.1161/ATVBAHA.114.304365.)

Key Words: activator protein-1 ■ aneurysm ■ extracellular matrix ■ matrix metalloproteinases-12

Aneurysms frequently lead to high morbidity and mortality as a result of rupture or dissection without symptoms.1 Aortic aneurysms, including abdominal aortic aneurysm (AAA), thoracic aneurysms, and carotid aneurysms, are highly common conditions, with AAA being 3 times more prevalent than thoracic aneurysm.2 Although they are typically regarded as being distinct entities, vascular inflammation is a common pathogenic factor in them.3,4 Pathological features of aneurysmal diseases include transmural inflammatory cell infiltration, noticeable breakdown of elastic lamellae, smooth muscle cell loss, endothelial cell death and detachment, neovascularization, calcium deposition, and focal aneurysmal dilation of the vessel wall.5,6 However, the specific cellular mechanisms that underlie aneurysm formation and progression are poorly understood.

Increasing evidence7,8 points to an important role for innate immune cells in the pathobiology of aneurysms. Monocytes/macrophages infiltrate the vessel wall and release proteases, including elastase (matrix metalloproteinase-12 [MMP-12]) and metalloproteinases that compromise the integrity of the vascular wall through degradation of the extracellular matrix (ECM). Monocytes/macrophages also secrete inflammatory cytokines in the media and adventitia of aneurysmatic vessels, such as tumor necrosis factor (TNF)-α, interferon-γ,
interleukin-1β,9–11 and interleukin-6,12 which are reported significantly increased in patients with AAA. As a central role in AAA, macrophages may be classically activated by toll-like receptor (TLR) ligands. Studies recently have confirmed that deficiency of TLR4 or its adaptor protein MyD88 reduced angiotensin II–induced AAA formation.13 Meanwhile, TLR signaling pathway has cross-talk with phosphatidylinositol 3-kinase (PI3K) during innate immune. Accumulating evidence has established the role of PI3K in the attenuation of TLR signaling.14–17

PI3Ks are a family of ubiquitously expressed enzymes that possess both lipid and protein kinase activities. Indeed, PI3Ks are a family of ubiquitously expressed enzymes that can be grouped into 3 different classes. Class I PI3Ks are heterodimeric enzymes, consisting of a p55, p85, or p101 regulatory subunit and a p110 catalytic subunit (classified as α, β, γ, and δ, respectively).18,19 The p110 catalytic subunit catalyzes the generation of phosphatidylinositol 3,4,5 trisphosphate (PIP3) and activates numerous downstream targets,21 including the serine–threonine kinase protein kinase B/Akt, which is involved in antiapoptosis, proliferation, and oncogenesis. PI3Kδ is a Class I family member and is low or absent in most cells but is predominantly expressed in leukocytes.22 The role of PI3Kδ in leukocytes has been confirmed in P110δ/D910A kinase-dead knock-in mice (P3K δ110δ/D910Ad910A).23 These PI3Kδ-inactivated mice showed defects in B- and T-cell signaling24 and impaired humoral immune responses. In p110δ/D910A mice, macrophages are hyperresponsive to TLR signaling and the augmented TLR signaling pathways elicits an exuberant inflammatory response.25 Besides, the direct inhibition of Akt in monocytes resulted in a proinflammatory phenotype similar to that observed with PI3K inhibition.26 Therefore, we hypothesized that inactivation of PI3Kδ/Akt signaling in macrophages play a crucial role in AAA development.

To test this hypothesis, we investigated the effect of PI3Kδ inactivation on artery architecture and AAA development in 2 distinct murine models in p110δ/D910A mice and in vitro experiments with macrophage migration and MMPs expression. Our results suggested that MMP-12 is the most important molecules influenced by p110δ inactivation in arteries and in vascular injury models. Furthermore, PI3Kδ inactivation promotes macrophage recruitment and upregulates MMP-12 expression via mediating activator protein (AP)-1 protein activation in macrophages.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

**Inactivation of PI(3)K p110δ Unexpectedly Induced Aortic Aneurysm Formation In Carotid Injury Model**

In carotid injury model, hematoxylin-eosin staining (Figure 1A, a1 and a2, c1 and c2) revealed that carotid ligation results in similarly significant neointima formation and neointima/media area ratios (data not shown) of injured carotid arteries were marked reduced in wild-type (WT) as well as in p110δ/D910A mice but the arrows in Elastica van Gieson staining images showed that wavy elastic fiber and fragments seemed only in p110δ/D910A mice not in WT mice. Beneath the ligation point, serial sections further showed that a remarkable loss of medial smooth muscle cells, the separation of the layers within the aortic wall, significant fragmentation of elastic lamellae, and significant matrix degradation under the adventitia of the carotid artery colonized with massive inflammatory cells in p110δ/D910A mice (Figure 1A, c3–c6). Furthermore, immunohistochemical staining showed that a large number of CD68-positive macrophages infiltrated under the adventitia (Figure I in the online-only Data Supplement). In contrast, the carotid arteries of WT mice with surgery showed foci of reduced vascular smooth muscle cells (VSMCs) and loosen media. However, no apparent inflammation was observed in the media of WT mice after surgery (Figure 1A, a3–a6; Figure I in the online-only Data Supplement). Besides, 4 weeks after ligation surgery, 27% of p110δ/D910A mice (n=25) unexpectedly exhibited characteristic aneurysms beneath the ligation point of the common carotid, whereas this did not occur in WT mice (n=25; P<0.001; Figure I).

