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Thymosin β4 and its degradation product, Ac-SDKP, are novel reparative factors in renal fibrosis

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Abstract

Previously we found thymosin β4 (Tβ4) is up-regulated in glomerulosclerosis and required for angiotensin II-induced expression of plasminogen activator inhibitor-1 (PAI-1) in glomerular endothelial cells. Tβ4 has beneficial effects in dermal and corneal wound healing and heart disease yet its effects in kidney disease are unknown. Here we studied renal fibrosis in wild type and PAI-1 knockout mice following unilateral ureteral obstruction to explore the impact of Tβ4 and its prolyl oligopeptidase tetrapeptide degradation product, Ac-SDKP, in renal fibrosis. Additionally, we explored interactions of Tβ4 with PAI-1. Treatment with Ac-SDKP significantly decreased fibrosis in both wild type and PAI-1 knockout mice, as observed by decreased collagen and fibronectin deposition, fewer myofibroblasts and macrophages, and suppressed pro-fibrotic factors. In contrast, Tβ4 plus a prolyl oligopeptidase inhibitor significantly increased fibrosis in wild type mice. Tβ4 alone also promoted repair and reduced late fibrosis in wild type mice. Importantly, both pro-fibrotic effects of Tβ4 plus the prolyl oligopeptidase inhibitor, and late reparative effects of Tβ4 alone, were absent in PAI-1 knockout mice. Thus, Tβ4 combined with prolyl oligopeptidase inhibition, is consistently pro-fibrotic, but by itself, has anti-fibrotic effects

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Disclosures

None.

in late stage fibrosis, while Ac-SDKP has consistent anti-fibrotic effects in both early and late stages of kidney injury. These effects of Tβ4 are dependent on PAI-1.

Introduction

Thymosin β4 is a highly conserved G-actin sequestering protein that has a wide range of extracellular functions, including cell migration, angiogenesis, and extracellular matrix (ECM) synthesis, some of which might be receptor-mediated [1–3]. Thymosin β4 is degraded by a two-step process to the anti-fibrotic tetrapeptide Ac-SDKP, with the initial step mediated by unknown protease(s) and then by prolyl oligopeptidase (POP). Moreover, POP can regulate the activity of the first step enzyme and thus auto-regulate the release of Ac-SDKP [4]. Ac-SDKP is further inactivated by angiotensin-converting enzyme (ACE). The beneficial effects of ACE inhibitors may, at least partially, relate to increased Ac-SDKP levels [5–9].

Thymosin β4 accelerates dermal wound healing with increased cell migration, tissue remodeling and accelerated collagen deposition [1, 10–12]. In contrast, thymosin β4 is anti-fibrotic in cultured human hepatic stellate cells [13, 14]. Thymosin β4 induces adult epicardium progenitor mobilization and neovascularization and phase II clinical trials to test for improved cardiac function after myocardial infarction (MI) are planned [15–19].

Whether thymosin β4 has beneficial or fibrotic effects in the kidney has not been established. Previously, we observed that thymosin β4 is increased in early glomerulosclerosis, and is required for angiotensin II (AII)-induced plasminogen activator inhibitor-1 (PAI-1) expression in glomerular endothelial cells [20]. PAI-1 inhibits matrix degradation and also alters cell migration [21, 22]. Accumulation of matrix and inflammation versus preservation of parenchymal cells may differentially affect repair versus fibrosis at early and late stages after injury. We investigated the effects of thymosin β4 and Ac-SDKP on renal interstitial fibrosis induced by the unilateral ureteral obstruction (UUO) model. Complete UUO starts a rapid sequence of events in the obstructed kidney, including reduced renal blood flow and glomerular filtration rate within 24 hours. This is followed by interstitial inflammation, tubular dilation, tubular atrophy and fibrosis within several days. The obstructed kidney progresses to a severely hydronephrotic kidney with marked loss of renal parenchyma and reaches end stage over 1- 2 weeks [23]. Therefore, we chose two time points, day 5 and day 14 after UUO, to assess early and late repair vs. injury, as previously reported [24, 25]. Our results demonstrate fibrosis is increased by thymosin β4 plus POP inhibitor at both early and late stages, while both thymosin β4 and Ac-SDKP ameliorate late matrix accumulation. The effects of thymosin β4 to enhance fibrosis when POP is inhibited, and to promote repair when given alone, are both PAI-1 dependent. These data suggest that thymosin β4 and in particular its degradation product Ac-SDKP are anti-fibrotic in the kidney.

Results

Thymosin β4 is increased in tubulointerstitial fibrosis

Immunostaining for thymosin β4 was increased in obstructed kidneys in WT mice at day 5 and day 14 compared with non-obstructed contralateral kidneys (Figure 1A–F). Thymosin β4 was present mainly in interstitial cells, and occasionally tubular epithelial cells (Figure 1A, 1C). Immunostaining for thymosin β4, alpha-SMA and F4/80 on serial sections confirmed that some of the thymosin β4-positive interstitial cells were myofibroblasts and macrophages (Figure 2A–D).

Thymosin β4 has divergent effects in early vs. late stage injury, while exogenous Ac-SDKP consistently promotes repair

At day 5, tubular dilatation, tubular atrophy, and interstitial collagen accumulation were present in obstructed kidneys in WT mice. Tubulointerstitial fibrosis assessed by Sirius red morphometry was significantly increased by 17% in obstructed kidneys treated with thymosin β4 plus POP inhibitor vs. untreated UUO. Neither thymosin β4 alone nor POP inhibitor alone significantly affected early fibrosis, although thymosin β4 numerically decreased this parameter (Figure 3A). In contrast, fibrosis was significantly less by 16% in obstructed kidneys treated with Ac-SDKP vs. untreated UUO (Figure 3A). Neither thymosin β4 nor Ac-SDKP administration affected POP activity (Table 1). POP activity was decreased in mice receiving POP inhibitor, and consequently, Ac-SDKP levels were decreased. However, treatment with thymosin β4 alone did not change Ac-SDKP levels (Table 1).

At day 14, compared to untreated UUO, fibrosis in WT mice was significantly increased by 15% in obstructed kidneys treated with thymosin β4 plus POP inhibitor, but decreased by 12% in the Ac-SDKP treated group (Figure 4A). Surprisingly, thymosin β4 alone significantly decreased fibrosis by 35%, even more than with Ac-SDKP treatment (Figure 4A). These data indicate that thymosin β4 is protective at only the late stage of fibrosis while Ac-SDKP reduces fibrosis at both stages.

We further examined the effects of different interventions on intrinsic thymosin β4 expression assessed by real time PCR and Ac-SDKP concentration assessed by ELISA in both the obstructed and the non-obstructed contralateral kidneys in WT mice at day 5 after UUO and compared with those of normal kidneys. Thymosin β4 mRNA expression was significantly higher in the obstructed kidneys vs. non-obstructed contralateral kidneys with the same treatment, while Ac-SDKP level was lower in the obstructed kidneys (Table 2). Both thymosin β4 mRNA and Ac-SDKP levels were similar in the non-obstructed contralateral kidneys from day 5 untreated control WT mice and normal kidneys (Table 2), while POP activity was lower in normal kidneys. These data indicate that intrinsic thymosin β4 gene expression was significantly increased in and associated with the early stage of tubulointerstitial fibrosis induced by UUO. Further, the non-obstructed contralateral kidneys from mice treated with POP inhibitor showed significantly lower POP activity vs. untreated mice, indicating the POP inhibitor was effective (Table 2). Ac-SDKP levels were also decreased in the contralateral kidneys treated with either POP inhibitor or thymosin β4 plus

POP inhibitor but increased as expected in response to Ac-SDKP treatment (Table 2). Although Ac-SDKP measurement cannot distinguish the intrinsic molecules from exogenous compounds, the data suggest that increased Ac-SDKP has beneficial effects on fibrosis. PAI-1 and thymosin β4 mRNA levels in the non-obstructed contralateral kidneys were reduced by Ac-SDKP, but not affected by POP inhibitor with or without thymosin β4 (PAI mRNA: Control 3.41±0.57, POP inhibitor 2.99±0.44, thymosin β4 plus POP inhibitor 2.60±0.89, thymosin β4 1.70±0.70, Ac-SDKP 1.48±0.30, $p<0.05$ only for Ac-SDKP vs. control; thymosin β4 mRNA in Table 2). TGF-β1 mRNA did not differ in these contralateral kidneys amongst groups. These data indicate that the short-term effects of exogenous thymosin β4 and Ac-SDKP on non-obstructed contralateral kidneys are minimal.

