

RESEARCH PAPER

Key role of PI3K γ in monocyte chemotactic protein-1-mediated amplification of PDGF-induced aortic smooth muscle cell migration

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BACKGROUND AND PURPOSE

Vascular smooth muscle cell (SMC) migration within the arterial wall is a crucial event in atherogenesis and restenosis. Monocyte chemotactic protein-1/CC-chemokine receptor 2 (MCP-1/CCR2) signalling is involved in SMC migration processes but the molecular mechanisms have not been well characterized. We investigated the role of PI3K γ in SMC migration induced by MCP-1.

EXPERIMENTAL APPROACHES

A pharmacological PI3K γ inhibitor, adenovirus encoding inactive forms of PI3K γ and genetic deletion of PI3K γ were used to investigate PI3K γ functions in the MCP-1 and platelet-derived growth factor (PDGF) signalling pathway and migration process in primary aortic SMC.

KEY RESULTS

The γ isoform of PI3K was shown to be the major signalling molecule mediating PKB phosphorylation in MCP-1-stimulated SMC. Using a PI3K γ inhibitor and an adenovirus encoding a dominant negative form of PI3K γ , we demonstrated that PI3K γ is essential for SMC migration triggered by MCP-1. PDGF receptor stimulation induced MCP-1 mRNA and protein accumulation in SMCs. Blockade of the MCP-1/CCR2 pathway or pharmacological inhibition of PI3K γ reduced PDGF-stimulated aortic SMC migration by 50%. Thus PDGF promotes an autocrine loop involving MCP-1/CCR2 signalling which is required for PDGF-mediated SMC migration. Furthermore, SMCs isolated from PI3K γ -deficient mice (PI3K γ ^{-/-}), or mice expressing an inactive PI3K γ (PI3K γ ^{KD/KD}), migrated less than control cells in response to MCP-1 and PDGF.

CONCLUSIONS AND IMPLICATIONS

PI3K γ is essential for MCP-1-stimulated aortic SMC migration and amplifies cell migration induced by PDGF by an autocrine/paracrine loop involving MCP-1 secretion and CCR2 activation. PI3K γ is a promising target for the treatment of aortic fibroproliferative pathologies.

Abbreviations

AG-1478, N- (3- chlorophenyl)- 6, 7- dimethoxy- 4- quinazolinamine; AG-1296, 6, 7- dimethoxy- 2- phenyl- quinoxaline; AS-252424, 5- [5- (4- fluoro- 2- hydroxy- phenyl)- furan- 2- ylmethylene]- thiazolidine- 2, 4- dione; CCR2, CC-chemokine receptor 2; EGF, epidermal growth factor; MCP-1, monocyte chemotactic protein-1; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; RS102895, 1'-[2-[4-(trifluoromethyl)phenyl]ethyl]-spiro[4H-3,1-benzoxazine-4,4'-piperidin]-2(1H)-one hydrochloride; SMC, smooth muscle cell

Introduction

Vascular smooth muscle cells (SMCs) play important functions in normal and diseased blood vessels. In normal adult arteries, SMC displays a contractile phenotype to maintain vascular tone. In response to vascular injury, however, they switch to a synthetic phenotype characterized by increased proliferation and migration, two major steps in atherosclerosis progression and neointimal thickening after angioplasty (Zargham, 2008; Orr *et al.*, 2009). Among cytokines and growth factors released by injured vessels and inflammatory cells, the monocyte chemotactic protein-1 (MCP-1) plays a critical role in pathological vascular remodelling. MCP-1 amounts are particularly high in experimental mice during atherogenesis and after wire injury of the carotid artery. Monocyte recruitment is reduced when MCP-1 signalling is blocked as in MCP-1 knockout mice (Boring *et al.*, 1998; Gu *et al.*, 1998). The same applies to chemokine C-C motifs receptor 2 (CCR2) knockout mice, a GPCR, selectively activated by MCP-1. Importantly, the impaired MCP-1/CCR2 signal transduction axis reduces atherosclerotic lesions and prevents restenosis. These findings have prompted the design of anti-MCP-1/CCR2 therapeutic approaches, using for instance antibodies against MCP-1, CCR2 or an N-terminal truncated form of MCP-1. These treatments successfully prevented intimal hyperplasia in mice, rabbits and primates, suggesting that these therapies might have some potential to prevent arterial restenosis in humans (Egashira *et al.*, 2002; Horvath *et al.*, 2002; Mori *et al.*, 2002; Zhong *et al.*, 2008). The biological effects mediated by MCP-1 and its receptor, CCR2, are not restricted to the regulation of inflammatory processes but can also modify SMC functions. CCR2 mRNA has been detected in human vascular SMC (Hayes *et al.*, 1998). In addition, CCR2 itself is involved in the functional switch of SMC to the synthetic phenotype (Denger *et al.*, 1999) and induces migration of aortic SMC in rabbits and rats (Ma *et al.*, 2007; Grassia *et al.*, 2009). Moreover, in rats, aortic MCP-1 and CCR2 increase with age, as does the invasive ability of aortic SMC, suggesting that MCP-1/CCR2 signalling might play a role in age-associated vascular remodelling (Spinetti *et al.*, 2004). While MCP-1/CCR2 signalling pathways leading to cell migration have been studied intensively in monocytes, molecular mechanisms involved in MCP-1-induced SMC migration are poorly understood.

PI3Ks, a family of lipid kinases, are important regulators of cell survival and growth, as well as of migration through the direct binding of proteins to their lipid product. Based on their structural characteristic and substrate specificity, PI3Ks are divided into the three classes, I, II and III (Vanhaesebroeck *et al.*, 2001; Wymann *et al.*, 2003). Class IA and class IB of PI3K comprise a highly homologous 110 kDa catalytic subunit (α , β and δ for class IA; γ for class IB) and an associated regulatory subunit. Both these kinase classes act *in vivo* on phosphoinositol (4,5) bisphosphate (PI4, 5P₂) substrate to produce phosphoinositol (3,4,5) trisphosphate (PIP₃). Whereas class IA PI3Ks are typically recruited downstream of tyrosine kinase receptors, class IB is specifically activated by $\beta\gamma$ subunits of heterotrimeric G proteins conferring on PI3K specific functions downstream of GPCR activation (Wymann *et al.*, 2003; Fougerat *et al.*, 2008). A growing body of evidence suggests that PI3Ks are involved in SMC migration (Gon-

charova *et al.*, 2002; Irani *et al.*, 2002; Radhakrishnan *et al.*, 2008; Zhou *et al.*, 2009). In this study, we identified PI3K γ as the major PI3K involved in PKB (Akt) phosphorylation and migration downstream of CCR2 activation by MCP-1 in aortic SMC. Furthermore, we found that PI3K γ could also amplify platelet-derived growth factor receptor (PDGF)-induced migration by an autocrine/paracrine loop involving MCP-1/CCR2 signalling. This provides a new insight into the molecular mechanisms of, and signalling involved in, the aortic SMC migration process.

