Activation of Src Kinase in Platelet-Derived Growth Factor-B-Dependent Tubular Regeneration after Acute Ischemic Renal Injury

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We previously reported that the platelet-derived growth factor B-chain (PDGF-B)/PDGF receptor (PDGFR) axis is involved in tubular regeneration after ischemia/reperfusion injury of the kidney. In the present study, we examined the activation of Src tyrosine kinase, a crucially important signaling molecule for PDGFR, and assessed the role of Src in PDGF-B-dependent renal tubular regeneration after ischemia/reperfusion injury. Immunoblot using clone 28, a monoclonal antibody specific for the active form of Src kinases, demonstrated increased active Src expression in the injured rat kidney 6 hours after reperfusion with peak activation at 12 hours. *In vitro* kinase assay confirmed increased Src activity that concurred with PDGFR- β activation as detected by the increment of receptor-phosphorylated tyrosine. Immunohistochemistry using clone 28 demonstrated that active Src was preferentially expressed in the S3 segment of the proximal tubule in reperfused kidney, where it is not normally expressed. This enhanced expression of active Src was colocalized with the increased PDGFR expression in the tubular cells that were undergoing cell proliferation cycle. Trepidil administration suppressed Src and PDGFR- β activation in the reperfused kidney and resulted in deteriorated renal function. These findings suggest that active Src participates in PDGF-B-dependent regeneration of tubular cells from acute ischemic injury. (Am J Pathol 2003, 163:277–286)

injury involving the proximal tubule.¹ After an ischemic insult, damaged cells are desquamated, after which the dedifferentiated proximal tubular cells (PTCs) proliferate and migrate into denuded areas of the basement membrane to establish new epithelium.² Various growth factors are generally considered to regulate this repair process in a coordinated process involving sequential expression. For example, platelet-derived growth factor (PDGF)-B, epidermal growth factor, heparin-binding epidermal growth factor-like growth factor, insulin-like growth factor, and transforming growth factor- β have all been reported to stimulate renal tubular epithelial cell mitogenesis after ischemic injury.^{3–6} Specifically, PDGF-B exerts a wide range of biological effects on renal cells through stimulation of cellular proliferation and migration.^{7–13}

The biological activity of PDGF-B is mediated through the binding of its cell surface receptors, PDGFR- α and - β subunits, which transduce extracellular events into intracellular biochemical signals.¹⁴ PDGFRs associate with a variety of SH2 domain-containing substrates of protein tyrosine kinases, including the Src kinase family, for further signal relaying.⁷ Src family kinases are nonreceptor tyrosine kinases that regulate biological responses, including cell proliferation, migration, differentiation, and survival.¹⁵ Although the activation of Src kinase in response to a single stimulus often involves several mechanisms, dephosphorylation of Tyr-530 at its C-terminal regulatory domain is thought to be a necessary step in the initiation of Src activation.^{15–19} Src kinase activation has been reported to contribute to PDGF-dependent cell-cycle progression, mitogenesis, and chemotaxis through association with PDGFR- β *in vitro*.²⁰ It is, however, unknown whether PDGF-B-dependent Src activation is a trigger for these biological activities *in vivo*.

In a previous study,³ we clarified the role of PDGF-B and PDGFR expression in the kidney after ischemia/reperfusion injury. The mRNA expression of PDGF-B and PDGFRs was enhanced after injury. The results of immunohistochemical analysis and/or *in situ* hybridization revealed the expression of both PDGF-B and PDGFR- β in the S3 segment of the proximal tubule where expression

Regeneration of damaged proximal tubular epithelium through cell proliferation and differentiation is a fundamental mechanism of nephrogenic repair after ischemic

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is not normally observed. Inhibition of the PDGF-B/PDGFR axis resulted in a deterioration of renal functional recovery, abnormal regeneration, and suppressed proliferation of tubular epithelial cells. On the basis of these observations, we hypothesized that Src kinase may be activated by enhanced PDGF-B/PDGFR expression and thus, Src kinase might be involved in tubular regeneration after ischemic renal injury. Accordingly, the role of Src kinase was assessed in the present study by immunoblot and immunohistochemistry using active Src-specific monoclonal antibody (mAb), clone 28,²¹ together with the measurement of Src kinase activity in kidneys with ischemic injury. Further, the PDGF-B/PDGFR axis was inhibited by Trapidil²²⁻²⁵ and the correlation between the Src activation and the state of PDGFR- β activation was also examined.

