PDGF receptor-β modulates metanephric mesenchyme chemotaxis induced by PDGF AA

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1Department of Molecular Medicine, Institute of Biotechnology, Departments of 2Medicine and 4Pediatrics, University of Texas Health Science Center, and 3South Texas Veterans Health Care System/Audie L. Murphy Memorial Hospital Division, San Antonio, Texas; and 5Schepens Eye Institute, Harvard Medical School, Boston, Massachusetts

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Ricono JM, Wagner B, Gorin Y, Arar M, Kazlauskas A, Choudhury GG, Abboud HE. PDGF receptor-β modulates metanephric mesenchyme chemotaxis induced by PDGF AA. Am J Physiol Renal Physiol 296: F406–F417, 2009. First published November 19, 2008; doi:10.1152/ajprenal.09368.2008.—PDGF B chain or PDGF receptor (PDGFR)-β-deficient (−/−) mice lack mesangial cells. To study responses of α- and β-receptor activation to PDGF ligands, metanephric mesenchymal cells (MMCs) were established from embryonic day E11.5 wild-type (+/+) and −/− mouse embryos. PDGF BB stimulated cell migration in +/+ cells, whereas PDGF AA did not. Conversely, PDGF AA was chemotactic for −/− MMCs. The mechanism by which PDGFR-β inhibited AA-induced migration was investigated. PDGF BB, but not PDGF AA, increased intracellular Ca2+ and the production of reactive oxygen species (ROS) in +/+ cells. Transfection of −/− MMCs with the wild-type β-receptor restored cell migration and ROS generation in response to PDGF BB and inhibited AA-induced migration. Inhibition of Ca2+ signaling facilitated PDGF AA-induced chemotaxis in the wild-type cells. The antioxidant N-acetyl-L-cysteine (NAC) or the NADPH oxidase inhibitor diphenyleneiodonium (DPI) abolished the BB-induced increase in intracellular Ca2+ concentration, suggesting that ROS act as upstream mediators of Ca2+ in suppressing PDGF AA-induced migration. These data indicate that ROS and Ca2+ generated by active PDGFR-β play an essential role in suppressing PDGF AA-induced migration in +/+ MMCs. During kidney development, PDGFR-β-mediated ROS generation and Ca2+ influx suppress PDGF AA-induced chemotaxis in metanephric mesenchyme.

reactive oxygen species; calcium

PROLIFERATION AND MIGRATION are key biological processes involved in organ development, including development of the metanephric kidney. Two structurally similar transmembrane platelet-derived growth factor receptors (PDGFRs) with intrinsic tyrosine kinase activity have been identified, PDGFR-α and PDGFR-β (11, 43, 63). Biologically active platelet-derived growth factor (PDGF) is a dimer (AA, AB, BB, CC or DD). The α-receptor has greater affinity for PDGF AA than BB. PDGFR-β binds PDGF BB and DD with high affinity (5, 11, 26, 39, 52). Activation of PDGFR-β mediates proliferation, migration, survival, and differentiation in a variety of cell types (1, 18). The role of PDGF B chain and PDGFR-β in development of the renal glomerular microvasculature has been conclusively demonstrated in two studies utilizing mice carrying mutations in either PDGFR-β or PDGFB B chain ligand (38, 54).

Homozygous PDGFR-β-deficient mice (−/−) do not survive past birth. They are affected with anemia, thrombocytopenia, and form rudimentary glomeruli that lack mesangial cells (54). The phenotype is fully penetrant and observed in mature glomeruli of 16–18 days postconception (dpc) embryos. Mice carrying a null mutation in the gene encoding PDGF B chain exhibit a similar glomerular phenotype (38). In these studies, PDGFR-α was found to be intact and functional, yet not compensatory for the loss of PDGFR-β. Mice deficient in PDGFR-α (e.g., the Patch mutant) are characterized by maldevelopment of mesenchymal structures and have a deficiency in renal fibroblasts (6, 51), but there are no reports of mesangial cell abnormalities. Therefore, PDGFR-β has a principle role in mesangial cell development.

Binding of a PDGF dimer to the extracellular domain of the receptor induces homo- or heterodimerization (depending on the ligand) and subsequent transphosphorylation. Autophosphorylation of the receptors provides docking sites or activates intracellular effector proteins including phosphatidylinositol 3-kinase (PI3-K), phospholipase C-γ1 (PLC-γ1), and Ras. Downstream effects include activation of the mitogen activated protein kinase/extracellular regulated kinase kinase (MEK), and mitogen activated protein kinase (MAPK) pathways (1, 8, 27, 41). It is known that PDGFR-β-mediated chemotaxis depends on PI3-K (64). Cells expressing a PDGFR-β mutant incapable of activating PI3-K do not demonstrate chemotaxis to PDGF (59, 62). PDGF B chain and PDGFR-β are essential for mesangial cell development. Signaling through PDGFR-α does not compensate for the loss of PDGFR-β signaling. Therefore, the biological effects of PDGF AA and PDGF BB in wild-type (+/+) and PDGFR-β-deficient (−/−) MMCs were examined.

