# Platelet-derived growth factor-D induces expression of cyclooxygenase-2 in rat mesangial cells through activation of PI3K/PKB and PKCs

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**Abstract.** Platelet-derived growth factor (PDGF)-D is suggested to be a key factor in the development of several renal pathologies, including mesangioproliferative glomerulonephritis. Cyclooxygenase (COX)-2 is a protein involved in the biosynthesis of inflammatory prostaglandins. In this study, we investigated the effect of PDGF-D on the regulation of COX-2 expression in rat mesangial cells (RMCs). Treatment with PDGF-D induced COX-2 at both the protein and mRNA levels in RMCs, suggesting that the PDGF-D-mediated induction of COX-2 is due to COX-2 transcriptional upregulation. PDGF-D treatment also led to a rapid but transient activation of PKB and extracellular signal regulated kinase (ERK)-1/2. Activities of JNK-1/2 and p38 MAPK, however, were not influenced by PDGF-D in RMCs. Markedly, pharmacological inhibition studies showed that pretreatment with LY294002 (a PI3K/PKB inhibitor) or GF109203X (a pan-PKC inhibitor) suppressed the PDGF-D-induced expression of COX-2 protein and mRNA, while pretreatment with PD98059 (an ERK-1/2 inhibitor) or PP1 (an Src inhibitor) had no effect on it. These findings collectively demonstrate for the first time that PDGF-D induces COX-2 by transcriptional upregulation in RMCs and the induction is largely related to PI3K/PKB and PKCs activities.

## Introduction

Platelet-derived growth factor (PDGF) is a potent mitogen and chemotactic factor for numerous cell types of mesenchymal origin, including glomerular mesangial cells (1,2). To date, four PDGF family members have been identified: PDGF-A, -B, -C and -D (3-6). The PDGF family members exert their biological activities through the cell membrane PDGF receptor (PDGFR), which comprises the α (PDGFR-α) and β (PDGFR-β) chains, with different binding specificities and affinities (2,7). Physiologically, the PDGF system is important in embryonal development, wound healing, and adult maintenance (4,8,9). However, excessive signaling of the system has been implicated in various diseases, including cancer, vasculopathies, fibrosis, and renal pathologies (10-14). Among components of the PDGF system, PDGF-D signaling is suggested to be a key factor in the development of a variety of renal pathologies, including mesangial proliferative glomerulopathy, renal fibrosis, and mesangioproliferative glomerulonephritis (15,16).

Previous studies have suggested the involvement of prostaglandins (PGs) in renal physiology and/or pathology, such as vasodilatation, renin secretion, and sodium and water excretion, and/or renal failure (17-20). Cyclooxygenase (COX) is the rate-limiting enzyme involved in the biosynthesis of PGs and related eicosanoids from arachidonic acid. There are two isoforms of COX: COX-1 and COX-2. COX-1 is constitutively expressed in most cell types and the COX-1-derived PGs are involved in normal inflammatory responses, bone development, and wound healing (21-23). By contrast, COX-2 is an inducible enzyme and its expression is highly increased in cells following the exposure of extracellular stimuli, including interleukin-1β, lipopolysaccharide, manganese or growth factor (24-27). Clinical evidence indicates overexpression of COX-2 and its role in several inflammatory and neoplastic diseases (28). Previous studies have demonstrated an upregulation of COX-2 expression in proliferative glomerulonephritis (29) and an increased renal expression of COX-2 in nephropathies (19,30). It has been shown that inhibition of COX-2 by specific COX-2 inhibitors ameliorates renal ablation-induced changes in the kidney function (31) and reduces expression of several mediators of renal injury in a model of diabetes and hypertension (32). These previous findings suggest that exaggerated COX-2 expression and activity may be involved in various renal pathologies. PDGF-D regulation of COX-2 expression in renal cells, however, remains unknown.

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*Key words:* platelet-derived growth factor-D, cyclooxygenase-2, PKB, extracellular signal regulated kinase-1/2, PKC, rat mesangial cell

In this study, we investigated the effect of PDGF-D on COX-2 expression in rat mesangial cells (RMCs) and determined possible molecular and signaling mechanisms involved.