Materials and Methods

Animal Studies

All animal experiments were performed in accordance with our institution's regulations for the care of animals. Ischemic tubular injury was induced in male Sprague-Dawley rats weighing 200 to 250 g by clamping their bilateral renal arteries for exactly 30 minutes. During this process, their core body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ by placing them on a homeothermic table and monitoring them with a temperature-sensing rectal probe. After the clamp was released, their kidneys were reperused for the indicated time intervals. Under deep anesthesia with pentobarbital, a 22-gauge needle was inserted into the aorta caudal to the renal arteries and their kidneys were perfused with 250 ml of chilled Ringer's solution to remove all blood from the organ. The left kidney was excised, and the outer strip of outer medulla (OSOM) was dissected from sliced kidney on ice. Dissected OSOM was immediately frozen in liquid nitrogen and stored at -80°C for Western blot analysis. To perform morphological analysis, the right kidney of each rat was further perfused and fixed with 4% paraformaldehyde. After embedding into paraffin, glass-mounted 4- μm -thick tissue sections were prepared for immunohistochemical staining and for other stainings.

Western Blot Analysis

Frozen tissue was homogenized with a polytron homogenizer on ice in buffer containing 20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L ethylenediaminetetraacetic acid, 140 mmol/L NaCl, 1% Nonidet P-40, 50 $\mu\text{g}/\text{ml}$ aprotinin, 50 mmol/L NaF (all of the above were purchased from Nakarai, Kyoto, Japan), 1 mmol/L sodium orthovanadate (Wako, Osaka, Japan), and 1 mmol/L phenylmethyl sulfonyl fluoride (Wako). Homogenized tissue was centrifuged for 30 minutes at 15,000 rpm at 4°C . Supernatant was collected and protein concentrations were measured using the method of Bradford.²⁶ After 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, all protein was transferred to a nylon membrane (Amersham Phar-

macia Biotech, Buckinghamshire, England, UK). Blocking was performed for 2 hours in a phosphate-buffered saline solution (PBS) containing 5% (w/v) skim milk, followed with incubation with anti-active Src kinase monoclonal antibody clone 28²¹ (0.3 $\mu\text{g}/\text{ml}$) at 4°C overnight. Immunoblot for GAPDH (mAb for GAPDH, 2.73 $\mu\text{g}/\text{ml}$; Biogenesis, Poole, England, UK) was used as loading control for protein applied in each lane. After repeated washing with PBS containing 1% Triton (PBS-T), the filter was incubated with horseradish peroxidase-conjugated sheep anti-mouse Ig (dilution of 1:2000; Amersham Pharmacia Biotech) at room temperature for 2 hours. Immunoreactive protein was detected by enhanced chemiluminescence (Bio-Rad, Richmond, CA). Results were analyzed with NIH imaging software.

In Vitro Src Kinase Assay

Proteins were prepared as indicated in the Western Blot Analysis section. Clone 28 (2 μg) was added to protein lysate (500 μg) and the mixture was gently rocked for 2 hours at 4°C . Immune complexes were precipitated with protein A-Sepharose (Amersham Pharmacia Biotech). After washing the precipitate five times with the buffer indicated above, then twice with the kinase assay buffer (20 mmol/L HEPES, pH 7.2, 5 mmol/L MgCl_2 , 10 mmol/L MnCl_2 , 2 mmol/L dithiothreitol), we added [γ -³²P]ATP (10 μCi ; New England Nuclear, Boston, MA) and acid-denatured enolase (2 μg ; Sigma Chemical Co., St. Louis, MO) as substrate.^{21,27} The reaction was allowed to proceed at 30°C for 10 minutes and stopped immediately by adding sodium dodecyl sulfate sample buffer. The samples were analyzed in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

PDGFR- β Activity Assay

Immunoprecipitation was performed by treating 500 μg of the protein lysate with 4 μg of anti-PDGF receptor type B antibody (Upstate Biotechnology, Lake Placid, NY). After washing the precipitate three times with the buffer, the samples were analyzed in 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nylon membrane. Blocking was performed overnight in PBS containing 5% (w/v) bovine serum albumin, followed with incubation with horseradish peroxidase-conjugated antibody against phosphotyrosine (250 $\mu\text{g}/\mu\text{l}$ of anti-PYtr horseradish peroxidase; Transduction Laboratories, Lexington, KY) at room temperature for 2 hours. After multiple washes with PBS-T, the immunoreactive protein was detected by enhanced chemiluminescence.

