**The peroxisome proliferator-activated receptor γ agonist
pioglitazone prevents NF- κ B activation in cisplatin nephrotoxicity
through the reduction of p65 acetylation via the
AMPK-SIRT1/p300 pathway**

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Running title

Pioglitazone prevents cisplatin nephrotoxicity

ABSTRACT

The thiazolidinedione pioglitazone, which is also a PPAR- γ agonist, now is widely used in patients with hypercholesterolemia and hypertriglyceridemia. NF- κ B is a ubiquitously expressed transcription factor controlling the expression of numerous genes involved in inflammation. The aim of the present study was to evaluate whether the activation of PPAR- γ attenuates the cisplatin-induced NF- κ B activation in cisplatin nephrotoxicity. The results showed that the PPAR- γ agonist pioglitazone decreased the expression of NF- κ B p65 transcription target genes (e.g. IL-6, IL-1 β , and TNF- α) and inhibited histological injury and inflammatory cells infiltration in cisplatin nephrotoxicity. The suppression of NF- κ B activity following pioglitazone treatment inhibited the cisplatin-induced I κ B- α degradation and NF- κ B p65 subunit translocation. Translocation of the NF- κ B p65 subunit depends on p65 acetylation, which primarily regulated by SIRT1 or p300. Notably, AMP kinase (AMPK) activation not only decreased the phosphorylation, activation and p65 interaction of p300 but also increased SIRT1 expression, activation and p65 binding, thus leading to a significant reduction in p65 acetylation. Interestingly, the reduction of IL-6, TNF- α and IL-1 β , the inhibition of histological injury and the inflammatory cells infiltration following pioglitazone treatment in cisplatin nephrotoxicity were attenuated after treatment with the PPAR- γ antagonist GW9662. These results suggest that the PPAR- γ agonist pioglitazone prevents NF- κ B activation in cisplatin nephrotoxicity through a reduction in p65 acetylation via the AMPK-SIRT1/p300 pathway.

Keywords: cisplatin; acute kidney injury; PPAR- γ ; NF- κ B p65 acetylation

1. INTRODUCTION

Cisplatin is one of the most broadly effective chemotherapeutic agents used to treat a wide variety of malignancies. However, cisplatin exhibits latent toxicity in the kidneys and is involved in the cumulative decline in renal function. Approximately 25-35% of patients who were administered cisplatin treatment showed a mild and partially reversible decline in renal function after the first course of therapy [1]. The mechanisms underlying cisplatin-induced acute kidney injury include DNA damage, oxidative stress, and an uncontrolled inflammation response [2, 3]. Specifically, the uncontrolled inflammation response is considered a key mechanism of cisplatin nephrotoxicity; thus, inhibiting the inflammatory response might be an effective therapeutic intervention to decrease cisplatin-induced kidney injury [4, 5].

Nuclear factor κ B (NF- κ B), an important nuclear transcription factor, plays a major role in regulating the inflammatory response [6, 7]. In unstimulated cells, NF- κ B is sequestered in the cytoplasm via interactions with inhibitory I κ B proteins. However, when cells receive pathological stimuli, such as cisplatin, I κ B proteins are phosphorylated through activated I κ B kinase (IKK) [6, 7]. The phosphorylation of I κ B proteins results in ubiquitination and degradation, which in turn releases sequestered NF- κ B, leading to the translocation of this protein to the nucleus, where NF- κ B induces the expression of various pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin 6 (IL-6) [6-8]. The activation of NF- κ B mediated transcription requires the acetylation of the p65 subunit of NF- κ B. The modification of p65 relies on the balance between acetylation and deacetylation, and cisplatin induces the acetylation of the NF- κ B p65 subunit at lysine 310 [6-10], suggesting that the deacetylation of p65 at lysine 310 might attenuate cisplatin

nephrotoxicity.

As a NAD⁺-dependent class III histone deacetylase, SIRT1 is involved in many cellular and internal processes, such as metabolism, stress responses, hormone responses, and apoptosis [11]. SIRT1 interacts with the RelA/p65 subunit of NF-κB and decreases the acetylation of RelA/p65 at lysine 310 [11, 12]. The transcriptional co-activator p300 is a histone acetyltransferase (HAT) that integrates diverse pathways involved in different functions *in vivo* and *in vitro* [13-15]. The p300 acetyltransferase promotes histone acetylation and thus regulates promoter activity through the removal of chromatin dependent repression [16-18]. Previous studies have shown that the p300 acetyltransferase mediated acetylation of p65 (at lysine 310) stimulates NF-κB specific transcriptional activity [11, 12]. The regulation of p65 modification through SIRT1 or p300 could be a potential target for the treatment of inflammatory injury in cisplatin nephrotoxicity.

AMP-activated protein kinase (AMPK) is an evolutionarily conserved metabolic fuel gauge in eukaryotes that senses changes in the intracellular AMP/ATP ratio [19]. AMPK can be activated to modulate the NAD⁺/NADH ratio. SIRT1 deacetylase activity is regulated through NAD⁺ levels [20]. The AMP-kinase signaling pathway modulates p300 activities through the phosphorylation of p300, thus inhibiting the interaction of this protein with nuclear receptors, such as NF-κB [21]. AMPK modulates the activities of SIRT1 deacetylase and p300 acetylase [21, 22]. Studies have shown that PPAR-γ agonists (e.g., pioglitazone) phosphorylate and subsequently activate AMPK [23, 24].

The aim of the present study was to determine whether the PPAR-γ agonist pioglitazone could suppress NF-κB mediated inflammation in cisplatin nephrotoxicity. Considering the relevant

factors, we hypothesized that PPAR- γ agonists could inhibit NF- κ B activation through p65 deacetylation via AMPK activation, which increased SIRT1 expression and decreased p300 phosphorylation.

2. MATERIALS AND METHODS

2.1. Animals and drug treatments

Male C57BL/6 mice (HuaFukang Experimental Animal Center, Beijing, China), which were provided a standard laboratory diet and water ad libitum, were maintained under a protocol approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. Mice that were 8-10 weeks of age and weighed 20-25 g were used in the following experiments and there were 8-10 mice in each group. After a minimum 7-days acclimation period, the mice were administered a single intraperitoneal injection of either vehicle (saline) (Shuanghe Pharmaceutical Co., Ltd., Beijing, China) or cisplatin (Nanjing Pharmaceutical Co., Ltd., China). Different cisplatin concentrations (0, 10, 15, 20, 25, and 30 mg/kg) were administered at different times (0, 24, 48, 72, and 96h) to determine the optimal drug concentration and induction time for cisplatin nephrotoxicity in mice. In the mice with cisplatin nephrotoxicity, different concentrations of pioglitazone (0, 5, 10, 20, and 40 mg/kg/day/Po) (Sigma-Aldrich, St. Louis, USA) were administered to determine the best concentration for decreasing nephrotoxicity. Subsequently, we explored the best concentration of GW9662 (0, 1, 2, 4, and 8 mg/kg/day/Po) (Sigma-Aldrich, St. Louis, USA) to reverse the protective effect of pioglitazone on cisplatin nephrotoxicity mice. Pioglitazone and GW9662 were administered through oral gavage once a day from 3 days before the cisplatin injection. The mice were anesthetized using 1% sodium

pentobarbital (6 mg/kg) and subsequently sacrificed 0, 24, 48, and 72 h after cisplatin injection through cervical dislocation. The Animal Care and Use Committee of the Hubei Province Health Care System approved all experimental procedures in accordance with the Guiding Principles for the Care and Use of Laboratory Animals of the National Institutes of Health and Chinese Physiological Society. In the renal failure model, a period of 14 days after cisplatin injection was considered long-term survival, and 25 mice were used in each group.

