Original Article
Combined anisodamine and probucol prevents contrast-induced nephropathy in diabetic rats by inhibiting p38 MAPK and Akt/mTOR/P70S6K signaling pathways

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Abstract: Apoptosis is recognized as an important mechanism in contrast-induced nephropathy (CIN). As anisodamine and probucol have been found to be renoprotective and anti-apoptotic in multiple kidney injuries, we hypothesized that they would prevent CIN. An experimental model of CIN was established in rats. Serum creatinine, blood urea nitrogen, urinary kidney injury molecule-1 (KIM-1), interleukin (IL)-18, and neutrophil gelatinase associated lipocalin (NGAL) levels were measured to evaluate kidney function. Superoxide dismutase (SOD), malondialdehyde (MDA) levels were assessed to discuss the effect of anisodamine and probucol on oxidative stress. The pathological changes of kidney were observed by hematoxylin and eosin (HE) staining and immunohistochemistry (IHC) analysis. Apoptosis was assessed by transferease-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining. Phospho-p38 mitoge-activated protein kinase (MAPK) and Akt/mTOR/P70S6K protein expression was assessed by Western blotting. Anisodamine and probucol significantly attenuated the resulting renal dysfunction and renal tubular cell apoptosis. Mechanistically, anisodamine and probucol decreased the expression of MAPK and Akt/mTOR/P70S6K protein expression. In addition, anisodamine and probucol inhibited Bax protein expression while it upregulated Bcl-2. The results suggested that anisodamine and probucol can accelerate the recovery of renal function by reducing renal oxidative stress and influencing p38 MAPK and Akt/mTOR/P70S6K signal pathways. Combined anisodamine and probucol might be more effectively.

Keywords: Anisodamine, probucol, contrast-induced nephropathy, phospho-p38 mitoge-activated protein kinase, Akt/mTOR/P70S6K signals

Introduction

The prevalence of contrast-induced nephropathy (CIN) is rising due to the increased use of contrast medium (CM) in percutaneous coronary intervention (PCI). CIN is generally defined as an otherwise unexplained acute impairment in renal function, manifested as serum creatinine (Scr) increases of 44.2 μmol/L or ≥ 25% from the baseline value within 3 days after the administration of CM [1, 2]. To date, chronic kidney disease, dehydration, diabetes mellitus (DM), advanced age, increased volume of CM and recurrent administration are well-known risk factors of CIN [3]. CIN is the third most common cause of hospital-acquired acute renal failure, accounting for 10~25% of acute kidney injury cases, and nearly 150,000 patients are estimated to develop CIN each year worldwide [4, 5]. Despite the advent of advanced CM and improvements in preventive strategies, the prevention of CIN remains challenging, and no specific prevention besides adequate periprocedural hydration is available [6]. Thus, it is urgent to uncover the pathogenesis of CIN and to identify novel preventive therapies decrease CIN incidence and to improve clinical prognosis.

Although the exact pathophysiological mechanism of CIN still remains poorly understood, it has been generally demonstrated that CIN appears to be the result of combined effects of direct nephrotoxicity of CM and hypoxic renal
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injury. Mounting evidence has shown that impaired renal blood flow and ischemia-reperfusion injury plays an important part in the pathogenesis of CIN [7]. In addition, reactive oxygen species (ROS), inflammation and apoptosis also contribute to the renal tubular cell injury. Recently, clinical studies reported that several potent free radicals scavenger, for example, probucol can effectively reduce the incidence of CIN [8]. Anisodamine, which is a muscarinic acetylcholine receptor antagonist, has been used for clinical treatment of various types of shock in China since 1965 [9]. Recently, anisodamine is used in the treatment of acute glomerulonephritis and diabetic nephropathy, because it can improve renal microcirculation. It was reported that anisodamine treatment may protect against CIN in patients with type 2 diabetes and renal insufficiency undergoing coronary angiography [10]. As far as we know, there was no study in the literature to investigate combine anisodamine and probucol to prevent CIN in diabetic rats. So in the present study, we established diabetic CIN rat models, evaluated whether combine anisodamine and probucol have beneficial effects on CIN. Besides, the underlying mechanisms were also investigated.