Immunohistochemical Analysis

Immunohistochemical staining was performed by the streptavidin-peroxidase method. After deparaffinization and rehydration, sections were treated with distilled water containing 0.3% H_2O_2 and 0.1% N_2Na to quench internal peroxidase activity, incubated overnight with anti-active Src kinase monoclonal antibody (clone 28, 0.06 $\mu\text{g}/\text{ml}$) at

4°C, and then subjected to a three-step immunoperoxidase procedure (Histofine kit; Nichirei, Tokyo, Japan). Immunoreactive products were visualized using diaminobenzidine as a chromogen. To stain for both active Src kinase and proliferating cell nuclear antigen (PCNA), tissue sections were first incubated with mouse monoclonal antibody against PCNA (dilution of 1:2000; Organon Teknica, West Chester, PA). After the immunoreactive products of this procedure were detected as mentioned above and visualized using a diaminobenzidine-nickel mixture as a chromogen, the same sections were incubated with clone 28. The immunoreactive products for clone 28 were then detected as described above. Control staining was performed using nonimmune mouse serum and the appropriate secondary antibody. To differentiate various segments of the nephron from each other, kidney tissue was stained with hematoxylin and eosin and examined morphologically, as previously described.²⁸ To further identify individual nephron segments, Tamm-Horsfall protein and peanut agglutinin stainings were performed. Tamm-Horsfall protein was found to localize within the distal convoluted tubule, as well as the thick ascending limb. Positive staining for peanut agglutinin was found in the proximal convoluted tubuli, the distal tubuli, and the collecting duct, but not in the glomeruli or proximal straight tubuli.²⁹

To perform immunofluorescence staining for both PDGFR- β and active Src kinase, individual sections were incubated overnight at 4°C with the mixture of clone 28 (0.6 μ g/ml) and anti-PDGFR- β antibody, Ab-1 (0.5 μ g/ml; Oncogene Science, Uniondale, NY). Ab-1 is a rabbit polyclonal antibody raised against synthetic peptides corresponding to amino acids 425 to 466 of murine, or 1082 to 1101 of human, PDGFR- β . The specificity of Ab-1 has been validated in rat tissue by Western blot analysis (data not shown). After this, secondary reagents (2 μ g/ml, rhodamine-conjugated goat affinity purified antibody to mouse IgG and fluorescein isothiocyanate-conjugated goat affinity purified antibody to rabbit IgG; ICN Pharmaceuticals, Aurora, OH) were added for 2 hours at room temperature. Confocal laser-scanning microscopy was performed using a model 510 laser-scanning microscope (Carl Zeiss, Göttingen, Germany) and LSM image processing software.

Inhibition of PDGF-B Action by Trapidil

To examine whether active Src kinase expression is dependent on the PDGF-B/PDGFR axis and to characterize the contribution of PDGF-B/PDGFR axis-dependent active Src to the nephrogenic repair process, we examined the expression of active Src kinase in relation to renal function by inhibiting the PDGF-B/PDGFR axis with Trapidil.^{22–25} Trapidil was given daily by intraperitoneal injection with a dose of 90 mg/kg starting 2 days before the onset of ischemic injury. Blood samples were obtained from the tail at indicated time intervals. The concentration of serum creatinine was measured by an enzyme method (Boehringer-Mannheim, Mannheim, Germany).

Figure 1. Western blot analysis for Src kinase expression in the OSOM of the rat kidney after ischemia/reperfusion. **A:** Detection of the active form of Src kinase with clone 28. Representative results of three independent experiments are shown. Clone 28 detected a 60-kd protein that was found to correspond to the active form of Src kinase. **B:** Quantification of the signal intensities noted in independent experiments is summarized for the active form of Src kinase. Data are expressed as the mean $\pm$ SD ($n = 3$). Significant differences between the kidneys of sham-operated and injured rats are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

Statistical Analysis

Analysis of variance was followed by the Scheffé's test to detect significance in a test of multiple comparisons. Comparisons between the two groups were analyzed by the Student's unpaired t -test. P values of <0.05 were defined as statistically significant.