2.2. Biochemical analysis

To assess renal function, blood was collected from the mice to measure the blood urea nitrogen (BUN) and serum creatinine (Cr) concentrations in the core laboratory of Tongji Hospital (Wuhan, China).

2.3. Histological examination

The mouse kidneys were sectioned in blocks and fixed in 10% formalin (Wuhan goodbio technology CO.LTD, Wuhan, China), then dehydrated in a graded series of alcohol (Wuhan goodbio technology CO.LTD, Wuhan, China) and finally embedded in paraffin (Wuhan goodbio technology CO.LTD, Wuhan, China). The 3- μ m paraffin kidney sections were stained with periodic acid-Schiff (PAS) (Wuhan goodbio technology CO.LTD, Wuhan, China) stain, and subsequently, the percentage of cortical tubules showing epithelial necrosis was calculated and assigned the following scores: 0 for all normal tubules; 1 for <10% necrotic tubules; 2 for 10-25% necrotic tubules; 3 for 26-75% necrotic tubules; and 4 for >75% necrotic tubules.

2.4. Real-time PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's instructions, and cDNA was generated through reverse transcription using the PrimeScript RT Master Mix kit (TaKaRa Bio Inc, Kusatsu, Shiga, Japan). Equal amounts of cDNA were diluted and amplified through real-time PCR using All-in-One qPCR Mix(GeneCopoeia, Maryland, USA) in a 20- μ l reaction volume containing 10 μ l of 2X All-in-One qPCR mix (GeneCopoeia, Maryland, USA), 1 μ l of 2 μ M forward primer, 1 μ l of 2 μ M reverse primer, 1 μ l of cDNA, and 6 μ l of nuclease-free water. After an initial denaturation step for 10 min at 95°C, the conditions for cycling were 40 cycles of 10 s at 95°C, 20 s at 60°C, and 15 s at 72°C. For the normalization of each sample, GAPDH primers were used to measure the amount of GAPDH cDNA. The primers are list in Table 1. The relative fold-change in the target gene cDNA was determined using the $2^{-\Delta\Delta C_t}$ method as previously reported.

2.5. Immunoblotting and immunoprecipitation for p-PPAR- γ , PPAR- γ , NF- κ B p65, Ace-p65, I κ B- α , SIRT1, p-AMPK, AMPK, p-p300, and p300.

To obtain whole-cell lysates, the renal tissue was homogenized in RIPA buffer (Wuhan goodbio technology CO.LTD, Wuhan, China) containing phosphatase inhibitor (1:100, Wuhan goodbio technology CO.LTD, Wuhan, China) and protease inhibitor cocktail (1:50, Roche, Ltd, Basel, Switzerland). The homogenate was centrifuged at 12000rpm for 30 min at 4°C. The cytoplasmic and nuclear proteins were isolated using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, Shanghai, China). The total cytoplasmic and nuclear protein concentration was determined using the BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China), and the sample was subsequently

stored at -80°C for western blot analysis.

Whole-cell lysates were mixed with NF- κ B p65 primary antibody (ABclonal Biotech Co., Ltd, Cambridge, MA, USA) and were subsequently coupled to agarose beads (Cell Signaling Technology, Inc. Danvers, MA, USA). The immunoprecipitates were separated through SDS-PAGE, transferred to PVDF membranes (Roche, Ltd, Basel, Switzerland) and blotted with antibodies against acetyl-lysine (Cell Signaling Technology, Inc. Danvers, MA, USA), PPAR- γ (ABclonal Biotech Co., Ltd, Cambridge, MA, USA), SIRT1 (ABclonal Biotech Co., Ltd, Cambridge, MA, USA), p-p300 (Abcam, Cambridge, UK), p300 (ABclonal Biotech Co., Ltd, Cambridge, MA, USA), and p65 (ABclonal Biotech Co., Ltd, Cambridge, MA, USA). The bands were incubated in ECL-Plus reagent (Wuhan goodbio technology CO.LTD, Wuhan, China), and chemiluminescence was detected using BioMax MR Film (Kodak, Rochester, NY). The X-ray films were scanned using a Bioimaging System (UVP, California, USA), and the density of the bands was quantified using LabWorks image acquisition and analysis software (UVP, California, USA).

2.6. SIRT1 activity assay

The SIRT1 deacetylase activity was measured using a SIRT1 Fluorescent Activity Assay Kit (Enzo Life Science, Plymouth Meeting, PA) according to the manufacturer's instructions with fluorescence emission at 460 nm and excitation at 360 nm.

2.7. P300 HAT activity assay

The p300 HAT activity was measured using a HAT activity colorimetric assay kit from Bio-vision (BioVision, Inc, Mountain View, CA, USA). The assay is designed to measure the amount of the unbound form coenzyme A released from acetyl coenzyme A after the acetyl

group is coupled to a substrate peptide by HAT.

2.8. Statistical analysis

All statistical analyses were conducted using SPSS12.0 (SPSS, Chicago, USA). The values are expressed as the means±s.e.m. Group comparisons were performed using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. The threshold for statistical significance was set at $P<0.05$.

3. RESULTS

3.1. The effect of cisplatin on renal function in mice

Cisplatin, a commonly used chemotherapy drug, decreases renal function. To obtain a stable cisplatin-induced renal injury model, we induced cisplatin nephrotoxicity in mice. As shown in Figure 1, cisplatin nephrotoxicity indicated by the increase in the levels of serum creatinine (Cr) and BUN is concentration dependent (Figure 1A, B) and partially time dependent (Figure 1C, D). The standard for cisplatin nephrotoxicity in mice is a Cr concentration 100-180 $\mu\text{mol/l}$ [25, 26]. Therefore, the best concentration and induction time for cisplatin nephrotoxicity in mice are 25 mg/kg and 72 hours, respectively.

