Ibuprofen attenuates nephropathy in streptozotocin-induced diabetic rats

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Abstract. Ibuprofen, a commonly administered nonsteroidal anti-inflammatory therapeutic agent, is also a partial agonist of peroxisome proliferator-activated receptor γ (PPARγ). The present study investigated the effects of ibuprofen on type 1 diabetic nephropathy (DN) in rats, and the potential mechanisms associated with the activation of PPARγ. Diabetic rats were induced through a single intraperitoneal injection of streptozotocin before oral treatment with ibuprofen or pioglitazone for 8 weeks. The 24-h urine collection was performed for measurement of total protein content. The kidney was fixed in 10% formalin for periodic acid-Schiff and Masson's trichrome staining. Blood and residual kidney tissue samples were collected to measure the associated biochemical parameters. Chronic ibuprofen treatment decreased urinary protein excretion, blood urea nitrogen, glomerular basement membrane thickening and renal fibrosis, which were accompanied by increases in PPARγ protein expression, glutathione (GSH) level, and superoxide dismutase (SOD) activity, decreases in cyclooxygenase 2 and inducible nitric oxide synthase protein expressions, as well as a decreased interleukin 1β (IL-1β) level in the renal cortex of DN rats. Furthermore, the reduced IL-1β level, increased GSH quantities and stronger SOD activity in the rat serum were evaluated in ibuprofen-treated diabetic rats and were compared with untreated diabetic rats. Regarding GSH and IL-1β levels, ibuprofen was identified to be superior to the positive control, pioglitazone, while levels of the other indices were identified to be similar. Thus, ibuprofen was observed to prevent the development of DN, caused by type 1 diabetes, by anti-inflammatory and anti-oxidative action, potentially via PPARγ activation.

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Introduction

Diabetic nephropathy (DN), a major complication of type 1 and 2 diabetes, is the most common cause of advanced kidney disease, leading to a high number of diabetes-associated mortalities (1,2). However, investigating the mechanisms underlying this condition is complex due to its complicated pathogenesis, and various or numerous symptoms. At present, DN is widely hypothesized to involve tubulointerstitial fibrosis and glomerulosclerosis, triggered by oxidative stress, inflammation, the activation of the renin-angiotensin-aldosterone system, profibrotic factors, collagen cross-linking and epithelial mesenchymal transition, amongst other causes (3-5). However, to the best of our knowledge, the presence of a possible association between these factors remains to be elucidated.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the ligand-activated transcription factors of the nuclear hormone receptor superfamily, and is closely associated with the pathogenesis of numerous diseases, such as diabetes, obesity and inflammatory diseases, including colitis, steatohepatitis, chronic obstructive pulmonary disease and osteoarthritis (6). Regarding the critical role of PPARγ in regulating diverse biological processes, such as lipid metabolism, adipogenesis, and insulin sensitization in diabetes (7), the effects of PPARγ activation on DN are primarily based on type 2 diabetes (8-10). However, PPARγ activation prevents the progression of DN in type 1 diabetes (11). Rosiglitazone and pioglitazone, thiazolidinediones (PPARγ agonists), ameliorate DN by reducing the expression level of chemerin receptor 23 in the kidney of streptozotocin (STZ)-induced type 1 diabetes in rats (11). In addition, low doses of rosiglitazone halt the progression of experimental nephropathy induced by type 1 diabetes by decreasing renal oxidative stress without effecting lipid alteration in diabetic rats (12). Furthermore, a previous study demonstrated that in Finnish adults with type 1 diabetes, the mortality associated with diabetes was almost entirely confined to those with chronic kidney diseases (13). Additionally, evidence indicates that PPARγ agonists alleviate certain symptoms in various types of renal disease (10,14,15). Together, these studies suggested PPARγ activation as an essential therapeutic strategy for kidney diseases caused by type 2 and 1 diabetes.

Previous studies show that the pathogenesis of type 1 DN is associated with inflammation and oxidative stress (16-18).

Recently, it was proposed that PPARγ agonists exert independent actions on the kidney functions, which may assist with preventing diabetic kidney disease, including important effects on inhibition of inflammation (17,19), oxidative stress (18,20), and advanced glycation end products and their receptor interaction (19,20). Therefore, PPARγ may be associated with oxidative stress, inflammation and other factors during the pathogenesis of DN. In addition, suppression of cyclo-oxygenase 2 (COX-2)-mediated prostaglandin E2 production decreases inflammation and albuminuria in STZ-induced type 1 diabetic mice (21). Notably, previous studies show that, in addition to inhibition of COX-2, certain nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and indomethacin, partially activate PPARγ (22). Thus, due to this double action on inflammation, such NSAIDs may be more efficacious than PPARγ agonists, thiazolidinediones, in the treatment of diabetic kidney diseases.

Based on the above-mentioned findings, the present study aimed to investigate the effects of ibuprofen, a partial agonist of PPARγ, (a widely used NSAID with fewer and lighter side effects) on DN, inflammatory response and oxidative stress in STZ-induced type 1 diabetes in rats. The effects of ibuprofen were compared with those of thiazolidinediones, and the thiazolidinedione, pioglitazone served as a positive control.

Materials and methods

Animals. Male Sprague Dawley rats (age, 10 weeks, n=40) were bred at the Xuzhou Medical College Experimental Animal Centre (Xuzhou, China) . Rats were housed in cages (5 rats per cage, 2 cages per group) at 50±10% humidity (temperature, 24±1°C) under a 12-h light/dark cycle, with free access to water and rodent chow. All animal experiments were approved by the Animal Ethics Committee of Xuzhou Medical College before being performed according to the Guidelines for Ethical Conduct in the Care and Use of Animals (23). Every effort was made to minimise stress to the animals.