**Inactivation of PI(3)K p110δ Exacerbates CaCl₂-Induced AAA Development**

To further determine the role of p110δ inactivation in aneurysm development, we examined the pathology of CaCl₂-induced AAA model in WT and p110δ/D910A mice. Morphometric analyses of the abdominal aortae showed although CaCl₂-induced AAA pathology in WT and p110δ/D910A mice, significant dilation of the external and internal aortic diameters in the p110δ/D910A mice compared with WT or IC87114-treatment WT mice (Figure 2A–2C). Besides, the dilation of aorta was accompanied by thinning of the medial layer, calcium deposition, and massive disruption of the elastic lamellae, which showed limited dilation and foci of disruption of the elastic lamellae instead of extensive damage in WT mice. Similarly, IC87114, specific p110δ inhibitor, significantly promotes inflammatory cells recruitment under the adventitia and result in the thinner medial layer and larger aortic diameters after treatment for 4 weeks in comparison with CaCl₂-induced WT mice, suggesting p110δ inactivation probably alter inflammatory cells function to induce vascular injury...
during AAA development. Moreover, ≈50% CaCl$_2$-induced p110$\delta$D910A/D910A mice died within 42 days (Figure 2D).

**Inactivation of PI(3)K p110$\delta$ Dysregulated the Expression Profiles of ECMs and Metalloproteinases in the Aorta**

Considering PI3K$\delta$D910A/D910A mice are susceptible to inflammatory irritant, we doubted that PI3K$\delta$ inactivity might have an effect on normal vessel structure. Therefore, we examined the ultrastructure of the carotid artery from both types of mice using perfusion fixation at constant pressure ≈100 mmHg as we previously reported. In p110$\delta$D910A/D910A mice, electron microscopy showed significant migration and disarray of smooth muscle cells in the vessel wall, thinning of the wavy elastic lamellae and deposition of the amorphous matrix between VSMCs (Figure II in the online-only Data Supplement). Hematoxylin-eosin staining shows that the diameter of carotid arteries has no difference between both types of mice after perfusion fixation. In addition, immunohistochemical analyses showed collagen I and IV expressions in carotid artery remarkably reduced in p110$\delta$D910A/D910A mice compared with WT mice (Figure 3, a1–a3). In abdominal aorta, immunofluorescence and western blot assays revealed that the expression of $\alpha$-smooth muscle actin was lower than that of WT mice (Figure 3, b1). Moreover, reverse transcription polymerase chain reaction microarray assays for ECM and adhesion molecules using the abdominal aortic arterties from both types of mice showed that the genes expression of crucial ECM components, such as Col1a1, Col2a1, Col3a1, Col4a3, Fibronectin l, and Laminin $\gamma$1 were significantly downregulated, whereas ECM degradative enzymes, including MMP-12, MMP-3, MMP-9, and MMP-11 were upregulated (Figure 3, b2). In particular, western blot assay confirmed that the expression of MMP-12 was significantly higher than that of WT mice (n=10; $P<0.01$; $P<0.05$; Figure 3, b3). These data suggested that p110$\delta$ inactivation contribute to vascular injury probably because of dysregulating ECM/MMPs expression in vessels.

p110$\delta$ Inactivation Upregulates MMP-12 Activity and Expression in CaCl$_2$-Induced AAA Mice

Previous studies have reported that MMP-12 play a key role in the CaCl$_2$-induced AAA model and MMP-12 expression in patients with AAA significantly enhanced. Moreover, our results also revealed the link between p110$\delta$ inactivation and MMP-12 upregulation. Next, we further analyzed the
MMP-12 activity in serum from AAA mice. Casein zymography showed that CaCl$_2$ significantly increased MMP-12 activation compared with sham-operated mice but the activity of MMP-12 was higher in p110$\delta$$^{D910A/D910A}$ mice than WT-AAA mice. IC87114 also enhance MMP-12 activity in WT-AAA mice (Figure 4A, a1). Notably, in AAA foci, MMP-12 expression was significantly increased under adventitia in each group compared with MMP-9 or MMP-2 expression (Figure III in the online-only Data Supplement). We then examined the cellular distribution of MMP-12 in aneurismatic sections by confocal microscopy. We found that the cellular localization of MMP-12 (red) from aneurismatic segments in macrophages (CD68, in green; Figure 4, b1) and to a lesser extent, in VSMCs (green; Figure 4, b3). Furthermore, our results demonstrate p110$\delta$ (red) specifically colocalized with vascular macrophages (CD68, in green; Figure 4, b2). Therefore, these results suggested that PI3K$\delta$ inactivation may induce macrophages infiltration and activate MMP-12 expression in macrophages.