Materials and methods

Animal experimental protocol

The study protocol was approved by the Medicine Animal Ethics Committee of Tianjin Nankai Hospital (Tianjin, China). A total of 40 adult 8- to 10-week-old male Sprague-Dawley rats weighing 200–250 g from the Tianjin Acute Abdominal Diseases Institute Animal Research Center were used. Rats were housed in an air-conditioned room maintained at 23°C with a 12/12-hour light/dark cycle. Food and water were provided ad libitum except for the day of dehydration.

Experimental materials

Probucol was purchased from Chengde Jing Fukang Pharmaceutical Co. Ltd. (China); Anisodamine and urografin were purchased from Tianjin Jinyao Pharmaceutical Co. Ltd. (China); Superoxide dismutase (SOD), malondialdehyde (MDA) kit were purchased from Nanjing Jiancheng Company (China). The microscopic image acquisition and analysis system was from the Motic Med 6.0 CMIAS Image Analysis System (Motic China Group Co., Ltd).

Model and grouping

40 rats were randomly divided into 5 groups of 8 rats each: control (C) group, model (M) group, rats treated with anisodamine (A) group, rats treated with probucol (P) group, and rats treated with both anisodamine and probucol (A+P) group. First, we used streptozotocin (STZ, Sigma Company) to induce diabetic rats model. Diabetic rat model was established by abdominal single dose injection STZ 60 mg/kg. The blood glucose ≥ 16.7 μmol/L is used as the standard for the success of diabetic rats model. After ten weeks feeding, we use urografin to induce CIN. Rats in the P and A+P groups received intragastric administration of probucol (500 mg/kg) for 7 days [11]. Thereafter water forbidden was conducted for 24 hours, and then the diabetic SD rats were injected intravenously with urografin to induce CIN. Drugs administered consisted of urografin at a dose of 10 mg/kg [12]. A relative (≥ 25%) or an absolute (≥ 44.2 μmol/L) increase in Scr from the baseline is used as the standard for the success of CIN rats model [13]. Rats were anesthetized with 60 mg/kg pentobarbital. Pentobarbital sodium anesthesia was followed by CIN induction, which was performed with drug administration into a tail vein. After injecting CM, rats in the A and A+P groups received abdominal injection anisodamine (100 μg/100 g) one time per hour [14]. After injecting CM 4 times, anisodamine was used every 4 hours in the following 8 hours. At the same time, rats in the C, M and P groups received the same dose of abdominal injection saline. Baseline blood samples were collected from the tail vein under ether anesthesia for analysis of Scr and blood glucose. Baseline 24-hour urine samples were collected for determination of interleukin-18 (IL-18), neutrophil-gelatinase-associates-lipocalin (NGAL).

Observation indicators

General condition: The mental state, activity, body weight, food intake, and mortality of rats were observed after the procedure. After modeling, all rats were killed. After the rats were anesthetized for the modeling procedure, 1 ml of blood was obtained from the inner canthus for blood biochemical examination prior to modeling.
Detection of relevant renal oxidative stress indicators: After the rats were killed, the kidney tissues were quickly removed. In addition, 3 ml of blood was obtained from the abdominal aorta. Tissue blocks of the appropriate size were taken, placed in pre-cooled ice-cold normal saline, and made into tissue homogenate at a ratio of 1:9. The supernatant was removed after 15 min of centrifugation at 3000 rpm, and the purchased kits were used to measure the levels of SOD, MDA in renal tissues.

Renal pathological examination: Tissue blocks from rat kidney were fixed in 10% formalin and prepared for hematoxylin and eosin (HE) staining, transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining, and immunohistochemistry (IHC) analysis. HE-stained tissue sections were viewed using light microscopy. Caspase-3 protein expression was investigated using light microscopy by IHC. Caspase-3 staining were achieved using a Vectastain Universal Elite ABC Kit (Vector Laboratories, USA) following the manufacturers' protocols. Caspase-3 staining was confirmed by staining with a 3,3'diaminobenzidine substrate.

