Original Article

Rosiglitazone attenuates renal injury caused by hyperlipidemic pancreatitis

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Abstract: Hyperlipidemic pancreatitis (HP) is a serious inflammatory disease with very high mortality and multiple organ injuries including renal injury. Rosiglitazone (Ros), an agonist of peroxisome proliferator activated receptor-γ (PPAR-γ), was reported to show a protective role against pancreatitis. However, whether Ros has an effect on renal injury caused by HP is not yet clear. In the present study, the function of Ros was explored using ELISA, RT-PCR, western blot, PAS staining and immunohistochemistry. Results of this study showed that Ros could inhibit the activation of NF-κB and MAPK P38 signaling pathways, relieve inflammatory response and inhibit cell apoptosis, thus attenuating renal injury caused by HP. This study suggested that Ros might be a promising drug for the treatment of renal injury caused by HP and also laid theoretical foundation for the development of renal injury treatment.

Keywords: Rosiglitazone, hyperlipidemic pancreatitis, renal injury, inflammation, apoptosis

Introduction

Acute pancreatitis (AP) is an inflammatory disease of the pancreas caused by activation of digestive enzyme zymogens. Most AP patients present a mild and self-limited course with less complication, and the mortality is low. However, 15%-20% patients progress to severe acute pancreatitis, of which 60% will die [1]. In the recent years, along with the improving living level and changing diet, morbidity of hyperlipidemic pancreatitis (HP) is also increasing [2]. HP causes the same complications as other kinds of pancreatitis, but more serious and with higher morbidity [3]. Inflammation in severe acute pancreatitis (SAP) leads to systemic inflammatory response syndrome (SIRS) accompanied with multiple organ dysfunction subsequently including acute renal injury which was usually seen in SAP with death rate of 74%-81% [4, 5].

Rosiglitazone (Ros) is a ligand of peroxisome proliferator activated receptor-γ (PPAR-γ) maintaining homeostasis of glucose and lipids and is usually used as an insulin sensitizing drug for the treatment of diabetes. Ros can regulate cytokines, chemokines and adhesion molecules, thus having broad anti-inflammation, anti-proliferation and anti-oxidative stress functions [6-9].

Ros plays important roles in the repair of injured organs [10, 11]. It was reported to have a protective function in pancreatitis [12, 13]. However, whether Ros has an effect on renal injury caused by HP is not yet clear. In the present study, the function of Ros on renal injury caused by HP was explored. Results of this study showed that Ros attenuated renal injury caused by HP through the regulating of NF-κB and MAPK P38 signaling pathways. This study suggested that Ros might be a promising reagent for the treatment of renal injury caused by HP and also laid a theoretical foundation for the development of renal injury treatment.

Materials and methods

Animal experiment protocol

Male Sprague-Dawley (SD) rats weighing 180-200 g were obtained from experimental Animal Center of China Medical University (Shenyang, China) and maintained in a temperature-controlled environment with a 12 h-light/dark cycle.
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The rats were given food and water *ad libitum*. The rats were fed in metabolism cages of rats and the volume of urine was monitored every 6 h before and after AP operation. KIM-1 in urine and NGAL in urine and serum was also detected by ELISA.

Model of acute pancreatitis (AP) was induced by retrograde infusion of 5% sodium taurocholate into bile-pancreatic duct. Briefly, prior to AP operation, rats were fasted for 12 h, but with free access to water. Anesthesia was done by intraperitoneal injection of 10% chloral hydrate. AP was induced by a standardized pressure-controlled retrograde infusion of 5% sodium taurocholate (1 ml/kg) into the bile-pancreatic duct. Then the abdomen was closed, and rats were given normal saline (40 ml/kg) by subcutaneous injection for resuscitation.

Rats were randomly divided into six groups: normal group (Normal, n=6), acute pancreatitis group (AP, n=6), high fat diet group (HFD, n=6), hyperlipidemic pancreatitis group (HP, n=6), normal group treated with rosiglitazone (Normal +Ros, n=6), hyperlipidemic pancreatitis group treated with rosiglitazone (HP+Ros, n=6). The Normal, AP, and Normal +Ros groups were given normal food for 4 weeks before AP operation, and HFD, HP, and HP+Ros groups were given high-fat diet for 4 weeks before AP operation. AP, HP and HP+Ros groups were treated with retrograde infusion of sodium taurocholate. The Normal +Ros and HP+Ros groups were given 10 mg/kg rosiglitazone by intraperitoneal injection 1 h before and 24 h after AP operation, the other groups were given equal volume of solvent. 48 h after AP operation, rats were sacrificed for the subsequent experiments. All animal experiments were performed according to the Guide for Care and Use of Laboratory Animals and met the criteria of the Institutional Animal Care and Use Committee of China Medical University.