**Inactivation of PI(3)K p110$\delta$ Promotes Macrophages Migration, MMP-12 Expression, and AP-1 Phosphorylation**

Because macrophages are important innate inflammatory cells in AAA development and TNF-\(\alpha\) is thought to be involved in the pathogenesis of AAA,\textsuperscript{12} we next wondered whether p110$\delta$ inactivation enhanced macrophage recruitment. We cultured macrophages by use of a transwell system. p110$\delta$ inactivation significantly enhances macrophage recruitment compared with WT macrophages (Figure 5, a1 and a2; \(P<0.05\), \(**P<0.01\), \(***P<0.001\) versus control-WT macrophages). TNF-\(\alpha\) induce more p110$\delta$$^{D910A/D910A}$ macrophages recruitment than did the WT macrophages (Figure 5, a3 and a4; \(P<0.05\), \(##P<0.01\) versus control-p110$\delta$$^{D910A/D910A}$ macrophages). Besides, IC87114-treatment significantly enhance WT macrophages recruitment induced by TNF-\(\alpha\) compared with dimethyl sulfoxide-treatment WT macrophages but IC87114-treatment has no effect on p110$\delta$$^{D910A/D910A}$ macrophages recruitment induced by TNF-\(\alpha\) (Figure 5, a5 and a6; \(\Delta P<0.05\).
versus TNF-α–treated WT macrophages), which agrees with the in vivo observation that p110δ inhibition or p110δ inactivation induced inflammatory cell infiltration in the adventitia and disruption of elastic lamella in CaCl2-induced mice. In addition, we then examined the MMP-12 expression in both types of peritoneal macrophages. Our results showed that in p110δD910A/D910A macrophages, Akt phosphorylation was significantly suppressed compared with WT macrophages (n=3; P < 0.01 versus WT macrophages; Figure 5B). TNF-α treatment effectively inhibited Akt activation in both types of mice. Meanwhile, TNF-α induced remarkable increased MMP-12 expression in p110δD910A/D910A macrophages compared with WT macrophages (n=3; P < 0.01 versus WT macrophages).

Considering that TNF-α, which is known to increase the transcriptional level of MMP-12 and to enhance the mRNA stability of MMP-12, controls MMP-12 expression via AP-1 (c-Jun/c-Fos) activation,30 we further examined the effect of p110δ inactivation on c-Jun expression and activation. Notably, p110δ inactivation significantly induced c-Jun expression and phosphorylation compared with WT macrophages, and this was greatly enhanced by TNF-α treatment. Therefore, our results demonstrated that p110δ inactivation altered macrophages recruitment, upregulated MMP-12 expression, and enhanced the effect of TNF-α on macrophages.

**p110δ Inhibition Upregulated MMP-12 Expression by Activating AP-1 in Macrophages**

To further investigate the molecular mechanisms of whether p110δ inactivation regulate MMP-12 expression mediated by AP-1 activation, we use mouse macrophage (RAW 264.7) cells, which have features similar to macrophages and express elastolytic proteases such as MMP-1231 in vitro experiment. We also used IC87114 (10 μmol/L)32 to selectively inhibit PI3Kδ catalytic activity to address this question. Our data showed that IC87114 (10 μmol/L) effectively inactivated Akt in macrophages after treatment for 1 hour (n=6; P < 0.001 versus control; Figure 6A). We next detected the effect of IC87114 on AP-1 DNA-binding activity. The electrophoretic mobility shift assay assay demonstrated that DNA-binding activity of AP-1 was significantly increased after the treatment with TNF-α (10 ng/mL; P < 0.001) and TNF-α (20 ng/mL; P < 0.001). IC87114 alone markedly induced AP-1 DNA-binding activity after treatment for 1 hour. Furthermore, our results demonstrated that there was stronger AP-1 DNA-binding activity after costimulation of IC87114 (10 μmol/L) and TNF-α (0–20 ng/mL) than only treatment with TNF-α (0–20 ng/mL; n=5; P < 0.01; Figure 6B). Meanwhile, phosphorylated-c-Jun (p-c-Jun) antibody caused a characteristic supershift of the retarded complex. These data suggested that
inhibition of p110δ did indeed promote AP-1 DNA-binding activity and the phosphorylation of c-Jun, a component of the AP-1 protein complex. To further elucidate the link between p110δ and MMP-12, we detected the effect of IC87114 on both p-c-Jun expression and MMP-12 mRNA expression stimulation with or without TNF-α. In consistent with results from peritoneal macrophages, after treatment with TNF-α (10 ng/mL) or IC87114 (10 μmol/L) for 24 hours, MMP-12 mRNA expression was significantly enhanced compared with the control group (Figure 6C; n=6; P<0.05 for TNF-α treatment; P<0.001 for IC87114 treatment). Then, western blotting demonstrated that the expression of p-c-Jun was higher after cotreatment with IC87114 (10 μmol/L) and TNF-α (10 ng/mL) than stimulation with just one of them (n=6; P<0.001). Reverse transcription polymerase chain reaction analysis showed that MMP-12 mRNA was also significantly enhanced by cotreatment with IC87114 (10 μmol/L) and TNF-α (10 ng/mL; n=6; P<0.05; Figure 6C). IC87114 (10 μmol/L) alone did not further increase p-c-Jun expression (n=6; P>0.05 compared with the control group).

Figure 5. p110δ inactivation increased macrophages migration and MMP-12 expression and activator protein (AP)-1 phosphorylation. A, Representative images and cumulative data (right) showing migratory responses to tumor necrosis factor (TNF)-α–conditioned media in peritoneal macrophages pretreated with/without IC87114 for 1 h. *P<0.05, **P<0.01, ***P<0.001 vs control-wild-type (WT) macrophages; ΔΔP<0.01 vs TNF-α–treated WT macrophages. Data are mean±SEM. B, Effect of TNF-α on MMP-12 expression and AP-1 phosphorylation in WT and p110δD910A/D910A peritoneal macrophages. *P<0.05, **P<0.01, ***P<0.001 vs WT macrophages; #P<0.01 vs p110δD910A/D910A macrophages; ΔΔP<0.01 vs TNF-α–treated WT macrophages. Data are mean±SEM from ≥3 individual experiments. DMSO indicates dimethyl sulfoxide.

