Platelet-derived growth factor-BB (PDGF-BB) regulation of migration and focal adhesion kinase phosphorylation in rabbit aortic vascular smooth muscle cells: roles of phosphatidylinositol 3-kinase and mitogen-activated protein kinases

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Abstract

Objective: Phosphatidylinositol 3'-kinase (PI3-kinase) is implicated in cell migration and focal adhesion kinase (FAK) phosphorylation. In contrast, it has been proposed that mitogen-activated protein (MAP) kinases are essential for proliferation but may be dissociated from chemotactic signalling. We investigated the roles of PI3-kinase and p42/p44 MAP kinases in cell migration and FAK tyrosine phosphorylation induced by platelet-derived growth factor-BB (PDGF-BB) in rabbit aortic vascular smooth muscle cells (VSMCs). The roles of PI3-kinase and MAP kinase pathways in the chemotactic response to insulin-like growth factor-I (IGF-I) were also examined.

Methods: The roles of PI3-kinase and p42/p44 MAP kinases were assessed using the PI3-kinase inhibitors, wortmannin and LY294002, and an inhibitor of MAP kinase kinase, PD98059. PI3-kinase activity was measured by phosphatidylinositol phosphorylation in anti-phosphotyrosine immunoprecipitates and by thin layer chromatography of phosphorylated products. Phosphorylation was assessed by immunoprecipitation with anti-phosphotyrosine antibodies and Western blotting with FAK-specific antibody. Migration was evaluated in a chemotaxis chamber using polycarbonate filters with an 8-mm pore size.

Results: Neither wortmannin nor LY294002 significantly reduced PDGF-BB stimulation of FAK tyrosine phosphorylation, chemotaxis or immunofluorescent staining of focal adhesions in VSMCs. PD98059, a specific inhibitor of MAP kinase kinase, PD98059, PI3-kinase activity was measured by phosphatidylinositol phosphorylation in anti-phosphotyrosine immunoprecipitates and by thin layer chromatography of phosphorylated products. Phosphorylation was assessed by immunoprecipitation with anti-phosphotyrosine antibodies and Western blotting with FAK-specific antibody. Migration was evaluated in a chemotaxis chamber using polycarbonate filters with an 8-mm pore size. Results: Neither wortmannin nor LY294002 significantly reduced PDGF-BB stimulation of FAK tyrosine phosphorylation, chemotaxis or immunofluorescent staining of focal adhesions in VSMCs.

Conclusions: PDGF-BB stimulation of both FAK tyrosine phosphorylation and migration in VSMCs are not dependent on PI3-kinase activation. While PDGF-BB stimulation of FAK tyrosine phosphorylation is not dependent on p42/p44 MAP kinase activation, PDGF-BB and IGF-I both stimulate p42/p44 MAP kinase activity and the chemotactic response to these factors is partially dependent on MAP kinase activation. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: FAK; Paxillin; IGF-I; Wortmannin; Chemotaxis

1. Introduction

Migration of vascular smooth muscle cells (VSMCs) from the arterial media is a key event both in progressive intimal thickening leading to atherosclerosis and in vasculoproliferative complications resulting from angioplasty and by-pass graft surgery [1,2]. VSMC migration is regulated by a variety of factors, including platelet-derived growth factor-BB (PDGF-BB) and insulin-like growth factor-I (IGF-I) [1,2]. PDGF-BB is the most potent known chemoattractant for VSMCs in culture [1] and PDGF-induced migration of VSMCs is thought to play a major role in neo-intima formation in atherosclerosis and in restenosis [1–6]. IGF-I and IGF-I receptor expression are increased in the arterial wall following balloon angioplasty

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[7,8], and IGF-I is expressed and secreted by arterial VSMCs, macrophages and platelets [9–12]. IGF-I receptors are highly expressed in arterial VSMCs while insulin receptor levels are low [13]. Relative to PDGF-BB, IGF-I has been reported to be a weak mitogen for human aortic VSMCs, but a potent and effective chemoattractant [14]. IGF-I also promotes motility, membrane ruffling and lamellipodia formation in other cell types [15,16].

Previous work from our laboratory showed that, in VSMCs, PDGF-BB acting through the β-receptor, stimulates migration and tyrosine phosphorylation of focal adhesion components, including the non-receptor protein tyrosine kinase focal adhesion kinase (FAK) and the FAK-associated component, paxillin [2,17–20]. In several cell types, including VSMCs, increased FAK and paxillin tyrosine phosphorylation correlate with the chemotactic response to migration factors, including PDGF-BB, hepatocyte growth factor, hyaluronan, RANTES and vascular endothelial growth factor [2,16,20–24]. Support for a central role of FAK in controlling rates of cell movement has come from gene knock-out and over-expression studies [25–27]. The downstream targets for FAK are not fully understood, but tyrosine phosphorylation of FAK is associated in several cell types with that of paxillin, and paxillin has been reported to associate with FAK and is a putative substrate for FAK [28,29].