Experimental design. The rats were fasted for 12 h and subjected to a single intraperitoneal injection of 60 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, USA), freshly dissolved in 0.1 mol/l sodium citrate buffer (pH 4.5; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Age-matched healthy rats (n=10) received sodium citrate buffer alone. The development of diabetes was assessed in accordance with non-fasting blood glucose (nFBG) levels using a reagent kit (Jiancheng Bioengineering Institute, Nanjing, China). Subsequent to 7 days of STZ treatment, diabetic rats (those with nFBG ≥16.7 mmol/l) (24) were successfully obtained, and randomly divided into three groups (n=10) as follows: Diabetic (DM); ibuprofen-treated (DM + IB; 40 mg/kg); pioglitazone-treated (DM + PI; 25 mg/kg). The administered volume of ibuprofen (purity >99%; Huayida Technology Co., Ltd., Wuhan, China) and pioglitazone (purity >99%; Zhongke Yitong Chemical Co., Ltd., Jinan, China) was 10 ml/kg, with the concentrations of 4 and 2.5 mg/ml, respectively, prepared in 1% w/v sodium carboxymethyl cellulose (Sinopharm Chemical Reagent Co., Ltd.). Ibuprofen and pioglitazone were administered orally once per day for 8 weeks. The rats were weighed weekly and underwent blood glucose tests, for which the nFBG levels

were measured using a reagent kit (Jiancheng Bioengineering Institute), according to the manufacturer's instructions, and ultraviolet-visible (UV-Vis) spectrophotometry (722N UV-Vis spectrophotometer; INESA Analytical Instrument Co., Ltd., Shanghai, China). Eight weeks later, the rats were placed in metabolic cages for 24-h urine collection and consequent albuminuria measurement prior to being sacrificed under ethyl ether (Sinopharm Chemical Reagent Co., Ltd.) anesthesia, using cotton balls soaked with ether. The albuminuria levels were measured using a urine protein test kit (Jiancheng Bioengineering Institute; cat. no. C035-2), according to the manufacturer's instructions, and UV-Vis spectrophotometry. Their blood samples (~3 ml) were collected via femoral vein bleeding, and the serum was collected following centrifugation at 4°C at 1,500 x g for 10 min, for approximately 1 ml of blood. Bilateral kidneys were removed and the left kidney was decapsulated and fixed in 4% buffered formalin (Sinopharm Chemical Reagent Co., Ltd.) for 24 h prior to paraffin (Beyotime Institute of Biotechnology, Nantong, China) embedding. In addition, the renal cortex was rapidly isolated. The samples were stored at -70°C prior to use.

Renal function assessment. Renal function can be evaluated through measurement of urinary protein and blood urea nitrogen (BUN). Excretion of urinary protein was quantified using a urine protein test kit (Jiancheng Bioengineering Institute) through the Coomassie (Beyotime Institute of Biotechnology) brilliant blue method, while BUN was examined using a BUN assay kit (Jiancheng Bioengineering Institute) according to a diacetyl oxime colorimetric method (25).

Renal pathological changes by periodic acid-Schiff (PAS) staining and Masson's trichrome staining. Renal PAS staining (Sigma-Aldrich) and Masson's trichrome (Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China) staining were performed, as previously described (26). Kidney tissue samples were fixed in a 10% buffered formalin solution and embedded in paraffin for histological analysis. The 3-μm thick paraffin sections were dewaxed through a series of graded ethanol baths, to displace the water, and subsequently infiltrated with water. The sections were stained with PAS or Masson's trichrome, cleared in xylene (Sinopharm Chemical Reagent Co., Ltd.) and mounted with neutral balsam (Sinopharm Chemical Reagent Co., Ltd.) prior to examination under an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan). The three typical views of each imperfect section were analyzed. Linear measurements were obtained using an image analysis system (Image-Pro Plus 4.0; Media Cybernetics, Inc., Silver Spring, MD, USA).

PPARγ protein expression in rat kidneys by immunohistochemistry. Kidney sections (4 μm) were deparaffinized and endogenous peroxidase was blocked by the addition of 3% H_2O_2 (Zhongshan Golden Bridge Biotech Co., Ltd.; OriGene Technologies, Inc., Beijing, China) for 10 min. The sections were incubated overnight at 4°C with a rabbit polyclonal anti-PPARγ antibody (1:1,000; Bioworld Technology, Inc., St. Louis Park, MN, USA; cat. no. AP0688), then a polymer helper for 20 min, followed by a polyclonal horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody

(Zhongshan Golden Bridge Biotech Co., Ltd.; OriGene Technologies, Inc.; cat. no. PV-9001) at room temperature for 20 min. The peroxidase was visualized through the addition of 3,3'-diaminobenzidine (Zhongshan Golden Bridge Biotech Co., Ltd.; OriGene Technologies, Inc.) in the dark for 3 min. The sections were counterstained with hematoxylin (Beyotime Institute of Biotechnology), dehydrated, and observed under an Olympus CX22 light microscope (Olympus Corporation). A minimum of 3 relatively intact kidney tissue sections were selected at random and further incubated with either a primary or a secondary antibody to determine the binding specificity of PPARγ, without any positive staining. Five sections were analyzed for each rat, and 10 images were obtained from a randomly selected site per slide. The optical density (OD) of PPARγ immunostaining in the cell nucleus was quantified using Image-Pro Plus 4.0 software.