Western blot

Kidneys in each group were obtained and protein in kidneys was extracted using RIPA lysis buffer (Beyotime, Shanghai, China). The concentration of protein was measured using a BCA Protein Assay Kit (Beyotime). Equal amount of protein was subjected to SDS-PAGE for electrophoresis. The separated protein was transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blockade with 5% skim milk or 5% BSA, the membranes were incubated with primary antibodies against Bax, Bcl-2, cleaved Caspase-3, PPAR-γ, P65, p-P65, P38, p-P38 and ß-actin (1:1000, Waneibio, Shenyang, China) at 4°C overnight. Then the membranes were incubated with the corresponding horse-radish peroxidase (HRP) conjugated-secondary antibody (1:5000, Beyotime) at 37°C for 45 min. After washing with TBST, the membranes were visualized with Enhanced ECL Detection System and analyzed with Gel-Pro-Analyzer software using ß-actin as reference.

Quantitative real time PCR (RT-PCR)

Total RNA was extracted from each sample using RNA Simple Total RNA Kit (Tiangen, Beijing, China) according to the manufacturer’s instruction. The extracted total RNA was reverse transcribed to cDNA using Super M-MLV Reverse Transcriptase (BioTeke, Beijing, China) and Oligo(dT)15. The relative mRNA levels of monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) were detected by RT-PCR (SYBR Green method) using cDNA as templates and primers as follows:

- TNF-α-F: TGGCGTGTTTCATCGGTCTCT;
- TNF-α-R: CCACACTTTCGCTCGCTGCT;
- IL-6-F: GTGTCCTTGGGACTGATG;
- IL-6-R: TACCTGCTGGTGTTGCTG;
- IL-1β-F: TCCAGTGCTGTCTCTGCTC;
- IL-1β-R: TCACTAGCTGTGAGATT.

The relative mRNA level was calculated using 2-∆∆Ct method [14] with ß-actin as an internal reference.

Periodic acid Schiff stain (PAS)

Kidneys obtained from each group were embedded in paraffin after fixation and cut into 5 μm sections. The paraffin sections were stained with periodic acid Schiff reagent (Baso, Zhuhai, China) and observed under microscopy after counterstaining with hematoxylin. Damage in kidney was scored as follows: 0= normal; 1≤10%; 2=10%-25%; 3=26%-75%; 4≥75%.

Immunohistochemistry (IHC)

Paraffin sections were dewaxed in xylene, hydrated with decreasing concentration of etha-
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Figure 1. Renal injury was caused in HP model. A. The concentration of KIM-1 in urine was detected by ELISA. B. ELISA was used to detect the concentration of NGAL in serum. C. The concentration of NGAL in urine was detected. ***P<0.001 compared with Normal group.

Figure 2. Ros decreased the serum lipid level in HP rat model. A. ELISA was used to measure the concentration of total cholesterol (TC). B. The concentration of Triglyceride (TG) was detected by ELISA. C, D. The concentration of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) was detected using ELISA. *P<0.05, **P<0.01, ***P<0.001 compared with Normal group, ##P<0.01, ###P<0.001 compared with HP group.

Endogenous peroxidase was quenched by 3% H₂O₂, and unspecific sites were blocked with normal goat serum (Solarbio, Beijing, China). Then, the sections were incubated with primary antibody against neutrophils (1:100,
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Wanleibio) and F4/80 (1:50, Santa Cruz, Dallas, TX, USA) overnight at 4°C. Then the sections were incubated with biotin labeled-secondary antibody followed by incubation with HRP labeled streptavidin. Thereafter, the sections were colorated with a 3, 3'-diaminobenzidine (DAB), counterstained with hematoxylin and observed in an inverted digital image light microscopy.

**TUNEL assay**

The TUNEL assay was performed with In Situ Cell Death Detection Kit (Roche, Penzberg, Germany) according to the manufacturer’s instruction. After dewaxing and rehydrating, the sections were permeabilized with 0.1% Triton X-100 followed by blockade with 3% H$_2$O$_2$. Then the sections were incubated with a mixture of TUNEL reaction buffer, enzyme solution and label solution in dark at 37°C for 1 h followed by incubated with Converter-POD. The sections were colorated with DAB detection system (Solarbio, Beijing, China) and taken photos with microscope after counterstaining with hematoxylin. TUNEL-positive cells and total cell number per view were counted and the percentage of apoptosis cells was calculated.