3.2. The effect of pioglitazone on cisplatin nephrotoxicity in mice

Previous studies have shown that PPAR- γ agonists can confer protection against renal cisplatin toxicity [27, 28]. The results of the present study also showed that pretreatment with the PPAR- γ agonist pioglitazone prevented cisplatin-induced renal injury, and the PPAR- γ antagonist GW9662 could completely reverse this effect. As shown in Figure 2A-B, the effect of pioglitazone on cisplatin nephrotoxicity is partially concentration dependent, and the best

203 pioglitazone concentration is 20 mg/ kg/day/Po at 72 h. As shown in Figure 2C-D, GW9662
204 attenuates the protective effect of pioglitazone (20 mg/kg/day/Po) on cisplatin nephrotoxicity
205 in a partially concentration-dependent manner, and the best concentration of GW9662 is 4
206 mg/kg/day/Po. A time-dependent experiment (Figure 2E-F) revealed lower Cr and BUN
207 levels in the P (cisplatin+ pioglitazone) group than in the C (cisplatin) group from 48 h after
208 cisplatin injection. Moreover, pretreatment with GW9662 attenuated the protective effect of
209 pioglitazone that reduced renal dysfunction, and higher levels of Cr and BUN were observed
210 in the G group (cisplatin+pioglitazone+GW9662) than in the P group from 48 h after cisplatin
211 injection. The level of kidney injury molecule-1 (KIM-1), a new specific marker for renal
212 tubule cell injury, was also significantly increased after cisplatin injection, peaking at 48 h
213 after cisplatin injection. Moreover, pretreatment with GW9662 attenuated the protective effect
214 of pioglitazone. Higher KIM-1 mRNA levels were observed in the G group than in the P
215 group (Figure 2G). The mortality rate was approximately 80% in the cisplatin-treated group
216 but decreased to approximately 20% with pioglitazone pretreatment, indicating that the
217 survival rate reached approximately 80%. However, GW9662 pretreatment reversed the
218 effects of pioglitazone treatment, and the survival rate in the GW9662 group decreased to
219 approximately 10% (Figure 2H). The morphology and ultrastructure of kidney cells were
220 nearly intact in the control group (N, untreated normal mice); 72 hours after the cisplatin
221 injection, the kidney tissue in the C group (C72) exhibited had increased cellular swelling,
222 tubule degeneration, protein cast, necrosis and multiple inflammatory cells infiltration (Figure
223 2J). However, pioglitazone administration protected the kidney from cisplatin injury at 72h
224 (P72), and GW9662 pretreatment completely eliminated this effect at 72h (G72) (Figure 2J).

The semi-quantitative assessment of the histological changes in the mice showed a significantly lower score in the P72 group than in the C72 and G72 groups (Figure 2I).

3.3. Pioglitazone pretreatment induced PPAR- γ activation and decreased the expression of the pro-inflammatory cytokines in cisplatin nephrotoxicity

To elucidate the mechanisms underlying the pioglitazone protection against cisplatin-induced renal injury in mice, we examined PPAR- γ activation because pioglitazone is a ligand for PPAR- γ . Unexpectedly, the cisplatin, pioglitazone and GW9662 treatments had no effect on PPAR- γ expression (Figure 3A, lower panel). However, the amount of activated PPAR- γ (PPAR- γ phosphorylation, p-PPAR- γ) in the mice after the cisplatin insult increased significantly in a time-dependent manner (Figure3A, upper panel). Nevertheless, the administration of pioglitazone further promoted PPAR- γ activation, as indicated by higher levels of p-PPAR- γ in pioglitazone-treated mice than in cisplatin-treated mice. In addition, GW9662 treatment attenuated the effect of pioglitazone in promoting PPAR- γ activation. CD36 acts as downstream of PPAR- γ and can thus be used as another measure of PPAR- γ activation. The mRNA level of CD36 was consistent with the p-PPAR- γ results (Figure3C).

Inflammation plays an important role in the development and progression of ischemia/reperfusion (I/R)-induced renal injury [29]; thus, important pro-inflammatory cytokines and indicators were also tested (Figure3D-K). As shown in Figure 3D-E, more inflammatory cells infiltration, as measured by myeloperoxidase (MPO), was found in the C72 group than in the N group (Figure 3D, E). Pioglitazone reduced the MPO expression in the P72 group, and GW9662 treatment reduced the effect of pioglitazone in decreasing inflammatory cell infiltration (Figure 3D, E). The expression of the pro-inflammatory

cytokines TNF- α , IL-1 β and IL-6 in renal tissue was measured through realtime-PCR and ELISA at 0, 24, 48, and 72h after cisplatin injection. Cisplatin-treated mice (C group) expressed higher levels of TNF- α , IL-1 β , and IL-6 at 24, 48, 72h than at 0h (Figure 3F-K); pioglitazone (P group) decreased the expression of the pro-inflammatory cytokines in cisplatin nephrotoxicity. While pioglitazone reduced inflammatory cytokine expression, the opposite effects were observed with GW9662 (G group) (Figure 3F-K). These results indicated that pioglitazone decreased the expression of the pro-inflammatory cytokines in cisplatin nephrotoxicity through the activation of receptors.

3.4. Pioglitazone attenuates NF- κ B activation through the reduction of p65 acetylation in cisplatin nephrotoxicity

Inflammatory cytokine expression depends on the activation of NF- κ B, a nuclear transcription factor regulating the expression of a large number of genes. Inactive NF- κ B is sequestered in the cytoplasm, bound by the I κ B family of inhibitor proteins, including I κ B α , I κ B β , I κ B γ , and I κ B ϵ . The various stimuli that activate NF- κ B also induce I κ B phosphorylation, resulting in the exposure of the nuclear localization signals (NLS) on NF- κ B subunits and subsequent translocation of this molecule to the nucleus [7]. Recent evidence demonstrated the fascinating translocation of acetylated p65 [8, 30]. In the present study, cisplatin, pioglitazone and GW9662 treatment did not influence the expression of total p65 (Figure 4B, F). However, cisplatin decreased I κ B- α expression (Figure 4A middle panel, D) and increased p65 and I κ B- α activation in a time-dependent manner, with higher levels of p-I κ B- α (I κ B- α phosphorylation, Figure 4A up panel, E), Ace-p65 (p65 acetylation, Figure 4B up panel, G), p65 in the nucleus (N-p65) and a lower level of p65 in the cytoplasm (C-p65) in the C

group(0 hour) than in the C group (24, 48, 72 hour) (Figure 4C, H, I). However, kidneys in the P group expressed lower levels of p-I κ B- α , Accep65, and p65 in the nucleus and higher level of p65 in the cytoplasm compared with the C group. Moreover, GW9662 reversed the effects of pioglitazone in cisplatin nephrotoxicity (Figure 4A, B, and C). These results indicated that pioglitazone attenuates NF- κ B activation through the reduction of p65 acetylation and sequential p65 migration in cisplatin nephrotoxicity.

