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Biochemical Pharmacology 67 (2004) 1215–1225
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Pancreatic stellate cell migration: role of the phosphatidylinositol 3-kinase (PI3-kinase) pathway

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Received 29 April 2003; accepted 18 November 2003

Abstract

Pancreatic stellate cells (PSCs) are implicated as key mediators of pancreatic fibrogenesis and are found in increased numbers in areas of pancreatic injury. This increase in number may be due to increased local proliferation and/or migration of PSCs to affected areas from surrounding tissue. We have recently shown that PSCs can migrate and that this migration is stimulated by PDGF in a predominantly chemotactic manner [Gut 52 (2003) 677]. However, the signalling mechanisms responsible for PDGF-induced PSC migration are not known. *Aims*: (i) To determine whether PDGF-induced PSC migration is mediated by the PI3-kinase pathway. (ii) To investigate whether cell migration is influenced by cell proliferation and whether an interaction exists between the PI3-kinase pathway and the ERK1/2 pathway (known to mediate cell proliferation) in PSCs exposed to PDGF. *Methods*: (i) PI3-kinase activity was assessed by measuring the activation (phosphorylation) of its downstream substrate Akt in rat PSCs incubated with PDGF (10 ng/mL) for 5 min, 15 min, 60 min, and 24 hr in the presence or absence of the specific PI3-kinase inhibitor wortmannin. (ii) The role of the PI3-kinase pathway in PSC migration was examined by assessing PSC migration through a porous membrane after exposure to PDGF in the presence and absence of wortmannin for 24 hr. (iii) The relationship between migration and proliferation was assessed by examining migration of PSCs exposed to PDGF in the presence and absence of mitomycin C, an inhibitor of cell proliferation. (iv) The interaction between PI3-kinase and ERK1/2 was examined by incubation of PSCs with PDGF in the presence and absence of wortmannin, followed by assessment of ERK1/2 activation by western blot. *Results*: PDGF increased Akt activation in PSCs as early as at 5 min of incubation and this increase was sustained for 24 hr. Inhibition of PI3-kinase by wortmannin decreased basal as well as PDGF-induced migration and also inhibited ERK1/2 activation. Inhibition of PSC proliferation with mitomycin C significantly reduced (but did not abolish) basal and PDGF-induced PSC migration. *Conclusions*: (i) The PI3-kinase pathway is induced in PSCs after exposure to PDGF and this induction is sustained for at least 24 hr. (ii) The PI3-kinase pathway plays a role in PDGF-induced PSC migration and is partially involved in mediating ERK1/2 activation. (iii) PSC migration is dependent, at least in part, on cell proliferation.

Keywords: Pancreatic stellate cells; Migration; Platelet derived growth factor; PI3-kinase; Extracellular-signal regulated kinase 1/2; Wortmannin

1. Introduction

Pancreatic fibrosis is a key histopathological feature of chronic pancreatitis and of pancreatic cancer. It is now generally accepted that fibrosis is not merely an end point of chronic injury, but an active process that may be reversible in its early stages. Therefore, the pathogenesis of pancreatic fibrosis has received increasing attention in recent years. Considerable progress has been made with respect to insights into pancreatic fibrogenesis, particularly

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Abbreviations: αSMA, alpha smooth muscle actin; BrdU, 5-bromo-2′-deoxyuridine; DAB, di-aminobenzidine; ECM, extracellular matrix; ERK1/2, extracellular-signal regulated kinases 1/2; HSCs, hepatic stellate cells; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PSCs, pancreatic stellate cells; PI3-kinase, phosphatidylinositol 3-kinase.

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doi:10.1016/j.bcp.2003.11.013

following the recent identification and characterisation of a specific cell type in the pancreas—the pancreatic stellate cell—now thought to be a major effector cell in the fibrogenic process.

Recent studies have demonstrated that PSCs become activated (as indicated by proliferation, increased expression of the cytoskeletal protein αSMA and synthesis of ECM proteins) when exposed to factors known to be up regulated during pancreatic injury such as platelet-derived growth factor (PDGF), proinflammatory cytokines and oxidant stress [2–5]. Increased numbers of PSCs have been demonstrated in fibrotic areas of pancreas in tissue sections from patients with chronic pancreatitis and from experimental models of pancreatic fibrosis [6–8]. This increase in PSC number may be a result of increased local proliferation and/or migration of cells to affected areas from surrounding tissue. The ability of PSCs to proliferate when activated by factors such as PDGF and proinflammatory cytokines is well documented [2,3,9]. We have now established that rat PSCs are also capable of migration and that this migration is stimulated by PDGF in a predominantly chemotactic manner [1].

Cell signalling pathways mediating PDGF-induced responses such as migration and proliferation have been characterised recently using vascular smooth muscle cells and meningioma cells [10,11]. It has been demonstrated that binding of PDGF to its receptor leads to receptor dimerisation accompanied by activation of intrinsic tyrosine kinase activity and receptor autophosphorylation [12,13]. The phosphorylated sites on the PDGF receptor act as high affinity binding sites for several enzymes that are involved in signal transmission [14–18]. One of the enzymes that binds to these sites is phosphatidylinositol 3-kinase (PI3-kinase). Upon activation, PI3-kinase phosphorylates phosphatidylinositol to produce several phosphoinositols, leading to Akt activation [14–18]. Akt, a serine/threonine protein kinase, is a core component of the PI3-kinase signalling pathway and has been shown to be a powerful promoter of cell survival and may also play a role in mediating other cellular functions such as cell migration [19].

Another signalling pathway that is activated by the binding of PDGF to its receptor is the extracellular-signal regulated kinase (ERK1/2) pathway [15,20,21]. ERK belongs to the family of mitogen-activated protein kinases (MAPK). Two isoforms have been identified and are referred to as ERK1 (or p44 MAPK) and ERK2 (or p42 MAPK). Upon translocation to the nucleus, ERK1/2 mediates a diverse array of fundamental cellular processes by phosphorylating a variety of transcription factors [22]. Interestingly, studies with hepatic stellate cells (HSCs) and swiss 3T3 cells have indicated that PI3-kinase may contribute to the activation of ERK1/2 when exposed to PDGF [23,24].