Activation of phosphatidylinositol 3′-kinase (PI3-kinase) and p42/p44 mitogen-activated protein (MAP) kinases are rapid responses to PDGF in several cell types [20,30,31]. PI3-kinase has been implicated in the migratory and membrane ruffling responses to PDGF [32,33]. PI3-kinase has been shown to associate with FAK [34–36] and is implicated in PDGF-induced activation of the FAK pathway in murine Swiss 3T3 cells [37]. Though the role of the MAP kinase cascade in VSMCs chemotactic signalling has not been extensively studied, it was reported that IGF-I stimulates migration of VSMCs without activating MAP kinases and it has been proposed that MAP kinases can be dissociated from chemotactic signalling mechanisms [14].

In the present paper, we studied the role of PI3-kinase and the MAP kinase cascade in migratory responses of rabbit aortic VSMCs to PDGF-BB and IGF-I. The results show that activation of PI3-kinase can be dissociated from PDGF-BB stimulation of FAK tyrosine phosphorylation and PDGF-BB-induced cell migration. In contrast, PDGF-BB-stimulated VSMC migration is partly dependent on activation of p42/p44 MAP kinases. IGF-I also stimulates PI3-kinase and MAP kinase activities and, similar to PDGF-BB, IGF-I-induced migration is also partly mediated through a MAP kinase-dependent pathway.

2. Methods

2.1. Cell culture

VSMCs were cultured by explant outgrowth from the thoracic aortas of healthy New Zealand White rabbits (aged nine weeks) as described previously [20]. Rabbits were killed by lethal intravenous injection of sodium pentobarbitone (500 mg/animal) and suffered no obvious or additional discomfort. In this and other respects the investigation conforms with the Guide for the Care and use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Explants were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 4 mM l-glutamine, penicillin/streptomycin (10 units/ml and 100 mg/ml, respectively) and 20% fetal calf serum (FCS). Cultured cells were identified as VSMCs on the basis of morphology and expression of VSMC-specific α-actin using α-actin antibodies (Sigma). For experimental purposes, primary cultures of aortic VSMCs were rendered quiescent by incubation with DMEM containing 0.5% FCS for 40 h [20]. Murine Swiss 3T3 cells were cultured and maintained as previously described [20].

2.2. Assays of cell migration

Cell migration was measured in a modified Boyden chemotaxis chamber (NeuroProbe, Cabin John, USA) essentially as described [20]. Test chemoattractants were diluted in DMEM supplemented with 1% (w/v) bovine serum albumin (BSA; Sigma) and placed in the bottom wells of the chamber. Polycarbonate filters with 8-mm pores (Polymicro) were preincubated in a 0.1% solution of collagen type I (Sigma) and placed between the chemoattractants and the upper chambers. Cells were trypsinized and washed twice in DMEM and resuspended into each well in the upper chamber, and the chemotaxis chambers were routinely incubated at 37°C for 6 h. After the incubation, unmigrated cells were removed from the upper side of the filters and migrated cells were stained with ‘Pro-Diff’ (Braudwood Laboratories, Beckenham, UK). Filters were mounted onto microscope slides and stained cells were counted at 200× magnification in four fields per well. In each individual experiment, chemotaxis was performed in four separate wells for each concentration of a given test substance under a specified condition.

2.3. Immunoprecipitations

Quiescent cultures of cells (1.5×10⁵) were washed twice with DMEM, treated with peptide factors in 1 ml of this medium, as indicated, and lysed at 4°C in 1 ml of a solution containing 10 mM Tris–HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM di-sodium pyrophosphate, 50 mM NaF, 0.1 mM Na₂VO₃, 1 mM phenylmethyl-sulphonyl fluoride, 0.5% NP-40 and 1% Triton X-100 (lysis buffer). Lysates were clarified by centrifugation at
15,000 g for 10 min and precleared by incubation with albumin–agarose for 1 h at 4°C. After removal of albumin–agarose by brief (10 s) centrifugation, immunoprecipitations were performed by incubating lysates with 1 mg/ml of primary antibody for 2 h at 4°C. Immunocomplexes were collected either by incubating lysates with protein A–agarose beads for a further 1 h or by incubating them with 5 mg per lysate of anti-mouse IgG for 1 h followed by a 1-h incubation with protein A–agarose beads. Immunoprecipitates were washed three times with lysis buffer, proteins were extracted with 2× sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and further analysed by Western blotting.

2.4. Western blotting

After SDS–PAGE, proteins were transferred to Immobilon membranes (Millipore). Membranes were blocked using 5% non-fat dried milk in phosphate-buffered saline, pH 7.2, and incubated for 1 h in phosphate-buffered saline–0.1% Tween-20 containing antibodies (1 mg/ml of each) as indicated. Immunoreactive bands were visualized either by chemiluminescence using horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG and ECL™ reagent. Autoradiograms were semi-quantified by scanning densitometry using an LKB Ultrascan XL densitometer. The values shown in the figures have been calculated from the peak areas corresponding to individual protein bands.