Protein expression of COX-2 and inducible nitric oxide synthase (iNOS) in the renal cortex by western blotting. The preserved renal cortex was weighed and homogenized using an electronic tissue homogenizer (GF-1; Kylin-Bell Lab Instruments Co., Ltd., Haimen, China) in 10 vol (w/v) Tris-buffered saline (50 mmol/l; pH 7.4; Beyotime Institute of Biotechnology) containing 0.6 mmol/l phenylmethylsulphonyl fluoride (Beyotime Institute of Biotechnology), 1 mmol/l Na_3VO_4 (Sangon Biotech Co. Ltd., Shanghai, China) and 50 mmol/l NaF (Sinopharm Chemical Reagent Co., Ltd.) in an ice bath. The resulting homogenates were maintained at 4°C for ≥60 min prior to centrifugation at 4°C for 15 min at 10,000 x g to obtain the supernatant for western blot analysis. Protein concentrations in the supernatant were determined using the bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology).

Protein samples (80 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (10% gel; run at 100 volts for 80 min; Beyotime Institute of Biotechnology) and transferred to BioTrace nitrocellulose membranes (Merck Millipore Ireland BV, Co Cork, Ireland). The membranes were blocked with 5% blocking buffer (Beyotime Institute of Biotechnology) for 120 min and incubated overnight at 4°C with primary antibodies, including rabbit polyclonal anti-COX-2 (1:1,000; ProteinTech Group, Inc., Chicago, IL, USA; cat. .no. 55070-1-AP), rabbit polyclonal anti-iNOS (1:100; Abcam, Inc., Cambridge, UK; cat. .no. ab3523) and rabbit polyclonal anti-β-actin antibody (1:2,000, Bioworld Technology, Inc.; cat. no. AP0060). The blots were detected using alkaline phosphatase-conjugated affinipure goat anti-rabbit secondary antibody (1:1,000; Zhongshan Golden Bridge Biotech Co., Ltd.; OriGene Technologies, Inc.; cat. no. ZB-2308). The membranes were exposed to a 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (NBT) alkaline phosphatase color developing reagent (Beyotime Institute of Biotechnology) for 15 min. Signal densities on the blots were measured with Image J software, version 1.48u (http://imagej.nih.gov/ij/) and normalized against rabbit anti-β-actin (Bioworld Technology Inc.), which served as an internal control ($OD_{protein}$ / $OD_{internal\ control}$).

Determination of interleukin-1β (IL-1β) levels in the rat kidney and serum by enzyme linked immunosorbent assay (ELISA.

The level of IL-1β in the rat renal cortex and serum was estimated using a commercial ELISA kit (ExCell Biology, Inc., Shanghai, China) according to the manufacturer's instructions.

Assay of superoxide dismutase (SOD) activity and reduced glutathione (GSH) level in the rat kidney and serum. SOD activity was measured using the Total Superoxide Dismutase (T-SOD) assay kit (Hydroxylamine method; Jiancheng Bioengineering Institute; cat. no. A001-1) according to the manufacturer's instructions. Briefly, tissue was prepared in a 10% solution (100 mg tissue in 1,000 µl normal saline) and mixed with the reagents of the kit using a vortex mixer. The solution was incubated at 37°C for 40 min prior to the addition of the chromogenic agent. Samples were incubated at room temperature for 10 min and the OD was then determined at a wavelength of 550 nm. SOD activity (U/mg prot) was calculated as follows: $SOD_{activity} = (OD_{control}-OD_{test})/OD_{control} \div 50\% \times TRV/SV \div PrCn$, where TRV is the total reaction volume (ml), SV is the sample volume (ml) and PrCn is the protein concentration of sample (mg prot/ml). The reduced glutathione (GSH) assay (Jiancheng Bioengineering Institute; cat. no. A006-2) was performed according to a previously reported spectrophotometric method (27), using the 722N UV-Vis spectrophotometer. One unit of SOD was defined as the quantity of enzyme causing 50% inhibitory rates of NBT reduction. The tissue GSH level was expressed as nanomoles of GSH per milligram protein, and the serum GSH level was expressed as micromoles of GSH per liter.

Statistical analysis. The results are expressed as means ± standard deviation. Intergroup variation was measured by one-way analysis of variance followed by Tukey's test. The analysis was performed using SPSS Statistical Software, version 13.0 (SPSS, Inc., Chicago, IL, USA) and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of ibuprofen on nFBG and the body weight of diabetic rats. The serum glucose levels of experimental rats were measured at weeks 0 and 8 weeks after treatment with 40 mg/kg ibuprofen and 25 mg/kg pioglitazone (the positive control). Compared with the age-matched healthy rats, higher nFBG levels were observed in the diabetic rats throughout the treatment ($P<0.01$). However, nFBG remained at a high level in diabetic rats treated with either ibuprofen or pioglitazone, without any significant difference between the treated and untreated groups (Table I). Furthermore, the body weights of the experimental rats were measured from week 1 to week 8 following treatment with 40 mg/kg ibuprofen and 25 mg/kg pioglitazone. Diabetic rats continually demonstrated reduced body weights in comparison to the age-matched healthy rats following the STZ injection ($P<0.01$). Ibuprofen or pioglitazone treatment produced no obvious effect on the body weights of diabetic rats (Table II).

Effects of ibuprofen on renal dysfunction in diabetic rats. Fig. 1 illustrates the effects of ibuprofen and the positive control, pioglitazone on rat serum BUN (Fig. 1A), and urinary protein excretion (Fig. 1B) in the healthy and experimental rats.