PSC proliferation in response to PDGF has been reported to be mediated by the ERK1/2 pathway [9]. However, the cell signalling pathway responsible for PDGF-induced migration of PSCs is not known. Therefore, the aims of this study were (i) to examine the effect of PDGF on the PI3-kinase signalling pathway in PSCs; (ii) to determine whether the PI3-kinase pathway plays a role in mediating PDGF-induced PSC migration; and (iii) to assess whether PI3-kinase contributes to the activation of ERK1/2 in PSCs exposed to PDGF.

2. Materials

All general chemicals were of analytical reagent grade and were purchased from the Sigma Chemical Company. Collagenase P was purchased from Roche. Protease Type XIV (from *Streptomyces griseus*) was obtained from the Sigma Chemical Company. DNase was purchased from Pharmacia Biotech. Nycodenz was obtained from Nycomed Pharma. Cell culture reagents were purchased from Gibco Invitrogen Corporation. Cell culture inserts with porous membranes and companion culture plates were purchased from Becton-Dickinson. Human recombinant PDGF-BB, wortmannin, mitomycin C, diaminobenzidine (DAB), and 5-bromo-2′deoxyuridine (BrdU) were purchased from the Sigma Chemical Company. Antibodies for phospho-Akt (Ser473), phospho-ERK1/2 (Thr202/tyr204), total Akt, and total ERK1/2 were purchased from Cell Signaling. A monoclonal mouse anti-5-bromo-2′-deoxyuridine (BrdU) antibody and a secondary goat-anti-rabbit antibody was purchased from DAKO Corporation. The avidin-biotin-peroxidase complex (ABC Kit) was purchased from Vector Laboratories.

3. Methods

3.1. Isolation and culture of PSCs

Rat pancreatic stellate cells were isolated by density gradient centrifugation as detailed previously [25]. This technique yields a preparation of PSCs that is devoid of contamination by endothelial cells or macrophages, as evidenced by negative staining for the markers factor VIII and ED1, respectively [6]. Freshly isolated stellate cells were cultured in 25 cm^2 plastic culture flasks containing Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% foetal bovine serum (FBS) at 37° in humidified 95% air/5% CO_2 atmosphere. At confluence, cells were harvested, counted and replated at equal seeding densities for use in the experiments described below. All experiments were performed with culture-activated cells (passages 1–3).

3.2. Assessment of PI3-kinase activity in PSCs

PI3-kinase activity in PSCs was assessed by measuring the phosphorylation of its downstream target Akt.

Cells were cultured in 100 mm petri dishes until the monolayers were 80–85% confluent. Incubations were performed in serum-reduced medium containing 0.05% foetal bovine serum (0.05% medium) to avoid any confounding effects of serum when assessing the effects of PDGF on PI3-kinase activity. Cells were then exposed to PDGF (10 ng/mL) for 5 min, 15 min, 60 min, and 24 hr. Cells incubated with 0.05% medium alone for 24 hr served as controls. To measure activated Akt, PSCs were washed with ice-cold PBS (pH 7.4) and lysed in ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM PMSF; pH 7.5) for 45 min with gentle agitation at 4°. Samples were then sonicated on ice and centrifuged at 1400 *g* for 10 min at 4° to remove cell debris. The supernatant was harvested and stored at −80° until analysis. Protein levels in cell lysates were determined using a Pierce BCA assay following the manufacturer's instructions (Pierce Endogen). Equal amounts of cell lysate protein (20 μg) were subjected to gel electrophoresis using a 10% (w/v) SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane using a commercial blotting apparatus (Biorad). The membrane was incubated with the primary antibody (polyclonal rabbit anti-phospho-Akt specific (Ser473) antibody, 1:1000) overnight at 4°. This was followed by incubation with the secondary antibody (goat anti-rabbit antibody, 1:2000) for 1 hr. Activated phospho-Akt was detected by the enhanced chemiluminescence (ECL) technique using the Amersham ECL kit, and quantified using densitometry (BioRad GelDoc Image One software). The blots were then stripped and re-probed for total Akt expression with rabbit polyclonal anti-Akt antibody (1:1000), which recognises both the phosphorylated and nonphosphorylated forms of the enzyme and used as a measure of equal protein loading.

3.3. PSC migration

Migration studies were performed using cell culture inserts placed in 12-well culture plates. The base of the inserts consisted of an uncoated, porous membrane (pore size 8 μm, membrane diameter 10.5 mm). PSCs suspended in culture medium containing 10% serum (10% medium) were placed in the inserts (30,000 cells per insert) and the inserts, in turn, were placed in culture wells and incubated for 24 hr in the presence of various agents. Cells incubated with culture medium alone served as the controls. At the end of the incubation period, culture medium was removed and the cells adherent to the membrane were fixed in 100% methanol and subjected to Giemsa staining [26]. Briefly, membranes treated with 100% methanol for 20 min were air dried and stained for 5 min in May Grunwald's solution. This was followed by immersion of the membranes in dilute Giemsa solution for 25 min and washing with buffered distilled water (pH 6.8) for a further 60 s. Membranes were then rinsed in tap water for 60 s, mounted on glass slides, covered with glass cover slips and examined by light microscopy. For each membrane, at least five noncontiguous randomly selected microscopic fields were examined using an Olympus BX60 microscope and a 40× objective. By changing the focus of the objective, images (magnification 400×) of Giemsa stained cells on the upper surface and the undersurface of the membrane in the same microscopic field were captured with a Spot Cooled Color Digital camera that had been calibrated with a reference measurement slide (Diagnostic Instruments). The narrow depth of field of the 40× objective readily permitted differentiation between the upper and undersurface of the membranes. Once all the images were acquired, the number of cells on the upper surface and undersurface of the membrane in the same microscopic field were counted and the cell number per millimeter square of the membrane was calculated. The rate of migration of PSCs was expressed as a migration index (%): (number of cells on undersurface of membrane divided by total number of cells on both surfaces of the membrane) × 100.