Methods

Materials

Cell culture reagents were purchased from Invitrogen (Grand Island, NY, USA). Human recombinant EGF was from Peprotech (Rocky Hill, NJ, USA), human recombinant PDGF-BB and mouse MCP-1 were from R&D Systems (Lille, France), and swine recombinant MCP-1 was from Kingfisher Biotechnologies (Euromedex, Mundolsheim, France). The inhibitors AS-252424, wortmannin, RS-102895, AG1478, AG1296 and DAPI were from Sigma-Aldrich (Saint-Quentin Fallavier, France). The PKB (Akt) inhibitor (PKB inhibitor VIII, Isozyme-Selective, PKBi-1/2) was from Tebu Bio (Le Perray en Yvelines, France). The pig MCP-1-neutralizing antibody was from Bethyl Laboratories, Inc. (Euromedex, Souffelweyersheim, France). Rabbit monoclonal antibody against phosphorylated PKB (serine 473), rabbit monoclonal antibody against phosphorylated PKB (threonine 308), rabbit polyclonal antibody against PKB and HRP-conjugated anti-rabbit antibody were from Cell Signaling Technologies (Danvers, MA, USA); anti-myc clone 9E10 was from BD Biosciences (Le Pont de Claix, France), anti-GFP was from Roche (Meylan, France), cyanin-2 conjugated secondary antibody was from Jackson ImmunoResearch Laboratories (Suffolk, UK) and HRP-conjugated anti-mouse antibody was from Sigma-Aldrich. The enhanced chemiluminescence (ECL) system was from Amersham Biosciences (Munich, Germany).

Animals

PI3K γ -deficient mice (PI3K $\gamma^{-/-}$) and PI3K $\gamma^{KD/KD}$ mice were generated as previously described (Hirsch *et al.*, 2000; Patrucco *et al.*, 2004). Both mutant and control mice were derived from 15 generation backcrosses to the C57Bl/6 genetic background.

All animal care and experimental procedures were in accordance with institutional guidelines on Animal Experimentation and with the French Ministry of Agriculture license. Moreover, they conformed to US NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and our institution's ethical policy is listed as A5326-01 on office of laboratory animal welfare (NIH website).

Adenoviral production and infection

The PI3K γ KR construct was PCR modified from ADNc PI3K γ KR (Bondeva *et al.*, 1998) to insert a myc-tag and inserted into the multiple cloning sites of the pShuttle-CMV. Then, PI3K γ KR-myc adenoviruses were generated by homologous recombination using the pAdEasy system (He *et al.*, 1998).

Infection with adenovirus was achieved by incubating a cell suspension with 150 IP per cell in serum-free medium. After 1 h, the cells were incubated in 10% fetal calf serum (FCS) culture medium for 72 h. Cells were then placed in serum-free medium for 24 h before stimulation by different agonists or seeded in a 96-well plate for migration experiments.

Cell culture

Aortic SMCs were prepared from the thoracic aorta of a 6-week-old pig. First, the pig was sedated using ketamine hydrochloride (25 mg·kg⁻¹ i.m.) and anaesthesia was induced by i.v. injection of sodium pentobarbital (20–30 mg·kg⁻¹). After a thoracotomy, the aorta was dissected and removed. Then, cells were isolated by using an explant technique and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and antibiotics. SMCs at 80% confluence from passages 3 to 6 were used in the experiments. Before stimulation, cells were incubated for 48 h in serum-free medium. Human aortic SMCs were from ATCC (Manassas, VA, USA; CRL-1999).

Mouse aortic SMCs were isolated from 8-week-old PI3K $\gamma^{+/+}$ and PI3K $\gamma^{-/-}$ or PI3K $\gamma^{KD/KD}$ mice. First, mice were killed using sodium pentobarbital in a 1 mg·mL⁻¹ solution at 0.10 mL·10 g⁻¹ body weight dosage injected into the left peritoneal cavity. Then, aortas were dissected out from their origin at the left ventricle to the iliac bifurcation. After being flushed with sterile PBS, the aortas were placed in DMEM supplemented with 10% FCS and 1 μ g·mL⁻¹ Fungizone. The adventitia was then totally removed from the aorta under a dissecting microscope. The aorta was placed in DMEM supplemented with 10% FCS and cut into small pieces of 1–2 mm. These pieces were then digested in DMEM supplemented with 10% FCS containing 1.36 mg·mL⁻¹ collagenase type II (LS004174, Worthington Biochemical Corporation, Serlabo technologies, Entraigues, France) for 4 h in a humidified 5% CO₂ atmosphere at 37°C. Cells were centrifuged and resuspended in DMEM, 10% FCS, in a single well of a 24-well plate. After 5 days, the medium was replaced systematically until confluence was reached. Mouse SMCs from the 5th to 10th passages were used in experiments.

Western blot analysis

Proteins from whole cells were dissolved in Laemmli buffer, boiled, separated by SDS-PAGE and transferred onto PVDF membrane. Immunodetection was achieved using the relevant primary antibody, anti-cmyc (1:10 000), anti-GFP (1:1000), anti-phosphorylated Ser⁴⁷³PKB (1:2000), anti-phosphorylated Thr³⁰⁸PKB (1:1000) or anti-PKB (1:1000) overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody (anti-rabbit 1:5000 and anti-mouse 1:10 000) was incubated for 1 h at room temperature and immunoreactive proteins were detected with ECL reagents according to the manufacturer's instructions.

Cell migration assay

The wound-healing assay was performed using the Oris cell migration kit (Oris™ Cell Migration Assay-Fibronectin Coated, Platypus Technologies, Tebu Bio) according to the

manufacturer's instructions. Formatted for a 96-well plate, this assay uses silicone stoppers, which restrict cell seeding to the outer annular regions of the well. When the stoppers are removed, cells can migrate in a 2 mm-diameter region in the centre of the well. We seeded 3.5 × 10⁴ cells of pig SMC or 5 × 10⁴ cells of mouse SMC per well. After attachment, cells were treated with PDGF-BB (10 ng·mL⁻¹) or MCP-1 (10 ng·mL⁻¹), alone or together with AS-252424 (100 nM), RS-102895 (5 μ g·mL⁻¹), PKB inhibitor (20 μ M) or pig MCP-1-neutralizing antibody (5 μ g·mL⁻¹). Cells were allowed to migrate for 48 h at 37°C then stained with DAPI and counted under a fluorescence microscope. The number of fluorescent nuclei in each well was counted using Image J Software (NIH).