Figure 4. p110δ inactivation upregulated MMP-12 activity and expression in abdominal aortic aneurysm (AAA) mice. A, Representative casein zymogram is shown for MMP-12 activity in serum. Equivalent amount of samples were loaded on the basis of protein content (from 3 individual experiments). IC, IC87114, specific p110δ inhibitor. B, Images of double immunofluorescence staining are shown for macrophages (b1, CD68 in green) with MMP-12 (red), with p110δ (b2, in red), and α-smooth muscle actin (SMA; b3, green) with MMP-12 (red). Yellow in merged images indicates overlapping localization of the red and green signals. Data are mean±SEM. *P<0.05, **P<0.01 vs wild-type (WT)-sham mice; #P<0.05 vs WT-AAA mice from ≥3 individual experiments.
μmol/L) also effectively inhibited p110δ catalytic activities (Akt phosphorylation) in macrophages with or without TNF-α treatment for 24 hours (n=6; P<0.001). To determine whether p110δ inactivation regulated MMP-12 mRNA expression via activation of AP-1 in macrophages, we first screened siRNA sequences to effectively silence c-Jun expression (Figure IV in the online-only Data Supplement). Both siR1 and siR2 significantly reduced c-Jun expression 24 hours (P<0.01) and 48 hours (n=6; P<0.001) after transfection compared with the control. Consequently, MMP-12 mRNA expression was significantly decreased after silencing c-Jun (Figure 6D, Lane 3; n=5; P<0.01 versus control). In addition, knockdown of c-Jun significantly suppressed the effect of TNF-α or IC87114 on MMP-12 mRNA expression (Figure 6D, Lanes 5 and 6) compared with the TNF-α treatment groups (Lane 4; n=5; P<0.01). However, after c-Jun knockdown, both TNF-α and IC87114 cotreatment also increased p-c-Jun expression and MMP-12 mRNA expression (Figure 6D, Lane 7) compared with only TNF-α (Lane 5) or IC87114 (Lane 6) treatment (n=6; P<0.05). These results demonstrated that p110δ inactivation indeed upregulated MMP-12 mRNA expression by strengthening AP-1 DNA-binding activity and activation of c-Jun in macrophages. Immunofluorescence analysis also showed that macrophages specifically colocalized with p-c-Jun in AAA arteries (Figure 6E).

Discussion

In this study, we demonstrated that in vivo p110δ inactivation induced significantly aneurismal pathology and macrophages recruitment in 2 distinct models and exacerbated CaCl2-induced AAA development via dysregulation of MMP-12 expression. Besides, MMP-12 mainly colocalized with CD68-positive macrophages in aneurismal segment. In vitro experiment, we demonstrated that p110δ inactivation facilitates macrophages migration and pharmacological specific inhibitor of p110δ also has similar effect on WT macrophages. Moreover, p110δ inactivation or inhibitor marked induced MMP-12 upregulation via phosphorylation of AP-1. Besides, p110δ inactivation enhances the effect TNF-α/TNFR on MMP-12 expression mediated by activation of AP-1 protein (Figure 7). Like atherosclerosis, aneurysms are a progressive inflammatory vascular condition. Depletion of macrophages or...
preventing them from expressing ECM degrading enzymes such as MMPs protected mice from developing aneurysm.30,34 The most prominent feature is irreversible degeneration of the elastic media induced by inflammatory response.35 Carotid ligation model can induce vascular inflammation and neointima lesion formation.36 Here, we use this model to investigate the effect of leukocytes-specific p110δ on vascular remodeling. Interestingly, p110δD910A/D910A mice showed 2 distinct pathologies including significant neointima formation, luminal stenosis and unexpectedly typical arterial aneurysm formation beneath the carotid ligation point. Notably, both the pathologies display elastic fiber fragments. Serial sections with immunohistochemical staining discovered massive CD68-positive macrophages recruitment under adventitia and hematoxylin-eosin staining showed remarkable dilation in aneurysmal section suggesting that p110δ inactivation may promote macrophage infiltration induced by carotid ligation and consequently causes characteristic aneurysm formation. However, the incidence of aneurysm in p110δD910A/D910A mice was only 27%, which suggested that multiple PI3K/Akt activity in vessel cells or other PI3K classes probably involve in protection against inflammatory injury. By contrast, WT mice only showed similar neointima formation and stenosis but without elastic fiber fragments and aneurysmal pathology. Furthermore, in CaCl2-induced AAA model, p110δD910A/D910A mice present markedly reduced VSMCs in media, inflammatory cells infiltration, massive calcium deposition under adventitia, and loosened adventitia compared with WT mice, suggesting that p110δ inactivation actually exacerbates vascular inflammatory response and result in abnormal pathology. Similarly, treatment with IC87114 for 4 weeks leads to marked inflammatory cells recruitments, destruction in media, and focal calcium deposition in CaCl2-induced WT AAA mice. Besides, in sham-operated groups, p110δD910A/D910A mice also showed abnormal ultrastructure in carotid arteries including wavy elastic fiber and VSMC derangement compared with WT mice. Subsequently, reverse transcription polymerase chain reaction microarray assay from abdominal aorta tissues showed p110δ inactivation significantly induced MMP-12 mRNA expression in comparison with WT sham-operated mice. Western blot analysis further demonstrated marked reduced Akt phosphorylation and enhanced MMP-12 expression in abdominal aorta and carotid artery in p110δD910A/D910A mice. Casein zymography further demonstrated that p110δ inactivation results in elevated MMP-12 activity in serum, and they are significantly higher in p110δD910A/D910A mice than that of in WT mice after CaCl2-challenged. Moreover, MMP-12 expression in each group was higher than MMP-9 or MMP-2 expression in AAA foci. Therefore, the effect of p110δ inactivation on inflammatory cells recruitment and MMP-12 dysregulation in arteries probably contribute to abnormal carotid artery architecture and aortic aneurysm development.