3.4. Effect of wortmannin on PDGF-induced PI3-kinase activity/PSC migration

3.4.1. PI3-kinase activity

To assess the effect of the specific PI3-kinase inhibitor wortmannin (a fungal metabolite which binds to PI3-kinase and noncompetitively inhibits its activity) on PI3-kinase activity, cells were plated at equal seeding densities into 100 mm petri dishes until they were 80–85% confluent. The cells were incubated in serum-reduced medium (0.05% medium) for 24 hr, and then preincubated with 100 nM wortmannin for 60 min in 0.05% medium. The concentration of wortmannin (100 nM) selected for use in this study was based on published work [35] as well as on the results of our initial studies (N = 3) using a range of concentrations of wortmannin (50–200 nM). These studies identified 100 nM as the optimum concentration for inhibition of migration. Although wortmannin at both 50 and 100 nM inhibited migration the effect with 100 nM was more consistent. The higher concentration of 200 nM did not have any additional effect on migration. This was followed by a further incubation for 60 min with PDGF (10 ng/mL) in the continued presence of wortmannin. Cells incubated with 0.05% medium with an equivalent amount of vehicle (0.0043% DMSO) served as controls. To ensure that assessment of PI3-kinase activity and PSC migration were performed under comparable culture conditions, the above experiments were also performed in serum supplemented medium (10% medium). Trypan blue staining was used to assess cell viability. Cell lysates were collected as described earlier and equal amounts of protein (20 μg)

were separated by gel electrophoresis using a 10% (w/v) SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Activation of Akt was assessed using the protocol described previously.

3.4.2. PSC migration

To assess the effect of wortmannin on PSC migration, cells were seeded into culture inserts (30,000 per well) containing 10% medium, allowed to settle and then preincubated with wortmannin (100 nM) or the vehicle (DMSO) for 1 hr. The cells were then incubated for 24 hr in the presence of PDGF (10 ng/mL), with or without wortmannin (100 nM), and the migration index calculated as described above.

3.5. Assessment of ERK1/2 activation in PSCs

A previous study by Jaster *et al*. [9] demonstrated that PDGF-induced PSC proliferation is mediated via the ERK1/2 pathway. In this study, we examined whether PI3-kinase may play a role in mediating PDGF-induced ERK1/2 kinase activation in PSCs. Rat PSCs were exposed to PDGF in the presence or absence of wortmannin and ERK1/2 activation assessed by western blotting. Briefly, cells were incubated in serum-reduced medium (0.05% medium) for 24 hr, and then treated with 100 nM wortmannin for 60 min. This was then followed by a further incubation for 60 min with PDGF (10 ng/mL). Cells incubated with 0.05% medium with an equivalent amount of vehicle (0.0043% DMSO) served as controls. The above experiments were also performed in serum-supplemented medium (10% medium). Cell lysates were collected as described above and equal amounts of protein (10 μg) were separated by gel electrophoresis using a 10% (w/v) SDS–polyacrylamide gel and transferred to nitrocellulose membrane. The nitrocellulose membrane was incubated with the primary antibody (polyclonal rabbit anti-active ERK1/2 kinase, 1:1000), which detects the dually phosphorylated amino acid residues corresponding to Thr202 and Tyr204 of ERK1/2 kinase. This was followed by incubation with a secondary antibody (goat anti-rabbit antibody, 1:2000) for 1 hr. Activated ERK1/2 was detected using ECL and quantified using densitometry as described above. To confirm equal protein loading, blots were stripped and reprobed for total ERK1/2 expression with a rabbit polyclonal anti-ERK1/2 kinase antibody (1:1000), which recognises both the phosphorylated and nonphosphorylated forms of the enzyme.

3.6. Effect of wortmannin on PDGF-induced PSC proliferation

To assess the effect of wortmannin on PDGF-induced cell proliferation, the rate of cell proliferation was assessed by measuring the incorporation of [^{3}H]thymidine 3×10^3 cells per well) in 10% medium were preincubated with wortmannin (100 nM) or the vehicle (0.0043% DMSO) for 1 hr. The cells were then incubated for 24 hr in the presence of PDGF (10 ng/mL), with or without wortmannin (100 nM). For the last 16 hr of the incubation, cells were pulsed with [^{3}H]thymidine at a concentration of 1 μCi per well. At the end of the incubation period, the medium was aspirated and the cells were washed twice with Hank's balanced salt solution. Ice cold 10% trichloroacetic acid (TCA) was then added to the wells, and the cells were incubated for 10 min on ice. This was followed by a further two incubations with 5% TCA for 10 min on ice. Cells were solubilised in 1 M NaOH and radioactivity was measured using a liquid scintillation counter.

3.7. Effect of mitomycin C on PDGF-induced PSC migration

To assess whether PSC migration is influenced by cell proliferation, PSCs were incubated with mitomycin C, a compound known to inhibit cell proliferation via inhibition of DNA synthesis. Mitomycin C covalently forms cross-links between complementary strands of DNA, thereby preventing their separation and inhibiting DNA replication [28,29]. PSCs were passaged and seeded at 30,000 cells per well onto culture inserts in 10% medium. The cells were allowed to settle for 20 min and then treated with 10 μg/mL of mitomycin C for 60 min. This was then followed by a further incubation for 24 hr with or without PDGF (10 ng/mL). Cells incubated with 10% medium alone served as controls. Trypan blue exclusion studies were performed to assess cell viability. At the end of the incubation period cells were stained with Giemsa as described above and the migration index calculated. The effect of mitomycin C on cell proliferation was assessed using BrdU incorporation [27]. This method involves labeling of cell nuclei with BrdU. Briefly, PSCs were incubated in 10% culture medium containing 5×10^{-5} M BrdU for 16 hr. Cells were then washed in cold phosphate-buffered saline (PBS) and fixed in ethanol:acetic acid (95:5, v/v) for 30 min at 37°. The DNA from fixed cells was denatured using formamide dissolved in 0.8 M NaCl, 20 mM Tris–HCl (pH 8.0) for 45 min at 70°. Cells were then washed and incubated with a monoclonal antibody for BrdU (1:100) for 1 hr at room temperature followed by incubation with a biotinylated secondary antibody (1:100) for 1 hr at room temperature. After washing in PBS, cells were incubated with an avidin-biotin-peroxidase complex. Immunocomplexes were visualised by diaminobenzidine (DAB) staining. Five randomly selected fields were examined and the number of BrdU positive cells on the upper surface and undersurface of the membrane in these fields was counted. Data are expressed as the proportion of BrdU positive cells on each side of the membrane.