2.5. Assays of PI3-kinase activity

PI3-kinase activity was determined by measuring phosphatidylinositol (PI) phosphorylation in anti-phosphotyrosine [anti-Tyr(P)] immunoprecipitates, as described previously [38,39]. Immunoprecipitates were washed three times with lysis buffer, once in 50 mM Heps, pH 7.5, and once in PI3-kinase assay buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA). Immunoprecipitates were preincubated in 25 ml of PI3-kinase assay buffer and 10 ml of a 1 mg/ml solution of PI for 20 min at 4°C. In some experiments, inhibitors of PI3-kinase were also added to immunoprecipitates for this preincubation period. Reactions were initiated by the addition of 15 ml of assay cocktail containing 10 mCi [γ-32P]ATP, 100 mM ATP, 10 mM MgCl2, and incubations were routinely performed for 10 min at room temperature. Reactions were terminated by the addition of 100 ml of 1 M HCl followed by the addition of 200 ml of a 1:1 mix of CHCl3 and methanol. Samples were vortex-mixed for 20 s and the phases were separated by centrifugation at 15,000 g for 2 min. The lower CHCl3 phase was collected, washed with 80 ml of a 1:1 mix of 1 M HCl and methanol, and the phases were separated by centrifugation as before. The lower phase was collected and applied to LK6DF silica gel thin layer chromatography (TLC) plates (Whatman), which had been presprayed with 1% (w/v) potassium oxalate and allowed to dry prior to sample application. TLC plates were routinely developed for 45 min using a 29.2:180:10.8:140 mixture of H2O, CHCl3, NH2OH and methanol, respectively. Developed TLC plates were dried and exposed to X-ray film for one to three days.

2.6. MAP kinase assay

Cells were treated with factors as indicated, washed rapidly twice with ice-cold phosphate-buffered saline (PBS) and immediately extracted by the addition of boiling 2× SDS–PAGE sample buffer. Cell extracts were collected by scraping, heated to 95°C for 10 min and run on 12.5% acrylamide SDS–PAGE gels. Following transfer to Immobilon membranes, proteins were immunoblotted with an antibody that specifically recognizes p42 and p44 MAP kinases (ERK1 and ERK2) activated by phosphorylation at Tyr204 or with an antibody that specifically recognizes the dually phosphorylated active forms [40]. Autoradiograms were semi-quantified by scanning densitometry, as described in Section 2.4.

2.7. Immunofluorescent staining

Freshly trypsinized VSMCs were replated onto glass coverslips that had been precoated with collagen. Following treatments, cells were washed three times with ice-cold PBS and then fixed in 3% paraformaldehyde in PBS for 10 min at room temperature. Aldehyde groups were quenched by incubation with 50 mM NH Cl for 10 min at room temperature. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature and then were washed three times in PBS. Fixed and permeabilized cells were incubated with primary antibody (2 mg/ml) for 30 min at room temperature, washed three times in PBS, and then incubated for 30 min at room temperature with a secondary antibody conjugated to fluorescein isothiocyanate (FITC). Cells were finally washed three times in PBS. Coverslips were mounted onto microscope slides using Vectashield mounting medium. Immunofluorescent staining was observed and photographed using a Zeiss Axiohot epifluorescence microscope fitted with an X63 (N.A. 1.4, oil) objective lens.

2.8. Statistical analysis

Results of chemotaxis experiments are presented as the mean fold increases in migrated cells above migration in the absence of chemoattractant±S.E.M. Each n value in the figure legends refers to the number of individual experiments. Statistical analysis of the difference between means was performed using one-way ANOVA. P<0.05 was considered to be statistically significant.
2.9. Materials

PDGF-BB and IGF-I were obtained from R and D systems. Cytochalasin D was from Sigma (St. Louis, MO, USA). Wortmannin was purchased from either LC laboratories or from Calbiochem. LY294002 and PD98059 were purchased from Calbiochem. Py20 anti-Tyr(P) monoclonal antibody (mAb), and mAbs to FAK, paxillin and p85a were from Transduction Laboratories. 4G10 anti-Tyr(P) mAb was from TCS Biologicals. Vinculin antibody was obtained from Sigma. Antibody to the tyrosine phosphorylated and dually phosphorylated forms of p42/p44 MAP kinases were obtained from New England Biolabs and Promega. Protein A–agarose and goat-anti-mouse IgG were from Oncogene Science. ECL™ reagents and HRP-conjugated secondary antibodies were from Amersham, UK. Secondary and FITC-conjugated reagents for immunofluorescent staining were obtained from Dako. All other reagents used were of the purest grade available.

3. Results

To investigate whether or not PDGF-BB stimulation of FAK tyrosine phosphorylation in VSMCs was dependent on PI3-kinase activity, studies were performed using selective inhibitors of PI3-kinase activity. The fungal metabolite wortmannin has been widely employed as an inhibitor of PI3-kinase and is useful for probing the role of PI3-kinase in a variety of cellular processes and cell types.
Since recent findings indicate that this agent is not specific [43], use of wortmannin was complemented by studies with a recently identified and specific inhibitor of PI3-kinase, LY294002 [44].

Treatment of VSMCs with wortmannin inhibited PDGF-BB-stimulated PI3-kinase activity in a concentration-dependent manner. The half-maximum effect of wortmannin was obtained at approximately 10 nM and the maximum inhibitory effect (95% inhibition) was obtained at 25–50 nM (Fig. 1A). LY294002 also inhibited PDGF-BB stimulation of PI3-kinase activity in VSMCs in a concentration-dependent manner. Inhibition was greater than 80% at 30 mM LY294002 and maximum inhibition (90%) was obtained at 60 mM (Fig. 1A).