## Materials and methods

*Materials*. RPMI-1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from WelGENE (Daegu, Korea). Enzyme-linked chemiluminescence (ECL) western detection reagents were bought from Thermo Scientific (Waltham, MA, USA). Nitrocellulose membrane was bought from Millipore (Rockford, IL, USA). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). PDGF-D was purchased from R&D (Minneapolis, MN, USA). Protease inhibitor cocktail (100X) and PP1 were purchased from Calbiochem (Madison, WI, USA). PD98059, LY294002 and GF109203X were purchased from Biomol (Plymouth Meeting, PA, USA). Antibody of COX-2 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Antibodies of phospho-extracellular signal regulated kinase-1/2 (p-ERK-1/2), total-ERK (T-ERK-1/2), phospho-c-Jun N-terminal kinase-1/2 (p-JNK-1/2), T-JNK-1/2, p-p38 MAPK, T-p38 MAPK, p-PKB and T-PKB were purchased from Epitomics (Burlingame, CA, USA). Other reagents, including anti-actin mouse monoclonal antibody, were purchased from Sigma (St. Louis, MO, USA).

*Cell culture*. RMCs were grown at 37˚C in a humidified condition of 95% air and 5% $CO_2$ in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin.

*MTS assay*. To measure the effect of PDGF-D and/or pharmacological inhibitors or agents on the viability of RMCs, cells were grown in 96-well plates at a density of $1x10^4$ cells/well in 100 $\mu$l volume and were serum-starved for 24 h. Cells were then treated without or with PDGF-D in the absence or presence of LY294002, PD98059, GF109203X or PP1 for 24 h, at which time point cells were incubated with MTS (20 $\mu$l/well) for 1.5 h at 37˚C. The absorbance was measured at 595 nm using a microplate reader. The MTS assay was performed in triplicates. Data are the means ± standard error (SE) of three independent experiments.

*Preparation of whole cell lysates*. To measure the effect of PDGF-D on the expression of COX-2 protein in RMCs, cells were plated in 6-well plates at a density of $1x10^6$ cells/well in 2 ml volume and were serum-starved for 24 h. Cells were then treated without or with different concentrations of PDGF-D for 24 h. Control or the PDGF-D-treated cells were washed with PBS and exposed to cell lysis buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, proteinase inhibitor cocktail (1X)]. The cell lysates were collected in a 1.5 ml tube and centrifuged for 20 min at 4˚C at 12,000 rpm. The supernatant was saved and protein concentrations were determined with the Bradford reagent.

*Western blotting*. Proteins (50 $\mu$g) were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes (Millipore). The membranes were washed with TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (vol/vol) Tween-20 (TBST) followed by blocking with TBST containing 5% (wt/vol) non-fat dried milk. The membranes were incubated overnight with antibodies specific for the protein of interest at 4˚C. The membranes were exposed to secondary antibodies coupled to horseradish peroxidase at room temperature for 2 h. The membranes were washed, and immunoreactivities were detected by ECL reagents.

*Reverse transcription-polymerase chain reaction (RT-PCR)*. To measure the expression levels of PDGFR-α and PDGFR-β in RMCs, cells were plated in 6-well plates at a density of $1x10^6$ cells/well in 2 ml volume overnight. To measure the effect of PDGF-D on the expression of COX-2 mRNA in RMCs, cells were plated in 6-well plates at a density of $1x10^6$ cells/well in 2 ml volume and serum-starved for 24 h. Cells were then treated without or with different concentrations of PDGF-D for 24 h. Total RNA from the conditioned cells above was isolated using the RNAzol-B (Tel-Test, Inc.). Three micrograms of total RNA were reverse transcribed using a random hexadeoxynucleotide primer and reverse transcriptase. Single stranded cDNA was amplified by PCR with the following primers: PDGFR-α, forward, 5'-GGC TTC AAC GGA ACC TTC AG-3' and reverse, 5'-CGC TGT CTT CTT CCT TAG CC-3'; PDGFR-β, forward, 5'-GAG TGC CCT CCC GCA TTG-3' and reverse, 5'-GGT AGA CCA GGT GAC ATT TG-3'; COX-2, forward, 5'-CTG TAC TAC GCC GAG ATT CCT GA-3' and reverse, 5'-GTC CTC GCT TCT GAT CTG TCT TG-3'; GAPDH, forward, 5'-GGT GAA GGT CGG TGT GAA CG-3' and reverse, 5'-GGT AGG AAC ACG GAA GGC CA-3'. The PCR conditions were: PDGFR-α, 30 cycles of denaturation at 94˚C for 30 sec, annealing at 50˚C for 30 sec, and extension at 72˚C for 1 min; PDGFR-β, 35 cycles of denaturation at 94˚C for 30 sec, annealing at 50˚C for 30 sec, and extension at 72˚C for 1 min; COX-2, 35 cycles of denaturation at 94˚C for 30 sec, annealing at 56˚C for 30 sec, and extension at 72˚C for 30 sec; GAPDH, 27 cycles of denaturation at 94˚C for 30 sec, annealing at 57˚C for 30 sec, and extension at 72˚C for 30 sec, respectively. GAPDH was used as an internal control to evaluate the relative expression of COX-2.