In addition, to determine whether mitomycin C changes any of the characteristics of culture-activated PSCs, cell

morphology was assessed by phase contrast microscopy and αSMA expression examined by western blotting of cell lysates using a monoclonal mouse antibody to αSMA as described in our previous study [4].

3.8. Statistical analysis

Results are expressed as means ± SEM for three to five separate cell preparations per experimental protocol. Data were analysed using analysis of variance (ANOVA). Fisher's protected least significant difference was used for comparison of individual groups provided the F test was significant. Where indicated, data were analysed by Student's paired t-test and $P < 0.05$ was considered significant.

4. Results

4.1. Effect of PDGF on the PI3-kinase pathway

Incubation of PSCs with PDGF (10 ng/mL) caused a significant increase in Akt activation at 5 min, 15 min, and 60 min ($P < 0.001$, N = 4 separate cell preparations; Fig. 1). After 24 hr, the level of Akt activation decreased although it remained higher than control levels ($P < 0.01$, N = 4 separate cell preparations; Fig. 1). Re-probing of the membranes with an antibody against total Akt demonstrated that total Akt expression was unchanged by PDGF treatment, indicating that PDGF had a specific effect on Akt phosphorylation.

4.2. Effect of wortmannin on PDGF-induced PI3-kinase activity

Incubation of PSCs with the specific PI3-kinase inhibitor wortmannin (100 nM) inhibited the PDGF-induced increase in Akt activation by 95.00 ± 3.05% ($P < 0.001$, N = 4 separate cell preparations; Fig. 2A). To ensure that assessment of PI3-kinase activity and migration were performed under comparable culture conditions, the above study (which had been performed under serum-reduced conditions) was repeated with serum supplemented (10% serum) medium. The results obtained in these experiments were similar to our findings with serum-reduced medium, that is wortmannin inhibited the PDGF-induced increase in Akt activation by 100.00 ± 0% ($P < 0.001$, N = 3 separate cell preparations; Fig. 2B). Total Akt expression was unchanged by the treatments. Trypan blue exclusion studies confirmed cell viability.

4.3. Effect of wortmannin on PDGF-induced migration

PDGF (10 ng/mL) significantly increased PSC migration ($P < 0.02$, N = 4 separate cell preparations; Fig. 3), confirming our previously published results [1]. In the

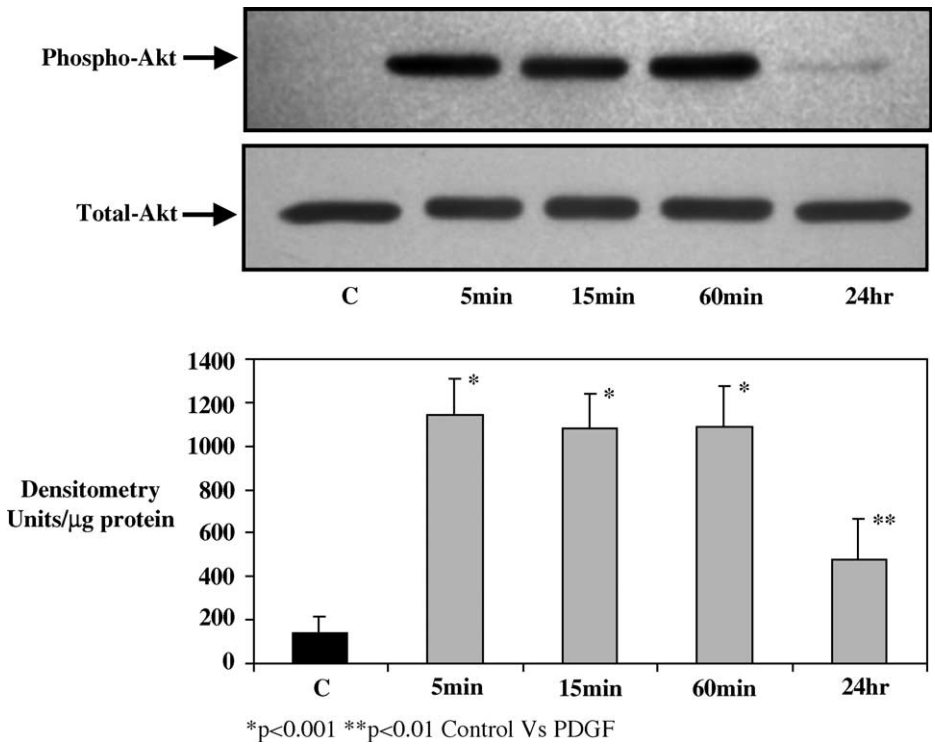

Fig. 1. Activation of Akt by PDGF in rat PSCs. A representative western blot for Akt activation showing an increase in the expression of activated Akt in PSCs exposed to 10 ng/mL of PDGF for 5 min, 15 min, 60 min, and 24 hr compared to control cells (C). Assessment of total Akt expression confirmed equal loading on the gel. Densitometry analysis of western blots showed a significant increase in Akt activation in PSCs treated with 10 ng/mL of PDGF for 5 min, 15 min, 60 min, and 24 hr compared to controls (C) (N = 4 separate cell preparations).