In this study, we established wild-type and −/− MMCs. We hypothesized that defective α-receptor signaling and aberrant cell migration may explain the inability of the α-receptor to compensate for the β-receptor in mesangial cell development. To our surprise, PDGF AA and α-receptors signal normally and induce MMC migration in the PDGFR-β −/− cells. In wild-type metanephric mesenchymal cells, PDGFR-β negatively regulates PDGF AA-induced cell migration through reactive oxygen species (ROS)- and Ca2+ -mediated signals. ROS are generated via PDGFR-β and stimulate extracellular...
Ca\(^{2+}\) influx, suppressing PDGF AA-induced migration in wild-type cells.

**MATERIALS AND METHODS**

**Materials.** Tissue culture materials were purchased from GIBCO BRL (Rockville, MD). Recombinant PDGF AA and PDGF BB were obtained from R&D Systems (Minneapolis, MN), BAPTA, A23187, fura 2, and 2',7'-dichlorofluorescein diacetate (DCFDA) were purchased from Molecular Probes (Eugene, OR). Primary antibody to PDGFR-β (958) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PDGFR-α antibody (C-20) was from Cell Signaling (Boston, MA). Antibodies against vimentin, pan cytokeratin, E-cadherin, and α-smooth muscle actin were purchased from Sigma (St. Louis, MO). Secondary FITC-conjugated antibodies were obtained from Chemicon (Temecula, CA). Lipofectamine Plus was purchased from GIBCO BRL. Protein measurement and polyacrylamide gel reagents were purchased from Bio-Rad (Hercules, CA). Agarose gel products, SeaKem GTG agarose, and NuSieve GTG low-melting-temperature agarose were obtained from FMC BioProducts (Rockland, ME). All other reagents were high-quality analytical grade.

**Establishment and characterization of metanephric mesenchymal cells in culture.** Primary cultures of mouse metanephric mesenchymal cells (MMCs) were prepared as recently described for rat MMCs (3). All animal protocols were reviewed by the Alexion Institutional Animal Care and Use Committee. Heterozygous PDGFR-β-deficient mice were mated. At postcoital day 11.5, pregnant mice were anesthetized by intramuscular injection with a mixture of ketamine (30%) and Rompun (20%) in 0.9% NaCl, and embryos were collected. The age of the embryo was counted from the day of the vaginal plug (day 0). Embryos were dissected in 1× phosphate-buffered saline using an Olympus SZH stereo zoom model microscope. Metanephric blastemas were collected from each embryo individually by removing the embryonic kidney and separating the metanephric mesenchyme from the ureteric bud. Tissue from each embryo was also collected for genotyping. Once metanephric mesenchyme was collected, cells were propagated in Dulbecco’s modified Eagle’s medium (GIBCO BRL) including 10% fetal calf serum and grown at 37°C in a 5% CO\(_2\) atmosphere. Cells from litters of the same genotype were pooled to propagate MMCs.

**DNA synthesis.** MMCs were plated at 7.5 × 10\(^4\) cells/24-well dish, grown to confluency, and serum-deprived for 48 h. Cells were stimulated with PDGF isoforms. One microcurie of \(^{3}H\) thymidine was added to each well. DNA synthesis was measured as incorporation of \(^{3}H\) thymidine into trichloroacetic acid (TCA)-insoluble material (23).

**Chemosatosis assays.** Cellular chemotaxis in response to PDGF was determined using blind well (modified Boyden) chamber assays. Polycarbonate filters (14 μm, Osmonics, Minnetonka, MN) were coated with rat tail collagen (4 μg/filter, Becton-Dickinson, Becton, MA). Confluent MMCs were serum-deprived overnight, and the monolayer of cells was briefly trypsinized and resuspended in serum-free media. The cell suspension (2 × 10\(^5\) cells/ml) was added to the top chamber, while PDGF was added to the bottom chamber of the apparatus. After 4 h at 37°C, the filters were inverted on glass slides and fixed with methanol, stained with Giemsa (Fischer Scientific), dried, and mounted in Permount. Cells were counted in 10 high-power fields (magnification, ×450) in the center of each filter. The data are presented as number of cells per high-power field (24). Cells in suspension were treated with 5 nM EGF, 20 μM BAPTA, or 20 mM N-acetylated-cysteine (NAC) for 2, 15, or 20 min and then added to the top chamber. Alternatively, cells were treated with 1 μM A32187 or 200 μM H\(_2\)O\(_2\) for 1 or 20 min and washed with serum-free media before being added to the top chamber.

**Immunohistochemistry.** Both +/+ and PDGFR β-deficient cells were grown to near-confluence on coverslips and fixed with methanol. Direct and indirect immunofluorescence staining was used to examine the expression of vimentin, α-smooth muscle actin, cytokeratin, E-cadherin, and *D. biforis* (rhodamine-conjugated lectin specific for urinary bud) as previously described (3). The primary antibodies were omitted or replaced by normal mouse or rabbit IgG (5 μg/ml) as controls. Sections were viewed and photographed with an Olympus AX70 research microscope.

**PCR.** Genomic DNA was isolated from each embryo or from cultured cell lines. PCR for PDGFR-β was performed using two forward primers, P1: 5'-ACA ATT CGG TGC CGA GTG ACA-3' and P2: 5'-AAA AGC AAT CAG GAA ACC CGG TCG G-3', and one reverse primer, P3: 5'-ATC AGC CTC GAC GGT TGC GTC TTC TAG-3'. Cycling temperatures were as follows: denaturing, 93°C, 30 s; annealing, 58°C, 30 s; and extending, 65°C, 45 s; for 35 cycles. PCR products were separated in 1% SeaKem GTG agarose (FMC BioProducts) with 2.7% NuSieve GTG low-melting-temperature agarose TEA gel (60).