Real-time quantitative PCR

SMCs were treated with PDGF-BB (10 ng·mL⁻¹) for 0.5–4 h. Total RNA was extracted with Trizol reagent (Invitrogen), phase separation was performed with chloroform and RNA precipitation with isopropanol. RNA concentrations were determined using a spectrophotometer. One microgram of total RNA was used for cDNA synthesis with hexamers. The cDNA was amplified by quantitative real-time PCR with SYBR Green (Roche). Primers to amplify MCP-1 RNA were designed using the Primer Express software (Life Technologies SAS, Villebon sur Yvette, France) with the following sequences: 5'-GCTGTGATCTTCAAGACCATTGTG-3' (F) and 5'-GAA TCCTGAACCCACTTCTGCTT-3' (R). All real-time PCR reactions were performed on a LC480 (Roche), with the following thermal cycling parameters: annealing at 60°C for 40 s, amplification at 95°C for 10 s and dissociation at 95°C for 10 s. The PCR programme was followed by a melting curve and values were normalized to the relative amounts of hypoxanthine-guanine phosphoribosyltransferase (HPRT). C_T values of HPRT were constant in all samples.

Quantification of MCP-1 by ELISA

SMCs were stimulated by PDGF-BB (10 ng·mL⁻¹) for 2–48 h. Cell culture supernatants were used to determine the concentrations of MCP-1 by the specific Pig CCL2 ELISA Kit (Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions.

Immunofluorescence

Mouse aortic SMCs were fixed with ice-cold methanol, washed in PBS and incubated with anti-smooth muscle- α -actin primary antibody (1:1000, clone 1A4, Sigma, Saint-Quentin Fallavier, France) for 1 h. After being washed three times with PBS, cells were incubated with a secondary cyanin-2 conjugated anti-mouse antibody and DAPI (1 μ g·mL⁻¹). Pictures were obtained using a fluorescence microscope (TE2000-E, Eclipse, NIKON, Melville, NY, USA).

Statistical analysis

For cell migration experiments, each individual experiment was performed in 8 wells for each condition and repeated at least three times. Experimental data were analysed by Mann-Whitney test. Results are expressed as means $\pm$ SEM. Differences were considered significant at $P < 0.05$.

Results

MCP-1-induced PKB phosphorylation does not require tyrosine kinase receptor transactivation

To investigate the involvement of the PI3K/PKB pathway in MCP-1-induced activation of aortic SMC, we first analysed the phosphorylation of PKB after MCP-1 stimulation in primary pig aortic SMC. Because most signalling pathways activated by GPCR agonists in SMC require tyrosine kinase receptor activation, such as EGF receptor or PDGF receptor (Kalmes *et al.*, 2001; Voisin *et al.*, 2002; Tanimoto *et al.*, 2004), we first analysed the effect of MCP-1 on PKB phosphorylation in the presence of the EGF receptor inhibitor AG1478 and PDGF receptor inhibitor AG1296. We observed that AG1478 and AG1296 did not modify MCP-1-induced PKB phosphorylation on both serine 473 and threonine 308 (Figure 1A). However, AG1478 effectively inhibited the EGF pathway and AG1296 similarly inhibited PDGF (Figure 1B). This indicates that PI3K activation by MCP-1 does not require tyrosine kinase receptor transactivation. The same results were obtained in human aortic SMC stimulated by MCP-1 (Supporting Information Figure S1). In combination, these results suggest that PI3Ks are directly activated downstream CCR2 activation in vascular SMC.

PI3K γ mediates MCP-1-induced PKB phosphorylation

To investigate the potential role of PI3K γ activity in PKB phosphorylation induced by MCP-1, aortic SMCs were treated with a selective inhibitor of PI3K γ (AS-252424) (Condliffe *et al.*, 2005). PKB phosphorylation induced by MCP-1 was reduced by 85% by AS-252424 and eliminated by wortmannin, a pan PI3K inhibitor (Figure 2A). This indicates that PI3K activity is required and that PI3K γ is the major isoform involved in this process although other class I PI3K (PI3K α and β) is also expressed in these cells (Supporting Information Figure S2). To control AS-252424 specificity, we investigated PKB phosphorylation after treating cells with EGF and PDGF, which are known to recruit class IA PI3K. We did not observe any modification of PKB phosphorylation by either treatment (Figure 2B), demonstrating the selectivity of the inhibitor against class IB PI3K. Similarly, infection of aortic SMC with an adenovirus encoding an inactive form of PI3K γ (PI3K γ KR) completely blocked PKB phosphorylation induced by MCP-1 (Figure 2C) whereas adenovirus encoding a GFP (Ad-GFP) as a control did not significantly modify the level of PKB phosphorylation, confirming the involvement of PI3K γ in MCP-1-induced PKB phosphorylation in aortic SMC.