3.5. Pioglitazone decreased SIRT1 inactivation and p300 activation in cisplatin nephrotoxicity

As previous studies have suggested that SIRT1 and p300 regulate NF- κ B activation [11, 12], we examined the effect of pioglitazone on the SIRT1, p300 and their interaction with NF- κ B in cisplatin nephrotoxicity. As shown in Figure 5A-B, cisplatin up-regulated p65-p300 and down-regulated p65-SIRT1 at 72h (C72), which was opposite in pioglitazone treated mice (P72); And, GW9662 (G72) reversed the effect of pioglitazone. Furthermore, cisplatin decreased the protein and mRNA levels of SIRT1 in a time-dependent manner with the down-regulation of SIRT1 deacetylase activity (Figure 5G-J). Notably, pioglitazone pretreatment inhibited cisplatin-induced SIRT1 degradation and inactivation, and this effect could be eliminated through treatment with GW9662 (Figure 5G-J). As shown in Figure 5C-E, cisplatin, pioglitazone and GW9662 treatment all had no effect on total p300 expression, but cisplatin increased the activation of p300 in a time-dependent manner, as higher levels of p-p300 were detected in the C group at 24,48,72hour than at 0hour after cisplatin injection. However, pioglitazone pretreatment inhibited cisplatin-induced p300 activation. In contrast, GW9662 reversed these effects. The p300 histone acetyltransferase (HAT) activity test

showed similar results for p-p300 expression (Figure 5F). It is demonstrated that pioglitazone may decrease SIRT1 inactivation and p300 activation to regulate NF- κ B activation in cisplatin nephrotoxicity

3.6. Pioglitazone further promotes AMPK activation in cisplatin nephrotoxicity

As a regulator of both p300 and SIRT1 activation, pioglitazone also stimulates AMPK activation [23, 24]. In the present study, pioglitazone promoted AMPK activation, manifested as a higher level of p-AMPK in the P group than in the C and G groups (Figure 6A-C) (* p <0.05). These results indicated that pioglitazone pretreatment further induced AMPK activation in cisplatin nephrotoxicity, and subsequently regulated p300/SIRT1 activation.

4. DISCUSSION

Cisplatin-induced nephrotoxicity is an important problem in cancer chemotherapy, which greatly limits the use and efficacy of cisplatin [1]. Thus, it is urgent and vital to solve this problem. Several mechanisms, such as DNA damage, apoptosis, inflammatory injury, and oxidative stress, play an important role in cisplatin-induced renal cell injury [1-4]. Notably, nuclear factor- κ B (NF- κ B) mediates inflammatory injury and plays a vital role in cisplatin nephrotoxicity [31-33]. Moreover, the transcriptional activation of NF- κ B is dependent on the migration of the subunit p65, and p65 migration relies on p65 structural modifications [7, 9]. Therefore, reducing p65 structural modifications might be an effective intervention strategy to decrease cisplatin nephrotoxicity.

Pioglitazone, a PPAR- γ agonist, is widely used in clinical settings because this molecule decreases lipid levels in patients with hypercholesterolemia and hypertriglyceridemia. A

313 recent study showed that pioglitazone decreases I/R-induced kidney injury through the
314 suppression of inflammation [25, 28]. However, the role of this compound in cisplatin
315 nephrotoxicity remains unclear. Thus, we induced cisplatin nephrotoxicity in mice by
316 injecting different concentrations of cisplatin and examined the renal function and the survival
317 rate at different times after cisplatin injection. The results showed that cisplatin-induced renal
318 injury is dose dependent and partially time dependent. Moreover, cisplatin leads to significant
319 renal dysfunction (manifested as higher levels of Cr, BUN, and KIM-1), pathological changes
320 (widespread cellular swelling, vacuolar degeneration, protein cast, necrosis and inflammatory
321 cells infiltration) and mortality compared with the normal group. Notably, preconditioning
322 mice with the PPAR- γ agonist pioglitazone significantly attenuated the renal dysfunction,
323 mortality and pathological changes. Moreover, GW9662, a PPAR- γ antagonist, reversed the
324 effect of pioglitazone on cisplatin nephrotoxicity. Consistent with these results, several pre-
325 vious studies have suggested that treatment with a PPAR- γ agonist resulted in significantly
326 less cisplatin nephrotoxicity [26, 27, 34]. However, in vitro results from Genc, G, et al
327 indicated no beneficial effect of pioglitazone on cisplatin-induced HK-2 cell death [35]. In fact
328 this was not controversial to in vivo studies, because various kinds of cells in the kidney can
329 be affected by medicine treatment. This study told us that pioglitazone may benefit tubular
330 cells through other cells and their products (for example, cisplatin induced infiltrated
331 neutrophils, macrophages or lymphocytes, and in situ vascular cells, pericytes or the rare
332 interstitial cells), rather than benefit tubular cells directly. As a whole organ, all cells in the
333 kidney affect each other and survive in the microenvironment; Separate cell culture
334 experiments may not always be consistent with in vivo results. On the other hand, different

335 dose and during time of pioglitazone administration should be tested. Taken together, these
336 results suggested that pioglitazone might be a novel effective therapeutic agent for the preven-
337 tion and treatment of cisplatin nephrotoxicity in clinical practice.

338 NF- κ B-mediated inflammatory injury plays a vital role in cisplatin nephrotoxicity [31-33].
339 Therefore, we examined the expression of the pro-inflammatory cytokines TNF- α , IL-1 β and
340 IL-6. The results showed that pioglitazone decreases the cisplatin-induced expression of
341 TNF- α , IL-1 β and IL-6 and that GW9662 suppresses the effect of pioglitazone in decreasing
342 pro-inflammatory cytokines. Therefore, the suppression of NF- κ B-mediated inflammation is
343 involved in the pioglitazone-mediated decrease in renal dysfunction in cisplatin
344 nephrotoxicity.

345 The transcriptional activation of NF- κ B is dependent on subunit p65 migration, and I κ B- α
346 activation [7, 9]. I κ B- α is quickly phosphorylated and degraded after activation of canonical
347 NF- κ B signaling leading to release of NF- κ B subunits, and p65 migration relies on p65
348 structural modifications [36]. Therefore, we examined I κ B- α and p65 expression and p65
349 structural modifications. In the present study, cisplatin, pioglitazone and GW9662 all showed
350 no influence on the expression of p65; instead, they altered the translocation of p65 and I κ B- α
351 through structural modifications. Cisplatin increased the levels of p-I κ B- α , Accep65, and p65
352 in the cytoplasm and decreased the p65 level in the nucleus compared with those in the 0 hour
353 groups, and these effects were reduced through pioglitazone treatment. It was noteworthy that
354 pioglitazone suppressed p65 acetylation, translocation to nucleus, and then would
355 down-regulated NF- κ B DNA binding, thus resulted in less releasing of NF- κ B signaling
356 pathway factors, for example TNF- α , and less I κ B- α proteins were activated (less p-I κ B- α),

357 finally further reduced p65 translocation. Pioglitazone administration formed this feedback
358 loop. Simultaneously, GW9662 could overturn the effect of pioglitazone in cisplatin
359 nephrotoxicity. These results indicated that pioglitazone attenuates p65 migration through a
360 reduction in p65 acetylation, resulting in the suppression of NF- κ B activity.