*Statistical analysis*. Cell count analysis was performed in triplicates and repeated three times. Data are expressed as the means ± standard error (SE). Significance ($P<0.05$) was determined by one-way ANOVA.

## Results

*PDGF-D induces expression of COX-2 at both the protein and mRNA levels in RMCs*. There are two types of PDGF receptor (PDGFR); PDGFR-α and PDGFR-β. PDGF-D binds predominantly to PDGFR-β. We thus initially measured the expression levels of PDGFR in RMCs using RT-PCR analysis. RMCs expressed mRNA expressions of both PDGFR-α and PDGFR-β (Fig. 1A). However, there were much higher expression levels of PDGFR-β than PDGFR-α. Expression levels of GAPDH mRNA were used as a loading control. After determining the PDGFRs expressed in RMCs, we next analyzed the effect of PDGF-D on the induction of COX-2 protein

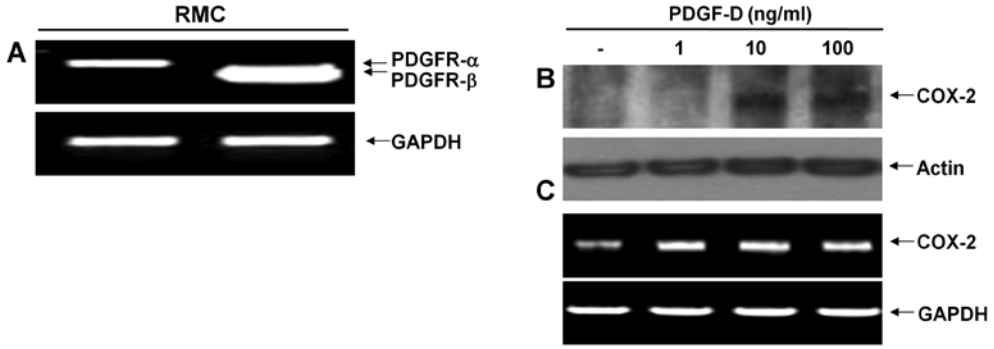


Figure 1. Expression of PDGFRs and effect of PDGF-D on the expression of COX-2 protein and mRNA in RMCs. (A) RMCs were plated in $1x10^6$ cells/2 ml/well/6-well plate overnight. Total RNA was prepared and subjected to RT-PCR analysis using specific primers of PDGFR-α and PDGFR-β, respectively. The image is representative of three independent experiments. (B and C) RMCs seeded in $1x10^6$ cells/2 ml/well/6-well plate the day before treatments were serum-starved for 24 h. Cells were then treated without or with PDGF-D for the indicated concentrations for an additional 24 h. (B) Whole cell lysates and total RNA were prepared, and subjected to western blotting (C) using specific antibody of COX-2 and actin and RT-PCR analysis with specific primers of COX-2 and GAPDH, respectively. The image in (B and C) is representative of three independent experiments.