Anti-fibrotic effects of Ac-SDKP are linked to decreased fibronectin, macrophages, myofibroblasts, and profibrotic factors

At day 5 in WT mice, fibronectin was significantly increased by 35% in obstructed kidneys treated with thymosin β4 plus POP inhibitor vs. untreated UUO, but decreased by 40% in response to Ac-SDKP (Figure 5A, Table 3). Since monocyte/macrophage infiltration occurs early after UUO, we evaluated F4/80 positive macrophages. At day 5, F4/80 positive macrophages were significantly decreased by 52% in obstructed kidneys treated with Ac-SDKP vs. untreated UUO (Figure 5B, Table 3). Further, α-SMA-positive myofibroblasts, a key contributor to extracellular matrix, were also significantly decreased by 65% at day 5 in response to Ac-SDKP. In contrast, thymosin β4 plus POP inhibitor tended to increase α-SMA- positive cells (Figure 5C, Table 3). Thymosin β4 whole kidney protein was similar in all day 5 obstructed groups regardless of treatment. Thymosin β4 mRNA at day 5 in obstructed kidneys was significantly reduced by 41% in response to Ac-SDKP, but not by other interventions. PAI-1 whole kidney protein, a key profibrotic factor, was significantly decreased by 52% in response to Ac-SDKP treatment in obstructed kidneys at day 5. Phosphorylated Smad3, a marker of TGF-β1 signaling activation, was not detected by either immunostaining or Western blot in obstructed kidneys at day 5 (data not shown). These data show that various indicators of early fibrosis are reduced at day 5 by Ac-SDKP.

We next examined late stage fibrosis. Collagen I and PAI-1 whole kidney protein levels were significantly increased at day 14 by thymosin β4 plus POP inhibitor, but decreased by Ac-SDKP or thymosin β4 alone (Figure 6). Phosphorylated Smad3 was increased in tubular epithelial cells and some interstitial cells similarly in obstructed kidneys in all groups (Figure 7A). Phosphorylated Smad3 whole kidney protein was also significantly increased by thymosin β4 plus POP inhibitor, but decreased by Ac-SDKP or thymosin β4 alone (Figure 7B).

Both profibrotic and reparative effects of thymosin β4 in fibrosis are PAI-1 dependent

PAI-1 is increased in fibrotic kidney diseases, and may contribute to scarring both by decreasing extracellular matrix proteolysis and affecting infiltrating cells [21, 22]. We therefore assessed impact of PAI-1 deficiency on effects of thymosin β4 and Ac-SDKP in both early and late fibrosis. At both stages (day 5 and 14 after UUO), tubulointerstitial fibrosis was similarly increased in PAI-1−/− vs. WT compared to non-obstructed kidneys (Figure 3B and 4B). Ac-SDKP significantly decreased fibrosis in both WT and PAI-1−/− mice at early and

late stages. However, at both early and late stages, PAI-1−/− mice had no further increase in fibrosis with thymosin β4 plus POP inhibitor treatment, in contrast to WT (Figure 3B and 4B). Thymosin β4 alone did not affect early fibrosis in PAI-1−/− mice, as observed in WT mice. However, late stage fibrosis was not decreased by thymosin β4 alone in PAI-1−/− mice, in contrast to less fibrosis in WT treated with thymosin β4 (Figure 3B and 4B).

We next examined POP enzyme activity and Ac-SDKP levels in the obstructed kidneys. POP activity was decreased at day 5 and day 14 in both WT and PAI-1−/− mice in response to the combination treatment compared to untreated UUO. Neither Ac-SDKP nor thymosin β4 changed POP enzyme activity in either WT or PAI-1−/− mice (Table 1). Ac-SDKP kidney levels were similarly reduced by the combination treatment and increased by exogenous Ac-SDKP in both WT and PAI-1−/− mice. At day 14, in WT, but not in PAI-1−/− mice, treatment with thymosin β4 alone increased Ac-SDKP levels vs. untreated UUO, but not as much as with exogenous Ac-SDKP treatment (Table 1).

Early injury markers at day 5, i.e. fibronectin, macrophages and myofibroblasts, were decreased in PAI-1−/− mice treated with Ac-SDKP (Table 3). Ac-SDKP treatment also significantly decreased macrophages at late stage day 14 in both WT and PAI-1−/− mice. Thymosin β4 and phosphorylated Smad 3 immunostaining expressions were similar in WT and PAI-1−/− mice at day 14 in obstructed kidneys in all groups (data not shown). Neither thymosin β4 alone nor combination treatment affected macrophage number in WT or PAI-1−/− mice (Table 4). Further, at day 14, Ac-SDKP treatment tended to reduce collagen I and phosphorylated Smad 3 protein in PAI-1−/− mice (Figure 8).

Taken together, these data demonstrate that the effects of thymosin β4 on fibrosis and its mediators are dependent on the presence of PAI-1.

Discussion

In the current study, we examined the roles of thymosin β4 and Ac-SDKP in modulating early versus late tubulointerstitial fibrosis and investigated possible mechanisms. Ac-SDKP has protective effects in hypertensive kidney disease models of glomerulosclerosis [26–28]. Endogenous thymosin β4 is up-regulated in the kidney ischemia-reperfusion model [29], but the role of exogenous thymosin β4 in the kidney has not been defined. We now demonstrate that thymosin β4 is up-regulated in kidneys after obstructive injury, along with increased expression in macrophages, fibroblasts, and occasional tubular cells. We also show that exogenous administration of thymosin β4 plus POP inhibitor, which prevents metabolism of thymosin β4 to Ac-SDKP, exacerbated both early and late interstitial fibrosis in WT mice. In contrast, Ac-SDKP treatment decreased both early and late fibrosis, with less total collagen and fibronectin deposition, decreased myofibroblasts and monocyte/macrophages, and suppressed profibrotic factors, PAI-1 and TGF-β1. Late phase fibrosis was also decreased by thymosin β4 alone in WT mice. These effects of thymosin β4, namely to enhance fibrosis when POP is inhibited, and to promote repair when given alone, were both PAI-1 dependent. Taken together, these data indicate that thymosin β4 has divergent effects on early vs. late stages of injury, while Ac-SDKP promotes repair and decreases fibrosis at both early and

late stages. Whether higher amounts of thymosin β4 could have similar effects on early fibrosis awaits further study.

In dermal wound healing, thymosin β4-accelerated collagen/matrix deposition is beneficial. In kidneys, excess extracellular matrix accumulation is usually detrimental. However, accumulation of matrix and macrophages may be a provisional wound healing-like response, which may ultimately promote resolution of scarring to allow less parenchymal loss. We therefore further investigated whether effects of thymosin β4 differ in early vs. late stages of renal fibrosis. When thymosin β4 metabolism to Ac-SDKP was inhibited, both early and late tubulointerstitial fibrosis increased with more total collagen and fibronectin accumulation, but no change in macrophages. We recognize that POP has numerous targets in addition to thymosin β4 [30]. Importantly, POP inhibitor by itself did not enhance early fibrosis, supporting that the effects of the combination of thymosin β4 and POP inhibitor are attributed to specific thymosin β4 activity. In contrast, Ac-SDKP treatment ameliorated both stages of fibrosis with less collagen and fibronectin accumulation. We also showed that short-term treatment with thymosin β4 with or without POP inhibitor, or Ac-SDKP, had minimal effects in the non-injured contralateral kidneys.

We next investigated the possible mechanisms underlying effects of thymosin β4 and Ac-SDKP in renal fibrosis. Myofibroblasts, one of the major contributors of interstitial extracellular matrix, may arise from different sources, including quiescent fibroblasts or pericytes [31–33]. We found that α-SMA expression, a marker of activated myofibroblasts, tended to be increased by the thymosin β4 and POP inhibitor combination, but was significantly decreased by Ac-SDKP. Macrophages have numerous functions, including removal of debris in injury, progression of fibrosis, and repair, and can be phenotypically switched in different environments [34]. Macrophage infiltration was significantly decreased by Ac-SDKP treatment, but not affected by exogenous thymosin β4 with or without POP inhibitor at early or late stage of injury. Future investigation to determine the phenotypes, functions, and responses of these macrophages will be of interest.

