Mitogenic Signaling via Platelet-Derived Growth Factor β in Metanephric Mesenchymal Cells

Brent Wagner,* Jill M. Ricono,*† Yves Gorin,* Karen Block,* Mazen Arar,‡ Dan Riley,*§ Goutam Ghosh Choudhury,*§ and Hanna E. Abboud*§

Departments of *Medicine and ‡Pediatrics, University of Texas Health Science Center, †Department of Molecular Medicine, Institute of Biotechnology, and the §Geriatric Research Education and Clinical Center, South Texas Veterans Health Care System, San Antonio, Texas

ABSTRACT

Mice deficient in either platelet-derived growth factor (PDGF) B chain or PDGF receptor (PDGFR) β lack mesangial cells. PDGF stimulates proliferation and migration of metanephric mesenchymal cells, from which mesangial cells are derived. Binding of PDGF to PDGFR-β induces autophosphorylation at specific tyrosine residues and activates various effector proteins, including phosphatidylinositol-3-kinase (PI3-K). This study explored the role of PI 3-K and reactive oxygen species (ROS) in PDGF-mediated signaling using cells established from wild-type and PDGFR-β−/− metanephric blastemas at 11.5 days post-conception. PDGF-induced effects that were dependent on PI3-K activation were determined using PDGFR-β−/− cells made to express “add-back” mutant PDGFR-β capable of binding PI3-K. We found that PDGF is mitogenic for mesenchymal cells expressing PDGFR-β, and PI3-K is an important regulator of PDGF-induced DNA synthesis. Activation of ERK1/2 is partially dependent on PI3-K, and both the PI3-K and MEK-ERK1/2 pathways contribute to PI3-K–dependent mitogenesis. In addition, PDGF-induced DNA synthesis in wild-type cells was found to be dependent on ROS that are generated downstream of PI3-K activation. Using antisense oligonucleotides and small interfering RNA, we determined that the NAD(P)H oxidase Nox4 produces these ROS that activate Akt and the MEK-ERK1/2 mitogenic cascade. In conclusion, the present study demonstrates Nox4 involvement in PDGF-induced DNA synthesis in metanephric mesenchymal cells and provides the first evidence that PDGF-induced PI3-K activity enhances production of ROS by Nox4.


A substantial portion of the mature nephron, including mesangial cells (MC), is derived from the condensation of metanephric mesenchyme during nephrogenesis. MC development depends on PDGF receptor β (PDGFR-β)-expressing metanephric mesenchyme.1 PDGF-BB triggers PDGFR-β dimerization. Intracellular tyrosines are autophosphorylated, serving to activate SH2 domain–containing effectors, including the regulatory p85 subunit of phosphatidylinositol-3-kinase (PI3-K),2 triggering cell migration, proliferation, and differentiation. PDGF-BB–induced migration and proliferation of metanephric mesenchymal cells into the cleft of the maturing glomerulus is PI3-K dependent.3

Reactive oxygen species (ROS) play important roles in agonist-mediated signal transduction. NAD(P)H oxidases are major sources of ROS. Several homologues of the phagocytic NAD(P)H oxidase (gp91phox/Nox2), termed Nox proteins, have
been discovered in nonphagocytic cells including kidney cells. Evidence is accumulating that PDGF activates a multicomponent NAD(P)H oxidase system.

Given the abundance of the Nox4 homologue in the fetal kidney, the goal of this study was to elucidate the cell signaling and potential role of this oxidase in nephrogenesis. Our data indicate that Nox4 is a target of PI3-K, and the subsequent generation of ROS plays a role in MC development.

RESULTS

Establishment and Characterization of Metanephric Mesenchymal Cells in Culture

Metanephric mesenchymal and ureteric bud cells from +/+ and PDGFR-β null (−/−) mouse embryos at 11.5 dpc were isolated and characterized. Tissue was collected at the time of dissection to determine the genotype by PCR. Genotypic analysis demonstrated −/−, heterozygous (+/−), and +/+ alleles (Figure 1A, left). Metanephric blastemas of the same genotype were pooled to propagate +/+ and −/− cells, and pure cultures were established (Figure 1A, right).