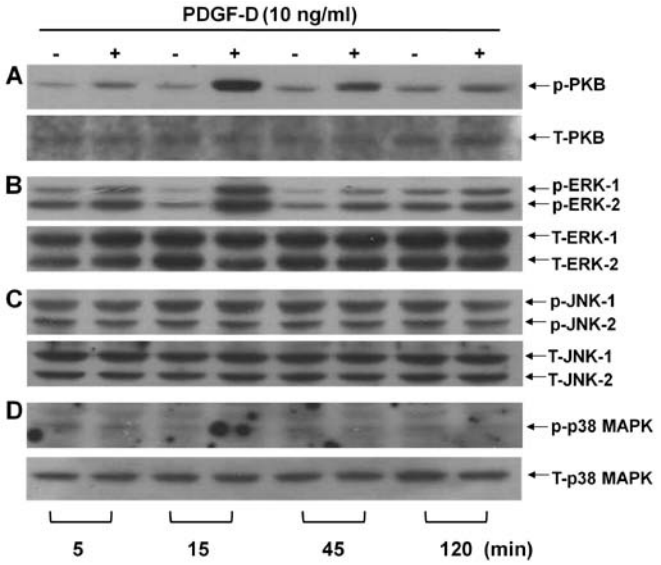


Figure 2. Time course experiments on activation of PKB and the family of MAPKs in the PDGF-D-treated RMCs. RMCs seeded in $1x10^6$ cells/2 ml/well/6-well plate the day before treatments were serum-starved for 24 h. Cells were then treated without or with PDGF-D (10 ng/ml) for the indicated times (5-120 min). Whole cell lysates were prepared, and subjected to western blotting using specific antibody. This image is representative of three independent experiments. p-PKB, phospho-PKB; T-PKB, total-PKB; p-ERK-1/2, phospho-ERK-1/2; T-ERK-1/2, total-ERK-1/2; p-JNK-1/2, phospho-JNK-1/2; T-JNK-1/2, total-JNK-1/2; p-p38 MAPK, phospho-p38 MAPK; T-p38 MAPK, total-p38 MAPK.

and mRNA expressions in RMCs using western blotting and RT-PCR analysis, respectively. COX-2 protein was not detected in control RMCs or RMCs exposed to PDGF-D at 1 ng/ml for 24 h (Fig. 1B). However, there was an upregulation of COX-2 protein in RMCs treated with PDGF-D at 10 or 100 ng/ml for 24 h. Notably, there were low levels of COX-2 mRNA in control RMCs (Fig. 1C). Treatment with PDGF-D even at 1 ng/ml further enhanced the COX-2 mRNA levels. However, there was no further enhancement by PDGF-D treatment at 10 or 100 ng/ml. Expression levels of control actin protein and GAPDH mRNA remained constant in RMCs treated without or with PDGF-D.

*PDGF-D leads to a rapid but transient activation of PKB and ERK-1/2 in RMCs*. Due to the strongest COX-2 inducing effect, the 10 ng/ml concentration of PDGF-D was chosen for further studies. Time course experiments were next used to examine the effect of PDGF-D on the activation of intracellular signaling proteins, herein PKB and the family of MAPKs, in RMCs. Treatment with PDGF-D led to a time-dependent increase in PKB phosphorylation in RMCs (Fig. 2A). PDGF-D treatment even at 5 min slightly enhanced phosphorylation of PKB. Maximal PKB phosphorylation occurred at 15 min, followed by a gradual decline at 45 or 120 min. PDGF-D treatment also resulted in an enhancement of ERK-1/2 phosphorylation in a time-dependent manner (Fig. 2B). In particular, strong ERK-1/2 phosphorylation was induced by 15 min treatment with PDGF-D, followed by a sharp decline thereafter. However, treatment with PDGF-D at the times tested did not change the phosphorylation levels of JNK-1/2 (Fig. 2C) and p38 MAPK (Fig. 2D) in RMCs. Total expression levels of PKB and each member of the MAPK family were not changed by treatment with PDGF-D at the times tested (Fig. 2, low panels). These results suggest that PDGF-D treatment specifically increases the phosphorylation levels of the pre-existing PKB and ERK-1/2 without *de novo* synthesis of PKB and ERK-1/2.