361 In the present study, we further explored the mechanisms of the pioglitazone-mediated
362 decrease in p65 acetylation. To this end, we examined the effect of pioglitazone on SIRT1,
363 p300 and their interaction with p65. The results indicated that pioglitazone inhibits SIRT1
364 inactivation and p300 activation in cisplatin nephrotoxicity, as indicated by increased SIRT1
365 expression and activity and decreased p-p300 expression and p300 activity in the P group
366 compared with the C group. Furthermore, pioglitazone decreased the p300 interaction with
367 p65 and increased the SIRT1 interaction with p65 in cisplatin nephrotoxicity. Thus, we
368 propose that pioglitazone regulates the balance of acetylation and deacetylation through the
369 regulation of SIRT1 and p300 activation.

370 It has been reported that AMPK could regulate SIRT1, p300 [21, 22] and PPAR- γ activation
371 stimulates AMPK [23, 24]. The results of the present study showed that cisplatin and
372 pioglitazone did not influence the expression of PPAR- γ and AMPK; instead, cisplatin alone
373 induced the expression of p-PPAR- γ and p-AMPK. Unexpectedly, pioglitazone further
374 promoted the activation of PPAR- γ and AMPK, and this effect could be diminished through
375 GW9662 treatment. These results suggested that pioglitazone promotes AMPK activation
376 through PPAR- γ activation. Therefore, the AMPK-SIRT1/p300 pathway is involved in the
377 pioglitazone-mediated induction of NF- κ B activation in cisplatin nephrotoxicity. There is no
378 unified conclusion in existed researches focusing on AMPK in kidney injury. Mia S, et al

379 showed that AMPK α 1 deficiency inhibited the UUO-induced tubular injury and apoptosis in
380 UUO [37]; but Chen KH, et al suggested that AMPK activation had therapeutic potential for
381 renal tubulointerstitial fibrosis [38]; In vitro, studies indicated a protective role of AMPK in
382 dysfunctional mesangial cells and kidney fibroblast cell line (NRK-49f) [39, 40]. The results
383 from Mount PF, et al showed renal injury was unchanged in AMPK- β 1 deficient mice [41].
384 So the role of AMPK is complicated in renal injury. The present study focused more on the
385 balance between p300-p65 and SIRT1-p65 and their combined effect on p65 acetylation, the
386 detailed interaction between AMPK and SIRT1/p300 would be discussed in the next stage of
387 our experiments. Notably, pioglitazone regulates additional pathways, such as the PI3K/AKT
388 kinase cascade, other than the AMPK-SIRT1/p300 signaling pathway to suppress NF- κ B
389 activation [42, 43]. Therefore, further studies addressing the pathways involved in
390 suppressing NF- κ B activation through pioglitazone after cisplatin insult are needed,
391 meanwhile the AMPK knockout mice in the following studies to investigate how AMPK
392 activation interact with SIRT1 or p300 is also necessary.

393 How to prevent cisplatin induced nephrotoxicity is a popular and meaningful issue, more
394 researches on this are needed. There are some similar studies. Mahmoud M. F and Jesse C.R
395 gave us a brief description about the protection of pioglitazone on cisplatin nephrotoxicity in
396 rat and mice respectively [27, 34]. Mahmoud M. F was interested in inflammation related
397 factors change in cisplatin nephrotoxicity, histological improve and decreased gene
398 expression of NF- κ B and TNF- α were showed [27]. On the other hand, Jesse C.R focused on
399 the oxidative stress inhibited by pioglitazone [34]. On the basis, we conducted a very different
400 study. Gradient doses of cisplatin, PPAR- γ agonist pioglitazone and PPAR- γ antagonist

GW9662 were all explored to search for the best concentration and we got the idea that the dose of pioglitazone was not the higher the better(Figure 1-2), and pioglitazone did function through PPAR- γ activation (Figure 3). In order to simulate the human drug use methods possible, pioglitazone was administered by oral gavage. 3 days pretreatment of pioglitazone before cisplatin injection implied that it could be used in advance in chemotherapy patients. Apart from the decreased gene expression of NF- κ B and TNF- α [27], innovatively we discovered a long signaling pathway involved in pioglitazone effects including AMPK, p300 and SIRT1, and we also discovered one key point of cisplatin nephrotoxicity as NF- κ B p65 acetylation and nucleus translocation. Pioglitazone regulated not only gene expression of related molecules, but also translation, post-translation modification, epigenetic modification and allosteric regulation. We can assume that all the related molecules may be new targets to prevent cisplatin nephrotoxicity, so more studies focusing on each molecule are needed in the future. Although the existed studies looks simple, they still gave us a clue to get more and our studies are also in turn to strong prove their results.

In summary, the results of the present study provided convincing evidence that pretreating animals with pioglitazone attenuated cisplatin-induced renal dysfunction through the suppression of NF- κ B inactivation and NF- κ B-mediated inflammation for the subsequent activation of AMPK-SIRT1/p300. Taken together, these data support pioglitazone as an alternative therapy for the prevention of cisplatin nephrotoxicity in clinical practice.

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References:

- [1] Merouani A, Shpall EJ, Jones RB, Archer PG, Schrier RW. Renal function in high dose chemotherapy and autologous hematopoietic cell support treatment for breast cancer. *KIDNEY INT* 1996;50:1026-31.
- [2] Thadhani R, Pascual M, Bonventre JV. Acute renal failure. *N Engl J Med* 1996;334:1448-60.
- [3] Schrier RW, Wang W, Poole B, Mitra A. Acute renal failure: definitions, diagnosis, pathogenesis, and therapy. *J CLIN INVEST* 2004;114:5-14.
- [4] Domitrovic R, Cvijanovic O, Pernjak-Pugel E, Skoda M, Mikelic L, Crncevic-Orlic Z. Berberine exerts nephroprotective effect against cisplatin-induced kidney damage through inhibition of oxidative/nitrosative stress, inflammation, autophagy and apoptosis. *FOOD CHEM TOXICOL* 2013;62:397-406.
- [5] Domitrovic R, Cvijanovic O, Pugel EP, Zagorac GB, Mahmutefendic H, Skoda M. Luteolin ameliorates cisplatin-induced nephrotoxicity in mice through inhibition of platinum accumulation, inflammation and apoptosis in the kidney. *TOXICOLOGY* 2013;310:115-23.
- [6] Kauppinen TM, Gan L, Swanson RA. Poly(ADP-ribose) polymerase-1-induced NAD(+) depletion promotes nuclear factor-kappaB transcriptional activity by preventing p65 de-acetylation. *Biochim Biophys Acta* 2013;1833:1985-91.
- [7] Hoesel B, Schmid JA. The complexity of NF-kappaB signaling in inflammation and cancer. *MOL CANCER* 2013;12:86.
- [8] Clavijo PE, Frauwirth KA. Anergic CD8+ T lymphocytes have impaired NF-kappaB activation with defects in p65 phosphorylation and acetylation. *J IMMUNOL* 2012;188:1213-21.
- [9] Diamant G, Dikstein R. Transcriptional control by NF-kappaB: elongation in focus. *Biochim Biophys Acta* 2013;1829:937-45.
- [10] Xu X, Woo CH, Steere RR, Lee BC, Huang Y, Wu J, et al. EVI1 acts as an inducible negative-feedback regulator of NF-kappaB by inhibiting p65 acetylation. *J IMMUNOL* 2012;188:6371-80.
- [11] Kauppinen A, Suuronen T, Ojala J, Kaarniranta K, Salminen A. Antagonistic crosstalk between NF-kappaB and SIRT1 in the regulation of inflammation and metabolic disorders. *CELL SIGNAL* 2013;25:1939-48.
- [12] Kim JW, Jang SM, Kim CH, An JH, Kang EJ, Choi KH. New molecular bridge between RelA/p65 and