**RT-PCR.** Monolayers of +/+ or -/- cells were washed, and total RNA was isolated with a TRIZol Plus RNA Purification Kit (Invitrogen). Primers for mouse PDGFR-α (determined by P3) were 5'-ggg gag aga ctc tga ggt gt-3' and 5'-ggt ggt gct gtg gat tg-3'.

**Western blotting.** Equal amounts of protein from cell lysates were separated on a 12.5% SDS-PAGE and transferred by electrophoresis to a polyvinyl membrane. The membrane was blocked with 5% nonfat milk prepared in TBST buffer, washed with TBST and incubated with PDGFR-β primary antibody (1:750 dilution, Santa Cruz Biotechnology). The membrane was then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. The blot was developed with ECL reagent according to the manufacturer’s directions.

**Immune complex kinase assay.** Serum-deprived confluent MMCs were treated with PDGF and lysed in RIPA with sodium orthovanadate (21). One hundred micrograms of protein were immunoprecipitated with 1 μg of the indicated antibody, and the immune complex kinase assay was conducted as described (10).

**Laser-scanning confocal microscopy.** Cells were seeded on slides, grown to < 30% confluency, washed with PBS, and fixed with 4% paraformaldehyde for 15 min. Membranes were permeabilized with 0.2% Triton X in PBS for 5 min, washed, and blocked with 5% BSA
in PBS at 4°C overnight. Anti-PDGFR-β and monoclonal anti-PDGFR-α (16A1) at 1:250 dilution were applied for 30 min, followed by 1:250 secondary antibody, FITC- and Alexa Fluor 680-conjugated anti-rabbit and -mouse, respectively. Images were obtained using an inverted microscope with a x60 oil objective (Olympus).

Flow cytometry. Serum-deprived monolayers were washed three times with PBS, detached from plates using nonenzymatic cell dissociation solution (Sigma), centrifuged, and fixed with 1% paraformaldehyde for 10 min. Cells were permeabilized with cold methanol for 10 min, rinsed, and blocked with 0.5% BSA in PBS. Cells were incubated with 1:400 primary antibody (rabbit IgG isotype control, anti-PDGFR-β, or C-20 anti-PDGFR-α) for 30 min, then 1:100 FITC-conjugated anti-rabbit (Jackson) for 15 min. Cells were sorted (FACS Calibur, Becton-Dickinson Immunocytometry Systems, San Jose, CA) with an excitation wavelength of 488 nm, 530/30-nm band-pass filter. Data were obtained by CellQuest (BDIS).

Ca²⁺ measurements. Cytosolic Ca²⁺ concentration ([Ca²⁺]i) was determined by spectrofluorometric measurements in cell suspensions as previously described (23). Briefly, cells were trypsinized and resuspended in growth media. Cells were washed with PBS and loaded with 1.2 µM Fura 2-AM at 37°C for 30 min with gentle agitation. Cells were brought to room temperature and washed two times to remove extracellular dye and resuspended at 1 x 10⁶ cells/ml. The cell suspension was placed in a fluorometer, and 10 ng/ml of PDGF BB or 100 ng/ml of PDGF AA was added. An excitation ratio of 340/380 was measured with a PTI Delta Scan spectrofluorometer (Photon Technology International, South Brunswick, NJ) using 340- and 380-nm wavelengths for excitation and 510 nm for emission. The following calculation was used: [Ca²⁺]i (nM) = Kd(R – Rmin)/(Rmax – R), where R is the fluorescent ratio of 340/380; Rmin and Rmax are minimal and maximal fluorescent ratios, respectively; and Kd is the dissociation constant (taken as 224 nM) of fura 2 for Ca²⁺.

Measurement of superoxide anion production in intact MMCs. Measurement of the superoxide anion (O₂⁻) released into the media of +/- and PDGFR-β-deficient MMCs was performed by detection of ferricytochrome c reduction, as described by Johnston et al. (30). Medium from growth-arrested MMCs grown in six-well plates (2 x 10⁶ cells/well) was aspirated and replaced with 1 ml of Hanks’ balanced salt solution without phenol red containing 80 µM cytochrome c with or without 100 ng/ml PDGF AA or 10 ng/ml PDGF BB. At the end of the incubation, the medium was removed at the times indicated and centrifuged for 2 min at 10,000 g at 4°C to stop the reaction. The optical density was measured by spectrophotometry at 550 nm and converted to nanomoles of cytochrome c reduced using the extinction coefficient ΔE₅₅₀ = 21.0 x 10³ M/cm. The reduction of cytochrome c that was inhibited by pretreatment with superoxide dismutase (SOD; 50 µg/ml) reflected O₂⁻ release.