For TUNEL staining, sections were stained using In Situ Cell Death Detection Kit (Roche Applied Science) according to the protocol. TUNEL-positive tubular cell numbers were counted at random in 20 non-overlapping cortical fields under a 400× magnification.

For IHC of caspase-3, sections (4 mm) were immersed in citrate buffer and autoclaved at 120°C for 10 min, then immersed in 3% aqueous hydrogen peroxide (H₂O₂). The sections were incubated with rabbit polyclonal antibody to caspase-3 (Cell Signalling Technology, 1:100), followed by incubation with secondary antibodies and visualization with 3,3'diaminobenzidine and counterstained with hematoxylin, dehydrated and fixed in xylene before mounting with DPX.

Western blotting analysis: Renal cortexes were lysed in lysis buffer. Proteins (30 μg) were subjected to electrophoresis on 6–12% polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% non-fat milk in TBST for 2 h at room temperature, and incubated with the primary antibodies overnight at 4°C. The bands were detected by GelDoc XR (BioRad, USA) after a second incubation with appropriate secondary antibodies. Protein expression was quantified by Image J1.45 software (Wayne Rasband, NIH, Bethesda, Md., USA) after scanning the film. Primary antibodies included the following: anti-phosphop ERK₁/₂ (Santa Cruz Biotechnology, 1:750); anti-phosphop JNK (Santa Cruz Biotechnology, 1:1000); anti-Akt/mTOR/P70S6K (Cell Signaling Technology, 1:1000); anti-phosphop38 MAPK (Cell Signaling Technology, 1:1000); anti-Bcl-2 (Santa Cruz Biotechnology, 1:1000); anti-Bax (Santa Cruz Biotechnology, 1:1000); anti-phosphop3 caspase-3 (Santa Cruz Biotechnology, 1:1000); anti-β-actin (Santa Cruz Biotechnology, 1:1000). All experiments were performed at least three times under the same conditions.

Statistical analyses

Continuous variables and categorical variables are expressed as the mean ± standard deviation (SD) and percentages, respectively. All samples were tested to ascertain if they followed a normal distribution. Categorical variables were compared using the χ² test or the Fishers exact test where appropriate. One-way analysis of variance (ANOVA) was applied for the analysis of continuous variables among the four groups. Two-tailed P values of P < 0.05 were considered statistically significant. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

General condition of the animals

There were no deaths among the rats involved in this study. The rats in the C group did not
show significant abnormalities in feeding or activity, although rats in the other groups showed various levels of malaise, slowed mobility, loss of appetite, and weight loss, with the M group of rats showing the most significant symptoms.

Comparison of renal function among various treatment groups

Scr, BUN, urinary IL-18 and NGAL levels in the rats in each group were all at the same baseline level before model establishment. After the procedure, the C group did not show significant changes in Scr, BUN, or urine protein levels in comparison to the pre-procedure levels. After the procedure, the M group showed significantly higher levels of Scr, BUN, and urine protein than the C group (\(P < 0.01\)). All the three drug treatment groups demonstrated significantly lower Scr, BUN, urinary IL-18 and NGAL levels compared to the M group (\(P < 0.05\)), rats treated with A+P group were even lower than those in A and P groups (Figure 1).