Effect of PDGF on DNA Synthesis

PDGF increased DNA synthesis in a dose-dependent manner in +/+ but not in −/− cells (Figure 2A). The PDGF inhibitors wortmannin (500 nM) and LY294002 (25 μM) abrogated PDGF-induced DNA synthesis in +/+ cells (Figure 2B). For validation of PD3-K as a principle effector of DNA synthesis, −/− cells were stably transfected with an “add-back” PDGFR-β mutant construct. The original PDGFR-β F5 mutant contains five tyrosine-to-phenylalanine mutations. This construct was used to add back the tyrosines 740 and 751 to restore the PI3-K–binding sites. This PI3-K add-back mutant PDGFR-β cDNA was subcloned into a mammalian expression vector containing a zeomycin resistance gene for the establishment of a stable receptor-bearing cell line, (+/−)PD3-K. Successful transfection of the −/− cells with the PD3-K add-back construct restored the expression of PDGFR-β (Figure 2C).

PDGF-Induced Activation of +/+ and −/− Cells Expressing the PD3-K Add-Back Mutant β Receptor

For detection of PDGF-dependent phosphorylation in the +/+ and (−/−)PD3-K cells, immune-complex kinase assays were conducted in the presence of [γ32P]ATP (Figure 2D). PDGF treatment led to phosphorylation of the PDGFR-β in +/+ and (−/−)PD3-K cells but not in −/− cells (Figure 2D, arrow). PD3-K activity was measured in anti-phosphotyrosine immunoprecipitates of lysates of PDGF-stimulated (+/+ and (−/−)PD3-K cells (Figure 2E). PDGF stimulated PD3-K activity in +/+ and (−/−)PD3-K cells but not in −/− cells. DNA synthesis increased in response to PDGF in +/+ but not in −/− cells (Figure 2F). PDGF induced a partial but significant restoration of DNA synthesis in (−/−)PD3-K cells. Inhibition of PD3-K with wortmannin or LY294002 suppressed PDGF-induced DNA synthesis in (−/−)PD3-K cells (Figure 2G). These data indicate that PDGF is mitogenic for cells expressing PDGFR-β and demonstrate that PD3-K is an important regulator of PDGF-induced DNA synthesis.

Activation of the PDGFR-β Downstream Effectors Akt, Mitogen-Activated or Extracellular Signal–Regulated Protein Kinase Kinase, and Extracellular Signal–Regulated Kinase Are PD3-K Dependent

The contribution of PDGF-induced PD3-K activation to these downstream effectors was explored. PDGF resulted in time-
dependent phosphorylation of Akt in +/+ cells, which was attenuated in the −/− cell line (Figure 3A). Phosphorylation of Akt in response to PDGF was restored in (−/−)PI3-K cells. In +/+ cells, there was a time-dependent increase in the levels of PDGF-induced mitogen-activated protein kinase (MEK) and extracellular signal–regulated kinase (ERK) phosphorylation (Figure 3, B and C). Compared with +/+ , −/− cells had lower cumulative levels of phosphorylation. The residual phosphorylation of these kinases in the −/− cells is likely due to the effect of PDGF on PDGFR-α. It is interesting that the levels of phosphorylation of ERK1/2 in response to PDGF was restored in the (−/−)PI3-K mutant-expressing cells. These data indicate that the activation of the ERK1/2 cascade in response to PDGF-BB is partially dependent on PI3-K. A MEK inhibitor, U0126, suppressed PDGF-induced DNA synthesis in +/+ and (−/−)PI3-K cells (Figure 3D). Together, these results indicate that both the PI3-K and MEK/ERK1/2 pathways are responsible for PI3-K–mediated PDGF-BB–induced mitogenesis.

**Effect of PDGF on NAD(P)H Oxidase Activity in Metanephric Mesenchymal Cells**

PDGF treatment led to a significant increase in NAD(P)H oxidase-dependent superoxide generation in +/+ but not −/− cells (Figure 4A). PDGF-induced NADPH-dependent superoxide generation was restored in the (−/−)PI3-K cells.
LY294002 reduced PDGF-induced NADPH-dependent ROS generation in +/+ cells to basal levels (Figure 4B). Together, these data indicate that PDGF-induced DNA synthesis was measured as described in the Concise Methods section. Inhibition of MEK1/2 with U0126 suppressed DNA synthesis in response to PDGF in +/+ and (-/-)PI3-K cells. Data are means ± SE of at least three independent experiments. **P < 0.01 versus control by one-way ANOVA; #P < 0.05, ##P < 0.01 versus PDGF-treated group by one-way ANOVA.