458 NF-kappaB target genes via histone acetyltransferase TIP60 cofactor. J BIOL CHEM 2012;287:7780-91.

459 [13] Yoshida T, Hashimura M, Mastumoto T, Tazo Y, Inoue H, Kuwata T, et al. Transcriptional upregulation of
 460 HIF-1alpha by NF-kappaB/p65 and its associations with beta-catenin/p300 complexes in endometrial
 461 carcinoma cells. LAB INVEST 2013;93:1184-93.

462 [14] Li Y, Li C, Sun L, Chu G, Li J, Chen F, et al. Role of p300 in regulating neuronal nitric oxide synthase
 463 gene expression through nuclear factor-kappaB-mediated way in neuronal cells. NEUROSCIENCE
 464 2013;248:681-9.

465 [15] Santer FR, Hoschele PP, Oh SJ, Erb HH, Bouchal J, Cavarretta IT, et al. Inhibition of the acetyltransferases
 466 p300 and CBP reveals a targetable function for p300 in the survival and invasion pathways of prostate cancer
 467 cell lines. MOL CANCER THER 2011;10:1644-55.

468 [16] Clarke DL, Sutcliffe A, Deacon K, Bradbury D, Corbett L, Knox AJ. PKCbetaII augments
 469 NF-kappaB-dependent transcription at the CCL11 promoter via p300/CBP-associated factor recruitment and
 470 histone H4 acetylation. J IMMUNOL 2008;181:3503-14.

471 [17] Chen W, Bacanamwo M, Harrison DG. Activation of p300 histone acetyltransferase activity is an early
 472 endothelial response to laminar shear stress and is essential for stimulation of endothelial nitric-oxide synthase
 473 mRNA transcription. J BIOL CHEM 2008;283:16293-8.

474 [18] Buerki C, Rothgiesser KM, Valovka T, Owen HR, Rehrauer H, Fey M, et al. Functional relevance of novel
 475 p300-mediated lysine 314 and 315 acetylation of RelA/p65. NUCLEIC ACIDS RES 2008;36:1665-80.

476 [19] Gowans GJ, Hardie DG. AMPK: a cellular energy sensor primarily regulated by AMP. Biochem Soc Trans
 477 2014;42:71-5.

478 [20] Nakahata Y, Sahar S, Astarita G, Kaluzova M, Sassone-Corsi P. Circadian control of the NAD⁺ salvage
 479 pathway by CLOCK-SIRT1. SCIENCE 2009;324:654-7.

480 [21] Zhang Y, Qiu J, Wang X, Zhang Y, Xia M. AMP-activated protein kinase suppresses endothelial cell
 481 inflammation through phosphorylation of transcriptional coactivator p300. Arterioscler Thromb Vasc Biol
 482 2011;31:2897-908.

483 [22] Lin JN, Lin VC, Rau KM, Shieh PC, Kuo DH, Shieh JC, et al. Resveratrol modulates tumor cell
 484 proliferation and protein translation via SIRT1-dependent AMPK activation. J Agric Food Chem
 485 2010;58:1584-92.

486 [23] Peeters A, Fraisl P, van den Berg S, Ver LVTE, Van Kampen A, Rider MH, et al. Carbohydrate
 487 metabolism is perturbed in peroxisome-deficient hepatocytes due to mitochondrial dysfunction, AMP-activated
 488 protein kinase (AMPK) activation, and peroxisome proliferator-activated receptor gamma coactivator 1alpha
 489 (PGC-1alpha) suppression. J BIOL CHEM 2011;286:42162-79.

490 [24] Shiota A, Shimabukuro M, Fukuda D, Soeki T, Sato H, Uematsu E, et al. Telmisartan ameliorates insulin
 491 sensitivity by activating the AMPK/SIRT1 pathway in skeletal muscle of obese db/db mice. CARDIOVASC
 492 DIABETOL 2012;11:139.

493 [25] Zou C, Hu H, Xi X, Shi Z, Wang G, Huang X. Pioglitazone protects against renal ischemia-reperfusion
 494 injury by enhancing antioxidant capacity. J SURG RES 2013;184:1092-5.

495 [26] Lee S, Kim W, Moon SO, Sung MJ, Kim DH, Kang KP, et al. Rosiglitazone ameliorates cisplatin-induced
 496 renal injury in mice. Nephrol Dial Transplant 2006;21:2096-105.

497 [27] Mahmoud MF, El SS. Pioglitazone protects against cisplatin induced nephrotoxicity in rats and potentiates
 498 its anticancer activity against human renal adenocarcinoma cell lines. FOOD CHEM TOXICOL
 499 2013;51:114-22.

500 [28] Hu H, Zou C, Xi X, Shi Z, Wang G, Huang X. Protective effects of pioglitazone on renal
 501 ischemia-reperfusion injury in mice. J SURG RES 2012;178:460-5.