**ELISA**

Levels of MCP-1, TNF-α, IL-6 and IL-1β in kidney tissues were detected by ELISA using corresponding ELISA kits (USCN, Wuhan, China) according to the protocols. Activity of myeloperoxidase (MPO) in kidney tissue was detected.
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using a MPO Detection Kit (Jianchengbio, Nanjing, China) according to the manufacturer’s instruction. And levels of creatinine, blood urea nitrogen (BUN) (Jianchengbio), total cho-

Figure 4. Ros relieved inflammatory response in kidney tissues. A-C. The concentrations of inflammatory cytokines TNF-α, IL-6 and IL-1β in kidney tissues were detected by ELISA. D-F. Quantitative real time PCR was used to detect the mRNA level of TNF-α, IL-6 and IL-1β in kidney tissues. The relative mRNA level was calculated using $2^{-\Delta\Delta Ct}$ method. **$P<0.01$, ***$P<0.001$ compared with Normal group, ###$P<0.001$ compared with HP group.
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Lesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) (Dongou, Wenzhou, China), low-density lipoprotein (LDL) (Beihuakangtai, Beijing, China), NGAL (USCN) in the serum and KIM-1, NGAL (USCN) in the urine were measured by corresponding detection kits.

Statistical analysis

The results were presented as mean ± SD. Differences between groups were analyzed with one-way ANOVA, Bonferroni’s Multiple Comparison. *P<0.05 was considered to be significant.

Results

HP caused renal injury

To explore whether the HP model in our study could cause renal injury, the concentrations of KIM-1 in urine and NGAL in serum and urine were detected. As shown in Figure 1, after AP and HP operation, the concentrations of KIM-1 and NGAL were increased significantly (Figure 1, *P<0.001). These results demonstrated that HP model in our study caused renal injury.

Ros decreased the blood lipid level in HP rat model

As abnormal lipid metabolism may contribute to pancreatitis, we detected the blood lipid level. As shown in Figure 2, the concentrations of TC, TG, and LDL in the serum were increased in HP group compared with the Normal group and the HDL concentration in the serum was decreased. However, after treatment with Ros, the concentrations of TC, TG, and LDL were decreased and the concentration of HDL was increased (Figure 2, HP+Ros vs. HP). These results indicated that Ros had an effect on the blood lipid level in HP.
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**Figure 6.** Ros reduced cell apoptosis caused by HP. A, B. Cell apoptosis in each group was detected by TUNEL assay. C, D. The expression level of Bax was detected by western blot. When the relative protein level of Bax was calculated, β-actin was used as internal reference. E, F. The protein level of Bcl-2 was detected using western blot with β-actin.
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As HP caused renal injury, pathological examination by PAS staining was carried out. As shown in Figure 3A, the kidney tissues of Normal group showed a normal architecture of kidney. The kidney tissues in the AP, HFD and HP groups showed marked edema and inflammatory cell infiltration. In the HP group, kidney tissue also showed an increased vacuolization. Whereas, after treatment with Ros, the pathological changes were reversed compared with the HP group, with a decreasing PAS score (Figure 3B). The concentrations of creatinin and BUN in the serum were measured. Compared with the Normal group, the concentrations of creatinin and BUN were elevated (Figure 3C and 3D), which indicated the injury of kidney. However, after Ros treatment, the levels of creatinin and BUN fell down (HP+Ros vs. HP). These results suggested that Ros treatment can attenuate the renal injury caused by HP.

Ros reduced cell apoptosis caused by HP

Cell apoptosis was usually seen in inflammatory part. In the present study, cell apoptosis was detected by TUNEL assay. As shown in Figure 6A, the HP group has a darker TUNEL staining than that of the Normal group, which indicated the increasing cell apoptosis in HP group. Whereas, the Ros treatment could decrease the cell apoptosis in HP group (Figure 6A and 6B, HP+Ros vs. HP). These result demonstrated that Ros could reduce cell apoptosis caused by HP. To further explore whether Ros has a protective role against cell apoptosis, the protein levels of Bax, Bcl-2 and cleaved Caspase-3 were detected by western blot. In the AP, HFD and HP group, the protein levels of Bax and cleaved Caspase-3 were higher than those of the Normal group, and the protein level of Bcl-2 was lower than that of the Normal group (Figure 6C-H). These results indicated the increasing cell apoptosis caused by HP. However, treatment with Ros reversed these changes, which suggested the protective role of Ros against apoptosis caused by HP and also consistent with the result of TUNEL assay.

Ros inhibited the activation of NF-κB and MAPK P38 signaling pathways

As Ros is an agonist of PPAR-γ, the protein level of PPAR-γ was measured by western blot. The HP group showed a lower PPAR-γ protein level than that of the Normal group. However, after treatment with Ros, the level of PPAR-γ was reversed to nearly normal level (Figure 7A and 7B). NF-κB and MAPK P38 signaling pathways have a close relationship with the inflammatory response and repair of injury, and the activation of these two signaling pathways was
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Figure 7. Ros inhibited the activation of NF-κB and MAPK P38 signaling pathways. A, B. The protein level of PPAR-γ was detected by western blot. When the relative protein level of protein was calculated, β-actin was used as internal reference. C, D. The phosphorylation of NF-κB P65 was evaluated by western blot with β-actin as internal reference.
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E. F. The phosphorylation of MAPK P38 was evaluated by western blot; β-actin was used as internal reference when the relative protein level of protein was calculated. *P<0.05, **P<0.001 compared with Normal group, #P<0.05, ###P<0.001 compared with HP group.

detected. In the HP group, the NF-κB P65 and MAPK P38 showed a high phosphorylation level, which indicated the activation of NF-κB and MAPK P38 signaling pathways. Whereas, treatment with Ros reversed the phosphorylation of NF-κB P65 and MAPK P38, inhibiting the activation of NF-κB and MAPK P38 signaling pathways (Figure 7C-F). These results demonstrated that Ros inhibited the activation of NF-κB and MAPK P38 signaling pathways.