Detection of intracellular H₂O₂. The H₂O₂-sensitive fluorescent probe DCFDA was used to assess the generation of H₂O₂. This compound is converted by intracellular esterases to 2',7'-dichlorofluorescein, which is then oxidized by H₂O₂ to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Both +/- and PDGFR-β-deficient MMCs were grown to near confluence in cover glass chambers and serum-deprived for 48 h. Cells were incubated with 10 µM DCFDA for 30 min at 37°C. The supernatant was removed and replaced with fresh media before treatment of MMCs with 100 ng/ml of PDGF AA or 10 ng/ml PDGF BB. Differential interference contrast images were obtained simultaneously using an Olympus inverted microscope with a x40 Aplanfluo objective and an Olympus fluoview confocal laser-scanning attachment (22). The DCF fluorescence was measured with an excitation wavelength of 488-nm light, and its emission was detected using a 510- to 550-nm band-pass filter. Cells were treated with 10 mM diphenylene iodophenium as described (21).

Statistical analysis. Results are expressed as means ± SE. Statistical significance was assessed by Student’s unpaired r-test. Significance was determined as P < 0.05.

RESULTS

Effect of PDGF receptor activation on migration and DNA synthesis in +/- and PDGFR-β-deficient MMCs. Metanephric blastemas were isolated from 11.5-dpc mouse embryos. MMCs were established from +/- and -/- embryos as described elsewhere (60). The ability of the PDGF isoforms AA and BB to induce DNA synthesis or cellular migration was examined in +/- and -/- cells. PDGF BB induced DNA synthesis only in +/- cells. As expected, PDGF BB had no mitogenic effect in -/- MMCs even at concentrations of 100 ng/ml (Fig. 1A). PDGF AA, even at doses up to 100 ng/ml, had...
no effect on DNA synthesis in either +/+ or −/− MMCs. These data indicate that PDGFR-β, but not PDGFR-α, is necessary for PDGF-induced mitogenesis in +/+ cells. PDGF BB induced migration of +/+ MMCs fourfold above basal levels, whereas PDGF AA did not have an effect (Fig. 1B). Surprisingly, PDGF AA stimulated migration in the −/− cells, whereas PDGF BB had no effect (Fig. 1B).

**Introduction of wild-type PDGFR-β into PDGFR-β-deficient cells inhibits PDGF AA-induced migration.** We next determined whether the differential migratory response between the +/+ and −/− MMCs to PDGF AA was due to PDGFR-β itself rather than to another alteration in the genetic program at early stages of development. Wild-type human PDGFR-β was transfected into the −/− MMCs and stable cell lines (−/−+/+) were established. PDGFR-β was expressed in the −/−+ cells, but not in cells transfected with an empty vector (−/−−) (Fig. 2A).

DNA synthesis and chemotaxis assays were performed on −/−− and −/−+ MMCs to examine whether the transfected PDGFR-β could rescue the phenotypes we observed in the −/−− MMCs. As expected, the −/−− cells demonstrated a lack of mitogenic response to either PDGF AA or BB (data not shown), similar to that in −/−− MMCs. In contrast, PDGF BB-induced DNA synthesis was restored to normal in the −/−+ cells (Fig. 2B). These data demonstrate that expression of wild-type PDGFR-β in the −/−− MMCs rescued the mitogenic effect of PDGF BB. PDGF AA induced migration fourfold over basal levels in −/−− MMCs, whereas PDGF BB had no effect, identical to the −/−− MMCs (Fig. 2C). PDGF BB induced cell migration in −/−−, whereas PDGF AA did not have an effect (Fig. 3C, right). In total, introduction of human wild-type PDGFR-β into −/−− MMCs restored the +/+ phenotype.

**PDGFR-α is not upregulated in −/−− MMCs.** PDGF AA is not a ligand for PDGFR-β, but it does activate PDGFR-α. The coexpression of the PDGFRs α and β may account for the lack of PDGF AA-induced migration in the +/+ cells. Because a greater level of PDGFR-α expression in the −/−− MMCs could explain PDGF AA-induced migration, we examined the extent of PDGFR-α-mediated signaling and the degree of its expression were examined.

The demonstration that PDGFR-α was not upregulated in the −/−− cells was performed by multiple techniques. RT-PCR was performed on +/+ and −/−− cells to assess the quantity of mRNA transcript for the PDGFR-α (Fig. 3A). There was a negligible difference between the cell lines. Immunoblots for PDGFR-α and β were performed on +/+ and −/−− lysates (Fig. 3B). There was no difference in the expression of PDGFR-α between +/+ and −/−− cells. The −/−− cells lacked PDGFR-β as detected by flow cytometry and did not appear to differ in the quantity of PDGFR-α expression from the +/+ cells (Fig. 3C). Laser-scanning confocal microscopy was employed to assess the expression of PDGFR-α among populations of +/+ and −/−− cells (Fig. 3D). PDGFR-β was again absent in the −/−− cells, and there was no discernable difference in PDGFR-α between the two cell lines. Furthermore, the −/−− cells were homogenous in the degree of PDGFR-α expression.

**Effect of PDGF isoforms on PI3-K-dependent signaling pathways in MMCs.** PI3-K has previously been shown to associate with tyrosine-phosphorylated PDGFRs and is a major component in PDGF-induced migration. Using the pharmacological PI3-K inhibitors wortmannin and LY294002, we have previously shown that PI3-K is the major pathway involved in PDGF BB-induced migration in rat MMCs (49). Both wortmannin and LY294002 attenuated PDGF BB- and AA-induced migration in +/+ and −/−− MMCs, respectively (data not shown). One of the downstream targets of PI3-K is the serine threonine kinase Akt (15). Because Akt has been implicated in
cell migration (44), we explored the activation of Akt pathway in MMCs. Cells were stimulated with PDGF AA, and lysate-derived protein was analyzed by immunoblotting using a specific antibody against the phosphorylated form of Akt. PDGF AA treatment led to higher phosphorylation of Akt in /H11001/H11002/MMCs over a similar time period (Fig. 3E).