Significant increases were observed in urinary protein excretion and serum BUN level in the diabetic rats compared with the healthy rats (P<0.01). Chronic treatment with ibuprofen significantly attenuated the damage resulting from renal function, as evidenced by decreases in urinary protein excretion (P<0.01) and serum BUN level (P<0.05) of diabetic rats. This finding was also observed in pioglitazone-treated diabetic rats (Fig. 1).

Effects of ibuprofen on glomerular basement membrane thickening and renal fibrosis in the kidney of diabetic rats. Glomerular basement membrane thickening is a pathological feature of DN. These changes were present in the kidneys of the healthy (Fig. 2Aa) and experimental (Fig. 2Ab-d) rats, and were visualized by PAS staining. Diabetic rats demonstrated obvious glomerular basement membrane thickening, which was characterized by a significant increase in the PAS-stained positive area, when compared with that of the healthy rats (P<0.01; Fig. 2B). However, treatment of the diabetic rats with ibuprofen or pioglitazone significantly reduced glomerular basement membrane thickening (P<0.01; Fig. 2B).

Renal fibrosis is another pathological feature of DN. Fig. 3 demonstrates the renal fibrosis of rats from the healthy (Fig. 3Aa) and experimental (Fig. 3Ab-d) groups using Masson's trichrome staining. Collagen fibers were stained blue, muscle fiber cytoplasm was stained red and nuclei were stained brown. The results reveal a marginal quantity of collagen fiber deposition in the healthy rats (Fig. 3B), but a significant increase in tubulointerstitial collagen in the diabetic rats (P<0.01; Fig. 3B). Diabetic rats that received ibuprofen or pioglitazone treatment exhibited less tubulointerstitial collagen when compared with the untreated diabetic rats (P<0.05; Fig. 3B).

Effects of ibuprofen on PPARγ protein expression in the kidney of diabetic rats. Ibuprofen and pioglitazone activate PPARγ. To examine the effects of the two therapeutic agents on activation of PPARγ, the activated form of PPARγ was identified in the kidney using immunohistochemistry (Fig. 4). The data indicates that the protein expression level of PPARγ in the cell nuclei was markedly reduced in the kidney of diabetic rats when compared with that of healthy rats (P<0.01; Fig. 4B), while chronic ibuprofen treatment prevented this reduction, with no significant difference when compared with pioglitazone (Fig. 4B).

Effects of ibuprofen on inflammatory responses in diabetic rats. The DN rats displayed marked inflammatory responses in the kidney; protein expressions of COX-2 and iNOS were significantly increased in the renal cortex of diabetic rats, compared with those of the healthy rats (P<0.01 and P<0.05, respectively). Chronic treatment with ibuprofen prevented the increase of COX-2 and iNOS, while treatment with pioglitazone significantly decreased the protein expression of iNOS (P<0.01 vs. DM), but not that of COX-2 (Fig. 5A). Furthermore, the level of IL-1β, an important pro-inflammatory cytokine, was markedly elevated in the renal cortex and serum (P<0.01) of DN rats compared with that of healthy rats, which was significantly attenuated following ibuprofen treatment (P<0.05). By contrast, pioglitazone treatment prevented the elevated IL-1β level in the serum of DN rats more effectively (P<0.05) than ibuprofen

Table I. Effects of IB on nFBG of streptozotocin-induced diabetic rats before and after treatment with IB.

| Group | n | nFBG (mmol/l) | |
		Before treatment	After treatment
Control	10	6.2±0.5	4.7±0.4
DM	9-10	36.8±5.4[a]	32.4±4.3[a]
DM + PI	10	36.6±4.6	29.9±4.2
DM + IB	9-10	37.1±5.7	27.1±6.1

Data are presented as means ± standard deviation. [a]P<0.01 vs. control. nFBG, non-fasting blood glucose; DM, diabetic group; PI, pioglitazone; IB, ibuprofen.

treatment, and although PI treatment decreased the IL-1β level in the renal cortex of DN rats, it exhibited a weaker effect than ibuprofen treatment (Fig. 5B and C).

Effects of ibuprofen on anti-oxidative function in diabetic rats. In addition to inflammatory responses, oxidative stress damage in DN rats was observed. Diabetes induced significant decreases in SOD activity (Fig. 6) and GSH level (Fig. 7) in the renal cortex of rats compared with healthy rats (P<0.01 and P<0.05, respectively), which were alleviated by chronic treatment with ibuprofen and pioglitazone (Figs. 6A and 7A), with no marked difference between the two. Furthermore, SOD activity and GSH level were markedly reduced in the serum of diabetic rats (P<0.01), while chronic treatment with ibuprofen enhanced SOD activity and the GSH level (P<0.01; Figs. 6B and 7B). Pioglitazone treatment had a similar effect on SOD activity, but a weaker effect on GSH level (P<0.05) when compared with ibuprofen treatment (Figs. 6B and 7B).

Discussion

Pathogenesis of DN is complex and multifactorial, involving different cell types, molecules, and factors, in particular oxidative stress and inflammatory cytokines (16). The present study demonstrated significant elevations of urinary protein excretion and BUN level, as well as glomerular basement membrane thickening and renal fibrosis in type 1 diabetic rats over a nine-week period. Diabetes produced large quantities of COX-2, iNOS, and IL-1β protein, but small quantities of PPARγ protein, SOD activity, and GSH levels in the kidney of rats. In addition, high IL-1β levels, and low SOD activity and GSH levels were observed in the serum of diabetic rats. However, the majority of the changes were markedly attenuated by treatment with ibuprofen and pioglitazone, which served as a positive control. These data indicated that ibuprofen prevents the progression of DN in an experimental rat model of type 1 diabetes, via suppression of inflammatory and oxidative damage, potentially through PPARγ activation.