Figure 1. Blood and urine of all rats were harvested for testing BUN, Scr, NGAL and IL-18. Diabetic SD rats were injected intravenously with urografin to induce CIN. They were randomly divided into 5 groups: control (C) group, model (M) group, anisodamine (A) group, probucol (P) group, combine anisodamine and probucol (A+P) group. The rats of M group showed significantly higher levels of BUN, Scr, NGAL and IL-18 than the C group. Those levels of rats in three drug treatment groups were decreased, and those in A+P group were lowest. \(^a\)P < 0.05 vs C group; \(^b\)P < 0.05 vs M group; \(^c\)P < 0.05 vs A group, \(^d\)P < 0.05 vs P group.
Detection of relevant indicators of oxidative stress in renal tissue

After the procedure, the SOD and MDA levels in the renal tissues of the C group showed no significant changes in comparison to the baseline levels. However, after the procedure, the SOD levels of the M group were decreased (P < 0.05), whereas the MDA levels were increased (P < 0.01). In addition, the SOD levels of all the three drug treatment groups were higher than those of the M group (P < 0.05), and the MDA levels of all the three drug treatment groups were lower than those of the M group (P < 0.01). (P < 0.05 vs C group; "P < 0.05 vs M group; "P < 0.05 vs A group, "P < 0.05 vs P group).

Renal morphological changes

HE staining of kidney tissues showed that the renal tubular epithelial cells of the C group presented a normal morphology and structure, without any luminal expansion or urinary casts (Figure 3). However, the M group showed obvious tubular epithelial vacuolar degeneration and disintegration and shedding of the brush border, as well as visible cell casts and protein casts in regions of the lumen. In comparison, the 3 drug treatment groups showed milder pathological changes than the M group, they presented slightly vacuolar degeneration and brush border loss. In addition, the A+P group showed milder pathological changes than those in the P group and A group.

Anisodamine and probucol mitigated renal apoptosis caused by CM

To explore whether anisodamine and probucol's beneficial effects on CIN were associated with apoptosis changes, we performed TUNEL staining and IHC analysis on the kidney sections from rats with CIN. In comparison to the C group, contrast injection led to elevated apoptosis, anisodamine and probucol could dramatically inhibit contrast induced apoptosis in kidney (Figure 4). Moreover, the expressions of Caspase-3, Bax and Bcl-2 levels were analyzed. Similar to TUNEL assay, western blotting analysis suggested that CIN led to substantial increase in cleaved Caspase-3, Bax expression and decrease in anti-apoptotic proteins Bcl-2 expression as indicated in Figures 5 and 6. Our data proved that Caspase-3, bax activation were effectively repressed while Bcl-2 expression was increased by anisodamine and probucol preconditioning. In summary, anisodamine and probucol can mitigate renal tubular cell apoptosis following CIN, and combine with the two agents maybe more effective.
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Effects of anisodamine and probucol on p38 MAPK and Akt/mTOR/P70S6K signaling pathways

To test the hypothesis that the p38 MAPK and Akt/mTOR/P70S6K pathways are activated in CIN in vivo, we examined p38 MAPK (including JNK, ERK) and Akt/mTOR/P70S6K signaling. Western blotting analysis suggested that CIN led to increase in JNK signals expression and decrease in ERK, Akt/mTOR/P70S6K signals expression. While pretreatment with anisodamine and probucol can reduce JNK signals expression and promote ERK, Akt/mTOR/P70S6K signals expression. Combine with the two agents maybe more effective (Figure 7).

Discussion

In the present study, we investigated the mechanism causing diabetic CIN as well as putative protective agent. This model in vivo is well established and involves water deprivation, and injection of 10 mg/kg urografin. The used dosage of urografin in vivo is similar to that used in the clinic.

CIN is a serious complication caused by radiological imaging. CIN results in increased health care costs, prolongs hospital length of stay, and increases both short- and long-term morbidity and mortality, even after adjustment for other comorbidities [15]. CIN has become the third leading cause of hospital acquired acute renal failure [16]. The diagnosis of CIN often relies on a relative (≥ 25%) or an absolute (≥ 44.2 μmol/L) increase in serum creatinine from the baseline value within 3 days after intravascular administration of CM [1]. However, the change of Scr levels fall behind the damage of kidney. Nor can the change demonstrate renal function in time. Recent studies indicate that the values of urine IL-18 and NGAL can early specific to predict the development and progression of CIN [17, 18]. Our data indicate that the urine IL-18, NGAL levels also have good sensitivity to diagnose early kidney injury. Pretreatment with anisodamine and probucol remarkably suppressed the increase in urine IL-18, NGAL, and Scr levels.