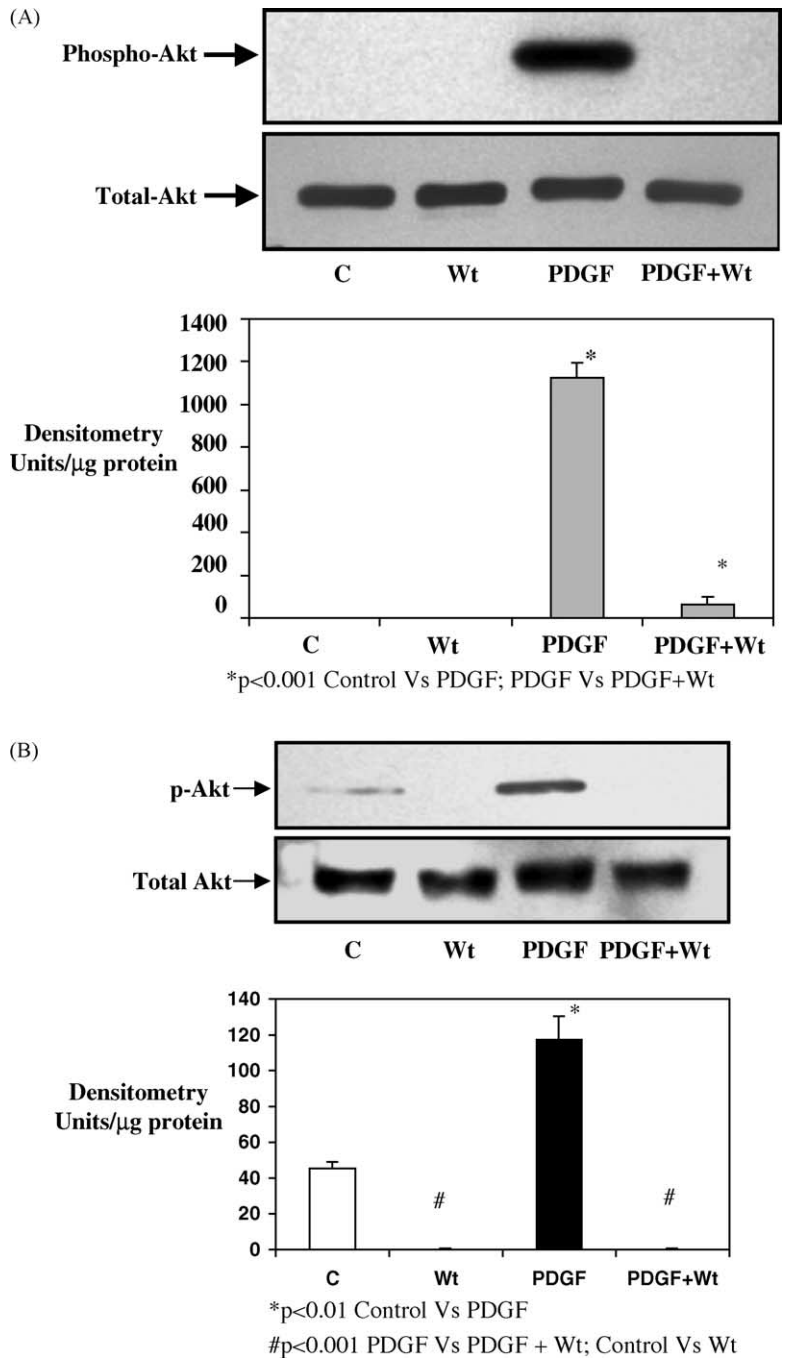

Fig. 2. (A and B) Effect of wortmannin on Akt activation in rat PSCs treated with PDGF in serum-reduced culture medium (A) and serum supplemented (10% serum) medium (B). Representative western blots for Akt activation showing increased Akt activation in PSCs treated with 10 ng/mL of PDGF for 60 min compared to control (C), and a marked decrease in Akt activation in the presence of 100 nM wortmannin (Wt). Densitometry analyses of western blots demonstrate a significant increase in Akt activation in PDGF-treated PSCs compared to control (C). This increase in Akt activation was abolished in the presence of 100 nM Wt ($N = 4$ separate cell preparations for serum-reduced culture medium, panel A; $N = 3$ separate cell preparations for serum supplemented medium, panel B).

presence of wortmannin (100 nM), this PDGF-induced increase in PSC migration was abolished ($P < 0.005$, $N = 4$ separate cell preparations; Fig. 3), suggesting that the PI3-kinase pathway plays a role in mediating the PDGF-induced migration of PSCs. Wortmannin alone also significantly decreased basal (unstimulated) PSC migration ($P < 0.02$, $N = 4$ separate cell preparations; Fig. 3).

To ensure that the observed decrease in wortmannin-induced migration was not due to a decrease in the adhesion of cells to the porous membrane, we estimated cell adhesion by calculating the number of cells per millimeter square of the membrane (upper surface and undersurface) at the end of the incubation period. Data obtained are as follows: (cells/mm^2, mean ± SE) control

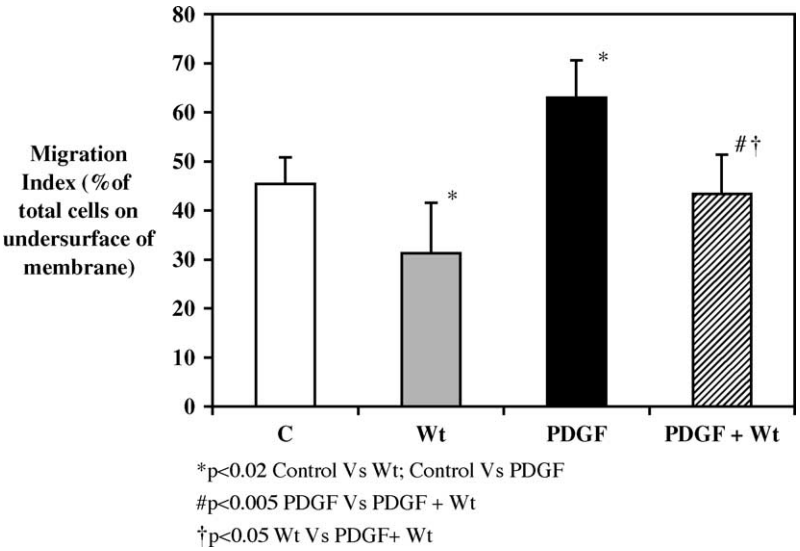

Fig. 3. Effect of wortmannin on PSC migration. The presence of 10 ng/mL PDGF in the culture medium significantly increased the rate of migration of PSCs compared to control cells not exposed to PDGF. In the presence of 100 nM wortmannin (Wt), this PDGF-induced migration was abolished (N = 4 separate cell preparations).

281.51 ± 81.10, wortmannin 374.18 ± 185.80 ($P = 0.50$; N = 4 separate cell preparations). Thus, wortmannin did not have any effect on adhesion of cells to the porous membranes.