Effects on two key profibrotic factors, PAI-1 and TGF-β1, likely contributed to the changes in fibrosis. At late stage in WT mice, treatment with thymosin β4 plus POP inhibitor resulted in significant upregulation of these molecules with increased fibrosis. In contrast, these factors were decreased with corresponding reduced fibrosis in response to Ac-SDKP or thymosin β4 alone. PAI-1 has effects both on proteolysis and cell migration, processes that are crucial for response to injury [21, 22]. Ultimate effects on fibrosis are determined by a balance of matrix modulation and repair, and are likely time- and context-dependent. We have previously shown that thymosin β4 was required for angiotensin II-induced PAI-1 expression in cultured glomerular endothelial cells [20]. Thymosin β4 also increases PAI-1 expression in human umbilical vein endothelial cells [35, 36]. We therefore examined PAI-1 dependence of thymosin β4 effects. Tubulointerstitial fibrosis was similar in PAI-1−/− and WT mice after 5 days and 14 days of UUO, and Ac-SDKP treatment decreased this fibrosis similarly in PAI-1−/− and WT mice. However, in contrast to the increased early and late fibrosis in WT induced by exogenous thymosin β4 plus POP inhibitor combination, PAI-1−/− mice showed no increase in fibrosis with this intervention. Further, the decreased late fibrosis in response to thymosin β4 alone was present only in WT, but not in PAI-1−/− mice.

These data show that both the pro-fibrotic effect of thymosin β4 combined with POP inhibitor at either early or late stage of fibrosis, and the reparative effects of thymosin β4 alone in late stage injury, were PAI-1 dependent. Of note, increased Ac-SDKP levels in thymosin β4-treated mice did not account for this protective effect. Thus, Ac-SDKP levels in thymosin β4-treated WT mice were numerically even lower than that in Ac-SDKP treated mice, although late fibrosis was even less with treatment with thymosin β4 alone than with Ac-SDKP treatment. Thymosin β4 induces vascular endothelial growth factor (VEGF) expression by an increase in the stability of hypoxia inducible factor (HIF)-1α protein [37, 38]. We postulate progressive fibrosis could result in increased hypoxia in the interstitium, and thymosin β4 beneficial effects could be contributed to by preserving peritubular capillaries. Whether thymosin β4 also affects macrophage phenotypic switch toward tissue repair and regeneration awaits further study. The mechanisms of beneficial effects of thymosin β4 in late kidney injury point to a novel reparative pathway for progressive kidney disease.

In summary, our study shows that Ac-SDKP has anti-fibrotic effects in both early and late stages of renal fibrosis induced by UUO. Thymosin β4 alone has context- and time-dependent effects on renal fibrosis and promotes PAI-1-dependent repair of late fibrosis.

Concise Methods

Animals

Adult male wild type (WT) and PAI-1 deficient mice (PAI-1−/−) on C57Bl/6 background were housed in microisolator cages in a pathogen-free barrier facility with standard chow and tap water ad libitum. All protocols were carried out in accordance with National Institutes of Health and Vanderbilt University animal care facility guidelines.

Experimental Protocol

Mice (25∼30 g; Jackson, Indianapolis, IN), age 8 to 10 weeks, underwent UUO under sterile conditions as described previously [39]. Mice were then randomized to groups as follows (n=6–8 in each group): untreated UUO control, UUO treated with prolyl oligopeptidase (POP) inhibitor (S17092, 40 mg/kg/d, mixed with peanut oil and administered by gavage), UUO with thymosin β4 (150 μg/d, i.p.), UUO with combination (thymosin β4 plus POP inhibitor), and UUO treated with Ac-SDKP (Calbiochem, Gibbstown, NJ, 1.6 mg/kg/d, delivered by micro-osmotic pump) (Alzet, model 1007D, DURECT Corp., Cupertino, CA). The doses and duration of POP inhibitor, thymosin β4 and Ac-SDKP were chosen based on previous reports and pilot studies [28, 40]. S17092 and thymosin β4 were kindly provided by Institut de Recherches Internationales Servier, France and RegeneRx Biopharmaceuticals, Inc. (Rockville, MD), respectively. Five or 14 days after UUO surgery, mice were sacrificed and kidneys were harvested for morphological, immunohistochemical, biochemical and molecular analyses.

Morphology and Immunohistochemistry

Qualitative tubulointerstitial injury was examined by Masson's trichrome staining. For quantitative assessment of fibrosis, 3-μm sections of tissues were stained with picrosirius red (0.1% Sirius red in saturated picric acid) for 16 h, followed by two changes of 0.5% acetic

acid, dehydrated in three changes of 100% ethanol, and coverslip mounting. Sections were examined by polarized light microscopy, as reported by other investigators[4142]. For each kidney, 12 consecutive, non-overlapping cortical × 40 fields were captured by an Olympus BX-41 microscope equipped with a digital camera, and morphometric analysis performed using NIH image (version 1.63). Arteries and periadventitial areas were excluded. Results are expressed as percentage fibrosis of total tubulointerstitial area.

Kidney sections were stained for fibronectin (1:400, Rockland Immunochemicals, Inc., Gilbertsville, PA), α-smooth muscle actin (α-SMA) (1:100, DAKO Corp., Carpinteria, CA), F4/80 (1:800, AbD Serotec, Raleigh, NC), thymosin β4 (1:300, Meridian Life Science, Inc., Saco, ME), phosphorylated Smad3 (1:100, Rockland Immunochemicals, Inc, Gilbertsville, PA) with Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) with diaminobenzidine as a chromogen. For assessment of fibronectin, α-SMA, and F4/80, 12 consecutive, non-overlapping cortical x40 fields were captured with digital images and the ratio of positively stained areas was analyzed using NIH image and expressed as percentages of total cortical area [41, 43]. Arteries were excluded for α-SMA staining analysis. All morphologic and morphometric assessments were examined without knowledge of the treatment protocol.

Tissue Preparation and POP Activity Assay

POP activity was assayed using the fluorogenic substrate, Suc-Gly-Pro-7-amino-4-methylcourmarin (Z-Gly-Pro-AMC, Bachem, Torrance, CA) as previously described [44]. In brief, frozen kidney samples (10mg) were homogenized in 90 μl assay buffer (0.1 M Na-K-phosphate buffer, pH 7.0), centrifuged at 13,000g at 4°C for 20 mins and the supernatant was stored at −80°C until determination of enzyme activity. Kidney homogenate supernatant (2 μl) was preincubated with 93 μl assay buffer at 30°C for 30 mins. The reaction was initiated by adding 5 μl of substrate (200μM Z-Gly-Pro-AMC) with 60 mins incubation at 30°C. The reaction was stopped by adding 100 μl 1 M sodium acetate buffer (pH 4.2). Formation of the AMC product was measured fluorometrically (excitation 360 nm and emission 460 nm). Results are expressed as picomole AMC per minute per milligram of total protein.

Kidney Ac-SDKP Measurement

Ac-SDKP in kidneys was measured with an enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI). Tissue was weighed, homogenized with cold methanol, centrifuged, and the supernatant was evaporated to air dry and stored at −20°C [30]. Samples were then reconstituted and processed according to the manufacturer's recommendations.

Real time PCR

Total RNA from non-obstructed kidneys was analyzed by real-time quantitative reverse transcriptase-polymerase chain reaction for mouse TGF-β1, PAI-1, thymosin β4, and GAPDH using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), with primer sequences listed in Table 5, as previously described [45]. Relative quantification of each target mRNA level was normalized to GAPDH.

Western Blot Analysis

Frozen kidney tissues were lysed for Western blot measurements of PAI-1 (1:500, R&D Systems, Inc, Minneapolis, MN), collagen I (1:8,000, Abcam, Cambridge, MA), phosphorylated Smad3 (1:100, Rockland Immunochemicals, Inc, Gilbertsville, PA) and normalized to β-actin (1:10,000, Sigma, St. Louis, MO), as previously described [45].

Densitometric quantitation was performed using NIH image (version 1.63).

Statistical analysis

Results are expressed as mean±standard error of the mean (SEM). Statistical difference was evaluated by single factor variance (ANOVA) followed by least significant difference (LSD) correction for multiple comparisons. Nonparametric data were compared by Mann–Whitney *U*-test. A p value <0.05 was considered significant.