LY294002 reduced PDGF-induced NADPH-dependent ROS generation in +/+ cells to basal levels (Figure 4B). Together, these data indicate that PDGF-induced NADPH oxidase-derived superoxide generation is mediated by and is downstream to PI3-K.

PDGF-induced DNA synthesis in +/+ cells was suppressed by the antioxidant N-acetyl-L-cysteine (NAC; Figure 4C). This suggests that PDGF-induced DNA synthesis in +/+ cells is ROS dependent. DNA synthesis in NAC-treated cells was also examined in the (-/-)PI3-K cells. Pretreatment with NAC led to a decrease in PDGF-induced DNA synthesis in (-/-)PI3-K cells, again implicating PI3-K–mediated ROS generation in stimulating mitogenesis.
Characterization of Nox4 Antibody
Cheng et al. reported that the NAD(P)H oxidase homolog Nox4 is expressed in fetal kidney. Affinity-purified Nox4 antibody was generated as described in the Concise Methods section (Figure 5A). HEK293 cells were used as a positive control. The estimated molecular weight of Nox4 is 70 kD. Preincubation of Nox4 antibody with Nox4 peptide blocked the appearance of the 70-kD band in cell lysates from +/+ and mouse MC14 (data not shown). Furthermore, the antibody recognizes Nox4 protein translated in vitro using rabbit reticulocyte lysate (TNT Quick Coupled kit; Promega, Madison, WI), as a 66-kD band (Figure 5B). The difference in the molecular weights between in vitro translated protein band and the protein band detected in cultured cells is likely due to posttranslational modification of Nox4.

Nox4 Regulates PDGF-Induced DNA Synthesis in Metanephric Mesenchymal Cells
Nox4 protein is expressed in these cells (Figure 5A). For validation that Nox4 message was present, +/+ cells were treated with Nox4 sense (S) and antisense (AS) oligonucleotides (Figure 5C). Reverse transcription–PCR (RT-PCR) for Nox4 and the housekeeping gene GAPDH revealed that treatment with AS oligonucleotides decreased Nox4 message. Treatment of +/+ cells with AS Nox4 oligonucleotides substantially reduced the expression of Nox4 protein (Figure 5D). PDGF increased DNA synthesis in control and S oligonucleotide-treated cells (Figure 5E). Treatment of +/+ cells with AS Nox4 oligonucleotides significantly inhibited PDGF-induced DNA synthesis. As Nox4 oligonucleotide treatment blocked PDGF-induced DNA synthesis in (−−)PI3-K cells. For substantiation that Nox4 is the NAD(P)H oxidase homolog mediating the effect, +/+ cells were transfected with small interfering RNA (siRNA) for Nox4. Using a mouse polyclonal Nox4 antibody, we detected a significant reduction in the expression of Nox4 in the siRNA-treated group when compared with control or cells transfected with scrambled (Scr) siRNA (Figure 5F). Nox4 siRNA abrogated PDGF-induced DNA synthesis in +/+ cells (Figure 5G). These data suggest that Nox4-derived ROS mediate the mitogenic effect of PDGFR-β activation.

For determination of whether PDGFR-β–activated mitogenic kinases were redox sensitive, the effects of hydrogen peroxide and antioxidants were investigated. Treatment of +/+ cells with H2O2 led to increased phosphorylation of Akt, MEK,
and ERK1/2 (Figure 6A). Pretreatment of +/+ cells with NAC reduced PDGF-induced Akt and ERK phosphorylation (Figure 6B). The flavoprotein NAD(P)H oxidase inhibitor diphenyliodonium also significantly reduced PDGF-induced Akt and ERK1/2 phosphorylation (Figure 6C).