- [29] Deftereos S, Angelidis C, Bouras G, Raisakis K, Gerckens U, Cleman MW, et al. Innate immune inflammatory response in the acutely ischemic myocardium. *MED CHEM* 2014;10:653-62.
- [30] Juan YS, Lee YL, Long CY, Wong JH, Jang MY, Lu JH, et al. Translocation of NF-kappaB and Expression of Cyclooxygenase-2 Are Enhanced by Ketamine-Induced Ulcerative Cystitis in Rat Bladder. *AM J PATHOL* 2015.
- [31] Sahu BD, Kalvala AK, Koneru M, Mahesh KJ, Kuncha M, Rachamalla SS, et al. Ameliorative effect of fisetin on cisplatin-induced nephrotoxicity in rats via modulation of NF-kappaB activation and antioxidant defence. *PLOS ONE* 2014;9:e105070.
- [32] Benedetti G, Fredriksson L, Herpers B, Meerman J, van de Water B, de Graauw M. TNF-alpha-mediated NF-kappaB survival signaling impairment by cisplatin enhances JNK activation allowing synergistic apoptosis of renal proximal tubular cells. *BIOCHEM PHARMACOL* 2013;85:274-86.
- [33] Jung YJ, Lee JE, Lee AS, Kang KP, Lee S, Park SK, et al. SIRT1 overexpression decreases cisplatin-induced acetylation of NF-kappaB p65 subunit and cytotoxicity in renal proximal tubule cells. *Biochem Biophys Res Commun* 2012;419:206-10.
- [34] Jesse CR, Bortolatto CF, Wilhelm EA, Roman SS, Prigol M, Nogueira CW. The peroxisome proliferator-activated receptor-gamma agonist pioglitazone protects against cisplatin-induced renal damage in mice. *J APPL TOXICOL* 2014;34:25-32.
- [35] Genc G, Kilinc V, Bedir A, Ozkaya O. Effect of creatine and pioglitazone on Hk-2 cell line cisplatin nephrotoxicity. *Ren Fail* 2014;36:1104-7.
- [36] Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. *CELL* 2008;132:344-62.
- [37] Mia S, Federico G, Feger M, Pakladok T, Meissner A, Voelkl J, et al. Impact of AMP-Activated Protein Kinase alpha1 Deficiency on Tissue Injury following Unilateral Ureteral Obstruction. *PLOS ONE* 2015;10:e135235.
- [38] Chen KH, Hsu HH, Lee CC, Yen TH, Ko YC, Yang CW, et al. The AMPK agonist AICAR inhibits TGF-beta1 induced activation of kidney myofibroblasts. *PLOS ONE* 2014;9:e106554.
- [39] Lv ZM, Liu Y, Zhang PJ, Xu J, Jia ZH, Wang R, et al. The role of AMPKalpha in high-glucose-induced dysfunction of cultured rat mesangial cells. *Ren Fail* 2012;34:616-21.
- [40] Luo X, Deng L, Lamsal LP, Xu W, Xiang C, Cheng L. AMP-activated protein kinase alleviates extracellular matrix accumulation in high glucose-induced renal fibroblasts through mTOR signaling pathway. *CELL PHYSIOL BIOCHEM* 2015;35:191-200.
- [41] Mount PF, Gleich K, Tam S, Fraser SA, Choy SW, Dwyer KM, et al. The outcome of renal ischemia-reperfusion injury is unchanged in AMPK-beta1 deficient mice. *PLOS ONE* 2012;7:e29887.
- [42] Ihle NT, Lemos R, Schwartz D, Oh J, Halter RJ, Wipf P, et al. Peroxisome proliferator-activated receptor gamma agonist pioglitazone prevents the hyperglycemia caused by phosphatidylinositol 3-kinase pathway inhibition by PX-866 without affecting antitumor activity. *MOL CANCER THER* 2009;8:94-100.
- [43] Sinagra T, Tamburella A, Urso V, Siarkos I, Drago F, Bucolo C, et al. Reversible inhibition of vasoconstriction by thiazolidinediones related to PI3K/Akt inhibition in vascular smooth muscle cells. *BIOCHEM PHARMACOL* 2013;85:551-9.

FIGURE LEGENDS

Figure 1 Cisplatin decreases renal function in mice

(A, B) Serum creatinine (Cr) and urea nitrogen (BUN) levels were measured 72 h after cisplatin injection. Cisplatin decreased the renal function in a dose-dependent manner. The values are presented as the means±s.e.m, * $P<0.05$, ** $P<0.01$ vs. control (cisplatin 0 mg/kg), $n=8-10$ for each group. **(C, D)** Serum Cr and BUN levels were measured at different times after 25 mg/kg cisplatin injection. Cisplatin decreased renal function in time-dependent manner. The values are presented as the means±s.e.m, * $P<0.05$, ** $P<0.01$ vs. control (0 h), $n=8-10$ for each group.

Figure 2 Pioglitazone administration protects renal function from cisplatin damage in mice

(A, B) Serum Cr and BUN levels were measured 72 h after cisplatin (25 mg/kg) injection and pioglitazone (mg/kg/day/Po) pretreatment at different doses for 3 days prior to the cisplatin injection. Pioglitazone significantly suppressed the cisplatin-induced renal injury in a dose-dependent manner. The values are presented as the means±s.e.m, * $P<0.05$, ** $P<0.01$ vs. control (pioglitazone 0 mg/kg), $n=8-10$ for each group. **(C, D)** Serum Cr and BUN levels were measured 72 h after cisplatin injection and pretreatment with both pioglitazone (20 mg/kg/day/Po) and different doses of GW9662 for 3 days prior to the cisplatin injection. GW9662 effectively diminished the pioglitazone protection in a dose-dependent manner. The values are presented as the means±s.e.m, * $P<0.05$, ** $P<0.01$ vs. control (GW9662 0 mg/kg), $n=8-10$ for each group. **(E, F)** Cr and BUN levels and **(G)** the relative mRNA expression of

KIM-1 was measured at different times for the C group, P group, and G group. Pioglitazone obviously reversed the cisplatin-induced renal injury, but GW9662 eliminated this effect. The values are presented as the means $\pm$ s.e.m, * P <0.05, ** P <0.01, P group vs. C and G groups, n =8-10 for each group. **(H)** Although cisplatin induced a high mortality rate similar to that in the G group, pioglitazone protected the kidneys from cisplatin toxicity, n =25 for each group. **(J)** PAS staining were conducted on kidney sections. The cisplatin-treated kidneys (C72) showed marked injury after 72 h, with cast formation, tubular epithelial cell sloughing, brush border loss and tubule dilation; similar damage level showed in the G group after 72 h (G72) and less damage in the P group (P72). The damage score according to the PAS staining**(J)** was showed in **(I)**, the percentage of cortical tubules showing epithelial necrosis was calculated and assigned the following scores: 0 for all normal tubules; 1 for <10% necrotic tubules; 2 for 10-25% necrotic tubules; 3 for 26-75% necrotic tubules; and 4 for >75% necrotic tubules. The abbreviations for groups: N group(untreated normal control), C group (cisplatin 25 mg/kg), P group (25 mg/kg cisplatin+20 mg/kg pioglitazone), and G group (25 mg/kg cisplatin+20 mg/kg pioglitazone+4 mg/kg GW9662) .

Figure 3 pioglitazone pretreatment suppressed inflammations in cisplatin-induced acute renal injury through PPAR- γ activation.