Pretreatment of VSMCs for 1 h with wortmannin at 100 nM had no detectable effect upon the increase in FAK phosphotyrosine content induced in VSMCs by a subsequent challenge with PDGF-BB (Fig. 1B). Even at a concentration as high as 1 mM, wortmannin had only a small effect on the ability of PDGF-BB to induce FAK tyrosine phosphorylation. Similarly, pretreatment with LY294002 at 30 mM, twice the concentration previously shown to cause marked inhibition of PDGF-BB-induced FAK tyrosine phosphorylation in Swiss 3T3 cells [37], also had no detectable inhibitory effect on PDGF-BB-stimulated FAK tyrosine phosphorylation in rabbit VSMCs (Fig. 1C). In five independent experiments, semi-quantification of FAK tyrosine phosphorylation showed that, in VSMCs pretreated with 100 nM wortmannin and 30 mM LY294002, the effects of PDGF-BB were, respectively, 106 and 120% of the maximum response in control cells that were untreated with PI3-kinase inhibitor. Pretreatment of VSMCs with LY294002 up to 50 mM, a concentration that was previously reported to inhibit proliferation of rabbit and human aortic VSMCs [44], also had little effect upon PDGF-BB stimulation of FAK tyrosine phosphorylation (results not shown). Similar results were obtained using wortmannin and LY294002 purchased from two different sources.

Since stimulation of FAK tyrosine phosphorylation is
crucially dependent upon the integrity of the actin cytoskeleton [45], whether or not the actin filament-disrupting agent cytochalasin D had any inhibitory effect upon PDGF-BB stimulation of the PI3-kinase pathway in VSMCs was investigated. Pretreatment with 2 mM cytochalasin D, a selective inhibitor of actin polymerization, inhibited PDGF-BB stimulation of FAK and paxillin tyrosine phosphorylation and chemotaxis, and caused a marked disruption of the actin filament network, as shown by staining with FITC-phalloidin ([20] and results not shown]. This pretreatment had no effect on PDGF–BB stimulation of either p85a tyrosine phosphorylation or of PI3-kinase activity (Fig. 2). It was verified that, in parallel samples, wortmannin completely inhibited PDGF-BB-induced PI3-kinase activity.

Whether or not inhibitors of PI3-kinase had any effect on the chemotactic response of VSMCs to PDGF-BB was examined next. Cells were pretreated for 1 h with either wortmannin or LY294002, trypsinized and resuspended in medium containing freshly added inhibitor. Trypsinized and resuspended VSMCs were then used to assay directed cell migration in a chemotaxis chamber. As shown in Fig. 3, PDGF-BB caused a striking increase in the migration of VSMCs. Wortmannin at 50 nM did not significantly attenuate PDGF-BB stimulation of VSMC migration (Fig. 3A; \( P > 0.05 \)). Similarly, treatment with LY294002 at either 30 or 60 mM caused no significant decrease in the migratory response of rabbit VSMCs to PDGF-BB (Fig. 3B; \( P > 0.05 \)).

Since the chemotactic response to PDGF-BB was determined using freshly trypsinized cells and membranes coated with collagen, the effects of PI3-kinase inhibitors on FAK phosphorylation was examined accordingly under similar conditions. Cells were pretreated for 1 h with wortmannin, trypsinized and replated onto collagen in the presence of the same concentration of freshly added inhibitor and in the presence of PDGF-BB. Tyrosine phosphorylation of FAK and paxillin was then assessed after 6 h. As shown in Fig. 4, PDGF-BB stimulated PI3-kinase activity and FAK and paxillin tyrosine phosphorylation in freshly trypsinized cells that had been replated onto collagen. In parallel cells, wortmannin had little effect on PDGF-BB stimulation of FAK and paxillin tyrosine phosphorylation (Fig. 4B).

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**Fig. 3.** Effects of PI3-kinase inhibitors on PDGF-BB stimulation of VSMC chemotaxis. Subconfluent cultures of VSMCs were washed twice in DMEM and then treated with either wortmannin (A) or LY294002 (B) at the concentrations shown for 1 h. The cells were trypsinized and resuspended in serum-free DMEM containing 1% BSA. Migration assays were carried out in the chemotaxis chamber. Migration of VSMCs towards the chemoattractant PDGF-BB was measured either in the presence or absence of PI3-kinase inhibitors that were freshly added to the cell suspensions. Cells were allowed to migrate for 6 h at 37°C and migrated cells were counted as described in Section 2. The values shown represent the mean fold increases\( \pm \) SEM \((n = 5)\) in the number of migrated cells above the control. Analysis of the differences between the means showed that the small decreases in PDGF-BB-induced migration in the presence of wortmannin and LY294002 were not statistically significant \((P > 0.5)\).
Fig. 4. Effect of wortmannin on PDGF-BB-stimulated FAK and paxillin tyrosine phosphorylation in VSMCs that had been freshly replated onto collagen. Subconfluent cultures of VSMCs were pre-treated with PI3-kinase inhibitors (WT, wortmannin) for 1 h and then were trypsinized and plated onto collagen-coated plates in serum-free DMEM either in the presence or absence of PI3-kinase inhibitors and PDGF-BB (25 ng/ml). The cells were allowed to attach for 6 h at 37°C. The cells were lysed into 1× lysis buffer, immunoprecipitated with an anti-Tyr(P) mAb and immunoprecipitates were either assayed for PI3-kinase activity (A) or were resolved by SDS–PAGE and then Western blotted either with an antibody specific to FAK (B, top) or paxillin (B, bottom). The results shown are representative of three independent experiments. C, control untreated; P, 25 ng/ml PDGF-BB; P+WT, 25 ng/ml PDGF-BB + 100 nM wortmannin; Pax, paxillin.