The effect of Nox4 AS oligonucleotides on Akt and ERK1/2 phosphorylation was examined (Figure 6, D and E). Treatment of +/+ cells with AS Nox4 oligonucleotides suppressed PDGF-induced Akt and ERK1/2 phosphorylation (Figure 6, D and E) compared with S oligonucleotide-treated cells. Nox4 siRNA treatment significantly suppressed PDGF-BB–induced ERK phosphorylation (Figure 6F). These data indicate that PDGF induces activation of Akt and the MEK/ERK1/2 mitogenic cascade via Nox4-generated ROS.

**DISCUSSION**

This study demonstrates the involvement of Nox4 in PDGF-induced DNA synthesis in metanephric mesenchyme and provides the first evidence that PDGF-induced PI3-K activity enhances ROS production via Nox4 NAD(P)H oxidase. We demonstrate that PDGF activates Nox4, and the subsequent

![Figure 7](image-url)
generation of ROS activates MEK/ERK1/2 and Akt signal transduction to induce mitogenesis in these cells (Figure 7).

Mice deficient for PDGF-B chain or PDGFR-β lack MC and have rudimentary kidney glomerular tufts and die before gestational day 18.5. The β receptor is localized to undifferentiated metanephric mesenchyme and at later stages is expressed in MC precursors in the cleft of the comma- and S-shaped bodies and in more mature glomeruli in a mesangial distribution. Conversely, at early stages, PDGF-B chain is expressed in epithelial cells and the mesangium and endothelium of maturing glomeruli. PDGF-BB acts in a paracrine manner to act on epithelial cells and the mesangium and endothelium of maturing glomeruli. PDGF-BB acts in a paracrine manner to stimulate the migration and proliferation of MC precursors from undifferentiated metanephric mesenchyme to the maturing glomerulus.

There are controversial reports in regard to PDGF-dependent cell-cycle progression in various cell lines. Hemizygous mouse embryos that express an extracellular β/intacellular α chimeric PDGFR (β/α) demonstrate a similar glomerular phenotype as PDGFR-β null animals (i.e., lacking an organized glomerular tuft). In contrast, homozygous β/α have normal glomeruli, and mice expressing an extracellular α/intacellular β mutant chimeric β receptor demonstrate normal MC recruitment. Mice that express mutant PDGFR-β lacking autophosphorylation sites requisite for PDGFR-β null animals (i.e., lacking an organized glomerular tuft) demonstrate normal glomerular tufts.

In adult MC, it is known that the PI3-K pathway is critical for cell cycling and PDGF-dependent mitogenesis. Our data demonstrate that PDGF triggers activation of PI3-K via PDGFR-β, with subsequent Nox4-dependent ROS generation, and downstream activation of Akt and ERK1/2 pathways culminating in increased DNA synthesis. Our unique findings are that (1) PI3-K contributes to ERK1/2 activation, (2) PI3-K is involved in Nox4 activation, and (3) Nox4 serves a mitogenic role in metanephric mesenchymal cells. We provide the first evidence that the absence of PDGFR-β leads to profound changes in the biologic responses of cells. The attributes of the −/− cells may explain the failure of β receptor–deficient cells to populate the developing glomerulus. Soriano and colleagues reported that mice homozygous for PDGFR-β with inactivating mutations at the PI3-K binding site (the F2 mutant) and the PLC-γ binding site (the F3 mutant) do not have deleterious MC development. They concluded that other and likely redundant PDGFR-β–mediated signals participate in the actions of the receptor (summarized by Tallquist and Kazlauskas). Because we found a partial restoration of PDGF-BB–induced mitogenesis in the (−/−)PI3-K cells, there are likely other mechanisms absent in the PI3-K add-back mutant PDGFR-β responsible for the remaining stimulating signals. Furthermore, our data are in accordance with findings in the F2 mutant β receptor of Akt activation by PDGF-BB in −/− cells, consistent with minute PDGFR-α activation of the PI3-K pathway. Redundant signaling triggered by PDGF in mice expressing the mutant F2 and F3 PDGFR-β may explain the normal embryonic phenotypes in these animals.