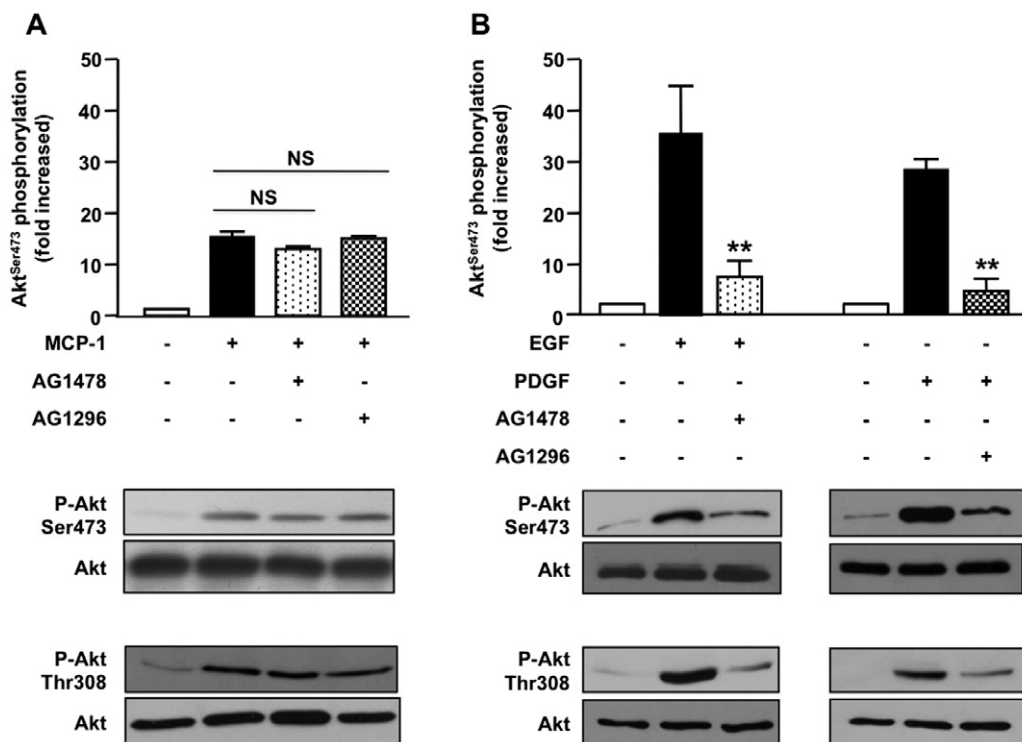


Figure 1

PKB (Akt) phosphorylation induced by MCP-1 in aortic SMCs does not require tyrosine kinase receptor transactivation. (A, B) Primary pig aortic SMCs were pretreated for 30 min with tyrosine kinase activity inhibitors (AG1478: 300 nM, AG1296: 10 μ M) and stimulated with MCP-1 (10 ng·mL⁻¹, 5 min) (A), EGF (10 ng·mL⁻¹, 2 min) or PDGF-BB (10 ng·mL⁻¹, 2 min) (B). PKB phosphorylation and expression were analysed with antibodies against phosphorylated PKB (serine 473 and threonine 308) and total PKB. Immunoblots shown are representative of three independent experiments ($n = 3$). ** $P < 0.01$ compared with EGF or PDGF stimulation alone.

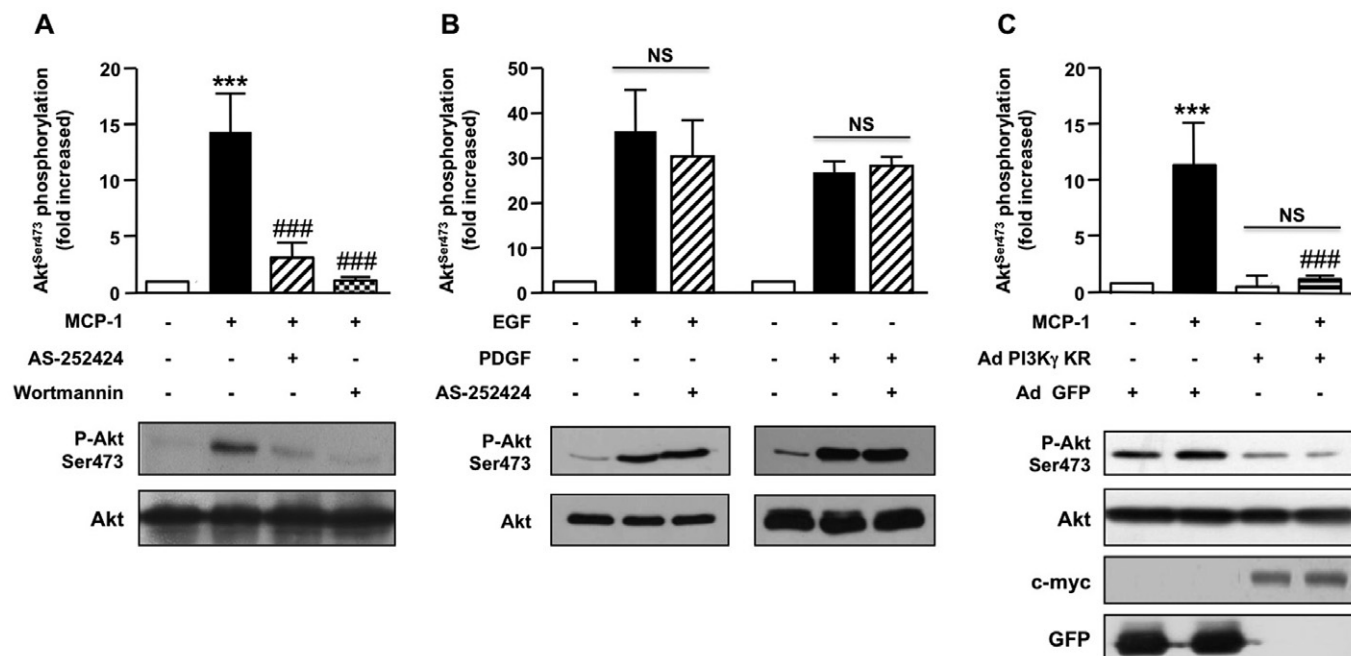


Figure 2

PI3K γ is required for PKB (Akt) phosphorylation in MCP-1-activated aortic SMC. (A, B) Pig aortic SMCs were pre-incubated (30 min) (A) with wortmannin (100 nM) or AS-252424 (100 nM) and stimulated with MCP-1 (10 ng·mL⁻¹, 5 min) or (B) with AS-252424 and stimulated with EGF (10 ng·mL⁻¹, 5 min) or PDGF (10 ng·mL⁻¹, 5 min). Lysates were analysed by Western blot using antibodies against phosphorylated PKB (serine 473) or total PKB. Immunoblots from representative experiments and densitometry analyses are shown (means $\pm$ SEM, $n = 4$). (C) Pig aortic SMCs were infected with PI3K γ KR-myc adenovirus (Ad PI3K γ KR) or a GFP encoding adenovirus (Ad-GFP) as a control and stimulated with MCP-1 (10 ng·mL⁻¹, 5 min). PKB phosphorylation and expression were analysed with antibodies against phosphorylated PKB (serine 473) or total PKB. Expression of PI3K γ KR was analysed with anti-c-myc antibody and control adenovirus with anti-GFP antibody. Immunoblots from representative experiments are shown ($n = 3$). ### $P < 0.001$ versus MCP-1 alone and *** $P < 0.001$ compared with unstimulated cells.

MCP-1/CCR2 signalling induces aortic SMC migration via a PI3K γ -dependent pathway

PKB, and especially PKB1, has been demonstrated to be a key event in SMC migration (Fernandez-Hernando *et al.*, 2009). Because MCP-1 is a chemoattractant for several cell types including SMC, we evaluated the possible involvement of PI3K γ /PKB pathway in MCP-1-induced aortic SMC migration. Using a wound-healing cell migration assay, we showed that MCP-1 induced aortic SMC migration at the dose of 5 ng·mL⁻¹ with a maximum efficiency at 10 ng·mL⁻¹ (Supporting Information Figure S3). This effect was totally abolished by a PKB inhibitor demonstrating the essential function of PKB in MCP-1-induced cell migration (Figure 3A). RS-102895, an inhibitor of the chemokine receptor CCR2 (Figure 3B), prevented MCP-1-induced migration indicating that the effect of MCP-1 on aortic SMC migration is mediated by CCR2 and PKB. We next investigated the involvement of PI3K γ in this process. Cells were pretreated with the PI3K γ inhibitor AS-252424 or infected by an adenovirus encoding an inactive form of PI3K γ . Basal migration was significantly decreased in both conditions indicating that a weak activation of PI3K γ persisted in the basal condition. Nevertheless, the PI3K γ inhibitor as well as an adenovirus encoding inactive PI3K γ totally prevented MCP-1-induced migration (Figure 3C,D). To verify the involvement of PI3K γ in migration processes induced by MCP-1, the chemotaxis of SMC

induced by MCP-1 was performed using a Boyden chamber assay. AS-25224 totally abolished the migration in this condition (Supporting Information Figure S4A). Moreover, BrdU staining showed that MCP-1 was not able to induce aortic pig SMC proliferation (Supporting Information Figure S4B). These data clearly indicate that PI3K γ activation downstream of MCP-1/CCR2 signalling plays a crucial role in aortic SMC migration.