Fig. 5. Effect of wortmannin on cell spreading and focal adhesion formation in freshly adherent VSMCs. Subconfluent cultures of VSMCs were pre-treated with 1 mM wortmannin and then were trypsinized and replated in serum-free DMEM onto collagen-coated coverslips in the presence of 25 ng/ml PDGF-BB and in the presence (PDGF-BB+WT) or absence (PDGF-BB) of 1 mM wortmannin. The cells were allowed to adhere for 2 h and then they were fixed in 3% (w/v) paraformaldehyde. Focal adhesions in the cells were visualised by immunofluorescent staining of the cells with specific antibodies (2 μg/ml) to paxillin, vinculin or anti-Tyr(P) (mAb 4G10), as described in Section 2. The photographs shown were taken using an X63 (N.A. 1.4, oil) objective lens. Size bar, 20 μm.
To test the possibility that PI3-kinase inhibitors could interfere with focal adhesion formation in freshly adherent cells, VSMCs were trypsinized and replated onto collagen in the presence of PDGF-BB with and without the addition of wortmannin. Focal adhesion formation was monitored by immunofluorescent staining of paxillin, vinculin and anti-Tyr(P). As shown in Fig. 5, neither cell spreading nor the overall pattern of paxillin, vinculin and anti-Tyr(P) immunofluorescent staining of focal adhesions in freshly adherent VSMCs were markedly affected by treatment with 1 mM wortmannin. FAK immunofluorescent staining for focal adhesions in freshly adherent VSMCs was weak, but was also not significantly altered by wortmannin at 1 mM (not shown).

Fig. 6. Effect of PD98059 on PDGF-BB stimulation of FAK tyrosine phosphorylation and MAP kinase activity. (A) VSMCs were pretreated for the times indicated with 25 ng/ml PDGF-BB, whole cell extracts were then prepared and, following SDS–PAGE analysis, were immunoblotted either with an antibody that specifically recognizes the activated form of p42/p44 MAP kinases (upper, pY-MAPK), or with an antibody that recognizes total (i.e. active plus inactive) p42/p44 MAP kinases (lower, MAPK). The results shown are representative of more than 20 independent experiments. (B) Confluent and quiescent cultures of VSMCs were pre-treated with PD98059 at the concentrations shown for 1 h and were subsequently treated with (+) or without (−) 25 ng/ml PDGF-BB for 10 min. Extracts were then prepared and, following SDS–PAGE analysis, were immunoblotted with an antibody that specifically recognizes the activated form of p42/p44 MAP kinases. Cells that were not pretreated with PD98059 received an equivalent volume of the solvent dimethylsulphoxide (DMSO). The result shown is representative of four independent experiments. (C and D) Confluent and quiescent cultures of either VSMCs (C) or Swiss 3T3 cells (D) were pre-treated with PD98059 at the concentrations shown for 1 h and were subsequently treated either with (+) or without (−) 25 ng/ml PDGF-BB (C) or with the indicated concentrations of PDGF-BB, in ng/ml (D), for 10 min. Cells were lysed, immunoprecipitated with an anti-Tyr(P) mAb and the immunoprecipitates were Western blotted using an antibody specific to FAK. Cells that were not pretreated with PD98059 received an equivalent volume of the solvent DMSO. The results shown in (C) and (D) are representative of three and two independent experiments, respectively.
PDGF-BB caused a striking activation of p42/p44 MAP kinases corresponding to ERK1 and ERK2 in VSMCs, which reached a maximum after 2–15 min and subsequently declined, reaching a lower level within 1 h that was sustained for up to 4 h (Fig. 6A). Western blotting of parallel samples with an antibody that recognizes both active and inactive forms of ERK1 and ERK2 showed that all cell extracts contained equivalent amounts of MAP kinases (Fig. 6A). PD98059, a specific inhibitor of MAP kinase kinase [46], caused a concentration-dependent inhibition of PDGF-BB-induced activation of ERK1 and ERK2 with a half-maximal inhibition at 20 mM and a maximum inhibitory effect at 40 mM (Fig. 6B). Pretreatment with PD98059 at 25 mM (Fig. 6C) or at 40 mM (results not shown) had no inhibitory effect on PDGF-BB stimulation of tyrosine phosphorylation of FAK. Pretreatment with PD98059 at 10 or 50 mM also had no inhibitory effect on PDGF-BB stimulation of biphasic FAK tyrosine phosphorylation [47] in Swiss 3T3 cells (Fig. 6D). It was noted in some experiments that PD98059 caused an apparent enhancement of FAK tyrosine phosphorylation (Fig. 6C–D). In contrast, pretreatment with PD98059 at either 5, 10 or 30 mM reduced PDGF-BB-stimulated VSMC migration to 77, 65 and 54% of the control.