Actually, inflammatory process involving MMP activities is essential for the vascular remodeling, entailing reorganization of the ECM scaffold of the vascular wall, and particularly mediating atherosclerotic plaque progression37 and AAA.38 Meanwhile, MMP-12 deficiency attenuated CaCl2-induced AAA probably via decreasing macrophage recruitment.28 Furthermore, it is known that macrophages are an important source of MMP-12, which is required for macrophage-mediated proteolysis and matrix invasion in vitro.39 In line with these studies, our results showed that significantly increased MMP-12 expression was mainly localized in CD68-positive macrophages of aneurysmal segments in CaCl2-induced WT mice, indicating that p110δ inactivation mediated vascular pathology probably via regulating MMP-12 expression in macrophages.

As mentioned, p110δ and p110δ isoforms of PI3-kinase are highly expressed in leukocytes such as macrophages, B and T cells. PI3Ks have emerged as important regulators of TLR signaling.40 The ability of TLRs to facilitate the activation of and induction of immunomodulatory cytokines from antigen-presenting cells, such as dendritic cells or macrophages is critical in shaping the adaptive immuneresponse.41 However, depending on the circumstances, PI3K can function either as a positive or negative regulator of TLR signaling.42 Indeed, PI3K activation enhances the immune response in mast cells and potential other granulocytes, but clearly dampens the inflammatory response in monocytes, macrophages, and dendritic cells.33 TLRs and the receptor for tumor necrosis factor (TNF-R) play an important role in innate immunity by regulating the activity of distinct transcription factors, such as nuclear factor-kB and AP-1 (cFos/cJun).43 TNF-α can bind to its receptor (TNF-R) and elicit diverse biological responses involved in AAA including activation and recruitment of immune cells to the sites of inflammation, the secretion of proinflammatory cytokines, and MMPs.44 In AAA tissue, TNF-α expression colocalizes mainly with CD68-positive macrophages in the medium and adventitia.45 Besides, MMP-12 expression (mRNA and protein) is controlled by AP-1 activation after TNF-α treatment in monocytes.31 Therefore, in vitro experiment, we used TNF-α as a stimulus to define the mechanism of p110δ inactivation on macrophage migration and MMP-12 expression. Besides, p110δ-specific inhibitor IC87114 was validated investigating neutrophil priming or B-cell responses in drug-treated cells versus cells isolated from mice harboring kinase-dead p110δ.24,46 Our results showed that genetic inactivation of p110δ or pharmacological inhibitor IC87114 has...
similar effect on significantly increasing macrophages migration. Other studies\(^5\) has showed that PI3K signaling is significantly diminished in PI3K p110\(^{\delta}\) bone marrow monocytes (BMMs) as demonstrated by decreased phosphorylation of the PI3K downstream target Akt in LPS-activated PI3K p110\(^{\delta}\) BMMs compared with WT BMMs. In addition, LPS-activated p110\(^{\delta}\) BMMs displayed that earlier activation and enhanced phosphorylation p38 mitogen-activated protein kinase but there were no significant differences in extracellular-signal–regulated kinases activation and nuclear factor-κB p65 phosphorylation in comparison with WT BMMs. Our results showed that TNF-α treatment induced marked enhanced MMP-12 expression and c-Jun phosphorylation in p110\(^{\delta}\) macrophages compared with WT macrophages, whereas Akt phosphorylation was significantly reduced in both types of macrophages, which indicate Akt inactivation probably involving in A AA development and negatively regulating AP-1 activation. Moreover, IC87114 significantly increased AP-1 DNA-binding activity in RAW cells and synergistically increased AP-1–binding activity with TNF-α. Subsequently, the effect of IC87114 on MMP-12 upregulation after TNF-α treatment was significantly suppressed by c-Jun knockdown. Meanwhile, we also demonstrated that p-c-Jun was specifically colocalized within nucleus of macrophage in aneurismal sections. Therefore, we demonstrate that p110\(^{\delta}\) inactivation increased MMP-12 expression at mRNA level and protein level in macrophages by inducing AP-1 activation, which probably results from c-Jun kinase activation. Likewise, studies have demonstrated that AKT2 deficiency\(^4\) and c-Jun N-terminal kinase activation\(^8\) aggregated AAA development, whereas c-Jun is the major target of c-Jun N-terminal kinase pathway. Besides, nuclear factor-κB also has been reported to regulate MMPs and promote the development of experimental AAA\(^8\) via AP-1 activation. p110\(^{\delta}\) inactivation may mediate these pathways during vascular injury.