We demonstrate that the greater levels of PDGF-induced phosphorylation of Akt, MEK, and ERK1/2 in +/+ cells depend on the PI3-K pathway activated by PDGFR-β. Furthermore, the results support a novel finding that ERK1/2 activation depends on PI3-K activation by PDGFR-β. There is increasing evidence for the involvement of PI3-K in PDGF-BB–induced ERK1/2 activation. In adult MC, inhibition of PI3-K by LY294002 or wortmannin reduces ERK1/2 activation, showing that ERK1/2 is PI3-K dependent. This seems cell-type specific, because ERK1/2 activation in TSC2 −/− cells is independent of PI3-K.

Activation of PI3-K and PLC-γ by PDGFR-α fails to lead to either mitogenesis or migration in human MC; therefore, specific attributes inherent to PDGFR-β are responsible for these biologic activities. PDGF-stimulated ROS generation has a potential role in cell signaling pathways that mediate cell proliferation, migration, and differentiation. This study demonstrates NADPH-dependent ROS generation and redox sensitivity of PI3-K–dependent effectors in metanephric mesenchyme. The NAD(P)H oxidase Nox4 is a PDGF-inducible source of these ROS. We provide the first evidence of Nox4 involvement in PDGF-induced proliferation. Our results place Nox4-derived ROS downstream of PI3-K in the cascade linking PDGF receptor to mitogenesis. Note that the mechanism described here is in contrast with recent findings by Kwan et al., who found that PDGF-induced NAD(P)H oxidase-derived ROS production is independent of PI3-K in primary rat MC. Our data do not indicate that Nox1 plays a role in PDGF-BB–mediated DNA synthesis in metanephric mesenchyme. This contrasts with the findings in vascular smooth muscle cells that Nox1 rather than Nox4 is the mediator of PDGF-induced superoxide. It is possible that Nox4 mediates PDGF-induced ROS generation in metanephric mesenchyme, whereas other Nox isoforms are involved in adult MC. Nox1 is expressed in adult MC and may be the accountable isoform for PDGF-induced ROS generation in the mature glomerulus.

Our data demonstrate that PDGF-BB–induced DNA synthesis in both +/+ and (−/−)PI3-K cells is PI3-K dependent. The effect of PDGFR-β is mediated through the generation of ROS via Nox4 (Figure 7). In this manner, Nox-derived ROS are relevant in the context of the regulation of organ development.

**CONCISE METHODS**

**Mouse Metanephric Mesenchymal Cell Culture and Cell Transfection**

All animal protocols were in accordance with National Institutes of Health guidelines and reviewed by the Alexion Institution Animal Care Committee.

**PDGFR β Cell Transfection**

Mouse metanephric mesenchymal cells were transfected with a recombinant adenovirus expressing GFP under the control of the PDGFR β promoter. Transfection efficiency was confirmed by immunofluorescence microscopy using an antibody directed against GFP. GFP-positive cells were analyzed for their ability to respond to PDGF-BB stimulation.

**PDGFR β Mouse Metanephric Mesenchymal Cell Culture**

Mouse embryonic mesenchyme was isolated from E14.5 mouse embryos and cultured as described previously. Mesenchymal cells were isolated from the metanephric mesenchyme and cultured in monolayer on fibronectin in serum-free medium.
Care and Use Committee. Heterozygous PDGFR-β-deficient mice, provided by Dr. Soriano,18 were mated to generate +/+ and −/− embryos. Primary cultures of metanephric mesenchymal cells were prepared as described for rat.4 Cells were grown for 3 days in growth media, and then transformed with a retroviral vector expressing the human papilloma virus 16 with the E6/E7 oncoproteins.

Mutant PDGFR-β with specific tyrosine-to-phenylalanine site mutations (F5) and add-back tyrosine residues have been described.11 The F5 mutant receptor is defined by tyrosine-to-phenylalanine inactivating mutations at amino acid positions 740, 751 (i.e., the PI3-K binding site), 771 (Ras guanosine triphosphatase [GTPase] activator protein [GAP]), 1009 (SH2-containing phosphatase [SHP]-2) and 1021.1 One receptor construct derived from the F5 mutant receptor has tyrosine residues restored at 740 and 750, hence referred to as a PI3-K add-back mutant PDGFR-β. This mutant receptor is auto- phosphorylated in the presence of PDGF and activates PI3-K but not GAP or SHP.11 Stable cell lines of −/− cells, (−/+)PI3-K, expressing this PI3-K add-back mutant PDGFR-β were established.