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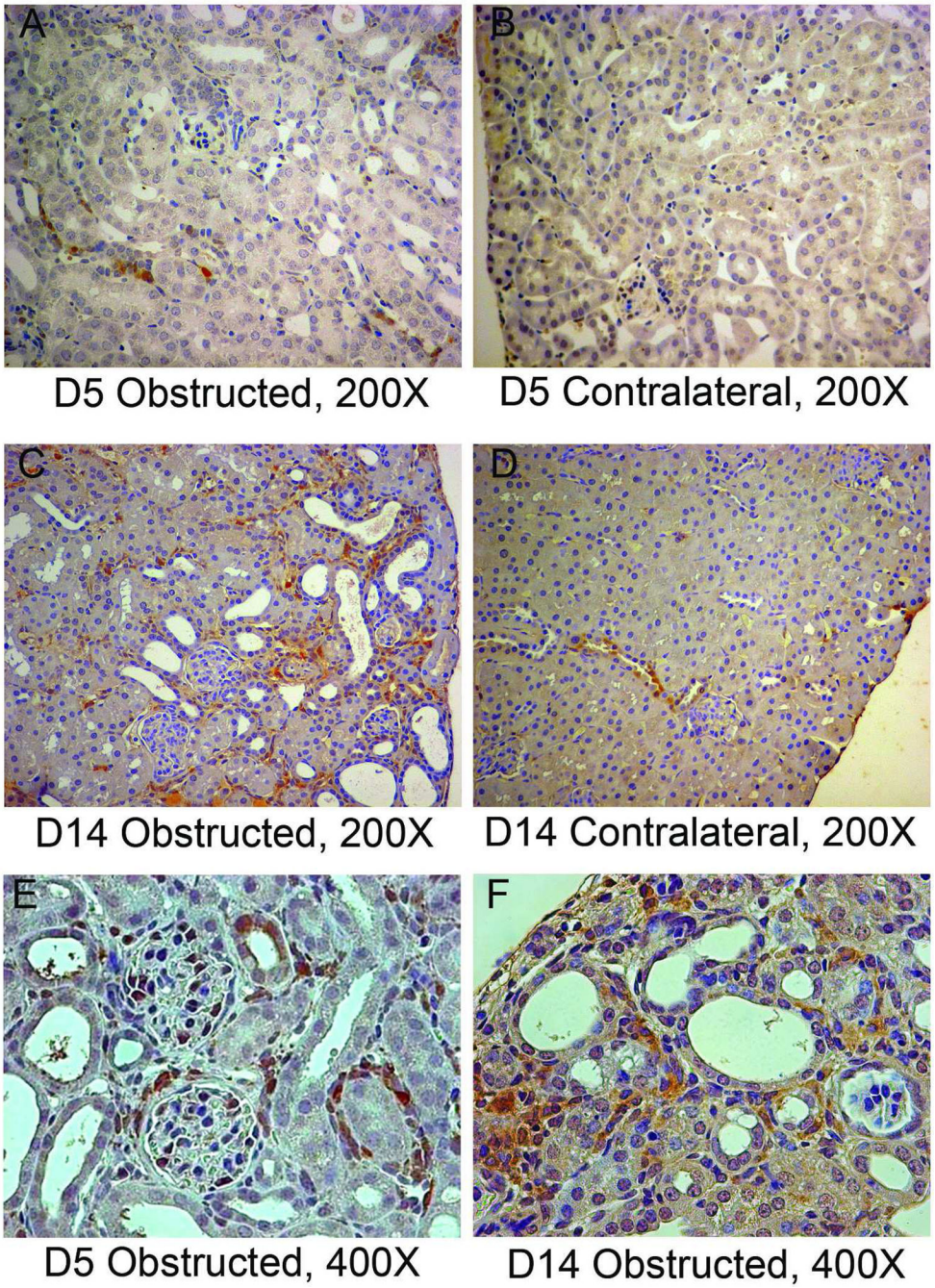

Figure 1.

Increased thymosin β4 expression in tubulointerstitial fibrosis at day 5 and day 14 in wild type mice. Thymosin β4 was significantly increased in obstructed kidneys compared with non-obstructed contralateral kidneys (anti-thymosin β4 immunostaining).

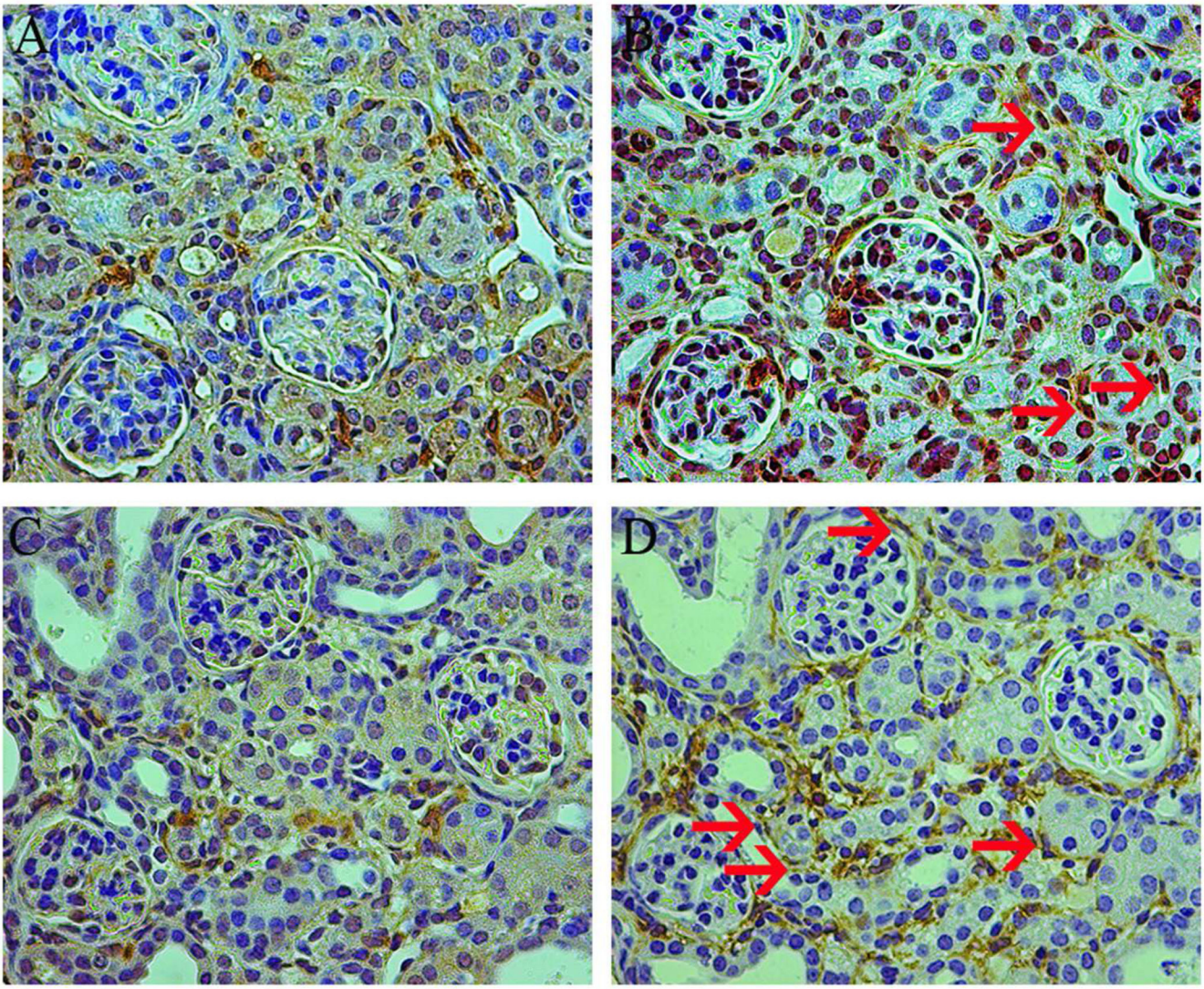

Figure 2.