Results

The Src Kinase Activation Was Increased in the Reperfused Ischemic Kidney and Was Partly Dependent on PDGF-B/PDGFR Signaling

We first examined the expression of active Src kinase in the reperfused ischemic kidney by immunoblot analysis with an active Src-specific monoclonal antibody, clone 28.²¹ Clone 28 recognizes a region adjacent to Tyr-530 in the C-terminal regulatory domain, and thus, is specific to the active form of Src kinase. Because ischemia/reperfusion has been reported to predominantly impair the S3 segments of proximal straight tubuli in the OSOM of the kidney,^{30,31} tissues corresponding to the OSOM were cut from sliced kidney tissue and analyzed. As shown in Figure 1A, clone 28 detected a 60-kd band corresponding to the active form of Src kinase as previously described.²¹ The kidney of a sham-operated rat showed only a slight band of active Src kinase (Figure 1, A and B). In the reperfused kidney, the expression of active Src kinase was significantly increased by 10-fold 6 hours after ischemic injury and demonstrated peak expression at 12 hours after reperfusion compared to the kidneys of sham-operated rats (Figure 1, A and B). Significant activation of Src kinase continued until 3 days after the ischemic insult.

Figure 2. The effect of inhibition of the PDGF-B/PDGFR axis on the expression of active Src kinase by Trapidil treatment in the OSOM of the reperfused ischemic kidney, as determined by Western blot analysis. **A:** A representative immunoblot analysis of active Src kinase expression 12 hours after insult. **B:** The mean results of three independent experiments are shown (mean $\pm$ SD, $n = 3$).

To further examine the role of Src kinase in PDGF-B-dependent nephrogenic repair, the effect of Trapidil, a competitive inhibitor of PDGF-B binding to PDGFR- β , which has been shown to inhibit PDGF-B/PDGFR activity including PDGF-induced mitogenesis,^{22–25} was examined in the reperfused ischemic kidney. The Western blot analysis using clone 28 revealed that Trapidil significantly suppressed the enhanced expression of active Src kinase that is observed in the reperfused kidney 12 hours after insult (Figure 2, A and B). However, the levels of active Src expression in Trapidil-treated animals were still higher than sham-operated control groups.

To confirm increased Src activation in the reperfused kidney, we determined the Src kinase activity in the protein fraction immunoprecipitated by clone 28. The activity of Src kinase in the kidney with ischemic injury at 12 hours after reperfusion significantly increased by 3.7-fold compared to the kidneys of sham-operated rats (Figure 3, A and B). Trapidil treatment suppressed increased Src activation after reperfusion, while the activity still remained at higher levels compared to sham-operated and Trapidil-untreated animals.

We also examined the activity of PDGFR- β by immunoprecipitating the receptor and immunoblotting with antibody against phosphotyrosine. The immunoblot showed 3.7-fold increase of phosphorylated PDGFR- β expression in the injured kidney at 12 hours after reperfusion, similar to Src kinase activation. This activation was again significantly suppressed by Trapidil treatment, although the inhibition was not complete.

These results show that Src activation is enhanced in the reperfused kidneys, and the activation is partly dependent on the PDGF-B/PDGFR signals. This was further confirmed by the assessment of PDGFR- β activation.

Figure 3. *In vitro* Src kinase assay in the OSOM of the rat kidney after ischemia/reperfusion. **A:** The result of the assay was detected as a 45-kd protein of enolase. Representative results of three independent experiments are shown. The band in lanes 2 and 3 were derived from the two different injured rats at 12 hours after reperfusion without treatment of Trapidil. The band in lane 4 corresponded to Trapidil-treated rats with ischemic injury at 12 hours after reperfusion. **B:** Quantification of the signal intensities noted in independent experiments is summarized for the active form of Src kinase. Data are expressed as the mean $\pm$ SD ($n = 3$). I/R, ischemia/reperfusion injury; T, Trapidil.