Chronic inflammation is significant in the development of diabetes and its late complications, including DN (28-30). Inflammatory markers are well known to be associated with the development of renal disease in diabetes (31). Anti-inflammatory therapeutic agents have been reported to prevent renal

Table II. Effects of IB on the body weight of streptozotocin-induced diabetic rats after treatment.

Group	Body weight (g)								
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Control	245±4	275±12	312±14	337±15	356±16	382±19	393±19	409±18	391±15
DM	185±17[a]	186±25[a]	173±29[a]	175±27[a]	174±32[a]	173±32[a]	166±31[a]	168±38[a]	158±34[a]
DM + PI	190±18	180±28	181±27	180±28	183±33	190±39	187±36	180±37	172±32
DM + IB	183±24	182±27	164±21	175±32	174±39	178±44	163±41	168±44	168±45

Data are presented as means ± standard deviation; n=9-10. [a]P<0.01 vs. control. DM, diabetic group; PI, pioglitazone; IB, ibuprofen.
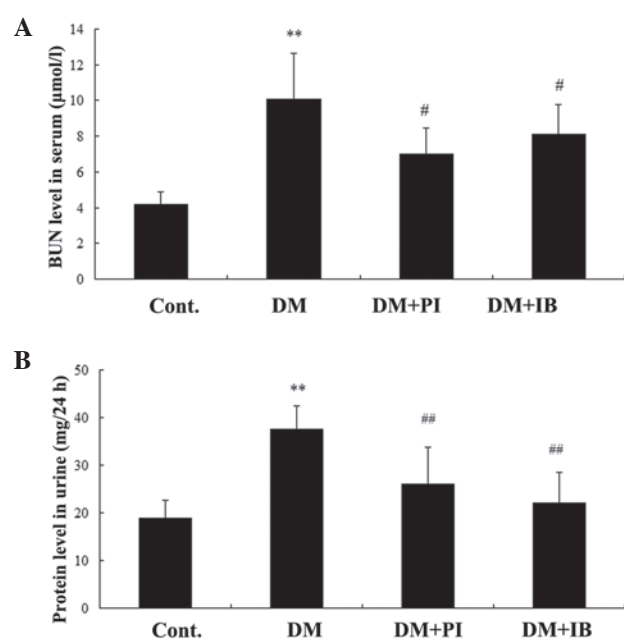

Figure 1. Effects of IB on (A) BUN and (B) urinary protein excretion. Cont., DM, DM + PI, and DM + IB represent healthy rats, diabetic rats, and diabetic rats treated with PI (25 mg/kg) and IB (40 mg/kg), respectively. Data are presented as means ± standard deviation; n=7. **P<0.01 vs. Cont.; #P<0.05 and ##P<0.01 vs. DM. BUN, blood urea nitrogen; Cont., control; DM, diabetic group; PI, pioglitazone; IB, ibuprofen.
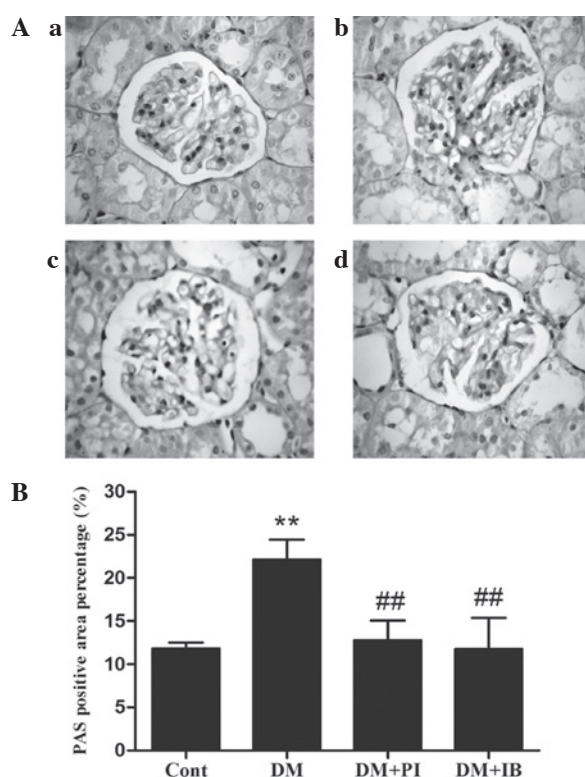

Figure 2. Effects of IB on glomerular basement membrane thickening in rat kidneys. Glomerular basement membrane thickening was examined by PAS staining. (A) Typical staining images (magnification, x400) of (a) Cont., (b) DM, (c) DM + PI and (d) DM + IB, and (B) quantitative analysis. The Cont., DM, DM + PI, and DM + IB groups represent healthy rats, and diabetic rats, diabetic rats treated with PI (25 mg/kg) and IB (40 mg/kg), respectively. Data are presented as means ± standard deviation; n=3. **P<0.01 vs. Cont.; ##P<0.01 vs. DM. PAS, Periodic acid-Schiff; Cont., control; DM, diabetic group; PI, pioglitazone; IB, ibuprofen.