Fig. 7. Effect of PD98059 on PDGF-BB stimulation of VSMC chemotaxis. Subconfluent cultures of VSMCs were pre-treated with PD98059 at the concentrations indicated for 1 h and then were trypsinized. Migration of VSMCs towards PDGF-BB (25 ng/ml) was measured either in the presence or absence of PD98059, which had been freshly added to the cell suspension at the same concentration as in the pre-treatment. Control and PDGF-BB-treated cells that were not treated with PD98059 received an equivalent volume of the solvent, DMSO. Other experimental details were as described in Section 2. The values shown represent the mean fold increases±SEM (n=4) in the number of migrated cells above the control. Analysis of the difference between the means showed that the inhibitory effects of 5, 10 and 30 μM PD98059 were statistically significant (P<0.001 at 5 μM and <0.00001 at 10 and 30 μM).

Fig. 8. IGF-I stimulation of FAK tyrosine phosphorylation and PI3-kinase effect on PDGF-BB stimulation of biphasic FAK tyrosine activity in VSMCs. Confluent, quiescent cultures of VSMCs were treated for 10 min with IGF-I at the concentrations indicated. Anti-Tyr(P) immunoprecipitates were prepared and were either used for assay of PI3-kinase activity (panel A) or immunoblotted with anti-Tyr(P) (panel B). The positions of PIP and the 97 kDa subunit of the IGF-I receptor (IGF-IR) are indicated on the right, and the positions of molecular weight markers (×10^{-6}) are indicated on the left. The results shown are representative of three–five independent experiments.
stimulated level, respectively (Fig. 7). Inhibition of PDGF-BB-induced migration was highly statistically significant at all concentrations of PD98059 tested ($P < 0.00001$).

It was next investigated whether PI3-kinase or p42/p44 MAP kinases were induced by IGF-I in VSMCs and could therefore play any role in the chemotactic response to this factor. Confluent cultures of rabbit aortic cells were treated with IGF-I, anti-Tyr(P) immunoprecipitates were prepared and PI3-kinase assays were preformed. As shown in Fig. 8A, IGF-I stimulated PI3-kinase activity in a concentration-dependent manner with a detectable increase at 2.5 ng/ml, and a maximum increase at 10 ng/ml, which was sustained at a concentration up to 100 ng/ml. The effect of IGF-I on PI3-kinase activity was markedly less than that of PDGF-BB measured in parallel cells and in the same assay. Anti-Tyr(P) Western blotting of anti-Tyr(P) immunoprecipitates showed that, within a similar concentration range, IGF-I induced a striking increase in the tyrosine phosphorylation of a prominent band of $M_r 97,000$, which corresponds closely with the previously reported mobility of the IGF-I receptor $\beta$-subunit (Fig. 8B).

IGF-I caused a marked increase in the activity of p42 and p44 MAP kinases, as judged by Western blot analysis using either an antibody that recognizes tyrosine phosphorylated activated ERKs 1 and 2 or an antibody recognizing dually phosphorylated activated MAP kinases (Fig. 9). The effect of IGF-I was significantly less than that obtained in response to 25 ng/ml PDGF-BB, obtained in parallel cells. Semi-quantification of activated MAP kinase immunoreactive bands showed that, in five independent experiments, IGF-I-induced MAP kinase activity was 40–50% of the effect of PDGF-BB. Activation of MAP kinases by IGF-I was completely abolished by pretreatment with 10 mM PD98059 (Fig. 9). Neither insulin nor IGF-II at concentrations up to 100 ng/ml had any detectable effect on activity of p42/p44 MAP kinases (results not shown). IGF-I-stimulated activation of p42/p44 MAP

![Image](https://example.com/image.png)