The Active Form of Src Kinase Is Expressed in the S3 Segments of Proximal Tubuli in the Reperfused Ischemic Kidney

We next investigated the distribution of enhanced active Src kinase expression within the reperfused kidney by immunohistochemical staining. In the kidneys of normal and sham-operated rats, immunoreactive products of ac-

Figure 4. PDGFR- β activity was examined by immunoprecipitating the receptor and immunoblotting with antibody against phosphotyrosine. **A:** The phosphorylated PDGFR- β was detected. Representative results of three independent experiments are shown. Middle band was derived from the nontreated injured rats at 12 hours after reperfusion. Right band corresponded to PDGFR- β activity in the kidney of Trapidil-treated rats with ischemic injury at 12 hours after reperfusion. **B:** Quantification of the signal intensities noted in independent experiments is summarized for phosphorylated PDGFR- β expression. Data are expressed as the mean $\pm$ SD ($n = 3$). I/R, ischemia/reperfusion injury; T, Trapidil.

Figure 5. A–F: Tissue distribution of the active form of Src kinase in the normal rat kidney. Expression of the active form of Src kinase was detected in the cytoplasm of the distal tubuli, the thick ascending limb, and the collecting ducts. However, the S3 segment of the proximal tubule did not stain positively for active Src kinase. The convoluted proximal tubule of the cortex was also completely negative for active Src kinase (**A**). The proximal tubule of the S3 segment in the outer stripe of the outer medulla was also negative (**B**). The thick ascending limb was positive in the inner stripe of the outer medulla (**C**). Strong immunoreactivity was observed in the collecting duct of the inner medulla (**D**). **E:** Tissue distribution of the active form of Src kinase 12 hours after reperfusion of the kidney after acute ischemic injury. The cytoplasm of injured PTCs in the S3 segment showed strong immunoreactivity for active Src kinase (**arrowheads**). **F:** The active Src expression in Trapidil-treated injured kidney at 12 hours after reperfusion was also localized in the S3 segments of PTCs of the OSOM (**arrowheads**). Note that the distribution was identical as seen in the postischemic kidneys without Trapidil treatment (**E**), however, the staining was weaker (**F**). CX, cortex; OS, outer stripe of the outer medulla; IS, inner stripe of the outer medulla; IM, inner medulla; G, glomerulus; DT, distal tubule; TAL, thick ascending limb; CD, collecting duct. A representative immunohistochemical staining from six independent experiments is shown. Original magnifications: $\times 100$ (**A–D**); $\times 200$ (**E** and **F**).

tive Src kinase were mainly distributed within the cytoplasm of tubular epithelial cells of the distal tubule and the thick ascending limb, and to a lesser extent, the collecting ducts (Figure 5; A to D). Significant staining was not observed in other parts of the kidney, including the glomerulus and the proximal tubule (Figure 5; A to D). Notably, significant staining was also not observed in the S3 segment of the OSOM, as indicated in Figure 5B.

After reperfusion injury, active Src staining was detected in some damaged proximal tubuli at 6 hours. Twelve hours after reperfusion, marked staining was observed most strongly in the S3 segments of proximal straight tubuli of the OSOM. Moreover, immunoreactive products were localized in the cytoplasm of surface-covering tubular epithelial cells but not in the cells desquamated and accumulated within the tubular lumen

(Figure 5E). These epithelial cells of proximal tubuli stained positively for active Src kinase on day 1 and day 2, but the staining gradually faded after day 3. Immunoreactivity for active Src kinase did not change significantly in other tubular systems after reperfusion injury.

Active Src Kinase and PDGFR- β Were Co-Localized in Epithelial Cells within Cell Proliferation Cycle in the S3 Segments in the Reperfused Ischemic Kidney

We previously demonstrated co-localization of PDGF-B and PDGFR- β in the S3 segments of proximal tubuli after reperfusion.³ To examine the relationship between active Src kinase and PDGFR- β in the reperfused ischemic kidney, we performed immunofluorescence staining for the detection of both PDGFR- β and active Src kinase. Similarly to our previous study,³ we observed green homogeneous fluorescence representative of PDGFR- β expression 12 hours after reperfusion injury in the cytoplasm of tubular epithelial cells from the S3 segment (Figure 6A). The red fluorescence observed in Figure 6B represents active Src kinase, which was observed in the same area as PDGFR- β (Figure 6B). When these two images were superimposed, yellow fluorescence was primarily observed together with small areas of green fluorescence, indicating co-localization of active Src kinase and PDGFR- β within the tubular epithelial cells of the S3 segment of the reperfused ischemic kidney (Figure 6C). This indicates that active Src kinase might participate in the PDGF-B/PDGFR- β signaling cascade that is activated in injured PTCs after acute renal ischemic injury.