Taken together, a genetic defect in specific intracellular signaling molecule contributes to global defects in adaptive homeostatic pathways in the intestine\(^25\) and in vessels. Pathogenesis of AAA was closely associated with innate immune disorder. However, PI3Ks have complicated cross-talk with TLRs and TNF-R pathways ultimately mediated inflammatory transcription factors, such as nuclear factor-κB and AP-1 activation, which may result in inflammatory cytokines release and MMPs expression. Elucidation of the details of PI3K-related molecular events may identify further potential therapeutic targets for inflammatory vascular diseases with destructive ECM metabolism.

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Disclosures
None.

References
Aneurysms are chronic vascular inflammation. Monocytes/macrophages infiltration and inflammatory cytokines release play key role in the development of aneurysms. We found that inactivation of phosphatidylinositol 3'-kinase (PI3Kδ) dysregulates the expression of extracellular matrix components and matrix metalloproteinases (MMPs) in arteries. Furthermore, PI3Kδ inactivation unexpectedly induced aneurysm formation and exacerbated aneurysm development in vivo closely related to macrophages infiltration and MMP-12 upregulation. In vitro, PI3Kδ inactivation promoted macrophages recruitment and upregulated MMP-12 expression by activating transcriptional factor activator protein-1. Our study provides pathological, molecular, pharmacological data supporting the novel role of PI3Kδ catalytic activity in preserving vascular architecture and protecting against aneurysm development by regulating macrophages recruitment and MMP-12 expression in macrophages. **Significance**
Inactivation of PI3Kδ Induces Vascular Injury and Promotes Aneurysm Development by Upregulating the AP-1/MMP-12 Pathway in Macrophages

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Inactivation of PI3Kδ Induces vascular injury and Promotes Aneurysm Development by Upregulating the AP-1/MMP-12 Pathway in macrophages


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Materials and Methods

This section is provided as an online supplement.

Animals

PI3K p110\textsuperscript{δD910A/D910A} mice and the corresponding wild-type (WT) C57BL/6J mice were kind gifts from the Institute of Biochemistry and Cell Biology (SIBCB, CAS, China). Mice were housed in specific pathogen free conditions (SPF) in accordance with the guidelines from the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal research was approved by the Guangdong Pharmaceutical University Animal Care and Use Committee.

Animal Model of Arterial Injury

Carotid injury was created as others reported previously\textsuperscript{1,2}. Briefly, 10-12-week-old male WT and p110\textsuperscript{δD910A/D910A} mice (25-27 g) were anesthetized using intraperitoneal injection of pentobarbital (60 mg/kg). Through a midline incision in the neck, 1 cm of the left common carotid artery was prepared. The left common carotid artery was looped proximally to the carotid bifurcation and ligated completely by a 7-0 propylene suture to disrupt blood flow. The mice were allowed to recover and were maintained on their assigned diet for 4 weeks after the surgery. At day 28 after the initial surgery, the mice were euthanized under pentobarbital anesthesia and perfusion fixed.

Animal Model of AAA and Treatment

Eight to 10-week-old male PI3K p110\textsuperscript{δD910A/D910A} (n=35) and their age-matched, male transgene-negative littermates (WT, n = 40) underwent surgical AAA (abdominal aortic aneurysm, AAA) induction. AAAs were induced by periaortic application of 0.5 M CaCl\textsubscript{2} as described previously\textsuperscript{3} with minor modifications. NaCl (0.9%) was substituted for CaCl\textsubscript{2}
in sham-operated mice (n = 25 for P110δD910A/D910A, n = 30 for WT). All tissues were collected at 6 weeks after surgery. After surgery 2 weeks, CaCl_{2}-induced WT mice were randomized into two groups (n = 5 per group) and injected i.p. daily with the selective PI3Kδ inhibitor IC87114 (50mg/kg/day) dissolved in PEG400 (Hampton Research, U.S.A) or the equivalent volume of PEG400 (vehicle control) for 4wks. This dose was chosen based on previous reports of its efficacy in vivo\textsuperscript{4,5}.

**Ultrasonography Measurement of Abdominal Aortic Diameter**  
At 42-day after CaCl_{2} treatment, survivor mice underwent ultrasonography with a Veov 2000 TM Imaging System (Visual sonics Ini, Toronto, Canada) in B-mode. The maximal suprarenal aortic diameter was measured at peak systole by the caliper measurement feature\textsuperscript{6}.

**Cell Culture and Drug Treatment**  
The murine macrophage cell line RAW264.7, obtained from the American Type Culture Collection (Manassas, VA, U. S. A), and peritoneal macrophages from both types of mice were maintained in Dulbecco’s modified Eagle's medium (DMEM, Sigma Chemical Co.) with 10% fetal calf serum (FCS, Cell Culture Technologies, Herndon, VA, U. S. A). Cultures were maintained at 37 °C in a humidified incubator in a 95% O_{2} plus 5% CO_{2} atmosphere. We treated cells with varied concentrations of TNF-α (sigma-aldrich, St. Louis, MO) and used IC87114 (ATP-competitive kinase inhibitor, Selleck, U.S.A) to inhibit PtdIns(3,4,5)P3-dependent phosphorylation of Akt before TNF-α stimulation at early time points (30 min).