In total, these data indicate that the PDGFR-α is neither overexpressed nor overactive in the /H11002/MMCs. Therefore, the differential biological activities in PDGFR-α/H9252/H11002/MMCs ob-
served in Fig. 2 were not due to alteration of any genetic program during development of these /H11002/MMCs; rather, they were due to an absence of the β-receptor. Thus we conclude that PDGF BB-induced proliferation and migration are mediated through PDGFR-β and that the presence of PDGFR-β inhibited PDGF AA-induced migration.

Involvement of Ca²⁺ in PDGF isoform-induced migration of MMCs. Between the PDGF receptors, activation of only the β isoform leads to DNA synthesis (Figs. 1A and 2B). There is clearly differential regulation of migration by PDGFR-α and -β in the MMCs (Figs. 1B and 2C). These data do not explain these differential effects in PDGF-induced cell migration in /H11001/MMCs. Therefore, we explored the effect of PDGF isoforms on Ca²⁺ flux in MMCs. Relative free cytosolic Ca²⁺ in the cells was measured by the ratio of fluorescence in fura 2-loaded cells (Fig. 4A). In /H11001/MMCs, PDGF BB induced a rapid initial increase in intracellular Ca²⁺ in a dose-dependent manner; this Ca²⁺ was likely from internal stores. This was followed by a sustained increase in intracellular Ca²⁺, likely due to influx from the extracellular compartment. In contrast, PDGF AA did not have any effect on intracellular Ca²⁺ in /H11001/MMCs. Neither PDGF AA nor PDGF BB induced

Fig. 3. PDGFR-α is not abundantly expressed in the /H11002/MMCs. A: PDGFR-α mRNA in /H11001/MMCs and /H11002/MMCs. RT-PCR was performed for PDGFR-α in the cells. 18S mRNA was used as a control. B: immunoblot for PDGFR-α in quiescent /H11001/MMCs and /H11002/MMCs. C: flow cytometry for PDGFR-α and -β in /H11001/MMCs and /H11002/MMCs. PDGFR expression (gray histogram) was compared with isotype control (unshaded) in nonstimulated, serum-deprived cells. D: PDGFR expression in /H11001/MMCs and /H11002/MMCs by laser-scanning confocal microscopy. E: equal amounts of total protein from the treated cell lysates were loaded and separated by SDS-PAGE. Akt phosphorylation was analyzed by Western blotting using a phospho-specific (Ser 473) anti-Akt antibody. Total Akt amounts are also shown.
an increase in intracellular Ca\(^{2+}\) in the \(-/-\) MMCs (Fig. 4B).

To determine whether \(-/-\) cells are capable of generating an intracellular Ca\(^{2+}\) flux, cells were stimulated with ATP, which did induce a rapid increase in intracellular levels of Ca\(^{2+}\) (Fig. 4B, inset). These results indicate that PDGFR-\(\beta\) is required for a PDGF BB-induced increase in intracellular Ca\(^{2+}\) and that neither PDGF AA nor PDGF BB could induce a response through the PDGFR-\(\alpha\) in the absence of PDGFR-\(\beta\).

EGTA and BAPTA are Ca\(^{2+}\) chelators and abolish sustained increases in cytosolic Ca\(^{2+}\) from the extracellular compartment. To determine the effects of EGTA on PDGF-induced intracellular Ca\(^{2+}\) flux, \(+/+\) MMCs were treated with EGTA before stimulation with PDGF BB, and cytosolic Ca\(^{2+}\) was measured (Fig. 5, A and B). Chelation of extracellular Ca\(^{2+}\) with EGTA slightly reduced the level of initial Ca\(^{2+}\) flux and completely abolished the sustained plateau of intracellular Ca\(^{2+}\) influx from extracellular stores.

To address the role of Ca\(^{2+}\) in the migration of MMCs, EGTA and BAPTA were used in combination with the PDGF isoforms in cell chemotaxis assays (Fig. 5C). PDGF BB induced migration of \(+/+\) MMCs, whereas PDGF AA had no effect (Fig. 5Ca). Treatment of these cells with the extracellular Ca\(^{2+}\) chelators did not affect PDGF BB-induced migration (Fig. 5C, b and c), indicating that the initial rapid peak of cytosolic Ca\(^{2+}\) from internal stores may be sufficient for PDGF BB-induced migration. However, in the presence of the extracellular chelators, PDGF AA induced migration in \(+/+\) MMCs to similar levels as PDGF BB in unpretreated cells. These data indicate that activation of cell migration in response to PDGF AA does not require sustained cellular Ca\(^{2+}\) signaling but is rather inhibited by it. EGTA and BAPTA themselves had no apparent effect on cell viability or basal migration.