Discussion

In the present study, the function of Ros in renal injury caused by HP as well as the underlying mechanism was explored. Results of this study showed that Ros could reduce inflammatory response in kidney tissues and inhibit cell apoptosis, thus attenuating the renal injury caused by HP.

Ros is usually employed in the treatment of diabetes. It also has an effect on blood lipid. Ezhumalai et al [15] and Yan et al [16] reported that Ros can regulate blood lipid to normal level. In our study, Ros showed an excellent function in balancing blood lipid. Hyperlipidemia was reported to aggravate pancreatitis. The effect of Ros on blood lipid may contribute to its application in the treatment of HP.

Renal dysfunction usually occurs in SAP with 14%-43% morbidity, secondary to lung dysfunction. Acute renal injury was a main reason for the death of SAP patients. As dysfunction of lipid metabolism also contributes to the renal injury, HP was reported to be more serious than SAP. Ros has been reported to attenuate the severity of acute pancreatitis [12, 13], but whether Ros has an effect on renal injury caused by HP was unknown. In our study, Ros was shown to relieve renal injury and to restore the function of kidney which was damaged by HP. Ros also show renal protective role in injury induced by cisplatin, adenine or type I diabetic [10, 11, 17]. At the meanwhile, it also has protective effects on pancreatitis-associated lung injury [13] and ischemia/reperfusion injury of myocardial [18].

Inflammatory response plays very important roles in the formation of pancreatitis [19]. In the present study, increase in levels of TNF-α, IL-6 and IL-1β as well as inflammatory cell infiltration in HP group was discovered, which indicated the inflammation in kidney tissue. Ros treatment was found to relieve this inflammation in our study. Ros was also found to inhibit inflammatory response in various tissues [7, 18, 20], and the inhibition of inflammation by Ros may be an important way for the protective function of Ros.

Ros has different roles in apoptosis of different kinds of cells. Ros inhibits apoptosis of pancreatic beta-cell, skeletal muscle cells, hepatocytes and neuronal cells via the modulation of the oxidative stress, endoplasmic reticulum stress and inflammatory response [21-24]. In this study, Ros performed its protective role through the inhibition of cell apoptosis induced by HP. Decrease in Bax level and increase in Bcl-2 level of HP+Ros group indicated the changes in mitochondrial permeability transition pore, which was consistent with the report of Wu et al [25].

Ros is an agonist of PPAR-γ. PPAR-γ regulates lipid metabolism and glucose homeostasis, as well as cell proliferation and differentiation, and plays an important role in inflammation regulation [20, 26-28]. Ros was also reported to promote the regeneration of pancreas and performed a protective role in pancreatitis through the activation of PPAR-γ [29, 30]. In our study, results showed that the protein level of PPAR-γ was down-regulated by HP, but reversed by Ros treatment. This result indicated that the protective function of Ros against renal injury caused by HP might through the regulation of PPAR-γ. Otherwise, the activation of NF-κB and MAPK P38 signaling pathways was also discovered in our study, which indicated that Ros performed its protective function through the regulation of NF-κB and MAPK P38 signaling pathways. Through other signaling pathways, such as AKT and AMPK, Ros also plays a protective role against injury induced by multiple factors [24, 25]. How exactly Ros performs its protective function needs further exploration.
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Mishra et al [31] reported that Ros has a cardiotoxicity by accelerated apoptosis of cardiomyocytes. However, in our study, we found that there was no significant difference between Normal+Ros and Normal group. This meant that Ros might have no effect on normal renal tissue and Ros attenuated renal injury caused by HP with no toxic on kidney, or maybe no side-effect on other organs. Consistent with our result, Dias and colleagues also reported that Ros did not induce acute kidney injury in normocholesterolemic rats [32].

In the present study, the effect of Ros was explored. Results of our study showed that Ros inhibited the activation of NF-κB and MAPK P38 signaling pathways, reduced inflammation and apoptosis, thus attenuating renal injury caused by HP. These results suggested that Ros may become a promising drug for the treatment of renal injury caused by HP and also laid theoretical foundation for the development of renal injury treatment.

Disclosure of conflict of interest

None.

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