4.4. Effect of wortmannin on PDGF-induced PSC proliferation

The effect of wortmannin (100 nM) on basal and PDGF-induced PSC proliferation was assessed by the incorporation of [^{3}H]thymidine into cellular DNA. As expected, PDGF (10 ng/mL) significantly increased PSC proliferation ($P < 0.001$, N = 4 separate cell preparations; Fig. 4), confirming our previously published results [3]. Wortmannin significantly reduced basal PSC proliferation ($P < 0.005$, N = 4 separate cell preparations; Fig. 4) but did not have a statistically significant effect on PDGF-induced proliferation.

4.5. Effect of wortmannin on PDGF-induced ERK1/2 activation

Incubation of PSCs with PDGF (under serum-reduced conditions) significantly increased ERK1/2 activation at 60 min compared to controls ($P < 0.001$, N = 4 separate cell preparations; Fig. 5A), confirming previously reported results [9]. However, this increase was inhibited in the presence of the PI3-kinase inhibitor wortmannin (100 nM) by 33.50 ± 5.70% ($P < 0.01$, N = 4 separate cell preparations; Fig. 5A). To ensure that assessment of PI3-kinase

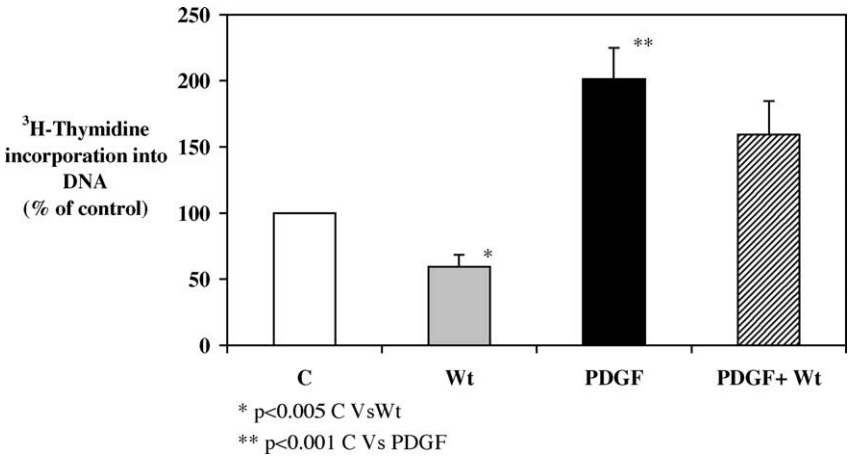

Fig. 4. Effect of wortmannin on basal and PDGF-induced PSC proliferation. Studies of [^{3}H]thymidine incorporation demonstrated that incubation of PSCs with PDGF increased PSC proliferation significantly compared to controls. Wortmannin (Wt) alone significantly decreased basal PSC proliferation but did not have a statistically significant effect on PDGF-induced proliferation despite a trend towards a decrease (N = 4 separate cell preparations).