injury in diabetic rats by reducing macrophage infiltration and the protein expression of transforming growth factor-β, type IV collagen and intercellular adhesion molecule 1 in renal cells (32). Furthermore, chronic COX inhibition by NSAIDs (non-selective COX inhibitor, ibuprofen and selective COX-2 inhibitor, NS-398) reduced diabetes-induced hyperfiltration, proteinuria and fibrosis in the kidney of Akita DN mice (33); with the effects of ibuprofen observed to be similar to, if not more beneficial than, COX-2 inhibition by NS-398. In the present study, chronic ibuprofen treatment at a low dose (40 mg/kg) attenuated proteinuria and renal fibrosis of diabetic rats, which was coupled with the reduced protein expression of COX-2 and iNOS (common inducible proteins in the presence of inflammation) in the renal cortex. In a previous study, IL-1 was hypothesized to increase vascular permeability, mesangial cell proliferation and extracellular matrix deposition, as well as glomerular basement membrane thickening (34). The present study

identified that glomerular basement membrane thickening occurred in the kidney of diabetic rats, with increased IL-1β levels observed in the serum and renal cortex. However, ibuprofen prevented glomerular basement membrane thickening, and decreased the IL-1β level in the blood and kidney. The above-mentioned studies and the results of the present study suggest that anti-inflammatory therapy alleviates functional and morphological impairments of diabetic animals. A previous study demonstrated that PPARγ agonists attenuated renal injury and inflammation in a mouse model of unilateral ureteral obstruction (35). Ohga et al (6) reported that
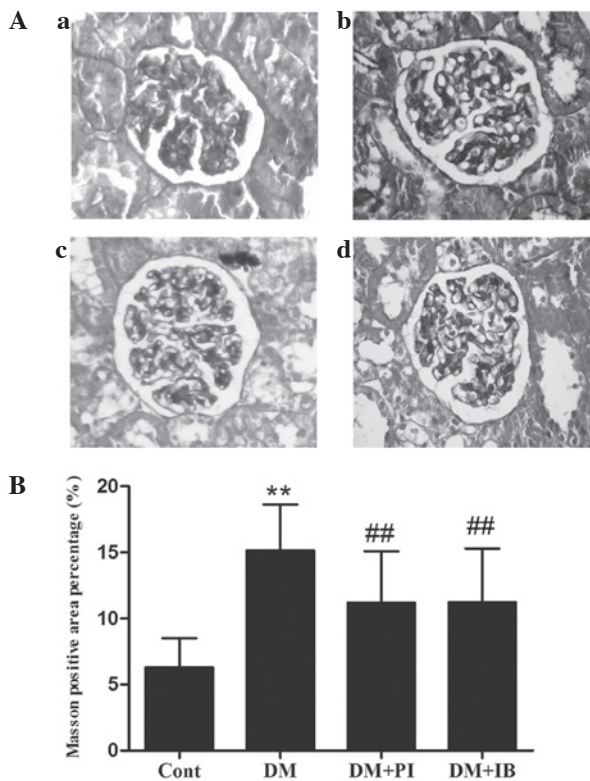
Figure 3. Effects of IB on rat kidney renal fibrosis, which was assessed by Masson's trichrome staining. (A) Typical staining images (magnification, x400) of (a) Cont., (b) DM, (c) DM + PI and (d) DM + IB, and (B) quantitative analysis. The Cont., DM, DM + PI, and DM + IB groups represent healthy rats, diabetic rats, and diabetic rats treated with PI (25 mg/kg) and IB (40 mg/kg), respectively. Data are presented as means ± standard deviation (n=3). **P<0.01 vs. Cont.; ##P<0.05 vs. DM. Cont., control; DM, diabetic group; PI, pioglitazone; IB, ibuprofen.

Figure 4. Effects of IB on the activated form of PPARγ in rat kidneys. Protein expression of PPARγ in the nucleus was assayed by immunohistochemistry. (A) Typical staining images (magnification, x400) of (a) Cont., (b) DM, (c) DM + PI and (d) DM + IB, and (B) quantitative analysis. The Cont., DM, DM + PI, and DM + IB groups represent healthy rats, diabetic rats, and diabetic rats treated with PI (25 mg/kg) and IB (40 mg/kg), respectively. Data are presented as means ± standard deviation; n=3. **P<0.01 vs. Cont.; ##P<0.01 vs. DM. PPARγ, peroxisome proliferator-activated receptor γ; Cont., control; DM, diabetic group; PI, pioglitazone; IB, ibuprofen.

pioglitazone ameliorated renal injury through the inhibition of nuclear factor κ-light-chain-enhancer of activated B cells activation, intercellular adhesion molecule 1 expression and macrophage infiltration in STZ-induced diabetic rats. The present findings showed that ibuprofen, a typical NSAID and partial agonist of PPARγ, decreased the level of inflammatory markers in the renal cortex, and elevated the activated PPARγ level in the kidneys of diabetic rats. These results demonstrated that the anti-inflammatory effect, possibly via PPARγ activation, was responsible for the alleviation of DN by ibuprofen in type 1 diabetes.