To further test the hypothesis that an increase in intracellular Ca\(^{2+}\) inhibits PDGF AA-induced migration in MMCs, \(-/-\) cells were treated with the Ca\(^{2+}\) ionophore A23187. This was used to enhance the influx of extracellular Ca\(^{2+}\) into the cytosol independently of sarco/endoplasmic reticulum Ca\(^{2+}\), ATPase (SERCA). Treatment of cells with A23187 completely inhibited PDGF AA-induced cell migration (Fig. 5D), whereas basal migration was unaffected, indicating that an increase in extracellular Ca\(^{2+}\) flux inhibits PDGF AA-induced migration in \(-/-\) MMCs.

PDGF-induced ROS regulate Ca\(^{2+}\) production and migration in MMCs. Second messengers such as ROS including O\(_2^*\) and H\(_2\)O\(_2\) have been implicated in PDGF-induced biological activity in various cells. A role for H\(_2\)O\(_2\) in PDGF-induced mitogenesis has been proposed (20, 56, 60). PDGF-induced H\(_2\)O\(_2\) production was measured in \(+/+\) and \(-/-\) MMCs with a fluorescence-based assay using a peroxide-sensitive fluorophore, DCFDA, and laser-scanning confocal microscopy. PDGF BB significantly increased the DCFDA fluorescence in \(+/+\) MMCs, indicating the production of H\(_2\)O\(_2\) in these cells (Fig. 6A, compare b with a). PDGF AA did not increase H\(_2\)O\(_2\) production in either \(+/+\) (Fig. 6Ac) or \(-/-\) MMCs (Fig. 6Af). One mechanism by which H\(_2\)O\(_2\) can be produced is the spontaneous dismutation of O\(_2^*\). We therefore assessed O\(_2^*\) generation in MMCs by measuring the SOD-inhibitable reduction of ferri-cytochrome c. Treatment of MMCs with PDGF BB resulted in a rapid and time-dependent increase in O\(_2^*\) generation only in \(+/+\) MMCs (Fig. 6B, left). This generation of O\(_2^*\) was maximal at 30 min and sustained for up to 4 h. PDGF AA did not have a significant effect on O\(_2^*\) generation. These data indicate that PDGF BB increases ROS through PDGFR-\(\beta\) in \(+/+\) MMCs, while in the absence of PDGFR-\(\beta\) neither O\(_2^*\) nor H\(_2\)O\(_2\) is produced in response to PDGF. We have previously demonstrated that the generation of ROS may be via an NAD(P)H oxidase (60); therefore, PDGF BB-stimulated \(+/+\) cells were pretreated with the flavoprotein inhibitor diphenylene iodonium (DPI) (Fig. 6B, right). DPI treatment suppressed PDGF BB-induced O\(_2^*\) generation without affecting basal levels. These data demonstrate that PDGF BB elicits ROS generation via a DPI-inhibitable source, such as an NAD(P)H oxidase.

Induction of ROS by PDGFR-\(\beta\) activation may be responsible for suppressing PDGF AA-induced migration in \(+/+\) MMCs. To address this, the MMCs were treated with the antioxidant NAC and PDGF-induced migration was assessed. NAC is an antioxidant thiol compound that works by increas-
ing glutathione stores. Treatment of +/+ MMCs with NAC resulted in a significant increase in PDGF AA-induced migration (Fig. 7A). Given that an antioxidant unmasked the inhibitory effect of PDGF BB on AA-induced migration in +/+ cells, the sensitivity of PDGF AA-induced migration to an oxidant was examined in −/− cells. Pretreatment with H2O2 completely blocked PDGF AA-induced migration (Fig. 7B). These results indicate that ROS negatively regulate PDGF AA-induced migration in +/+ cells. PDGF BB-induced increases in cytosolic Ca2+ or ROS generation are not key mediators in PDGF BB-induced migration (Figs. 5C and 7A). Nonetheless, these second messengers may play a significant role in suppressing PDGF AA-induced migration in MMCs when PDGFR-β is expressed.

To investigate cross talk between Ca2+ flux and redox pathways induced by PDGF BB, we examined the effect of Ca2+ chelators EGTA or BAPTA on PDGF BB-induced O2− production. There was no effect of either chelator on PDGF BB-induced O2− generation (Fig. 8A). These data indicate that the increase in Ca2+ flux does not regulate the redox pathway induced by PDGF BB. However, this observation does not rule out the possibility of redox regulation of Ca2+ by PDGF BB. Therefore, the effects of NAC and DPI on PDGF BB-induced intracellular Ca2+ fluxes were examined. Pretreatment of +/+ MMCs with either compound significantly inhibited PDGF BB-induced cytosolic Ca2+ (Fig. 8B). These data indicate that ROS are upstream regulators of the PDGF BB-induced increase in intracellular Ca2+.

**DISCUSSION**

Activation of PDGFR-β is typically promitogenic and chemotactic in a number of cell types, whereas PDGFR-α-medi-
migration is particular to specific cells (64). PDGF AA can inhibit cell migration in certain cell types (34, 45). In vascular smooth muscle cells and 3T3 fibroblasts, PDGFR-β mediates the mobilization of intracellular Ca^{2+} more efficiently than PDGFR-α (12, 13). It is possible that these differences reflect cell type-specific or context-dependent signaling mechanisms.