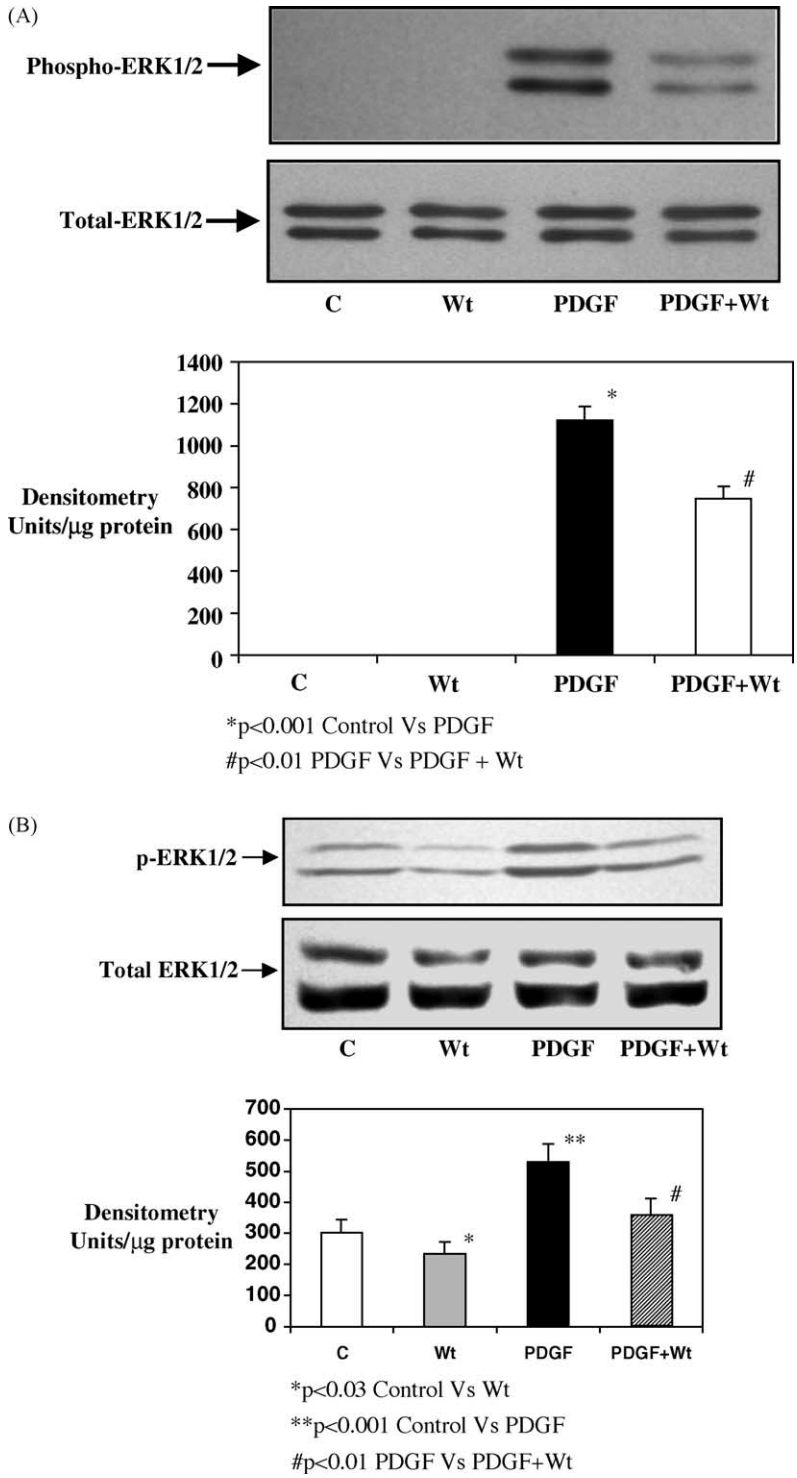

Fig. 5. (A and B) Effect of wortmannin on PDGF-induced ERK1/2 activation in serum-reduced culture medium (A) and serum supplemented (10% serum) medium (B). Representative western blots for ERK1/2 activation showing increased ERK1/2 activation in PSCs treated with 10 ng/mL of PDGF for 60 min compared to control (C), and a decrease in ERK1/2 activation in the presence of 100 nM wortmannin (Wt). Densitometry analyses of western blots demonstrates a significant increase in ERK1/2 activation in PDGF-treated PSCs compared to controls (C). This increase was significantly inhibited in the presence of 100 nM Wt ($N = 4$ separate cell preparations for serum-reduced culture medium, panel A; $N = 3$ separate cell preparations for serum supplemented medium, panel B).

activity and migration were performed under comparable culture conditions, the above study was repeated with serum supplemented (10% serum) medium. The results obtained in these experiments were similar to our findings with serum-reduced medium, that is wortmannin inhibited the PDGF-induced increase in ERK1/2 activation by $33.30 \pm 5.90\%$ ($P < 0.01$, $N = 3$ separate cell preparations; Fig. 5B). These findings suggest that PI3-kinase may

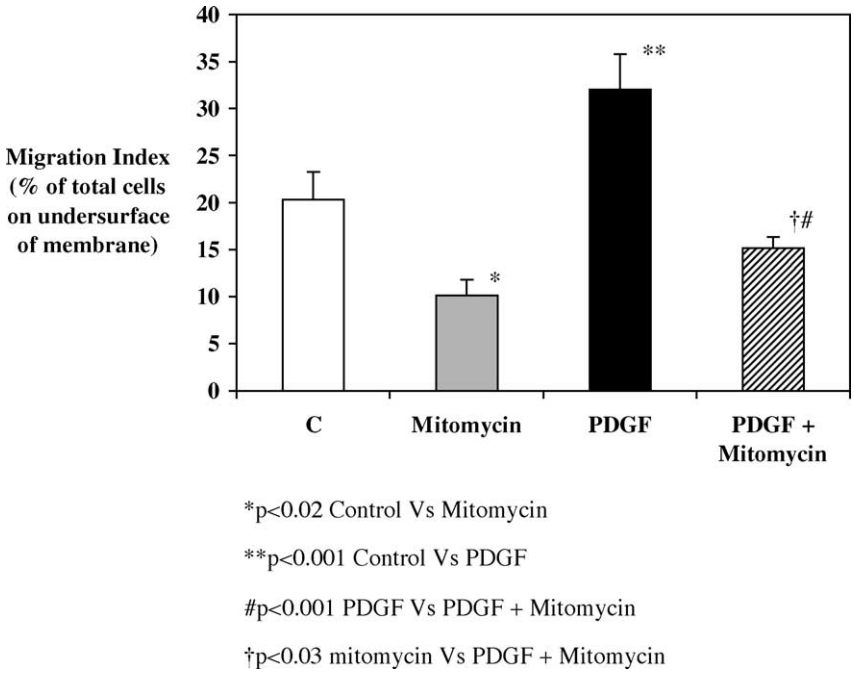

Fig. 6. Effect of mitomycin C on PSC migration. The presence of 10 μg/mL mitomycin C in the culture medium significantly decreased basal and PDGF-induced PSC migration (N = 5 separate cell preparations).

play a role, at least in part, in mediating PDGF-induced ERK1/2 activation. Re-probing of the membranes with an antibody against total ERK1/2 expression confirmed equal loading of protein on the gel.

4.6. Effect of mitomycin C on PSC proliferation and PDGF-induced migration

As expected, PSC proliferation was inhibited by mitomycin C as assessed by BrdU incorporation studies (% BrdU incorporation: Control 11.93 ± 7.1%, mitomycin 0 ± 0%, PDGF 10.3 ± 4.1%, PDGF + mitomycin 0 ± 0%; N = 3 separate cell preparations). Basal PSC migration was inhibited, but not abolished by mitomycin C ($P < 0.02$, N = 5 separate cell preparations; Fig. 6), indicating that migration is dependent, at least to some extent, on cell proliferation. PDGF-induced PSC migration was also decreased by mitomycin ($P < 0.01$, N = 5 separate cell preparations; Fig. 6). However, despite the inhibition of cell proliferation by the latter compound, PSCs responded to PDGF incubation with significantly increased migration (mitomycin vs. mitomycin + PDGF, $P < 0.03$, N = 5 separate cell preparations; Fig. 6) again supporting the concept that cell proliferation is an important, but not an essential factor for cell migration. To ensure that the inhibition of migration in cells exposed to mitomycin C was not due to a toxic effect of this compound, both cell viability and cell adhesion were assessed. Trypan blue exclusion studies confirmed cell viability. Cell adhesion (calculated as described earlier) of mitomycin C-treated cells was similar to that of control cells (cells/mm^2, mean ± SE: control 400.74 ± 52.95, mitomycin 344.46 ± 54.8, $P = 0.26$, N = 5 separate cell preparations). Therefore, mitomycin did not have any effect on cell adhesion.

In addition, morphological examination by phase contrast microscopy, and assessment of αSMA expression by western blotting indicated that mitomycin C did not have any direct effect on PSC activation. Both control and mitomycin C cells exhibited an activated phenotype with long cytoplasmic projections and the absence of vitamin A containing lipid droplets in the cytoplasm. Western blotting experiments indicated that there was no difference in αSMA expression in control and mitomycin-treated cells (densitometry units (% of control): mitomycin 103.45 ± 5.65%; N = 3 separate cell preparations).