In addition to chronic inflammation, oxidative stress was hypothesized to be vital in the pathological process of chronic diabetic complications, including DN (36,37). A unifying hypothesis has been proposed, where over-production of reactive oxygen species in mitochondria, in response to chronic hyperglycemia, may be the key initiator of various pathogenic pathways in diabetic complications (38). Gumien-iczek (39) reported that GSH reductase activity and the GSH level were diminished in the kidney of alloxan-induced diabetes in rabbits, and pioglitazone restored them to the control values. In the current study, SOD activity and GSH level, two important endogenous antioxidants, were significantly declined in the renal cortex and serum of STZ-induced diabetes in rats. Furthermore, oxidative stress and renal fibrosis in the kidney of STZ-induced diabetes in mice have
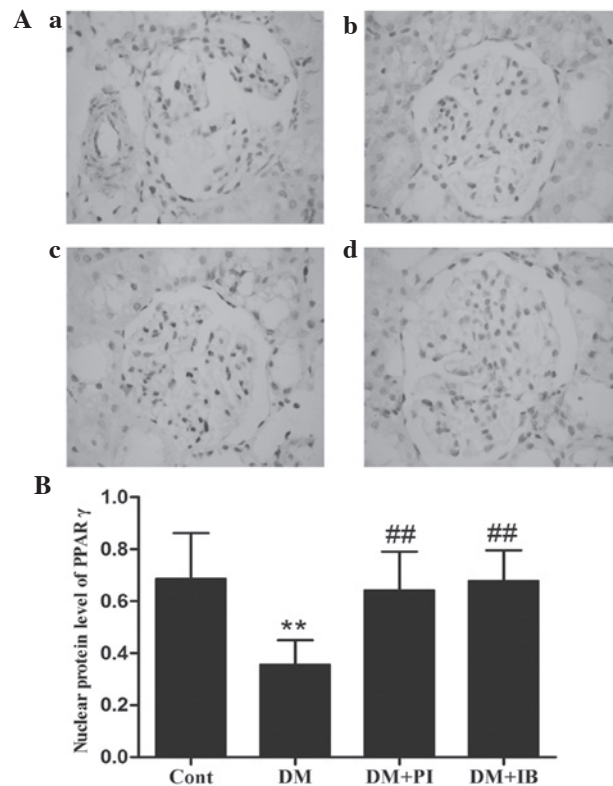
previously been associated with decreased protein expression of PPARγ and its coactivator PGC-1α, which may be attenuated by telmisartan (18). The present findings indicated that ibuprofen and pioglitazone relieved the oxidative damage in the kidney of diabetic rats, in combination with a marked increase in protein expression of nuclear PPARγ in the kidney. Notably, a previous study indicated that ibuprofen disrupted the signaling cascades that lead to microglial nicotinamide adenine dinucleotide phosphate-oxidase activation independently of COX inhibition, preventing oxidative damage in the brain (40). These results indicate that enhancement of anti-oxidation function, potentially via PPARγ activation, contributes to the renal protection of ibuprofen in type 1 diabetes.

Although in the current study ibuprofen or pioglitazone treatment did not influence the nFBG level and the body weight of diabetic rats, ibuprofen and pioglitazone showed significant attenuation on renal function and morphological changes in diabetic rats. There have been similar studies regarding these effects of PPARγ agonist; in experimental models of diabetes, PPARγ agonists have been shown to attenuate renal damage and reduce albuminuria, independent to blood glucose lowering (41). Previous studies have indicated that a low dose of pioglitazone ameliorated renal fibrosis and preserved renal function in an animal model of metabolic
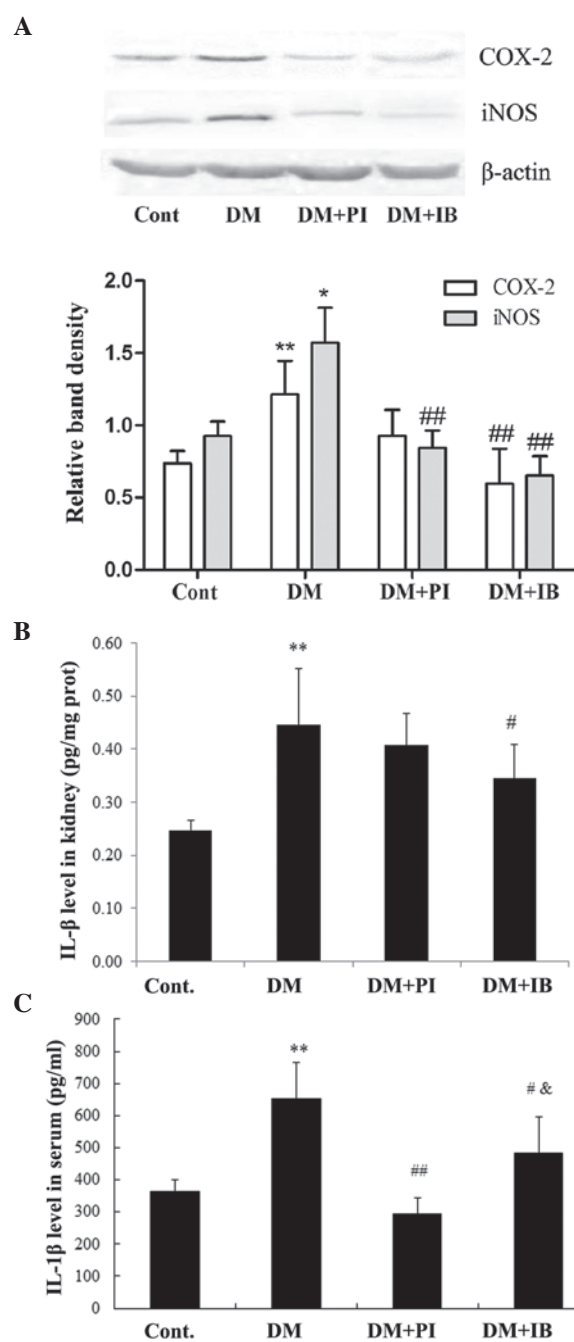
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B