We then investigated PCNA expression and its relationship with active Src kinase expression to characterize its role in proximal tubuli after ischemic insult. The proliferating cells detected by PCNA expression were found to be abundant in the S3 segment of the proximal tubuli, peaking 48 hours after reperfusion (data not shown). This observation is consistent with previously reported results.^{3,29} Staining for both active Src kinase and PCNA revealed that the nuclei of those PTCs that stained positively for active Src kinase in the OSOM, also stained positively for PCNA on day 2 (Figure 7A). However, the PTCs of the kidneys of sham-operated rats did not show concomitant expression of active Src kinase and PCNA (Figure 7B). Thus, we theorize that activation of Src kinase might lead to proliferation of injured tubular cells within the kidney after acute injury.

Trapidil Treatment Decreases the Active Src Expression and Aggravates Renal Failure after Ischemia

Because Trapidil partly suppressed the enhanced Src and PDGFR- β activation in the kidney after reperfusion, the effects of Trapidil treatment on renal regenerative process was assessed both morphologically and functionally. The immunohistochemical analysis for clone 28 detected the active Src expression in the injured tubular

Figure 6. Immunofluorescence staining for both active Src and PDGFR- β 12 hours after ischemia/reperfusion. PDGFR- β expression (green, **A**) and active Src kinase expression (red, **B**) were observed in the S3 segments of tubular cells. Superimposition of the active Src and PDGFR- β images produced by immunofluorescence staining showed yellow fluorescence in the cytoplasm of many cells, indicating co-localization of PDGFR- β and active Src kinase (**C**).

epithelial cells of S3 segments in Trapidil-treated and reperfused kidneys (Figure 5F). The distribution was identical as seen in the postischemic kidneys without Trapidil treatment, however, the staining was weaker, being compatible with the partial suppression of Src activation by Trapidil as detected in immunoblot and kinase assay (Figures 1, 2, and 3).

Moreover, the number of PCNA-positive cells in the proximal tubuli of the S3 segment of the reperfused kidney was suppressed by treatment with Trapidil (Figure 8A), as compared to reperfused kidney but not treated by Trapidil (Figure 8B). Finally, treatment with Trapidil

Figure 7. Staining for both active Src kinase and PCNA 48 hours after ischemia/reperfusion. Note that tubular cells in the S3 segment showed concomitant expression of PCNA (dark brown in color) in their nuclei and active Src kinase (light brown in color) in their cytoplasm (**A**; arrowheads). In the kidneys of sham-operated rats, tubular cells in the S3 segment did stain positively for neither active Src kinase nor PCNA (**B**). Original magnifications: $\times 200$ (**A**); $\times 100$ (**B**).

caused a significant elevation in serum creatinine levels, 6, 12, and 24 hours after reperfusion (Table 1). These findings suggest that inhibition of the PDGF-B/PDGFR axis with Trapidil abrogates the protective action of active Src kinase in the early phase of tubular regeneration and thereby, aggravates renal failure.

Discussion

In the present study, we assessed Src activation and its role in PDGF-B/PDGFR-dependent renal tubular regeneration after acute ischemic injury. Immunoblot for active Src and *in vitro* kinase assay demonstrated that the Src activation is enhanced in OSOM of the injured kidney. The expression of active Src kinase in association with PDGFR- β was detected within proliferating PTCs of the S3 segments of the reperfused ischemic kidney, in which segments neither the active Src kinase nor PDGFR- β expression was detected within the normal and sham-operated rat kidney. Furthermore, inhibition of the PDGF-B/PDGFR- β axis with Trapidil significantly suppressed enhancement of both Src kinase and PDGFR- β activation 12 hours after reperfusion, resulting in aggravated *in vivo* renal failure after ischemic injury. Our present findings provide evidence that active Src kinase might play a

Figure 8. The effect of inhibition of the PDGF-B/PDGFR axis on cellular proliferation in the OSOM of the reperfused ischemic kidney. Note that fewer PTCs in the S3 segment were positive for PCNA in the reperfused ischemic kidney after treatment with Trapidil (**A**), as compared to reperfused kidney but not treated by Trapidil (**B**). None of the cells were positively stained for PCNA in normal control kidney (**C**). Original magnifications: $\times 40$ (**A**, **B**, **C**) or $\times 100$ (**A-C**).

pivotal role in the acute phase of PDGF-B-dependent tubular regeneration.