Thymosin β4 localization in tubulointerstitial fibrosis. (A and B) Localization of thymosin β4 in fibrotic kidneys at day 14 in wild type mice (A). Serial section staining for α-smooth muscle actin (SMA) (B) indicates that thymosin β4 is expressed on myofibroblasts. (C and D). Thymosin β4 in fibrotic kidneys at day 14 in wild type mice (C). Staining for F4/80 on the adjacent section (D) indicates that thymosin β4 is expressed on macrophages (×400, immunostaining as indicated).

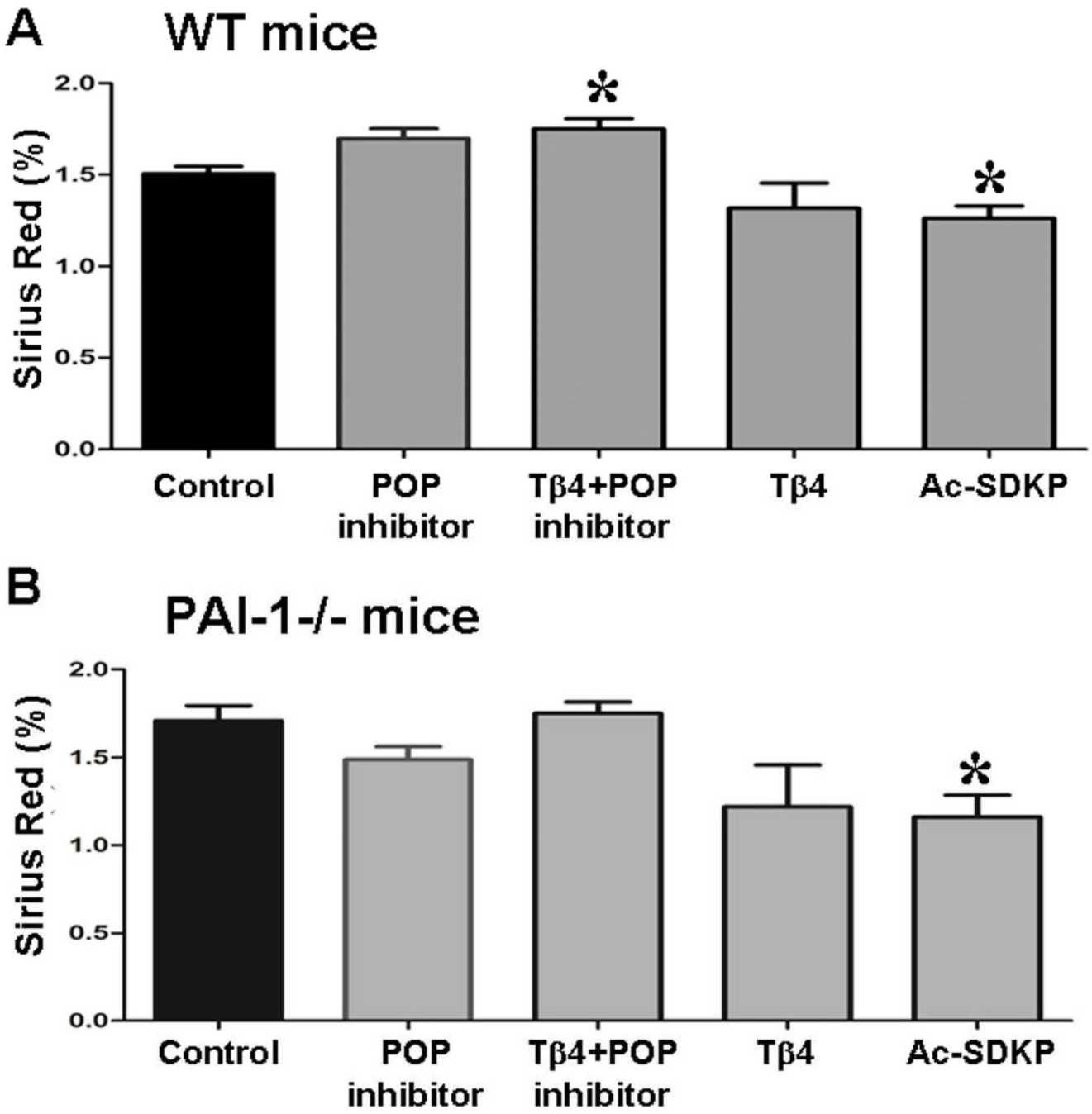

Figure 3.

Early tubulointerstitial fibrosis in wild type and PAI-1−/− mice. (A) Tubulointerstitial fibrosis in wild type mice at day 5 after UUO was significantly increased by thymosin β4 plus POP inhibitor (Tβ4+POPi) but was significantly decreased by Ac-SDKP (Control, n=8; POPi, n=6; Tβ4+POPi, n=6; Tβ4 n=6; Ac-SDKP, n=7; *p<0.05 vs control). (B) Tubulointerstitial fibrosis in PAI-1−/− mice at day 5 after UUO was significantly decreased by Ac-SDKP. In contrast to WT mice, neither thymosin β4 plus POP inhibitor nor thymosin

β4 alone affected fibrosis (Control, n=6; POPi, n=6; Tβ4+POPi, n=8; Tβ4 n=7; Ac-SDKP, n=5; *p<0.05 vs control).

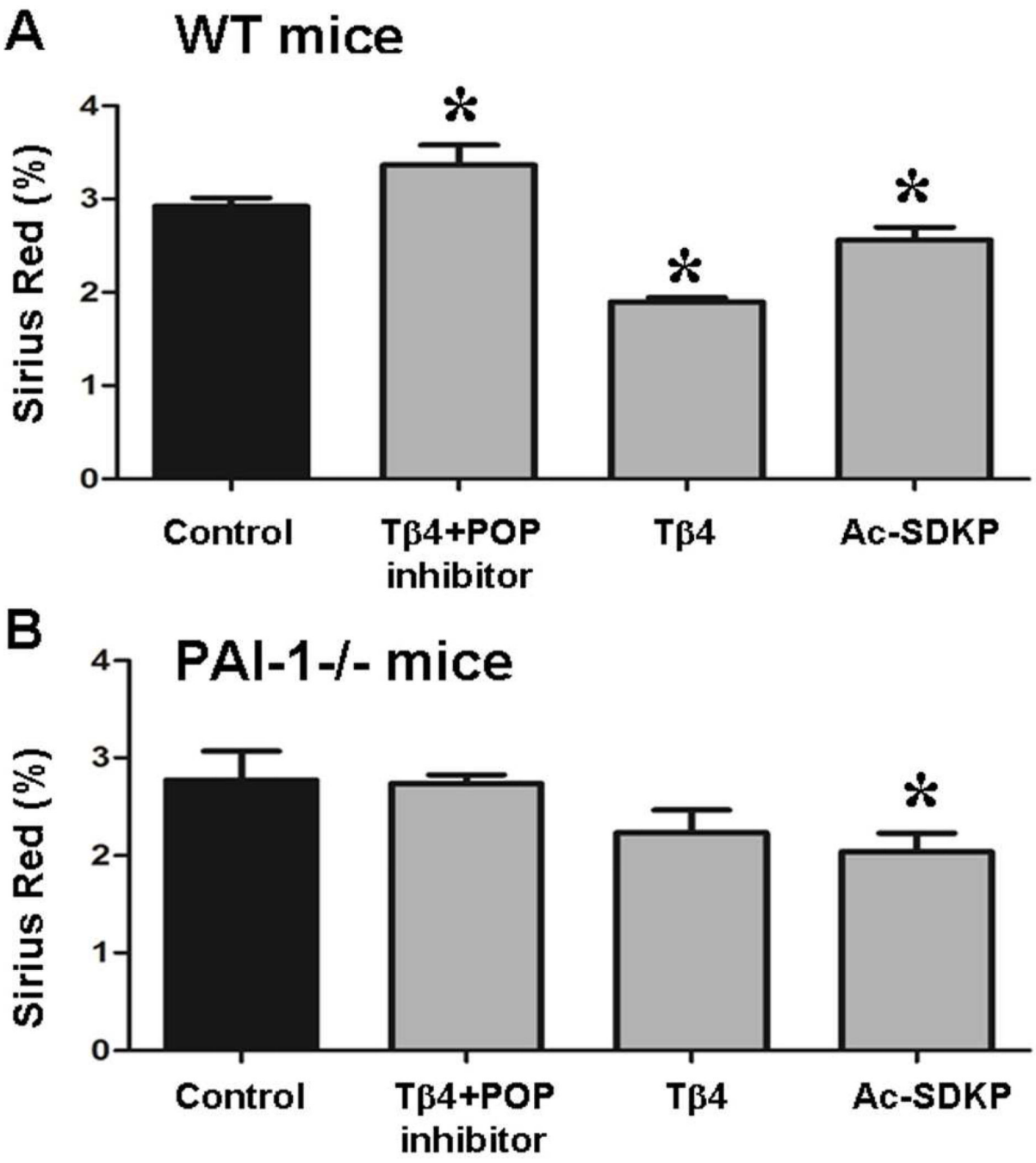

Figure 4.