PI3K γ can amplify PDGF-induced aortic SMC migration via an MCP-1/CCR2 pathway

MCP-1 mRNA is rapidly increased in arterial tissue after arterial injury and might be involved in both monocyte recruitment and the SMC migration process (Taubman *et al.*, 1992; Wysocki *et al.*, 1996). While the mechanism regulating CCR2 expression in SMC is not completely understood, induction of MCP-1 is well characterized. PDGF has been shown to induce MCP-1 accumulation in rat SMC not only by activation of a specific region in the promoter (Poon *et al.*, 1996; Bogdanov *et al.*, 1998) but also by increasing MCP-1 mRNA stability (Liu *et al.*, 2006). We therefore investigated whether PI3K γ is not just restricted to a role in MCP-1 signalling but could also amplify migration induced by PDGF through an autocrine/paracrine loop. To investigate this mechanism in our aortic SMC model, we first evaluated MCP-1 mRNA and protein level accumulation in response to PDGF. We showed

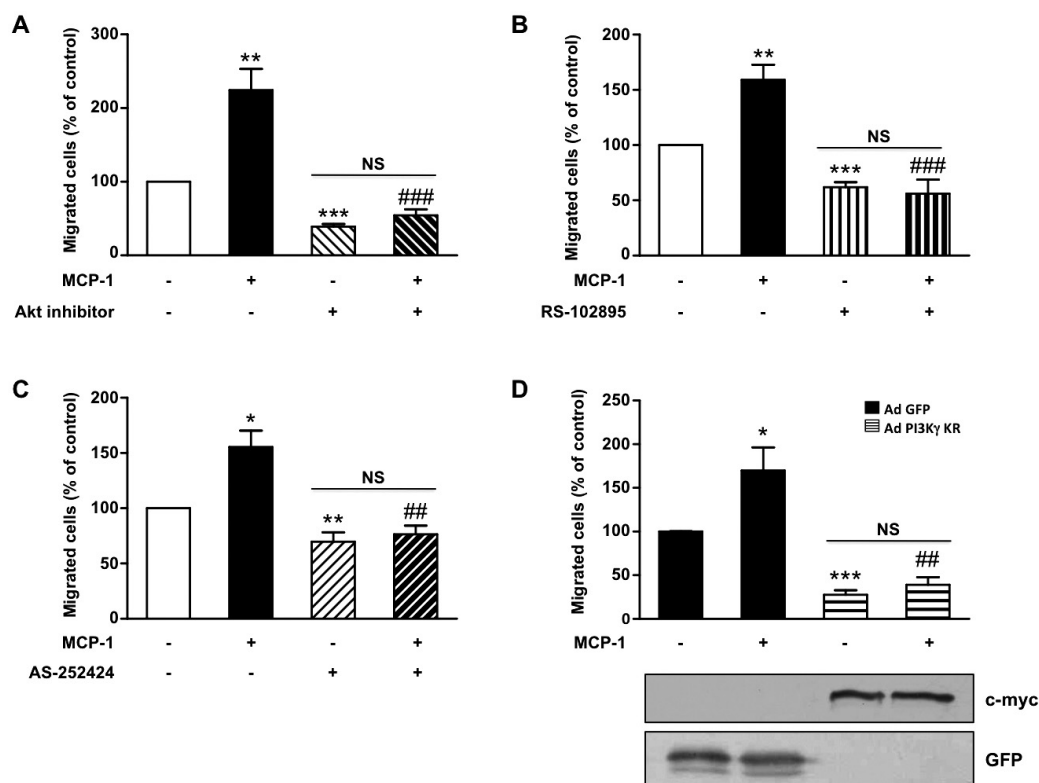


Figure 3

PI3K γ is essential for MCP-1-induced aortic SMC migration. Pig aortic SMC migration was quantified with a wound-healing assay using the Oris cell migration kit. Confluent SMCs were treated with MCP-1 (10 ng·mL⁻¹) in serum-free medium and allowed to migrate in the wound surface for 48 h. Migrated cells were stained with DAPI and counted under a fluorescence microscope. Results are expressed as a percentage of control. (A) SMCs were treated with MCP-1 (10 ng·mL⁻¹) only or together with PKB inhibitor (20 μ M) in serum-free medium. (B) SMCs were treated with MCP-1 (10 ng·mL⁻¹) alone or together with MCP-1 receptor inhibitor (RS-102895: 5 μ g·mL⁻¹). (C) SMCs were treated with MCP-1 (10 ng·mL⁻¹) alone or together with a specific PI3K γ inhibitor (AS-252424: 100 nM). (D) SMCs infected with GFP adenovirus (Ad-GFP) or with an adenovirus encoding a dominant negative form of PI3K γ (Ad PI3K γ KR) for 72 h were treated with MCP-1 (10 ng·mL⁻¹). Expression of PI3K γ KR was analysed with anti-c-myc antibody and control adenovirus with anti-GFP antibody. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with control, ## P < 0.01 compared with MCP-1 alone stimulated cells, ### P < 0.001 compared with MCP-1 alone stimulated cells.

an increase in mRNA encoding MCP-1 after as little as 1 h of PDGF stimulation (Figure 4A) and a 10-fold accumulation of MCP-1 protein after 48 h (Figure 4B). To explore a possible paracrine/autocrine loop in PDGF-induced SMC migration, aortic SMCs were incubated with a CCR2 inhibitor or an MCP-1-neutralizing antibody before stimulation with PDGF. Under these conditions, PDGF-induced aortic SMC migration was reduced by about 50% indicating an important function of the MCP-1/CCR2 pathway in PDGF-mediated migration (Figure 4C). To determine the possible involvement of PI3K γ in this process, the same experiments were performed in aortic SMC pre-incubated with AS-252424. Treatment with the PI3K γ inhibitor reduced by, about 40%, the migration (Figure 4C) and chemotactic response of SMCs (Supporting Information Figure S4C) induced by PDGF. To investigate the possible involvement of PI3K γ in the proliferation mechanism of PDGF in our wound healing experiments, we investigated SMC proliferation under the same conditions using BrdU staining. The results, presented in Supporting Information Figure S4D, showed that the PI3K γ inhibitor did not significantly modulate SMC proliferation