Src kinase undergoes several different activation stages before becoming fully activated to regulate a diverse array of receptor-induced biological activity.¹⁵ It is widely accepted that Src kinase activation is initiated by Tyr-530 dephosphorylation at its C-terminal regulatory domain.^{16–19} Clone 28 recognizes a region adjacent to the dephosphorylated form of Tyr-530 at its C-terminal

Table 1. The Effects of Trepidil, an Inhibitor of PDGF-B, on the Serum Creatinine Levels in Rats with Ischemic Injury

Treatment	6 hours	12 hours	Day 1
Trepidil (+), ischemia (–) <i>n</i> = 5	0.39 ± 0.01	0.39 ± 0.01	0.38 ± 0.01
Trepidil (–), ischemia (+) <i>n</i> = 5	1.54 ± 0.17	2.40 ± 0.15 [‡]	3.34 ± 0.14 [‡]
Trepidil (+), ischemia (+) <i>n</i> = 5	2.12 ± 0.06*	3.56 ± 0.15 [‡]	5.91 ± 0.94*

Trepidil significantly increased the serum creatinine at 6, 12, and 24 hours after injury. Significant differences: Trepidil (–), ischemia (+) versus Trepidil (+), ischemia (+), *, *P* < 0.05, †, *P* < 0.01; Trepidil (+), ischemia (–) versus Trepidil (–), ischemia (+), ‡, *P* < 0.01.

regulatory domain and therefore, can specifically identify active Src kinase.²¹ Its usefulness has been confirmed in a series of previously reported experiments and it has been shown to detect the active form of c-Src with high sensitivity in both normal and neoplastic tissue.^{32–34} For the first time, we have shown the distribution of active Src kinase in the normal rat kidney. In the kidneys of normal and sham-operated rats, active Src kinase expression, detected with clone 28, was observed in the distal tubuli, the thick ascending limb, and the collecting ducts, where PDGFR- β is also expressed.³ However, the physiological role of Src kinase in the normal kidney has yet to be clarified. Because Src kinase appears to be required for induction of cell-cycle progression and DNA synthesis,²⁰ Src kinase might be involved in the renewal of normal tubular cells either through the PDGF-B/PDGFR- β axis or in association with other receptor tyrosine kinases. Moreover, because collecting ducts express vasopressin receptors and vasopressin is reported to stimulate Src kinase activity *in vitro*,³⁵ Src kinase might also play a physiological role in the maintenance of vasopressin-dependent water balance in the normal kidney.

To investigate the signal relay cascade of PDGF-B-dependent tubular regeneration, we focused on the role of Src kinase, because Src kinase is reported to participate in PDGF-dependent cell proliferation *in vitro*.^{36–41} Src kinase activity is required at multiple stages of PDGF-dependent cell-cycle progression, sometimes during G₁, as well as during the M phase.^{37–40} In various Src mutant cell lines derived from Src knockout animals, PDGF-dependent DNA synthesis is blocked.⁴⁰ Furthermore, Src activity is required for a number of other PDGF-dependent responses. Phosphorylation of proteins that associate with PDGFR- β , such as Ras, GAP, PLC- γ , SHP-2, and SHC, is severely diminished in PDGFR- β receptor mutant cells, in which Src kinase cannot be activated.⁴¹ Thus, activated Src kinase might play a distinct role in the PDGF-PDGFR- β axis-mediated repair process of the postreperfused kidney through induction of proliferation of injured PTCs. Several observations from our laboratory support this insight. First, active Src kinase expression as detected by immunoblot by clone 28 was increased in the reperfused ischemic kidney, peaking 12 hours after the initial insult. This was further confirmed by activity assay of Src kinase, which showed 3.7-fold increase of Src activity after reperfusion in association with PDGFR- β activation. In light of previous findings that PDGF-B mRNA expression is induced to peak levels 6 hours after reperfusion in the kidney,³ it is possible that enhanced PDGF-B production might induce the activation of Src kinase as part of a PDGF-B-induced signal relay cascade. Secondly, concomitant expression of both active