Late tubulointerstitial fibrosis in wild type and PAI-1−/− mice. (A) Tubulointerstitial fibrosis in wild type mice at day 14 after UUO was significantly increased by thymosin β4 plus POP inhibitor (Tβ4+POPi) but was significantly decreased by thymosin β4 alone or Ac-SDKP (Control, n=10; Tβ4+POPi, n=7; Tβ4 n=5; Ac-SDKP, n=6; *p<0.05 vs control). (B) Tubulointerstitial fibrosis in PAI-1−/− mice at day 14 after UUO was significantly decreased by Ac-SDKP. In contrast to WT mice, neither thymosin β4 plus POP inhibitor nor thymosin

β4 alone affected fibrosis (Control, n=8; Tβ4+POPi, n=8; Tβ4 n=5; Ac-SDKP, n=7; *p<0.05 vs control).

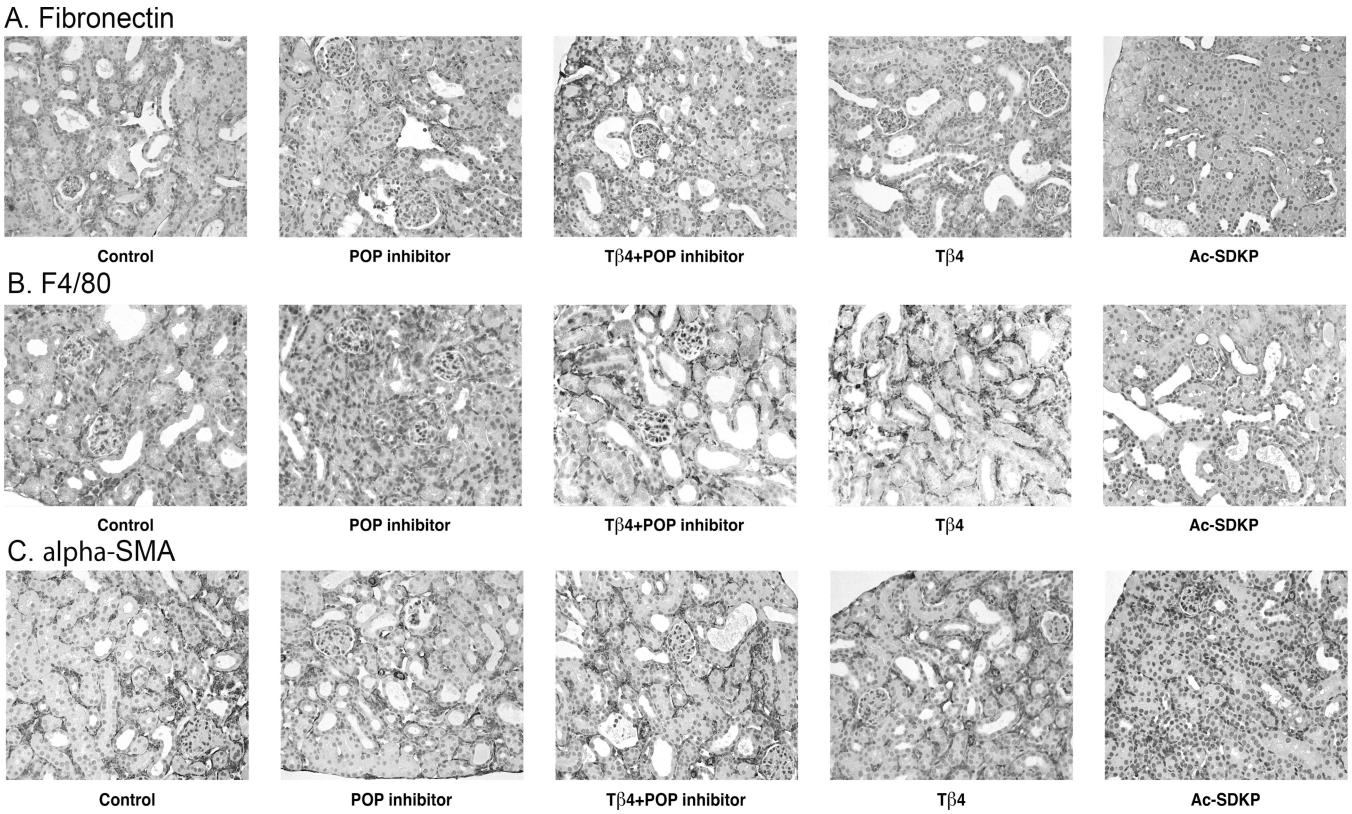

Figure 5.

Fibronectin accumulation, monocyte/macrophage infiltration and myofibroblast expression at early stage of fibrosis at day 5 after UUO in wild type mice. (A) Fibronectin (FN), one of the major extracellular matrices, was significantly increased by thymosin β4 plus POP inhibitor (Tβ4+POPi) but was significantly decreased by Ac-SDKP. Neither thymosin β4 (Tβ4) nor POP inhibitor (POPi) changed fibronectin accumulation. (B) F4/80 positive infiltrating cells were significantly decreased by Ac-SDKP, but not by other treatments. (C) α-SMA-positive myofibroblasts, a key contributor to extracellular matrix, tended to be increased by thymosin β4 plus POP inhibitor (Tβ4+POPi), and were significantly decreased by Ac-SDKP (×200, immunostaining as indicated).