**Peritoneal Macrophage Migration Assays**
The migration of macrophages toward TNF-α was assessed by Boyden transwell chamber assay with polycarbonate membrane inserts with 8-μM pores (Millipore). All experiments were performed in triplicate. Briefly, isolated peritoneal macrophages (10^5 cell) from mice were then added into the upper chamber of Transwells (Corning, U.S.A), which were treated with/without IC87114. The lower chambers were filled with DMEM contained DMSO/TNF-α. Cells on the inner side of the inserts were wiped by gentle abrasion with use of a cotton bud 16 hours later, and cells on the underside of the inserts were fixed and stained. The mean number of cells on the lower surface was counted from 4 randomly chosen low-power fields (10×) under fluorescence microscopy in 3 independent experiments.

**Nuclear Extracts and Gel Shift Assay**

RAW264.7 cells (1 × 10^8) in a 60-mm plate were incubated with TNF-α (0-20 ng/ml) for 1 h or pretreated with IC87114 (10 μM) for 30 min and then TNF-α (0-20 ng/ml) was added for 1 h. Nuclear extracts were prepared from the cells as described previously. Nuclear extracts (2 μg) were mixed with two double-stranded oligonucleotide probes containing a consensus binding sequence for AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3’) that were 3’ end-labeled with biotin (Beyotime, China). For competition assays, a 100-fold molar excess of unlabeled or mutant double stranded oligonucleotides was incubated with nuclear extracts for 10 min at room temperature before the addition of probe. For antibody supershift experiments, DNA probe/protein complexes were incubated with 1 μg of anti-p-c-JUN antibody (Cell Signaling Technology, U.S.A) for 20 min at room temperature before the addition of probe. Protein-DNA complexes were subsequently resolved in a 4% acrylamide gel in 0.5× TBE as the running buffer. The binding activity of
AP-1 to the probe was determined using a chemiluminescent EMSA kit (Pierce, U.S.A), according to the manufacturer’s protocol. Each band was scanned by densitometry analysis using ImageQuant software (Molecular Dynamics).

**siRNA Transfection**

The siRNA duplexes against the mouse c-JUN gene were synthesized with the following sequences as described previously. The sequence of JUN siRNA1 was 5′-r(CAGCUUCCUGCCUUUGUAA)dTT-3′. The scrambled counterpart, JUN siRNAscr1, had the sequence 5′-r(GAUUACUAGCCGU-CUCCU)dTT-3′. The sequence of JUN siRNA2 was 5′-r(GCGCAUGGGAACCGCAUU)dTT-3′, and its scrambled counterpart, JUN siRNA2scr2, was 5′-r(GCCGAACUAUAGGCCGUAG)dTT-3′. JUN scrambled RNA was used as a negative control. siRNA strands and Lipofectamine 2000 Transfection Reagent were diluted in serum and antibiotics-free OptiMEM (GIBCO). The final concentration of JUN siRNA was 100 nmol/L. The mixture was kept at room temperature for 10 min to form the transfection complexes. The complexes were then added to RAW cells and were swirled gently to ensure uniform distribution. After incubation for 6 h at 37°C, transfection complexes were replaced with normal DMEM containing 10% Fetal calf serum (Gibco, U.S.A.). After 24 and 48 hours, western blotting was performed to examine the effect of JUN siRNAscr and JUN siRNA on Jun protein expression. The intensities of bands of Jun and α-tubulin were quantified by digital densitometry using Quantity One software (Bio-Rad). Jun band intensities normalized to α-tubulin from JUN siRNA- and siRNAscr-transfected groups were compared on the same membranes.
RNA, Reverse Transcription, Quantitative Polymerase Chain Reaction, and the Polymerase Chain Reaction Array

Total RNA of aortic tissues from both P110δ^{D910A/D910A} and WT control mice were isolated using RNeasy Midi Kit (Qiagen). cDNA were prepared using the Qiagen First Strand Kit. The SYBR Green polymerase chain reaction master mix and the mouse polymerase chain reaction Array (PAMM-013z, SABiosciences) were used according to the manufacturers’ instructions. Bioinformatics analysis was performed using the Web-based RT_{2} Profiler PCR Array Data Analyzer (SABiosciences). Data are expressed as ΔΔC_{t}, and changes of ≥ 2-fold were considered significant.

RT-PCR Analysis

Total RNA was extracted from cells using Trizol reagent according to the manufacturer’s instructions (Invitrogen). One microgram of total RNA was reverse transcribed using the PrimeScript™ 1st Strand cDNA synthesis kit (Takara) according to the manufacturer’s instructions. The MMP-12 primers were synthesized by Invitrogen. MMP-12 primers: forward sequence, 5’-TGAAGCGTGAGGATGTAGACT-3’; reverse sequence, 5’TCAAGGATGGGGTTCACT-3’. GAPDH: forward sequence, 5’-GGTGAAGGTCGGTGAGC-3’; reverse sequence, 5’-CTCGCTCCTGGAAGATGTTG-3’. The expression values were normalized to unaltered control Gadph (encoding GADPH).

Tissue Preparation and Electron Microscopy Analysis

For electron microscopy analysis, after mice were deeply anesthetized with 80 mg/kg pentobarbital, mice were perfused transcardially with 0.1 M phosphate buffer, pH 7.4, with the pressure controlled at approximately 100 mmHg, followed by 4°C fixative solution
containing 4% freshly depolymerized paraformaldehyde, 0.25% glutaraldehyde and 15% saturated trinitrophenol in 0.1 M phosphate buffer at pH 7.4. The carotid artery was removed, the tissue blocks containing the carotid artery at midpoint were cut into cubes of 1 mm × 1 mm × 3 mm. Ultrathin sections with a thickness of 80-100 nm were prepared and stained with uranyl acetate and lead citrate and were then viewed under a transmission electron microscope (FEI TECNAI spirit G2, U.S.A).