*Activities of PI3K/PKB and PKCs are critical for the PDGF-D-induced COX-2 expression in RMCs*. Pharmacological inhibition studies were next performed to investigate the role of PKB and/or ERK-1/2 activation in the PDGF-D-induced COX-2 expression in RMCs. Pretreatment with LY294002 (LY, a PI3K/PKB inhibitor) strongly suppressed the PDGF-D-induced expression of COX-2 protein and mRNA, but pretreatment with PD98059 (PD, an ERK-1/2 inhibitor) had little effect on the PDGF-D-induced expression of COX-2 protein and mRNA in RMCs (Fig. 3A and B). Using additional pharmacological inhibitors, such as GF109203X (GF, a pan-PKC inhibitor) and PP1 (an Src inhibitor), we also determined the role of PKCs or Src in the induction of COX-2 expression by PDGF-D in RMCs. Pretreatment with GF largely inhibited the PDGF-D effect on the induction of COX-2 protein and mRNA, while pretreatment with PP1 did not (Fig. 3C and D).

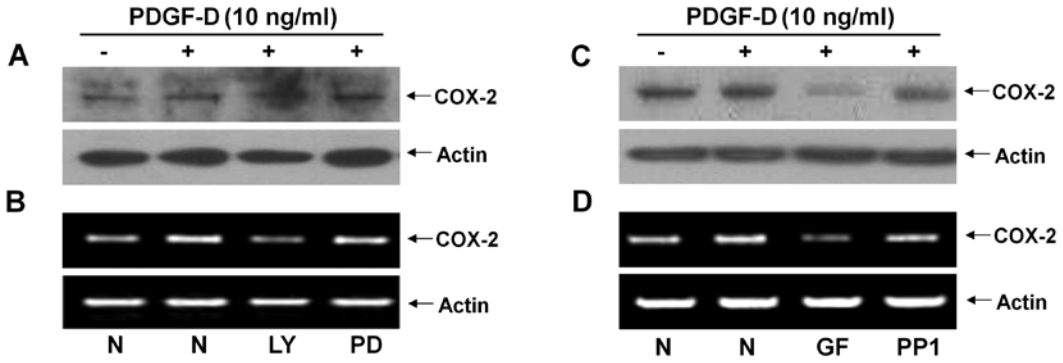


Figure 3. Effect of LY294002, PD98059, GF109203X or PP1 on the PDGF-D-induced expression of COX-2 protein and mRNA in RMCs. RMCs seeded in $1x10^6$ cells/2 ml/well/6-well plate the day before treatments were serum-starved for 24 h. Cells were pretreated without or with LY294002 (a specific inhibitor of PI3K/PKB), PD98059 (a specific inhibitor of ERK-1/2), GF109203X (a pan-PKC inhibitor ) or PP1 (a specific inhibitor of Src) for 1 h, and then treated without or with PDGF-D (10 ng/ml) in the absence or presence of each inhibitor for an additional 24 h. (A and C) Whole cell lysates and total RNA were prepared and subjected to western blotting (B and D) using specific antibody of COX-2 and actin and RT-PCR analysis with specific primer of COX-2 and GAPDH, respectively. This image is representative of three independent experiments.