Signaling through PDGFR-α does not compensate for the loss of PDGFR-β signaling in regulating the development of MMCs to mature mesangial cells. We show that PDGF BB is mitogenic and chemotactic through PDGFR-β only in −/− cells. In the absence of PDGFR-β, signaling through PDGFR-α may cause aberrant migration of MMCs during nephrogenesis and therefore may help explain the lack of mature mesangial cells in the glomerular tuft of PDGFR-β-deficient animals (54). We demonstrate that the lack of PDGF AA-induced migration in +/+ MMCs is specifically due to the presence of PDGFR-β and not due to differential activation of the Akt pathway, which is known to regulate PDGF-induced migration. We present evidence that PDGFR-β-induced generation of ROS and Ca^{2+} flux negatively regulates AA-induced migration and thus provide a mechanism by which specific signals generated by PDGFR-β suppress PDGF AA-induced migration of MMCs during kidney development.

Many studies have confirmed similarities as well as differences differences in the signaling events mediated by PDGFR-α and PDGFR-β. Both receptors are protein tyrosine kinases that activate similar sets of signaling molecules. However, in vivo studies of targeted deletions of the respective receptors reveal markedly different phenotypes. Mice lacking PDGFR-α die between embryonic days E8 and E16, with phenotypic abnormalities including cleft facies, skeletal defects, abnormal somite patterning, and hemorrhage (55). Mice lacking PDGFR-β die between E16 and birth, exhibiting cardiovascular, hematological, and renal defects including abnormal capillary formation within the glomerular tuft of the developing kidney and a total lack of mesangial cells (54). To test the specificity of PDGFR signaling in vivo, Klinghofer et al. (33) created complemental lines of knockin mice expressing mutant PDGFR-β with the intracellular signaling domains of PDGFR-α and mice expressing mutant PDGFR-α with the intracellular signaling domains of PDGFR-β. Both lines demonstrated substantial rescue of normal development, in particular the ability of the PDGFR-β intracellular domain to compensate for PDGFR-α. However, substitution of the PDGFR-β signaling domain with that of PDGFR-α resulted in varying degrees of vascular disease, including glomerular deoid of mesangial cells. These data conclusively demonstrated the importance of PDGF B-dependent β-receptor signaling in the development of mesangial cells.

Since both PDGFR-β and PDGF B-chain −/− mice die perinatally and exhibit a total lack of mesangial cells within the glomerular tuft, it is difficult to study the mechanisms of this pathway in the development of mature mesangial cells. Studies in the PDGF B-chain deficient mouse demonstrate PDGFR-β expression in the developing S-shaped nephron segment and in the early cup-shaped glomeruli. In late-stage glomerular development in the PDGF B-null kidney, PDGFR-β-positive cells are found only in the juxtaglomerular region. Lindahl et al. (40)
have generated chimeric mice from +/+ blastocysts injected with PDGFR-β-deficient embryonic stem cells to provide insight into the ontogeny of mesangial cells. They noted that the glomerular mesangium invariably expressed PDGFR-β, implying a direct effect of PDGF-B on the development of mesangial cell lineage. The glomerular pathology of the /-/- mice indicates that mesangial cells or their progenitors are critical targets for PDGF-B-mediated signals. The absence of mesangial cells in the mutant glomeruli may also be a result of indirect effects of PDGF-B on PDGFR-β signaling. The cells that populate the glomerulus may use PDGF-B and PDGFR-β signaling in the maintenance of cell survival during the transition from proliferating to terminally differentiating cells. Additionally, PDGF B/PDGFR-β may serve to suppress PDGF-A/ PDGFR-α signaling while other factors promote the differentiation program. Although many signaling pathways of PDGFR-α or -β may be redundant, certain signals are specific to each.

We report that PDGF BB induces ROS production and Ca2+ flux in +/+ MMCs, whereas PDGF AA does not. Neither PDGF AA nor BB increases ROS production or Ca2+ flux in /-/- MMCs. Increased cytosolic Ca2+ can activate downstream signals involved in localized structural changes and directed cell migration (53). Many reports have suggested that Ca2+ positively regulates the pathways involved in cell migration using the Ca2+/calmodulin signaling pathway. Previous reports have also suggested that the motility of endothelial cells can be sensitive or insensitive to extracellular Ca2+, depending on the immobilized extracellular matrix and integrins involved (37).

Fig. 7. PDGF-induced chemotaxis is redox dependent. A: quiescent +/+ MMCs were pretreated with 20 mM N-acetyl-L-cysteine (NAC; b), stimulated with PDGF AA or BB, and migration was assessed. B: quiescent /-/- MMCs were pretreated with H2O2, stimulated with PDGF AA or BB, and migration assessed as described in MATERIALS AND METHODS.