induced by PDGF indicating a specific role of PI3K γ in PDGF-induced migration in aortic SMCs (Supporting Information Figure S4D). Infection of SMCs with an adenovirus encoding the inactive form of PI3K γ confirmed these results (Figure 4D). In these experiments, PI3K γ inhibition reduced basal aortic SMC migration as effectively as the CCR2 inhibitor or MCP-1-neutralizing antibody treatment indicating that a weak activation of MCP-1/CCR2/PI3K γ pathway still occurred in our basal conditions. To confirm the involvement of PI3K γ in the amplification of aortic SMC migration upon PDGF stimulation, we isolated primary SMCs from aortas of PI3K $\gamma^{+/+}$, PI3K $\gamma^{-/-}$ mice or mice expressing a catalytically inactive form of PI3K γ (PI3K $\gamma^{KD/KD}$). These cells expressed CCR2 and PI3K γ as pig aortic SMCs (Supporting Information Figure S5). These cells stained positive for smooth muscle actin and did not show any difference in the number of positive cells in different genotypes (Figure 5A). In line with the results in SMCs treated with PI3K γ inhibitors, murine SMC derived from PI3K $\gamma^{-/-}$ or PI3K $\gamma^{KD/KD}$ mice exhibited a reduced migratory response to MCP-1 and PDGF compared with PI3K $\gamma^{+/+}$ cells. This demonstrates the importance of

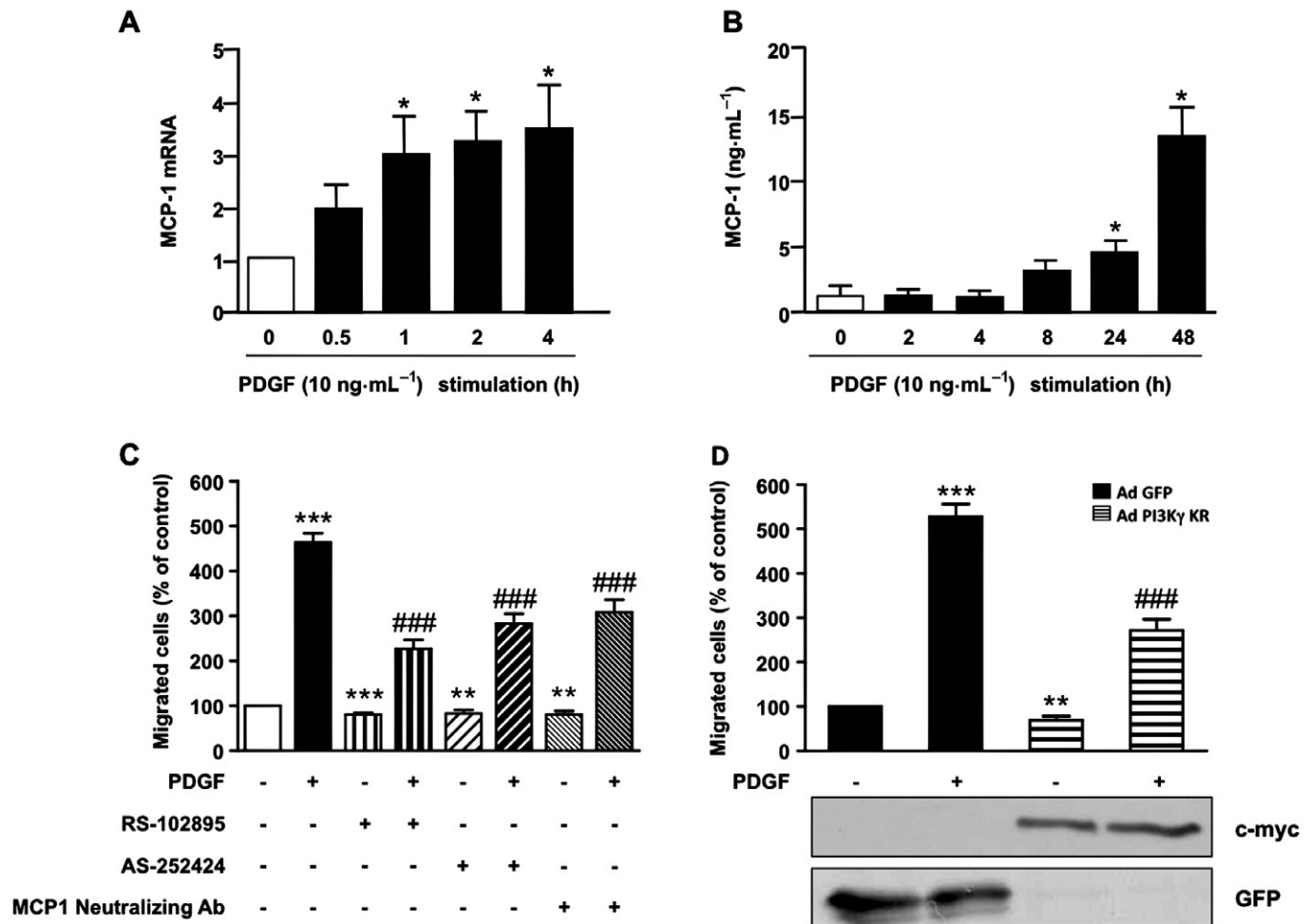


Figure 4

PI3K γ amplifies PDGF-stimulated aortic SMC migration by an MCP-1/CCR2 pathway. (A) Pig aortic SMCs were stimulated with PDGF-BB (10 ng·mL⁻¹) in serum-free medium for the indicated times. Total RNA was extracted and subjected to quantitative real-time PCR using specific primers for MCP-1. (B) Pig aortic SMCs were stimulated with PDGF-BB (10 ng·mL⁻¹) in serum-free medium for the indicated times. Cell supernatants were analysed for MCP-1 protein content by a specific ELISA kit as described in Methods. (C) Confluent pig aortic SMCs were treated with PDGF-BB (10 ng·mL⁻¹) alone or together with MCP-1 receptor inhibitor (RS-102895: 5 μ g·mL⁻¹) or a specific PI3K γ inhibitor (AS-252424: 100 nM) or MCP-1-neutralizing antibody (5 μ g·mL⁻¹) in serum-free medium. Cells were allowed to migrate for 48 h and migrated cells were stained with DAPI and counted under a fluorescence microscope. Results are expressed as a percentage of the control. (D) Pig aortic SMCs infected with GFP adenovirus or with an adenovirus encoding a dominant negative form of PI3K γ (PI3K γ KR) for 72 h were treated with PDGF-BB (10 ng·mL⁻¹) and analysed for migration as above. Expression of PI3K γ KR was analysed with anti-c-myc antibody and control adenovirus with anti-GFP antibody. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with control, ### P < 0.001 compared with PDGF alone stimulated cells.