5. Discussion

This study provides novel data with respect to the cell signalling mechanisms that mediate the responses of PSCs to PDGF. Our results indicate that the PI3-kinase pathway plays a major role in PDGF-induced PSC migration, as evidenced by the complete inhibition of PDGF-induced migration by the PI3-kinase inhibitor wortmannin. The study has also demonstrated that PSC migration is influenced by cell proliferation. In addition, we have shown that the PI3-kinase pathway participates in the PDGF-induced activation of ERK1/2, thereby suggesting an interaction between the two candidate signalling pathways stimulated by PDGF in PSCs.

Studies conducted over the past 5 years have identified that activated PSCs play a key role in fibrosis in pancreatic injury. It is well established that there are increased

numbers of PSCs in areas of injury [7,8,30]. Possible mechanisms to explain this finding include local proliferation of PSCs and/or migration of PSCs to areas of injury from surrounding areas. Work by our group and others have provided evidence to support both these possibilities. PSC proliferation in response to activating factors such as PDGF has been well established [6,9]. Most recently we have demonstrated that PSCs also have the ability to migrate and that this migration is stimulated by PDGF in a predominantly chemotactic manner [1].

Intracellular signalling events triggered by the interaction of PDGF with its receptor have been the focus of extensive investigation in recent years [31–34]. Studies in cell types such as meningioma cells, vascular smooth muscle cells, and HSCs have now established that PI3-kinase plays a major role in mediating the biological effects of PDGF [11,34,35]. However, the role of this pathway in the response of PSCs to PDGF had not been studied to date.

In the present study, we have demonstrated for the first time, the ability of PDGF to induce PI3-kinase activity in PSCs as early as 5 min of incubation. This increase was sustained at 30 and 60 min. It should be noted that this increase was also present at 24 hr of incubation, although the extent of activation was lower than at the early time points. We have also shown that treatment of PSCs with the specific PI3-kinase inhibitor wortmannin abolished PDGF-induced PI3-kinase activity. Our findings are in accord with reports in the literature using other cell systems. For example, Marra *et al*. [35] have reported that human HSCs exhibit increased PI3-kinase activity after exposure to PDGF for 1–30 min. This increase is prevented in the presence of wortmannin (100 nM). In addition, Taylor [36] has reported that treatment of porcine thecal cells with PDGF leads to a rapid (10 min) and sustained (after 48 hr) increase in PI3-kinase activity as assessed by the expression of activated Akt. This increase in Akt activation is blocked by wortmannin [36]. Our study further demonstrated that inhibition of PI3-kinase activity by wortmannin prevented basal as well as PDGF-induced increase in PSC migration, implicating PI3-kinase as a major pathway in PSC migration. The observed inhibition of PDGF-induced migration by wortmannin concurs with that reported by Marra *et al*. [35] in HSCs. Our findings are also in accord with another hepatic study by Reif *et al*. [37] demonstrating that the PI3-kinase/Akt pathway plays a role in mediating basal as well as PDGF-induced HSC migration.

Proliferation of PSCs in response to PDGF has been reported to be mediated by ERK1/2 [9]. In the present study, an interesting observation was noted with respect to PDGF-induced ERK1/2 activation in the presence of wortmannin. While our results confirmed that PDGF induces ERK1/2 activation in PSCs (as previously reported by Jaster *et al*. [9]), this ERK1/2 activation was found to be significantly reduced (but not abolished) in the presence of wortmannin, indicating that PI3-kinase may play a role, at least in part, in the activation of ERK1/2 by PDGF. Our findings concur with those of Marra *et al*. [23] and Duckworth and Cantley [24] which indicate that an interaction exists between the PI3-kinase and ERK1/2 pathways upon exposure of cells to PDGF. The study by Marra *et al*. [23] also indicates that PI3-kinase acts upstream of ERK1/2 since treatment of HSCs with PDGF in the presence of wortmannin (100 nM) significantly reduced the expression of activated (phosphorylated) ERK1/2 [23], but did not affect the activity of ERK1/2 itself (i.e. its ability to phosphorylate a downstream substrate).

When summarised, our experiments with wortmannin (as a PI3-kinase inhibitor) have established that this compound: (i) completely abolishes basal as well as PDGF-induced PI3-kinase activity but only partially inhibits basal and PDGF-stimulated ERK1/2 activation in PSCs; and (ii) does not have a significant effect on PDGF-induced cell proliferation, but completely reverses PDGF-induced migration to control (non-PDGF) levels. Taken together, these results strongly support a role for PI3-kinase in PSC migration.

To examine the influence of cell proliferation on PSC migration, studies were performed in the presence of mitomycin C, an inhibitor of cell proliferation. Basal and PDGF-induced proliferation was completely abolished by mitomycin C. This was associated with a significant (but not complete) inhibition of migration of PSCs in their basal as well as PDGF-induced state. It is to be noted that despite complete inhibition of cell proliferation by mitomycin, migration of mitomycin C-treated cells remained inducible in the presence of PDGF (see Fig. 6 mitomycin vs. mitomycin + PDGF; $P < 0.03$). These findings indicate that cell proliferation influences PSC migration, but is not an essential factor in the migratory process. In the liver, similar results were reported in a recent study by Yang *et al*. [38] demonstrating that PDGF-induced migration of human hepatic stellate cells is influenced by cell proliferation.

In conclusion, this study is the first to demonstrate that the PI3-kinase pathway plays a major role in mediating PDGF-induced PSC migration. Our findings also suggest that although cell proliferation influences migration, it is not essential for the migratory process. Furthermore, we have demonstrated that the PI3-kinase pathway may, at least in part, mediate PDGF-induced ERK1/2 activation in PSCs, suggesting an interaction between the two candidate signalling pathways (PI3-kinase and ERK1/2) known to regulate biological responses of a cell to PDGF.

Acknowledgments

Studies described in this manuscript were supported by grants from the Department of Veterans' Affairs Australia and the Health Research Foundation Sydney South West.

Joshua A. McCarroll was supported by a GlaxoWellcome/Gastroenterological Society of Australia Biomedical Postgraduate Research Scholarship.

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