**Histology and Immunohistochemistry Analysis**

The carotid artery and the abdominal artery were fixed in 4% phosphate-buffered formalin and later embedded in paraffin. For histological analysis, sections were stained with hematoxylin-eosin (H&E) or elastica van Gieson (E.V.G.) For immunohistochemistry, sections were incubated with anti-Collagen I antibody (1:100, Abcam), anti-Collagen IV antibody (1:100, Abcam), anti-MMP-12 (1:100, Abcam), anti-MMP-9 (1:50, Abcam) and anti-MMP-2 (1:100, Abcam), anti-CD68 (1:50, serotec, UK) at 4°C overnight, followed by incubation with a secondary goat anti-rabbit antibody. In negative controls, normal goat serum in PBS was substituted for the primary antibody. Positive immunostaining was determined with the Boster SABC kit and visualized with diaminobenzidine substrate (Boster, China), followed by counterstaining with hematoxylin (Sigma, U.S.A).

**Immunofluorescence Analysis**

Immunofluorescence was performed to detect the location and expression of MMP-12, CD68 and α-SMA. Dewaxed sections were boiled in 10 mM citrate (pH 6.0) in a pressure cooker for antigen retrieval. Then, sections were incubated with primary anti-mouse MMP-12 polyclonal antibody (1:100; Abcam, Cambridge, MA, U.S.A), CD68 polyclonal
antibody (1:50, serotec, U.K.), and α-SMA monoclonal antibody (1:100, Life technologies) at 4°C overnight, then with Alexa Fluor 594 goat anti-rabbit IgG (1:100, Life technologies) for MMP-12, Alexa Fluor 488 goat anti-mouse IgG (1:100, Life technologies) for α-SMA, and Alexa Fluor 488 goat anti-rat IgG (1:50) for CD68 at room temperature for 1 h. Sections incubated with anti-mouse IgG instead of the primary antibodies were used as negative controls. Double immunofluorescent staining to identify cells expressing MMP-12 was also performed as described in the supplementary materials. All sections were analyzed using a confocal system (Leica, TCSSP5, magnification ×630) and Adobe Photoshop CS5.

**Western Blot Analysis**

Western blotting was performed as described previously. Aortic arteries were homogenized, and protein contents were measured using the BCA protein assay (Pierce). After blocking for 1 h at room temperature in 5% milk solution, the membranes were probed overnight with one of the following primary antibodies: anti-MMP-12 (Abcam, Cambridge, MA), anti-p110δ (Santa Cruz), anti-Akt, anti-phospho-Akt, anti-phospho-c-Jun, and anti-c-Jun (all these antibodies were obtained from Cell Signaling Technology). After secondary antibodies were applied, final detection was carried out with the LumiGLO chemiluminescent reagent (New England Biolabs) according to the manufacturer's instructions. β-actin or α-tubulin was used as the loading control.

**Statistical Analysis**

Quantitative data are expressed as the mean ± s.e.m. For Western blotting, RT-PCR and immunohistology, n represents the number of independent experiments on different batches of cells or different mice. For the PCR array, n represents the number of different
mice in each group. Significance was determined by one-way ANOVA followed by the Bonferroni multiple comparison post hoc test with a 95% confidence interval or by an unpaired 2-tailed Student’s t test. For survival curves, log-rank tests were performed. For all tests were used GraphPad Prism (version 5.00) software. P values of less than 0.05 and 0.01 were considered significant and very significant, respectively.
References


SUPPLEMENTAL MATERIALS

Inactivation of PI3Kδ Induces vascular injury and Promotes Aneurysm Development by Upregulating the AP-1/MMP-12 Pathway in macrophages


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Supplemental Figures

Figure I  Effect of PI3Kδ inactivation on Macrophages recruitment in the subadventitia of carotid at 4 weeks after ligation. Carotid injury induced characteristic aneurysm formation in 27% of $p110^{\delta D910A/D910A}$ mice. Massive CD68-positive macrophages infiltration under the adventitia of carotid compared with WT mice (n=5).

Figure II  Transmission electron microscopy images of a carotid artery from $p110^{\delta D910A/D910A}$ mice. Carotid arteries were harvested and prepared after perfusion fixation at the physiological perfusion pressure. PI3Kδ
inactivation caused elastic lamella thinning, smooth muscle cell necrosis, disarrangement and amorphous matrix deposition in the intercellular space (n=5).

**Figure III** Effect of PI3Kδ inactivation on MMPs expression in CaCl₂-induced AAA foci. Abnormal arteries were harvested and prepared after perfusion fixation at the physiological perfusion pressure. The expression of MMPs was examined by IHC staining. MMP12 expression significantly increased compared with MMP9 or MMP2 in each group by two-way ANOVA analysis (*p<0.05, ***p<0.001). Data represent means±s.m.e. with at least five mice in each group.
Figure IV. Effect of JUN siRNAs on Jun protein expression in murine RAW macrophages. The cells were transfected with 0.2 μM of JUN siRNA-1, JUN siRNA-2 or siRNAscr and incubated in serum-free medium for 24 and 48 hours before determination of JUN protein expression. Data are representative of at least three independent experiments performed in triplicate and are the mean±s.e.m. ** p<0.01, ### p<0.001 compared with the no siRNA control by Student's t-test.