PI3K γ in both the MCP-1 and the PDGF-induced migration process (Figure 5B).

Discussion

MCP-1 has been shown to contribute to all steps of the arterial remodelling process by inducing monocyte infiltration and functional switching of SMCs to the synthetic phenotype, leading to increased proliferation and migration. Most of the effects mediated by MCP-1 appear to be mediated by its interaction with the GPCR CCR2 but downstream signalling pathways are not well characterized. The involve-

ment of PI3K lipid products, especially PIP₃, has been reported in restenosis processes such as SMC proliferation and migration (Goncharova *et al.*, 2002; Irani *et al.*, 2002; Mehrhof *et al.*, 2005; Radhakrishnan *et al.*, 2008; Zhou *et al.*, 2009). Here, we have investigated whether PI3K isoform(s) are involved in MCP-1-mediated SMC migration. Early signalling events occurring downstream of GPCR activation have been subject to intense investigation. It has been shown that GPCR agonists induce tyrosine phosphorylation of multiple substrates in target cells (Force *et al.*, 1991; Leeb-Lundberg and Song, 1991; Laffargue *et al.*, 1999). In SMCs, GPCR activation leads to rapid activation of tyrosine kinase receptors such as EGF and PDGF receptors that might be

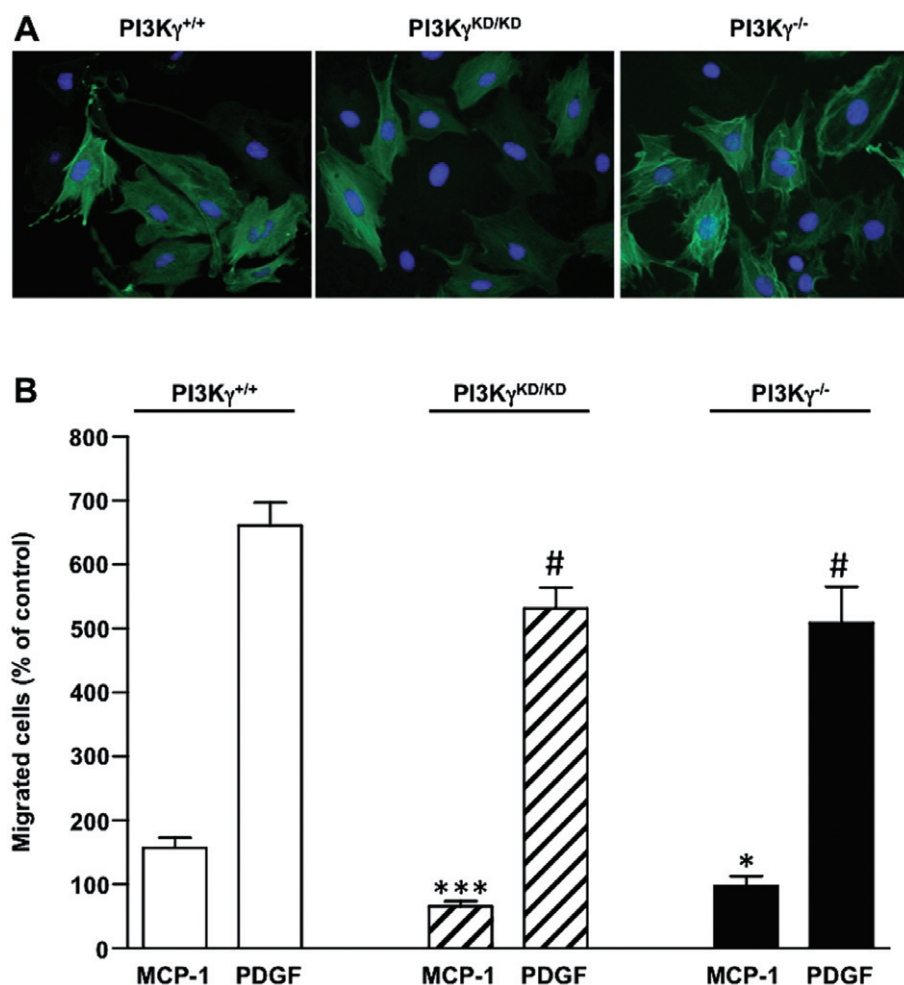


Figure 5

Effect of PI3K γ deficiency in MCP-1 and PDGF-induced migration of mouse aortic SMCs. (A) Aortic SMCs from wild-type (PI3K $\gamma^{+/+}$), PI3K γ -deficient mice (PI3K $\gamma^{-/-}$) or mice expressing a catalytically inactive PI3K γ (PI3K $\gamma^{KD/KD}$) were fixed and stained with an anti-smooth muscle α -actin antibody. Photomicrographs from representative experiments are shown. (B) Aortic SMCs from PI3K $\gamma^{+/+}$, PI3K $\gamma^{-/-}$ or PI3K $\gamma^{KD/KD}$ mice were stimulated with MCP-1 (10 ng·mL $^{-1}$) or PDGF-BB (10 ng·mL $^{-1}$) in serum-free medium and allowed to migrate for 48 h. Migrated cells were stained with DAPI and counted under a fluorescence microscope. Results are expressed as a percentage of PI3K $\gamma^{+/+}$, PI3K $\gamma^{-/-}$ or PI3K $\gamma^{KD/KD}$ non-stimulated cells, respectively. * $P < 0.05$, *** $P < 0.001$ compared with MCP-1-stimulated PI3K $\gamma^{+/+}$ cells; # $P < 0.05$ compared with PDGF-stimulated PI3K $\gamma^{+/+}$ cells.

involved in PI3K/PKB activation (Voisin *et al.*, 2002; Baudhuin *et al.*, 2004; Tanimoto *et al.*, 2004; Hsieh *et al.*, 2009). However, previous reports indicated that in SMCs, GPCR signalling can directly activate the PI3K γ isoform (Vecchione *et al.*, 2005). In this respect, our study demonstrated a direct link between MCP-1/CCR2 activation and the PI3K/PKB pathway. Furthermore, we have provided evidence that the γ isoform of PI3K is specifically involved in this MCP-1/CCR2 signalling pathway. Whereas PI3K γ has been widely reported to be involved in the migration of haematopoietic cells (Hirsch *et al.*, 2000; Hannigan *et al.*, 2002; Jones *et al.*, 2003; Del Prete *et al.*, 2004), our study is the first to demonstrate its involvement in SMC migration. Only a few studies have reported a role for PI3K γ in non-haematopoietic cell migration (Heller *et al.*, 2008; Guzman-Hernandez *et al.*, 2009). Heller *et al.* (2008) investigated the role of different isoforms of PI3K in sphingosine 1-phosphate-induced migration of endothelial cells and demonstrated that PI3K β and γ

are equally involved in this process. Whereas the structure of PI3K β predicted activation downstream of the tyrosine kinase receptor, recent data obtained from mouse mutants expressing a catalytically inactive PI3K β showed that PI3K β was more effective at activating the GPCR than tyrosine kinases (Ciraolo *et al.*, 2008; Guillermet-Guibert *et al.*, 2008; Jia *et al.*, 2008). Using PI3K β inhibitors, we also found that PI3K β is somewhat involved in MCP-1-induced aortic SMC migration (data not shown). This could explain the difference in PKB phosphorylation induced by MCP-1 in cells treated with a pan-PI3K inhibitor such as wortmannin compared with cells treated with a specific PI3K γ inhibitor. Even if PI3K γ is not the predominant isoform expressed in aortic SMCs, we clearly demonstrated that its involvement predominates in MCP-1-stimulated aortic SMC because PKB phosphorylation decreased by more than 85% in the absence of PI3K γ activity and MCP-1-induced migration was totally abolished in the same conditions.