Oligonucleotides and DNA Synthesis
Phosphothiolated S and AS oligonucleotides for Nox1 and Nox4 were used for transfection experiments. AS oligonucleotides were designed near the ATG start codon of native Nox4 (5'-AGCTCTCCAGGACACGCGCC-3'). The Nox1 S and AS oligonucleotide sequences were 5'-GGGAAACTGGCTGGTTAACC, and 5'-GGTTAACCACGCAGTTTCCCC, respectively (Integrated DNA Technologies, Coralville, IA). Twenty-four-well dishes were seeded with 50,000 cells per well with 1 µM of the AS or S oligonucleotides and incubated for 48 h. Monolayers were washed and incubated for another 48 h in serum-deprived medium containing 0 or 1 µM S or AS oligonucleotides. Unless otherwise indicated, PDGF-BB was used at a concentration of 2910 ng/ml. DNA synthesis was assessed by [3H]thymidine incorporation.37

Measurement of ROS
Reagents including lucigenin were purchased from Sigma (St. Louis, MO), and NADPH from Roche (Switzerland). Generation of ROS was measured by NADPH-dependent lucigenin assays.4 Recombinant human PDGF-BB was from R&D Systems (Minneapolis, MN).

PI3-K and Immune Complex Tyrosine Kinase Assay
Cells were lysed in radioimmunoprecipitation assay buffer.37 A total of 100 µg of protein were immunoprecipitated with 1 µg of monoclonal anti-phosphotyrosine antibody,23 and the immune complex kinase assay was conducted as described previously.12

Western Blot Analysis
Anti–phospho-Akt (Ser473), anti-Akt, anti–phospho-p44/p42 (ERK1/2) mitogen-activated kinase (Thr202/Tyr204), and anti–ERK1/2 were from Cell Signaling Technology (Beverly, MA). Anti–PDGFR-β (958) IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-phosphotyrosine antibody was from Upstate Cell Signaling Solutions (Waltham, MA). Immunoblotting was performed as described previously.37 Anti-Nox 4 antibody was generated in rabbits (ProSci, Poway, CA) from the sequence SKTLHSLSNRSNYGTFKFEY, a unique peptide.38

RNA Extraction and Analysis by RT-PCR
Total RNA was isolated and RT-PCR was performed as described previously.39

Nox4 siRNA
Cells were cultured in antibiotic-free medium until 30% confluent. Cells were treated with 400 nM scrambled or Nox4 siRNA (Dharmacon, Lafayette, CO) using X-tremeGENE transfection reagent (Roche). When confluent, monolayers were serum-deprived and treated with 400 nM siRNA. Nox4 antibody used for immunoblotting has been described.15

Statistical Analyses
Means ± SEM were analyzed by ANOVA with the Bonferroni all-pairwise multiple comparison post hoc test, α = 0.05.

ACKNOWLEDGMENTS
These studies were supported by the National Institute of Diabetes and Digestive and Kidney Diseases grant DK-33665 (H.E.A.). Y.G. is supported through a Scientist Development Grant from the American Heart Association. G.G.C. is supported by a VA Research Career Scientist Award. B.W. is a recipient of the National Institutes of Health Loan Repayment Program, National Kidney Foundation Research Fellowship, National Kidney Foundation Jack C. Kent Third Year Research Fellowship, and an American Heart Association Fellow-to-Faculty Transition Award.

We thank Dr. Philippe Soriano (Program in Developmental Biology and Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA) for providing the PDGFR-β-deficient mice. Andrius Kazlauskas (Schepps Eye Research Institute, Harvard Medical School, Boston, MA) provided the PDGF-β mutant constructs. We thank Bridget Fagg for reviewing and performing experiments for the revised manuscript and James Hernandez for help with RT-PCR. G.G.C. is supported by NIH RO1 DK50190 and VA Merit Review Programs.

DISCLOSURES
None.

REFERENCES