Src kinase and PDGFR- β has been observed in the proliferating epithelial cells of the S3 segments of proximal tubuli after acute injury, where they are not normally expressed. In addition, we previously reported an enhanced expression of PDGF-B and PDGFR- β in the kidney after ischemia/reperfusion injury and noted that both were expressed in the S3 segment of the proximal tubuli.³ When taken together, these results suggest that Src kinase might be involved in PDGF-B and PDGFR- β signal transduction activated by acute ischemic insult.

In the third place, PDGF-B, PDGFR- β , and activated Src kinase expression was noted within injured tubular cells undergoing cellular proliferation, as evaluated by staining for PCNA. Regenerating tubular cells also show immunoreactivity for vimentin, as previously described.³ Vimentin, a component of intermediate filaments in cells, is usually expressed in mesenchymal cells and is a reliable marker of dedifferentiation of the tubular epithelium.⁴² Src kinase activity is required for PDGF-B-induced tyrosine phosphorylation of vimentin, moreover, intermediate filaments require phosphorylation to modulate their assembly and distribution.⁴³ Thus, the enhanced expression of active Src kinase noted in the present study implies that active Src kinase might participate in PDGF-B-dependent nephrogenic repair by inducing proliferation and changes in the phenotype of injured tubular cells.

Finally, the enhancement of Src and PDGFR- β activation was not completely suppressed by Trepidil treatment, although Trepidil did diminish the level of enhanced expression after 12 hours in the postreperfused kidney and aggravated renal failure. In our previous study,³ Trepidil of 90 mg/kg significantly increased the serum creatinine of rats with ischemic injury at 24 hours after reperfusion, and the higher dosages of Trepidil increased the mortality of the rats after ischemia. Others showed that an oral dose of 50 mg/kg of Trepidil daily for a week was effective in inhibiting proliferation of several types of cells in rats.⁴⁴ Based on these findings, we treated animals with 90 mg/kg of Trepidil for the assessment of the function of increased PDGF-B/PDGFR signal cascades after ischemia. Trepidil suppressed the activation of both Src and PDGFR- β similarly and deteriorated renal function. Because Trepidil competes with PDGF-B binding to its receptor but does not directly affect the events downstream of PDGF binding to its receptor including Src activation, our present data show the linkage between PDGF-B/PDGFR signal and Src activation, and provide *in vivo* evidence to suggest that Src activation is involved in PDGF-B-dependent renal tubular regeneration after ischemic insult. Signaling cascades of PDGF-B/PDGFR axis are complex and Src independent events

of signaling also need to be further examined to understand the molecular mechanism of tissue regeneration.

On the other hand, other growth factors might cause the residual increase of active Src expression in Trepidil-treated ischemia/reperfusion kidney. Because Src kinase can communicate with a number of different receptor protein tyrosine kinases besides PDGFR, it is also possible that epidermal growth factor and colony-stimulating factor, which are reportedly involved in the nephrogenic repair process, might activate Src kinase through receptor binding.⁴⁵ Hepatocyte growth factor also associates with Src kinase and is induced in the reperfused ischemic kidney. However, it is unlikely that hepatocyte growth factor-induced renal regeneration occurs via a c-Src-dependent signaling pathway, because protein tyrosine kinase inhibitors do not block hepatocyte growth factor-induced tubulogenic cell differentiation.⁴⁶

In conclusion, the current study provides evidence to suggest the importance of active Src kinase in the early phase of PDGF-B-dependent nephrogenic repair after acute ischemia/reperfusion injury. We further identified the distribution of active Src kinase in the normal and reperfused kidney. However, further research is necessary to clarify the signaling cascade of PDGF-B-dependent tubular regeneration and the physiological role of Src kinase in the normal kidney and during various pathological processes. Thus, examination of Src kinase in relation to the PDGF-B/PDGFR- β axis is critical to clarify the pathogenesis of renal diseases and to pave the way for the generation of new treatment.

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