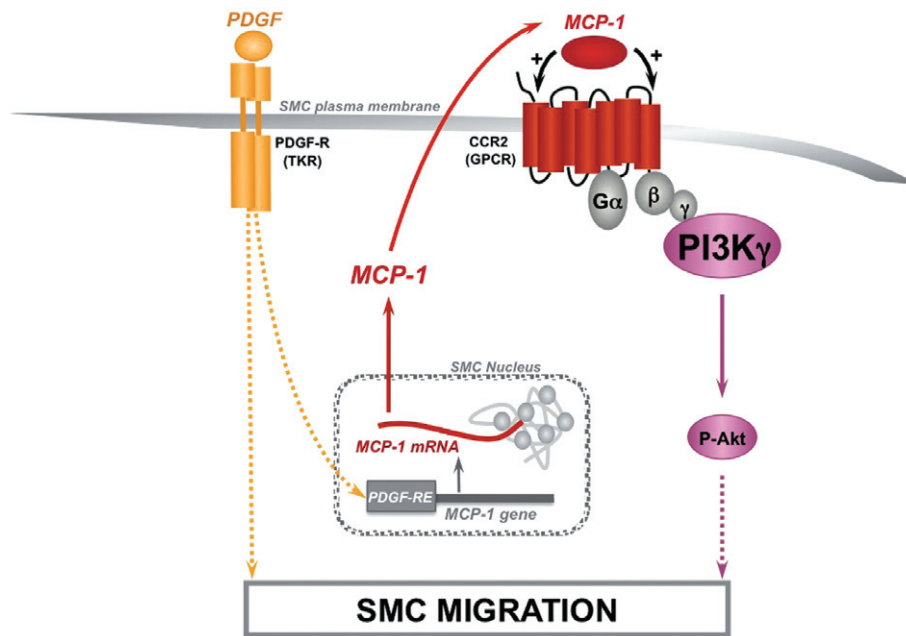


Figure 6

Proposed signalling model of the essential role of PI3K γ in MCP-1-stimulated aortic smooth muscle cell as an amplifier of PDGF-induced cell migration. In aortic SMC migration, PI3K γ is the major PI3K isoform implicated in MCP-1-induced PKB (Akt) phosphorylation. Moreover, PI3K γ could play a role as an amplifier of PDGF-induced SMC migration by an autocrine/paracrine pathway. MCP-1 mRNA level is increased upon PDGF stimulation leading to MCP-1 secretion and CCR2 activation. Dashed lines indicate potential signalling mechanism not fully established at this time. Abbreviation: TKR, tyrosine kinase receptor; PDGF-RE, PDGF-responsive-element.

High levels of MCP-1 are expressed not only by inflammatory cells but also by cells of the arterial wall during the development of atherosclerosis and after balloon injury (Taubman *et al.*, 1992; Wysocki *et al.*, 1996; Sako *et al.*, 2008). *In vitro*, PDGF could be responsible for part of this increase in MCP-1 secretion by SMCs (Bogdanov *et al.*, 1998; Liu *et al.*, 2006). Our data extended these observations and showed the involvement of the MCP-1/CCR2 pathway in PDGF-induced aortic SMC migration. In addition, our results demonstrated that PI3K γ , by this autocrine pathway, could amplify the migration process activated by PDGF. Other agonists, such as TNF- α or thrombin, are able to induce MCP-1 secretion in SMC (Kranzhofer *et al.*, 1996; Biswas *et al.*, 1998). Moreover, it has been shown that other cell types found in the arterial wall, such as inflammatory cells or endothelial cells, secrete MCP-1 in response to interleukin-6 (Biswas *et al.*, 1998; Rott *et al.*, 2003). It would be interesting to investigate the possible involvement of PI3K γ downstream of the effectors that are key players in intimal hyperplasia formation.

Although PI3K γ activation mechanisms require GPCR activation, other agonists acting via the tyrosine kinase receptor have been shown to activate PI3K γ . For example, migration induced by CSF-1, a tyrosine kinase receptor agonist, was altered in PI3K $\gamma^{-/-}$ macrophages (Jones *et al.*, 2003). It is also of interest that incubating cells with pertussis toxin to block Gi coupled receptors had a marked effect on cell morphology, indicating the involvement of a Gi-activated signalling pathway (Jones *et al.*, 2003). In the same cells, MCP-1-induced migration was markedly reduced in the absence of

PI3K γ . One explanation for this defect in CSF-1-induced migration of these PI3K $\gamma^{-/-}$ macrophages could be the presence of an autocrine/paracrine pathway involving secretion of chemokines. Hence, the pathway described in our study in aortic SMC could also be present in other cell types.

In summary, our study shows that PI3K γ is the major PI3K involved in PKB phosphorylation and migration induced by the chemokine MCP-1. Moreover, we demonstrated that the involvement of PI3K γ in aortic SMC migration was not restricted to MCP-1 because PI3K γ could amplify PDGF-induced cell migration by an autocrine/paracrine pathway involving MCP-1 secretion and CCR2 activation (Figure 6). These results together with the anti-inflammatory properties of the PI3K γ inhibitor in the arterial wall that we demonstrated previously (Fougerat *et al.*, 2008) make PI3K γ a promising target for the prevention of restenosis.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 PKB phosphorylation induced by MCP-1 in human aortic SMC does not require tyrosine kinase receptor transactivation.

Figure S2 PI3K isoforms are expressed in pig aortic SMC. PI3K isoform expression was analysed with specific antibodies against p110 α , p110 β , p110 γ in pig aortic SMC.

Figure S3 Dose-response of MCP-1-induced aortic SMC migration.

Figure S4 PI3K γ is essential to MCP-1 and PDGF-induced aortic SMC migration and not proliferation.

Figure S5 PI3K γ and CCR2 are expressed in mouse and pig aortic SMC.

Appendix S1 Supplementary Methods.

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