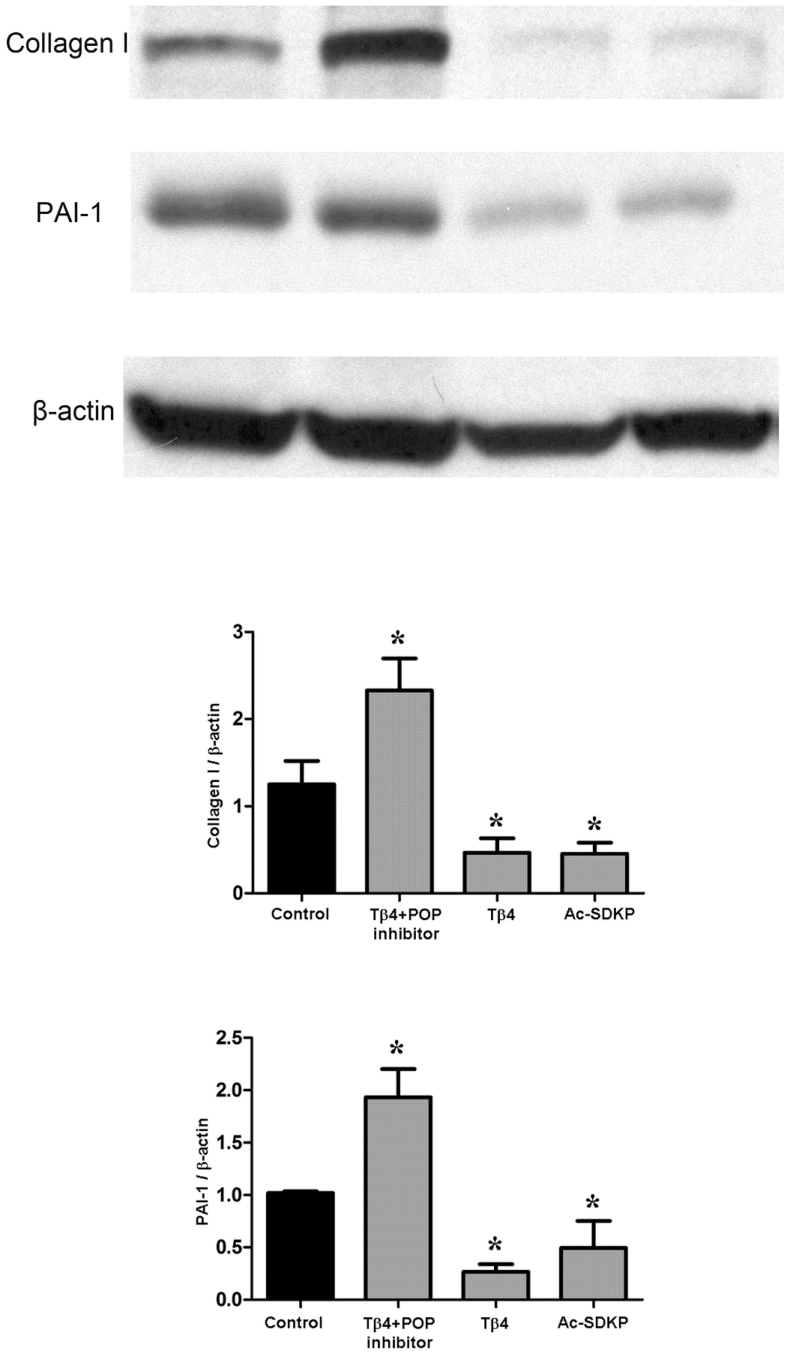

Figure 6.

Obstructed kidneys of wild type mice at day 14 after UUO. Collagen I protein was significantly increased by thymosin β4 plus POP inhibitor but significantly reduced by thymosin β4 alone and Ac-SDKP treatment; *p<0.05 vs control UUO, n=5 in each group. PAI-1 protein was significantly increased by thymosin β4 plus POP inhibitor but significantly reduced by thymosin β4 alone or Ac-SDKP treatment; *p<0.05 vs control UUO, n=5 in each group.

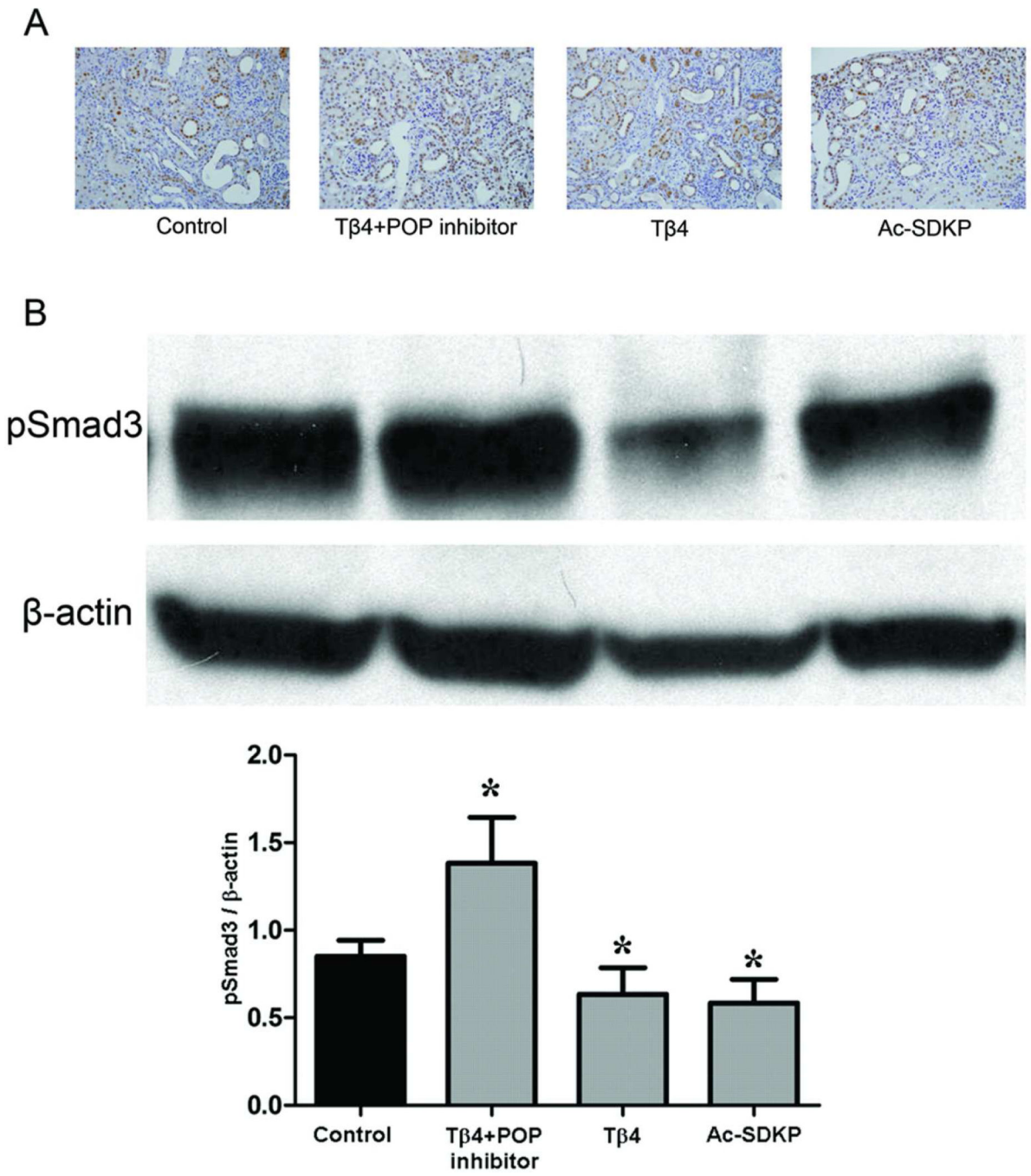

Figure 7.

TGF-β1 activation in obstructed kidneys of wild type mice at day 14 after UUO. (A) Phosphorylated Smad3 was present in tubular epithelial cells and some interstitial cells with similar expression amongst groups (×400). (B) Phosphorylated Smad3 whole kidney protein was increased by thymosin β4 plus POP inhibitor but reduced by thymosin β4 alone or Ac-SDKP treatment; *p<0.05 vs control UUO, n=5 in each group.