Fig. 8. ROS act as upstream mediators of Ca2+ flux. A: quiescent +/+ MMCs were treated with EGTA (5 mM) or BAPTA (2 mM) for 2 min before being stimulated with PDGF BB (10 ng/ml) or PDGF AA (100 ng/ml) for 120 min at 37°C in Hank’s balanced salt solution containing 80 μM ferricytochrome c. Superoxide-specific reduction of ferricytochrome c was calculated as in Fig. 7. Values are means ± SE of 3 separate experiments. B: serum-deprived +/+ MMCs were loaded with fura 2 and incubated with NAC or DPI for 20 min. After baselines were established, +/+ MMCs were treated with PDGF BB, and [Ca2+]i, was measured.
These studies focused on haptotaxis, whereas we now demonstrate sensitivity to Ca2+ in a chemotactic system. Other studies in various cell types have implicated Ca2+ as a mediator of migration, but only a few studies have shown that Ca2+ influx inhibits migration. Hodgson et al. (28) have reported that Ca2+ influx correlates with the phosphorylation of the cytoskeletal protein α-actinin in A2058 human melanoma cells. Horgan et al. (29) have shown G protein-mediated inhibition of neuronal migration requires Ca2+ influx. In the present study, we provide evidence that signals mediated by PDGFR-β, such as an increase in Ca2+ influx, block PDGF AA-induced migration of +/+ MMCs. In developing metanephroi, the absence of PDGFR-β-mediated Ca2+ influx in −/− MMCs allows PDGF AA-induced migration of these cells. This may result in aberrant localization of mesangial cell precursors in the −/− animals.

In nonphagocytic cells, there is considerable evidence that ROS act as classic second-messenger molecules in response to stimulation with a variety of growth factors and cytokines (17, 19). Cytosolic Ca2+ levels can be increased in response to ROS in various cell types through the mobilization of intracellular Ca2+ stores and/or through the influx of extracellular Ca2+ (14, 16, 17, 25, 35, 46–48). This suggests a physiological role of the redox state in the regulation of Ca2+ signaling (31, 32). The exact source of Ca2+ release in response to oxidants remains controversial, and molecular targets of oxidants have not yet been clearly defined (57). On the other hand, oxygen radicals may not be involved in Ca2+ influx (61), but rather the Ca2+ mobilization may be responsible for ROS production (58). We show that ROS are upstream mediators of Ca2+ influx in MMCs and that ROS-dependent Ca2+ influx negatively regulates PDGF AA-induced migration.

Activation of PDGFR-β stimulates production of O2− and H2O2 (4, 42, 56). The differential effect of the PDGF isoforms on ROS generation observed in MMCs correlates well with the data reported by Marumo et al. (42), who showed that PDGF BB, rather than PDGF AA, stimulated ROS generation in aortic smooth muscle cells. PDGF BB has been reported to elicit ROS generation in various cell types (2, 7, 36). For instance, Baé et al. (4) demonstrated that PI3-K and activation of Rac1 are required for PDGFR-β-induced H2O2 production in HepG2 cells. Cells from mice deficient for the mammalian 2-Cys peroxiredoxin type II (Prx II) demonstrate increased H2O2 production and increased PDGFR-β activity (9). In cultured mouse embryonic fibroblasts, overexpression of Prx II suppressed protein tyrosine phosphorylation (including of PLC-γ1) and inhibited PDGF-induced inositol-1,4,5-trisphosphate production and chemotaxis. The authors concluded that Prx II is a principle inhibitor of PDGFR-β signaling, as endogenous H2O2 amplifies PDGFR phosphorylation in a site-specific manner, and Prx II inhibits this phosphorylation and subsequent receptor activity (9). Our data indicate that PDGFR-β blunts activity of PDGFR-α via ROS. In vivo, there may be PDGF BB-induced redox-sensitive mediators that suppress PDGFR-α activation, such as the peroxiredoxins.

PDGFR-α may have a role in the development of interstitial cells, including adventitial fibroblasts (51). Mouse blastemas lacking this receptor (i.e., Patch mutation) have a severe phenotype, characterized by a diffuse paucity of fibroblasts (51) and deficiencies in renal interstitial mesenchyme, the precursors of vascular smooth muscle cells and mesangial cells (6). At later stages of nephrogenesis, PDGFR-β is expressed in MC precursors in the cleft of the comma-shaped and S-shaped bodies and in more mature glomeruli in a mesangial distribution (3). Because PDGFR-α-deficient mice have a deficit in renal interstitial mesenchyme (6, 39), it is our belief that PDGFR-α may serve as the means by which loose mesenchyme differentiates into interstitial mesenchymal cells before the S-shaped body phase, when PDGFR-β-expressing cells home to the developing glomerular cleft.

In this study, PDGF BB induced migration of +/+ MMCs, whereas PDGF AA had minimal effects. In the absence of PDGFR-β, PDGF AA induced migration in MMCs and the response was suppressed by H2O2 or a Ca2+ ionophore. By chelating extracellular Ca2+ or scavenging ROS in +/+ MMCs, PDGF AA seems to be capable of inducing migration. These results suggest that in the absence of PDGFR-β, PDGFR-α is capable of inducing migration, which does not normally occur in the presence of PDGFR-β, and that ROS and Ca2+ influx are involved in negatively regulating PDGFR-α-mediated migration (Fig. 9). These data may help explain why PDGFR-α is unable to compensate for the lack of PDGFR-β in the development of mesangial cells during embryogenesis. The factors involved in the regulation of mesangial cell development are multifactorial and likely depend on the temporospatial distribution of the PDGFs and ligands, the binding affinities of the ligands to their respective receptors, and the specificity of the signals. Further studies are needed to correlate the kinetics of ROS and Ca2+ signaling induced by PDGFR-β to particular cytoplasmic and nuclear events. A comprehensive examination of molecules activated by PDGFR-β may lead to an understanding of the mechanism by which PDGFR-β activation results in mesangial cell development and maturation.

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