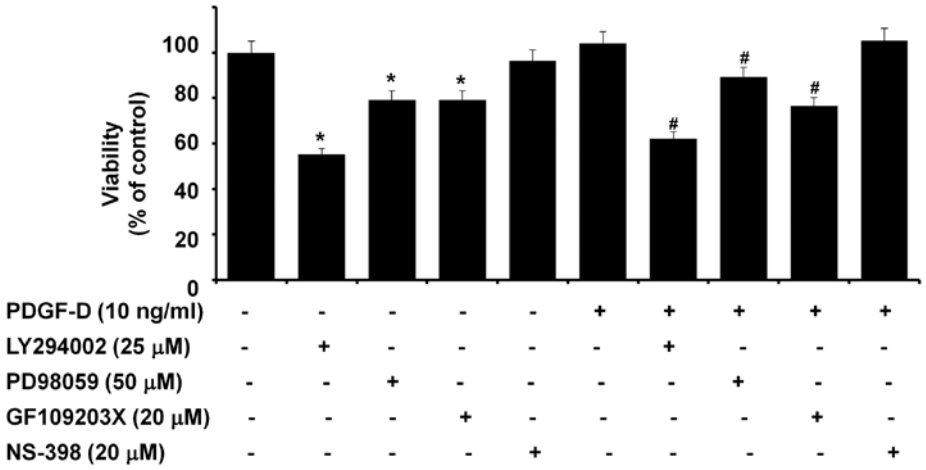


Figure 4. Effect of LY294002, PD98059, GF109203X or NS-398 on the viability of RMCs treated without or with PDGF-D. RMCs seeded in $1x10^4$ cells/100 $\mu$l/well/96-well plate the day before treatments were serum-starved for 24 h. Cells were pretreated without or with LY294002 (LY, a specific inhibitor of PI3K/PKB), PD98059 (PD, a specific inhibitor of MEK-1/2, an upstream activator of ERK-1/2), GF109203X (GF, a pan-PKC inhibitor ) or NS-398 (NS, a specific COX-2 enzymatic inhibitor) for 1 h and then treated without or with PDGF-D (10 ng/ml) in the absence or presence of each inhibitor for an additional 24 h, followed by measurement of the cell viability by MTS assay. Data are the means ± SE of three independent experiments. $^*P<0.05$ compared to the value in the absence of LY, PD or GF. $^\#P<0.05$ compared to the value of PDGF-D treatment in the absence of LY, PD or GF.

*Activities of PI3K/PKB, ERK-1/2 and PKC (but not COX-2) are necessary for the growth of RMCs*. The effect of PDGF-D on the growth of RMCs was next investigated by MTS assay. Treatment with PDGF-D slightly enhanced the viability of RMCs (Fig. 4). The role of COX-2, PKB, ERK-1/2 and/or PKCs induced or activated by PDGF-D in the growth of RMCs was then evaluated using pharmacological inhibitors, including NS-398 (NS, a COX-2 inhibitor). Pretreatment with LY, PD or GF reduced the viability of RMCs treated with PDGF-D. Notably, there was a similar suppressive effect by LY, PD or GF on the viability of RMCs that were grown in the absence of PDGF-D. However, NS pretreatment did not affect the viability of RMCs treated without or with PDGF-D.

## Discussion

In the present study, we demonstrated for the first time that PDGF-D induces the expression of COX-2 by transcriptional upregulation in RMCs. Moreover, our data suggest that the PDGF-D-induced COX-2 expression in RMCs is at least mediated through modulation of the PI3K/PKB and PKC activities.

Previous studies have shown that PDGF-B, a member of the PDGF family, induces expression of COX-2 in RMCs (33) or rat smooth muscle cells (34). However, PDGF-D regulation of COX-2 expression in renal cells remains unclear. In initial experiments, we have shown that RMCs express high levels of PDGFR-β, which binds predominantly to PDGF-D (Fig. 1A) and the exposure of PDGF-D into RMCs leads to induction of COX-2 protein expression (Fig. 1B). It is thus evident that the PDGF-D and PDGFR-β system is functional in inducing COX-2 in RMCs.

Expression of the *COX-2* gene is controlled at multiple levels, including transcription, post-transcription, and translation. It has recently been shown that the PDGF-B-induced COX-2 expression in rat smooth muscle cells is related to both COX-2 transcriptional and post-transcriptional upregulation

(34). However, there is evidence demonstrating that PDGF-B fails to induce expression of COX-2 mRNA in human gingival fibroblasts pretreated with interleukin-1β (35). The present study shows an upregulation of COX-2 transcripts in the PDGF-D-treated RMCs (Fig. 1C), indicating that the PDGF-D-induced expression of COX-2 protein in RMCs is primarily due to COX-2 transcriptional upregulation.