**Fig. 9.** IGF-I activates p42/p44 MAP kinase in rabbit VSMCs. (A) Confluent, quiescent cultures of VSMCs were either untreated (C), or were treated for 10 min with 25 ng/ml IGF-I, as indicated (I), or were treated for 10 min with 25 ng/ml PDGF-BB (P). Some cells were pretreated for 60 min with PD98059 at the concentrations indicated or at 10 $\mu$M prior to the addition of IGF-I (I+PD). Extracts were prepared and immunoblotted with antibodies that specifically recognize either the tyrosine phosphorylated (pY-MAPK) or dual threonine/tyrosine phosphorylated (pY/T-MAPK) forms of p42/p44 MAP kinases. The positions of p42/p44 MAP kinases are indicated on the right. The results shown are representative of four separate experiments. (B) Confluent, quiescent cultures of VSMCs were either untreated (C), or were treated for either 15 or 60 min with the indicated concentrations of IGF-I, or were treated for 10 min with 25 ng/ml PDGF-BB (P). The results shown are representative of two separate experiments. (C) VSMCs were treated with 25 ng/ml IGF-I for the times indicated or were treated for 5 min with 25 ng/ml PDGF-BB (P). The results shown are representative of three separate experiments.
kinases after a 15 min treatment was detectable as concentrations as low as 5 ng/ml, reaching half-maximal stimulation at 10 ng/ml and a maximum at 50 ng/ml. After 60 min, PDGF-BB-stimulated MAP kinase activation was still clearly evident, but IGF-I failed to cause a detectable increase in activity at concentrations up to 50 ng/ml (Fig. 9). The time-course for MAP kinase activation induced by IGF-I showed that, in contrast to PDGF-BB stimulation of p42/p44 MAP kinases, IGF-I-induced activation was slower, with a detectable increase as early as 10 min and a maximum effect at between 15–20 min after addition of the factor; IGF-I-induced activation declined after 20 min, reaching the basal unstimulated level of activity by 45 min after the addition of IGF-I.

IGF-I stimulated migration of rabbit aortic VSMCs in a concentration-dependent manner (Fig. 10A). A half-maximal stimulation was obtained at approximately 1.5 ng/ml and a maximum increase in migration was achieved at 3–10 ng/ml. In experiments in which the migratory responses to IGF-I and PDGF-BB were directly compared, the mean maximum chemotactic effect of IGF-I was 40% (n=4) that of the response to PDGF-BB (Fig. 10B). Pretreatment with PD98059 caused a significant inhibition of directed cell migration induced by 25 ng/ml IGF-I (P<0.016; n=8, Table 1). Pretreatment of VSMCs with wortmannin also caused a statistically significant decrease in IGF-I-stimulated VSMC chemotaxis (P<0.045; n=8).

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<th>Addition</th>
<th>Cell migration, mean fold increase in number of migrated cells±SEM</th>
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</tr>
<tr>
<td>IGF-I</td>
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<td>IGF-I+PD98059</td>
<td>1.76±0.19</td>
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<tr>
<td>IGF-I+wortmannin</td>
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VSMCs were pretreated with either 10 μM PD98059 or 50 nM wortmannin for 1 h, trypsinized and resuspended in serum-free medium containing the same concentrations of PD98059 and wortmannin. Resuspended cells were then used in assays of chemotaxis towards 25 ng/ml IGF-I, as described in Section 2. In each independent experiment, migrated cells were counted in quadruplet wells. The mean fold increases in the numbers of migrated cells above control were determined in eight independent experiments. Statistical analysis of the difference between means was performed using one-way ANOVA. P<0.05 was considered to be significant. P<0.0004 (n=8) for the difference in the mean numbers of migrated cells in control and IGF-I-stimulated cells. P<0.016 (n=8) for the difference in the mean numbers of migrated cells in cells treated with IGF-I with and without addition of PD98059. P<0.045 (n=8) for the difference in the mean numbers of migrated cells in cells treated with IGF-I with and without the addition of wortmannin.

4. Discussion

Previous findings have suggested that PI3-kinase plays a key role in the cell migration induced by the potent...
chemoattractant PDGF-BB [32,33], and that this pathway may mediate PDGF stimulation of FAK and paxillin tyrosine phosphorylation in the murine Swiss 3T3 cell line [37]. In the present paper, we report that, in primary cultures of rabbit aortic VSMCs, stimulation of FAK tyrosine phosphorylation and of cell migration by PDGF-BB, can be at least partially dissociated from PI3-kinase activation. This conclusion is based on several lines of evidence. Two different selective inhibitors of PI3-kinase failed to inhibit PDGF-BB stimulation of FAK and paxillin tyrosine phosphorylation at concentrations of the inhibitors that completely blocked PDGF-BB-induced activation of PI3-kinase. Conversely, inhibition of FAK tyrosine phosphorylation by the actin filament-disrupting agent cytochalasin D had no effect on PDGF-BB stimulation of PI3-kinase activity. Neither PI3-kinase inhibitor blocked the migratory response of VSMCs to PDGF-BB at concentrations that maximally inhibit PI3-kinase. Finally, PI3-kinase inhibitors had little or no effect on the formation of focal adhesions in freshly adherent VSMCs.

Our results contrast with the finding of Rankin et al. [37] that PI3-kinase was required for PDGF-stimulated FAK tyrosine phosphorylation in Swiss 3T3 cells. We have previously shown that FAK is differentially regulated by PDGF in rabbit aortic VSMCs and in Swiss 3T3 cells [20]. It is plausible therefore that differential effects of PI3-kinase inhibitors in the two cell types may reflect differences in the signalling pathways mediating activation of the FAK pathway. We do not preclude that PI3-kinase may make some contribution to the FAK pathway in VSMCs. For example, although our results suggested that PI3-kinase inhibitors did not affect the immunolocalization of paxillin and FAK in VSMCs, it is entirely plausible that inhibition of PI3-kinase may cause a more subtle alteration in the cellular distribution of these components in focal contacts and/or affect the rate at which they attach and disengage from focal adhesions.