C

Figure 5. Effects of IB on protein (prot) expression of COX-2 and iNOS in (A) rat kidneys, and IL-1 levels in rat (B) kidneys and (C) serum. COX-2 and iNOS protein expression levels were assayed by western blotting. The IL-1 level was measured by enzyme linked immunosorbent assay. The Cont., DM, DM + PI, and DM + IB groups represent healthy rats, diabetic rats, and diabetic rats treated with PI (25 mg/kg) and IB (40 mg/kg), respectively. Data are presented as means ± standard deviation; n=4 (COX-2 and iNOS); n=6-8 (IL-1). *P<0.05 and **P<0.01 vs. Cont.; #P<0.05 and ##P<0.01 vs. DM; &P<0.05 vs. DM + PI group. COX-2, cyclooxygenase 2; iNOs, inducible nitric oxide synthase; IL-1, interleukin-1; Cont., control; DM, diabetic group; PI, pioglitazone; IB, ibuprofen.
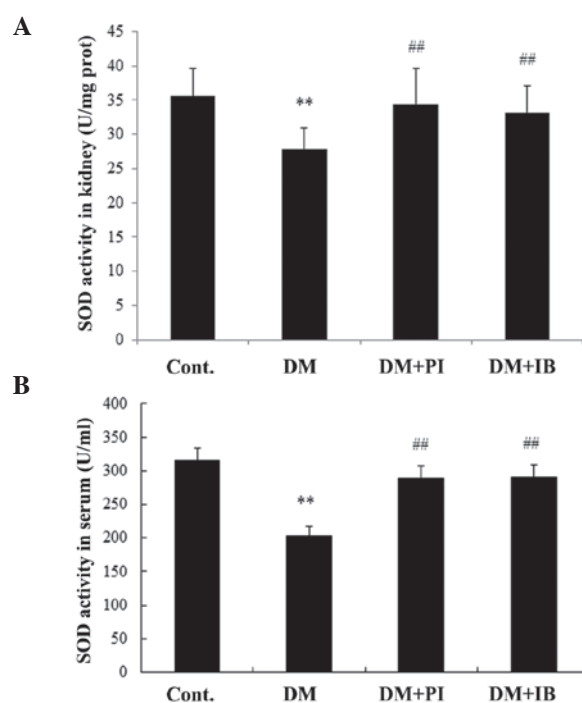
A

B

Figure 6. Effects of IB on SOD activity in rat (A) kidneys and (B) serum. SOD activity was measured using xanthine oxidase (the hydroxylamine method). The Cont., DM, DM + PI, and DM + IB groups represent healthy rats, diabetic rats, and diabetic rats treated with PI (25 mg/kg) and IB (40 mg/kg), respectively. Data are presented as means ± standard deviation; n=7-8. **P<0.01 vs. Cont.; ##P<0.01 vs. DM. SOD, superoxide dismutase; Cont., control; DM, diabetic group; PI, pioglitazone; IB, ibuprofen.
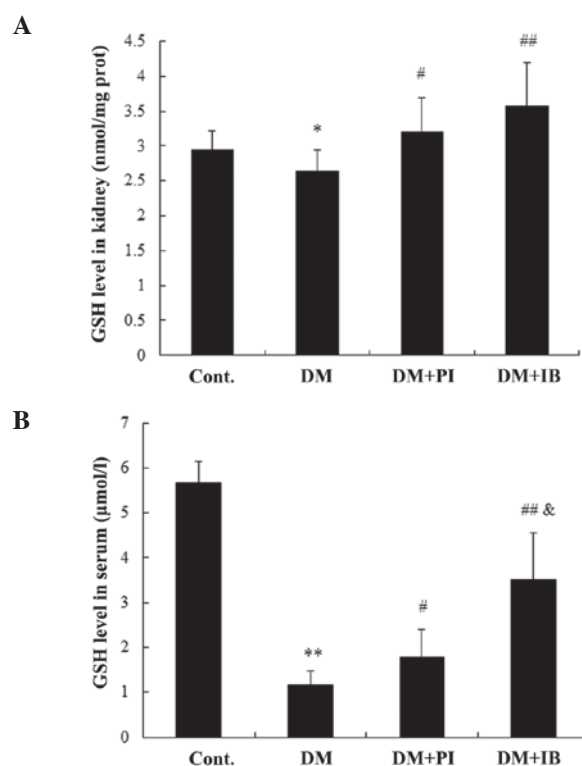
A

B

Figure 7. Effects of IB on reduced GSH levels in rat (A) kidneys and (B) serum. The GSH level was measured by spectrophotometry. Cont., DM, DM + PI, and DM + IB groups represent healthy rats, diabetic rats, and diabetic rats treated with PI (25 mg/kg) and IB (40 mg/kg), respectively. Data are presented as means ± standard deviation; n=7-8. *P<0.05 and **P<0.01 vs. Cont.; #P<0.05 and ##P<0.01 vs. DM; &P<0.05 vs. DM + PI. GSH, glutathione; Cont., control; DM, diabetic group; PI, pioglitazone; IB, ibuprofen.

syndrome, independently of hyperglycemic control or effects on body weight (12,42). A recent review demonstrated that PPARγ agonist-mediated renal protection resulted from numerous other efficacies beyond the glucose lowering effect (43). Together, these findings further confirmed the key role of anti-inflammatory and anti-oxidative actions of

ibuprofen via PPARγ activation in the prevention and treatment of type 1 diabetes-induced nephropathy.

In conclusion, the present study demonstrated that ibuprofen markedly attenuated the functional and morphological changes in the kidney of rats with STZ-induced type 1 diabetes, which was realized by anti-inflammatory and anti-oxidative action, potentially via COX-2 suppression and PPARγ activation, suggesting that ibuprofen may serve as a multi-target therapeutic agent for DN.

Acknowledgements

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