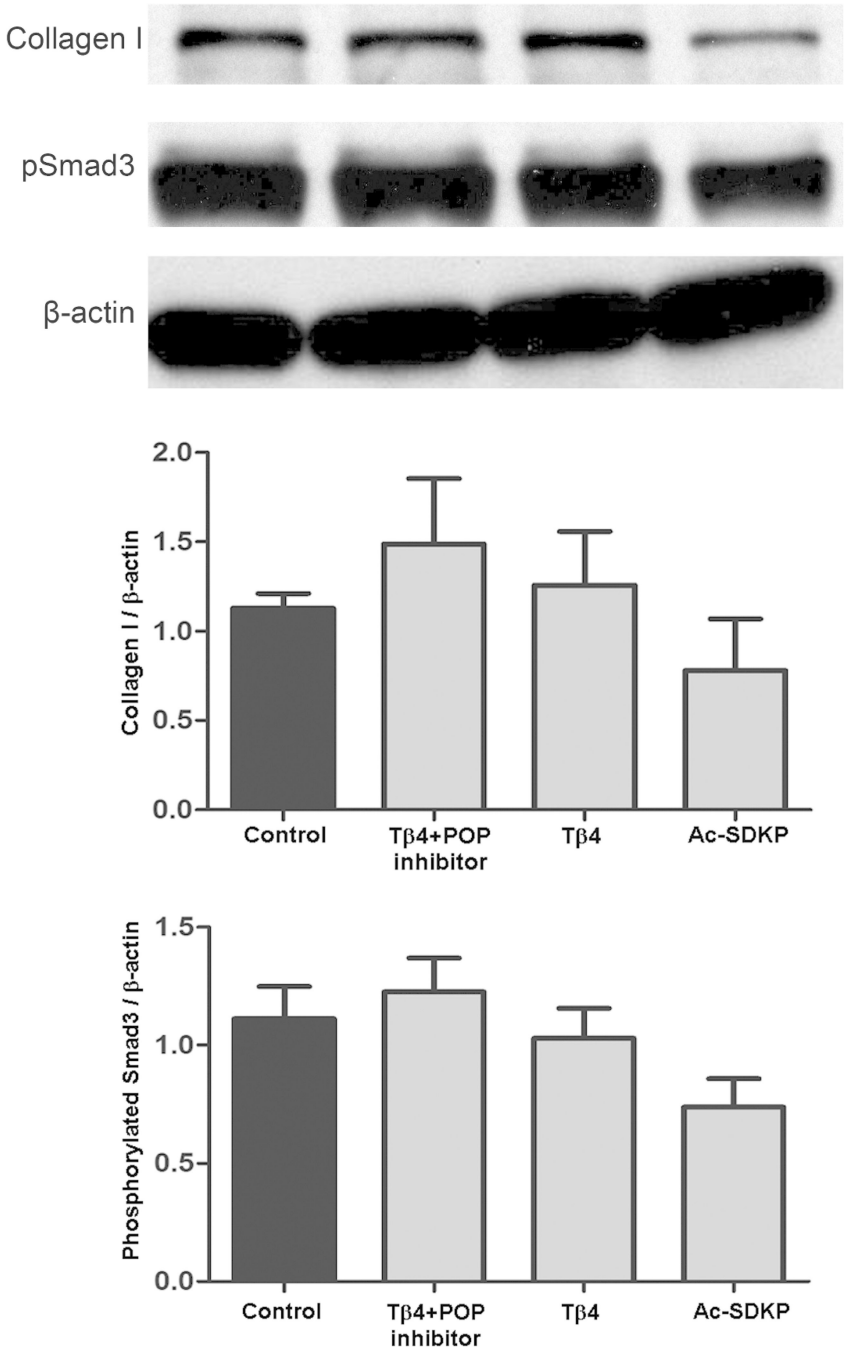

Figure 8.

Obstructed kidneys of PAI-1−/− mice at day 14 after UUO. (A) Collagen I protein was similar amongst groups, numerically highest in the thymosin β4 plus POP inhibitor combination group and numerically lowest in Ac-SDKP group; n=5 in each group. (B) Phosphorylated Smad3 protein was similar amongst groups, numerically highest in the thymosin β4 plus POP inhibitor combination group and numerically lowest in Ac-SDKP group; n=5 in each group.

Table 1

POP Activity and Ac-SDKP Concentration

	Day 5 UUO				Day 14 UUO			
	WT		PAI-1−/−		WT		PAI-1−/−	
Treatment	**POP activity**	**Ac-SDKP**	**POP activity**	**Ac-SDKP**	**POP activity**	**Ac-SDKP**	**POP activity**	**Ac-SDKP**
	pmol/ min* mg tissue	**pmol/ mg tissue**	**pmol min* mg tissue**	**pmol/ mg tissue**	**pmol/ min* mg tissue**	**pmol/ mg tissue**	**pmol/ min* mg tissue**	**pmol/ mg tissue**
Control	27.60±2.26	1.40±0.19	27.38±1.00	1.31±0.04	13.78±3.18	1.07±0.15	18.31±0.97	1.11±0.13
POPi	2.06±0.26*	0.66±0.08*	2.17±0.16*	0.61±0.10*	ND	ND	ND	ND
Tβ4+POPi	5.73±1.59*	0.86±0.09*	7.99±0.80*	1.00±0.15*	4.04±0.80*	0.54±0.13*	7.48±1.52*	0.47±0.05*
Tβ4	21.18± 1.61	1.09±0.12	23.29±1.58	1.26±0.32	12.24±1.51	1.58±0.17*	12.56±4.77	1.22±0.30
Ac-SDKP	31.88±1.18	2.41±0.08*	29.03±1.25	2.61±0.16*	16.68±2.40	2.11±0.23*	12.04±2.87	2.49±0.20*

Abbreviations: POPi, POP inhibitor; Tβ4+POPi, thymosin β4 plus POP inhibitor; Tβ4, thymosin β4; ND, not done.

Data are shown as mean ± SEM

*p<0.05 vs. its control, n=5–8 in each group.

Table 2

Thymosin β4 mRNA, POP Activity, and Ac-SDKP Concentration in Obstructed and Non-obstructed Contralateral Kidneys in WT Mice at Day 5 after UUO

	Thymosin β4 mRNA Thymosin β4/GAPDH		POP Activity (pmol/min*mg tissue)		Ac-SDKP (pmol/mg tissue)	
Treatment	**Obs**	**CL**	**Obs**	**CL**	**Obs**	**CL**
Control	10.81±0.89	1.29±0.27†	27.60±2.26	40.10±2.97†	1.40±0.19	2.41±0.22†
POPi	10.97±1.33	1.34±0.24†	2.06±0.26*	12.68±1.30*,†	0.66±0.08*	1.34±0.12*,†
Tβ4+POPi	9.14±0.68	1.27±0.26†	5.73±1.59*	18.35±1.42*,†	0.86±0.09*	1.48±0.26*,†
Tβ4	13.76±2.88	0.86±0.22†	21.18±1.61	46.88±3.51†	1.09±0.12	1.94±0.19†
Ac-SDKP	6.49±0.61*	0.57±0.03 *,†	31.88±1.18	25.40±2.63*,†	2.41±0.08*	4.06±0.34*,†
Normal	0.73±0.15#		31.39±3.20††		2.13±0.31#	

Abbreviations: Obs, obstructed kidneys; CL, non-obstructed contralateral kidneys; POPi, POP inhibitor; Tβ4+POPi, thymosin β4 plus POP inhibitor; Tβ4, thymosin β4.

Data are mean ± SEM, n=5–8 in each group.

*p<0.05 vs. its own control

†p<0.05 for the obstructed vs. the non-obstructed contralateral kidneys with same treatment

#p<0.05 for normal kidneys vs. the control obstructed kidneys

††p<0.05 for normal kidneys vs. the control non-obstructed contralateral kidneys.

Table 3

Fibronectin, Infiltrating Monocyte/Macrophages, and Myofibroblasts in WT vs. PAI-1−/− Mice at Day 5 after UUO

	Fibronectin positive area (%)		F4/80 positive area (%)		alpha-SMA positive area (%)	
Treatment	**WT**	**PAI-1−/−**	**WT**	**PAI-1−/−**	**WT**	**PAI-1−/−**
Control	2.35±0.23	2.08±0.08	1.70±0.11	1.30±0.07†	2.64±0.56	2.27±0.27
POPi	2.05±0.13	2.17±0.28	1.59±0.03	1.37±0.16	1.86±0.25	2.20±0.08
Tβ4+POPi	3.20±0.32*	2.06±0.07†	1.55±0.07	1.22±0.17	2.82±0.20	2.03±0.13†
Tβ4	2.32±0.26	1.69±0.27	1.86±0.07	1.12±0.06	1.73±0.13	1.86±0.21
Ac-SDKP	1.41±0.15*	1.36±0.15*	0.83±0.09*	1.06±0.07 *,†	0.92±0.05*	1.59±0.18*, †

Abbreviations: POPi, POP inhibitor; Tβ4+POPi, thymosin β4 plus POP inhibitor; Tβ4, thymosin β4

Data are given as percentage of immunostaining positive area, mean ± SEM, n=5–8 in each group.

*p<0.05 vs. its own control

†p<0.05 for WT vs. PAI-1−/− mice with same treatment.

Table 4

Infiltrating Monocytes/Macrophages in WT vs. PAI-1−/− Mice at Day 14 after UUO

Treatment	WT	PAI-1−/−
Control	2.75±0.41	2.54±0.38
Tβ4+POPi	2.51±0.36	2.85±0.18
Tβ4	2.78±0.27	2.36±0.07
Ac-SDKP	1.74±0.13*	0.98±0.11 *,†

Abbreviations: POPi, POP inhibitor; Tβ4+POPi, thymosin β4 plus POP inhibitor; Tβ4, thymosin β4.

Data are given as percentage of F4/80 positive area, mean ± SEM, n=5–8 in each group.

* $p<0.05$ vs. its own control

† $p<0.05$ for WT vs. PAI-1−/− mice with same treatment.

Table 5

Real Time RT-PCR Primer Sequences

	Forward	Reverse
Thymosin β4	gccccttttcacatcaaagaa	tttccttcaccaacagcaa
PAI-1	ccgatgggctcgagtatg	ttgtctgatgagttcagcatc
TGF-β1	gcaacatgtggaactctaccagaa	gacgtcaaaagacagccactca
GAPDH	gagaggccctatcccaactc	gtgggtgcagcgaactttat