(A, B, and C) The levels of total PPAR- γ , activated p-PPAR- γ and the PPAR- γ downstream molecule CD36 were examined using both WB and real-time PCR analyses in the C,P, and G groups at 0, 24, 48, and 72 h. Cisplatin up-regulated PPAR- γ activation (indicated as increased p-PPAR- γ and CD36 levels), and pioglitazone further elevated the p-PPAR- γ level; however, GW9662 reversed the effects of pioglitazone. The values are presented as the

means $\pm$ s.e.m, * P <0.05, ** P <0.01, P group vs. C and G groups, n =8-10 for each group. **B** is the quantification of the results shown in **A**. **(E,D)** Immunohistochemistry (IHC) showed that the MPO expression was in the normal (N group), C, P, and G groups after 72 h; MPO-positive area was quantified in **E**. The MPO expression was increased in the C group and decreased after pioglitazone treatment in the P group; GW9662 reversed this effect. The values are presented as the means $\pm$ s.e.m, * P <0.05, vs. C group and G group; # P <0.01, vs. N group, n =8-10 for each group. **(F-K)** The pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 were measured using real-timePCR (**F, H, J**) and ELISA (**G, I, K**) in the C, P, and G groups at 0, 24, 48, and 72 h. After cisplatin injection, the inflammatory cytokine levels increased over time and were decreased through treatment with pioglitazone; GW9662 reversed the effects of pioglitazone. The values are presented as the means $\pm$ s.e.m, * P <0.05, ** P <0.01, P group vs. C and G group, n =8-10 for each group. The abbreviations for groups: N group (untreated normal control), C group (cisplatin 25 mg/kg), P group (25 mg/kg cisplatin+20 mg/kg pioglitazone), and G group (25 mg/kg cisplatin+20 mg/kg pioglitazone+4 mg/kg GW9662) .

Figure 4 Pioglitazone decreased p65 acetylation in cisplatin injured mice.

(A)The cisplatin-induced I κ B- α phosphorylation was suppressed after pioglitazone administration, and this effect was eliminated after the GW9662 treatment. **D and E** show the quantification analyses of the results shown in **A**. The values are presented as the means $\pm$ s.e.m, * P <0.05, P group vs. C and G groups, n =8-10 for each group. **(B)** p65 acetylation was increased after cisplatin injection, and this effect was inhibited after pioglitazone administration. GW9662 could abolish the pioglitazone effect. The total p65

level was not changed in these groups. **F and G** show the quantification analysis of the results shown in **B**. The values are presented as the means $\pm$ s.e.m, * P <0.05, P group vs. C and G group, n =8-10 for each group. **(C)** Cisplatin promoted p65 translocation from the cytoplasm to the nucleus, and pioglitazone inhibited the cisplatin effect, whereas GW9662 induced the cisplatin effect. **H and I** show the quantification analyses for the results shown in **C**. The values are presented as the means $\pm$ s.e.m, * P <0.05, ** P <0.01, P group vs. C and G groups, n =8-10 for each group. The abbreviations for groups: C group (cisplatin 25 mg/kg), P group (25 mg/kg cisplatin+20 mg/kg pioglitazone), and G group (25 mg/kg cisplatin+20 mg/kg pioglitazone+4 mg/kg GW9662).

Figure 5 Pioglitazone inhibited p65 acetylation and regulated the interaction of p300 and SIRT1 with p65.

(A, B) The effect of pioglitazone on the p65-p300 and p65-SIRT1 interactions was tested using co-immunoprecipitation. Compared with the control (CT, untreated normal mice), cisplatin increased p65-p300 binding and decreased p65-SIRT1 binding. Pioglitazone eliminated the cisplatin effect, but GW9662 had effects similar to the cisplatin effects. **(C-F)** The total p300 protein (C) and mRNA (E) expression levels remained the same in all groups at all-time points. However, the p-p300 level increased after cisplatin treatment, partially decreased when protected by pioglitazone treatment, and subsequently increased with GW9662 treatment (C). The quantification of the results in **C** is shown in **D**. **F** shows that the p300 activity changed in a manner similar to the p-p300 changes. The values are presented as the means $\pm$ s.e.m, * P <0.05, P group vs. C and G groups, n =8-10 for each group. **(G-J)** The SIRT1 protein (**G, H**) and mRNA (**I**) relative levels decreased with cisplatin injury and

increased with pioglitazone protection; this effect was abolished through GW9662 treatment. The SIRT1 activity changed in a similar manner (J). The values are presented as the means±s.e.m, * $P<0.05$, ** $P<0.01$, P group vs. C and G groups, $n=8-10$ for each group. The abbreviations for groups: CT group (untreated normal control), C group (cisplatin 25 mg/kg), P group (25 mg/kg cisplatin+20 mg/kg pioglitazone), and G group (25 mg/kg cisplatin+20 mg/kg pioglitazone+4 mg/kg GW9662).

Figure 6 AMPK phosphorylation in cisplatin-induced renal injury is regulated through pioglitazone pretreatment.

(A)WB analysis showed that cisplatin stimulated AMPK phosphorylation, while pioglitazone further increased this effect, and GW9662 completely eliminated the pioglitazone effect. However, the total AMPK protein and mRNA showed no change (A, C). B showed the quantification of the results shown in A. The values are presented as the means±s.e.m, * $P<0.05$, ** $P<0.01$, P group vs. C and G groups, $n=8-10$ for each group. The abbreviations for groups: The abbreviations for groups: C group (cisplatin 25 mg/kg), P group (25 mg/kg cisplatin+20 mg/kg pioglitazone), and G group (25 mg/kg cisplatin+20 mg/kg pioglitazone+4 mg/kg GW9662).

Figure 7 Schematic diagrams illustrating the mechanism of pioglitazone protecting cisplatin nephrotoxicity through AMPK-SIRT1/p300 pathway.

Upon cisplatin induced inflammatory reaction activation, through activating PPAR- γ , pioglitazone promoted AMPK phosphorylation, then increased SIRT1 expression, SIRT1 deacetylase activity and its interaction with p65, at the same time, decreased p300 phosphorylation and its interaction with p65, finally decreased p65 acetylation and

651 translocation to the nucleus. Decreased NF- κ B p65 transport and binding with DNA led to
652 less following effect molecules release, for example, TNF- α and IL-1, then further reduced
653 p65 acetylation and translocation through down-regulating p65 inhibitory factor I κ B α
654 phosphorylation and degradation. Green arrows mean promotion and red arrow means
655 inhibition.

656

657

Table 1 Primers for realtime-PCR

Gene	Species	Sense	Antisense
TNF- α	mouse	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
IL-1 β	mouse	AGCTTCCTTGTGCAAGTGTCT	GACAGCCCAGGTCAAAGGTT
IL-6	mouse	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
KIM-1	mouse	ACATATCGTGGAATCACAACGAC	ACTGCTCTTCTGATAGGTGACA
β -actin	mouse	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT
AMPK	mouse	GTCAAAGCCGACCCAATGATA	CGTACACGCAAATAATAGGGGT
CD36	mouse	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
SIRT1	mouse	CAGCCGTCTCTGTGTCACAAA	GCACCGAGGAACTACCTGAT
p300	mouse	ACTGCCGGGATGGCTACTAT	TCTGGATTCGCTGGCTAATGG

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7