There are numerous reports suggesting the involvement of the activities of PKB and MAPKs in COX-2 transcriptional upregulation (26,36,37). Earlier investigations have shown activation of PKB, ERK1/2, JNK-1/2 or p38 MAPK and their role in many cellular changes in response to PDGF-D signal. For instance, it has been shown that treatment with PDGF-D (100 ng/ml, 10 min) activates ERK-1/2, which may enhance cell proliferation, in human schwannoma cells (38). In malignant mesothelioma cells, it has been demonstrated that treatment with PDGF-D (40 ng/ml, 10 min) leads to activation of Akt and ERK, which may facilitate cell chemotaxis (39). In cultured hepatic stellate cells and myofibroblasts, treatment with PDGF-D (100 ng/ml, 15 min) has been shown to induce phosphorylation of ERK1/2, JNK-1/2, p38 MAPK and PKB, which may contribute to matrix accumulation (40). Herein, we demonstrated that treatment with PDGF-D (10 ng/ml) rapidly but transiently induced phosphorylation of PKB and ERK-1/2 but did not influence that of JNK-1/2 and p38 MAPK in RMCs (Fig. 2). It is assumed that PDGF-D may differentially induce activation of the family of MAPKs in a cell type-dependent manner and/or that the differential effect of PDGF-D on activation of the family of MAPKs in previous studies and herein may be due to different experimental conditions used (such as, serum absence vs. presence, 100 ng/ml vs. 10 ng/ml of PDGF-D). Notably, in this study, we showed that activation of PI3K/PKB, but not ERK-1/2, is critical for the PDGF-D-induced COX-2 transcriptional upregulation in RMCs (Fig. 3A and B). Previous studies have suggested a role of PKC-δ in COX-2 expression induced by epidermal growth factor in gliomas (27) or of Src in COX-2 transcriptional and post-transcriptional upregulation induced by PDGD-B in rat smooth muscle cells (34). Although activities of PKCs and Src in response to PDGF-D treatment in RMCs are not measured herein, the present findings further indicate that activities of PKCs and Src are also necessary for the PDGF-D-induced COX-2 transcriptional upregulation in RMCs (Fig. 3C and D).

Evidence suggests that MC proliferation is an early event in various renal pathologies and PDGF-D is involved in MC proliferation *in vitro* (15,16). In support of the latter, in this study, we demonstrated that PDGF-D treatment leads to a slight increase of the viability of RMCs (Fig. 4). The COX-2-dependent and independent MC proliferation have previously been proposed. This notion is based on the fact that some COX-2 metabolites have antiproliferative effects on RMCs (41,42), while COX-2 exerts its antiproliferative effects on mesangial cells or 293 human embryonic kidney cells independently of COX activity (43,44). The present findings that treatment with NS-398, a selective COX-2 inhibitor, does not influence the viability of both control and PDGF-D-treated RMCs (Fig. 4) support no role of the basal or agonist-induced COX-2 (and their metabolites) in the viability of RMCs. There is a large body of evidence suggesting the importance of the activity of intracellular signaling proteins in MC proliferation. For instance, it has been shown that the activity of ERK-1/2 is necessary for MC proliferation induced by insulin-like growth factor-1, a major mitogenic growth factor for MC (45). It has also been shown that PI3K/PKB activity is linked to PDGF-B-induced MC proliferation (46) and PKC activity is critical for MC proliferation induced by lysophosphatidylcholine, a major component of oxidized-low density lipoproteins (47). The present study suggests that the basal (but not the PDGF-D-induced) activities of PI3K/PKB, ERK-1/2 or PKC are important for the growth of RMCs (Fig. 4). Given that MC proliferation has been largely attributed to the activity of PI3K/PKB, ERK-1/2 and/or PKC described above, it is suggested that single and/or combined treatments with pharmacological inhibitor of PI3K/PKB, ERK-1/2 or PKC may be useful against renal pathologies in which excess MC proliferation is problematic. Accordingly, a recent study suggested the potential utility of PKC inhibitor as a therapeutic strategy in glomerular disease, which is evidenced by the fact that PKC-β inhibition leads to the amelioration of the pathological findings of experimental mesangial proliferative glomerulonephritis (48).

In conclusion, we demonstrated that: i) PDGF-D induces expression of COX-2 through transcriptional upregulation in RMCs, ii) the PDGF-D-induced COX-2 expression in RMCs is at least mediated through the regulation of PI3K/PKB and PKCs activities, iii) the activities of PI3K/PKB, ERK-1/2 and PKCs, but not COX-2, are necessary for the growth of RMCs.

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