Recent studies have indicated a link between the MAP kinase and integrin signalling pathways [48–50] and this prompted us to assess the role of the MAP kinase pathway in FAK phosphorylation and migratory responses in VSMCs. The results show that FAK tyrosine phosphorylation induced by PDGF-BB is not inhibited by a selective inhibitor of the MAP kinase pathway, either in rabbit VSMCs or in Swiss 3T3 cells, indicating that regulation of the FAK pathway can be dissociated from activation of ERKs 1 and 2. This finding is consistent with the report that dominant negative Ras mutants block integrin-mediated MAP kinase activation without inhibiting FAK tyrosine phosphorylation or focal adhesion formation [50], with our recent finding that the MAP kinase inhibitor does not affect vascular endothelial growth factor-stimulated FAK tyrosine phosphorylation in endothelial cells [24] and with a recent study showing that abrogation of FAK tyrosine phosphorylation in Swiss 3T3 cells using either cytochalasin D or cell detachment was not accompanied by any attenuation of MAP kinase activation [51].

We also investigated the role of PI3-kinase and MAP kinase in signalling events induced by IGF-I. The results showed that IGF-I, relative to PDGF-BB, was a less effective chemotactic factor and activated both PI3-kinase and p42/p44 MAP kinases more weakly in rabbit aortic VSMCs. The results herein also showed that inhibition of both MAP kinase and PI3-kinase activation reduced the chemotactic response to IGF-I. This result suggests that, in contrast to PDGF-BB, the migratory response to IGF-I is dependent upon both MAP kinase and PI3-kinase pathways. It is plausible that the weaker chemotactic effect of IGF-I is dependent upon the cooperative effects of both of these signalling pathways. The effects of IGF-I on FAK tyrosine phosphorylation in VSMCs were not assessed in the present paper. Previous findings have shown that insulin causes dephosphorylation of FAK [52,53], while IGF-I stimulates tyrosine phosphorylation and lamellipodial immunolocalization of FAK in neuronal cells [16]. Our preliminary data shows that IGF-I produces a modest but variable increase in FAK tyrosine phosphorylation and has little detectable effect on paxillin tyrosine phosphorylation in rabbit aortic VSMCs (H. Abedi and I. Zachary, unpublished data). A weaker effect of IGF-I on the FAK/paxillin tyrosine phosphorylation pathway would also be consistent with the weaker chemotactic effect of IGF-I. The role of this pathway in the IGF-I chemotactic response is currently the subject of further investigation.

A salient feature of our results is that inhibition of p42/p44 MAP kinases by PD98059 markedly reduced the chemotactic response of rabbit aortic VSMCs to PDGF-BB. Previous findings have suggested that MAP kinases do not mediate VSMC migration in response to either PDGF-BB or IGF-I [14]. On the basis of these and other findings, it has been proposed that mitogenic MAP kinase signalling pathways propagated by receptor protein tyrosine kinase receptors may be functionally segregated from chemotactic signalling and that compartmentalization of distinct signalling pathways helps to determine the commitment of cells either to proliferation or migration [54]. The finding that inhibition of MAP kinase reduced VSMC migration in response to PDGF-BB suggests that MAP kinase activation does contribute to chemotaxis. This conclusion is further strengthened by our finding that IGF-I activated ERKs 1 and 2 and that inhibition of the MAP kinase pathway also partially blocked IGF-I-induced migration. Although IGF-I was a weaker activator of MAP kinase than PDGF-BB, the relative strength of this signalling response would be consistent with the weaker chemotactic response to this factor. Since the MAP kinase inhibitor only partially inhibited VSMC migration, we conclude that the MAP kinase cascade is probably not obligatory for cell migration, even when this biological response is induced by a potent activator of MAP kinase, such as PDGF-BB. Taken together, these findings nevertheless suggest that MAP
kinase activation may be a point of integration between mitogenic and chemotactic signalling networks. Compartmentalization and specificity of chemotactic versus mitogenic signalling may be determined by other signalling pathways [54]. In agreement with the present findings, it was recently reported that either PD98059 or anti-sense oligonucleotides to ERK-1 and ERK-2 markedly inhibited the chemotactic response of rat aortic VSMCs to PDGF-BB [55].

In summary, the findings presented here indicate that PDGF-BB stimulation of the FAK tyrosine phosphorylation pathway in rabbit aortic VSMCs can be dissociated from the activation of both the PI3-kinase and MAP kinase pathways. These results have implications for the signal transduction mechanisms that mediate FAK activation through the PDGF β-receptor and suggest that, at least in VSMCs, PI3-kinase may not be an obligatory pathway for chemotaxis. The divergence between the role of PI3-kinase in PDGF regulation of FAK tyrosine phosphorylation in VSMCs and in Swiss 3T3 cells further highlights cell type-specificity in the regulation of this pathway. Other signalling molecules, particularly small GTP-binding proteins of the rho/rac family, are implicated in mediating FAK tyrosine phosphorylation, but the role of these pathways in PDGF-BB regulation of FAK is as yet unclear. Elucidation of the role of these transduction pathways is likely to yield important insights into the mechanisms underlying the migratory response of VSMCs.

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