The pathophysiological mechanism associated with the development of CIN is complex, and remains poorly understood. Renal medullary...
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ischemia following CM induced intra-renal vasoconstriction, direct cytotoxicity, oxidative tissue damage and apoptosis are possible mechanisms implied for CIN [19]. First, calcium ions and adenosyl fragments mediate vasoconstriction, which is directly involved in the generation of oxygen radicals. Second, the glomerular basement membrane and mesangial cells are damaged and leukocyte chemotaxis is enhanced, further leading to oxygen radical production. Third, the xanthine oxidase activity in renal tissue is increased, which also increases the production of oxygen radicals [20]. In addition, iodine-containing contrast agents can also provide iodine atoms, which are directly involved in the generation of oxygen radicals. Oxygen radicals can cause toxic ischemia and immune-mediated tissue damage [21]. In the present study, the model group showed significantly decreased SOD and significantly increased MDA levels after modeling, consistent with the findings of Wang N et al. [11].

In China, anisodamine has been widely used in treatment of microcirculatory disorders. Intracoronary administration of 1000 μg of anisodamine was effective in reversing coronary no-reflow phenomenon during primary PCI in ST-segment elevation myocardial infarction, as reported by Fu et al. [22]. This effect maybe due to ameliorate the coronary microcirculation. The protective mechanisms of anisodamine on renal function work via dilating the arcuate artery, interlobular artery and afferent arteriole of the kidney in a dose-dependent manner. The glomerular blood flow increases by 15%-40% after administration of anisodamine, which can ameliorate renal ischemia and attenuate the damage of renal microvessels and tissue structure [23, 24]. Anisodamine is also helpful in recovery of renal blood flow in renal tissues by increasing the plasma prostaglandin I level [25]. The adverse effects of CM on renal function may involve the generation of ROS which may play a role as vasoconstrictors. The renal protective effects of anisodamine may be associated with activating SOD in renal tissues which can scavenge ROS and produce antioxidant effects [26]. In accordance with previous studies, our results demonstrate that anisodamine pretreatment was effective in attenuating the biochemical and histological changes of CIN.

Figure 4. TUNEL staining of kidney tissues (×400). In comparison to the C group, contrast injection led to elevated apoptosis, anisodamine and probucol could dramatically inhibit contrast induced apoptosis in kidney.
Probucol is a clinically used cholesterol-lowering drug. Besides its antioxidant properties, probucol was shown to protect against CIN by enhancing the endogenous antioxidant system including glutathione peroxidase, catalase and SOD [27]. Oxidative stress exerts both agonistic and antagonistic effects on apoptotic signaling through regulation of apoptosis, mediate cell proliferation and differentiation [28]. CIN fractionated the cellular signal traffics to mitochondria, activation of Bax in a caspase-dependent manner and initiates apoptosis. Bcl-2 regulates apoptosis and acts along intrinsic mitochondrial apoptosis pathway that is activated in response to oxidative stress [29, 30]. In this present study, tubular epithelial cell apoptosis in CIN was confirmed. The p38 MAPK and Akt/mTOR/P70S6K signals are involved in the apoptotic response to a variety of environmental stress and is critical for renal tubular cell apoptosis triggered by CM. Western blotting and IHC analysis revealed significant increase in JNK protein expression, while decrease in ERK and Akt and P70S6K protein expression and phosphrylation. The present data also confirmed CIN can increase expression of the proapoptotic protein Bax and decrease expression of the anti-apoptotic protein Bcl-2. Moreover, anisodamine and probucol inhibited signaling through the p38 MAPK and Akt/mTOR/P70S6K pathways and decreased apoptosis index.

A major strength of the present study is that the potential protective role of combine anisodamine and probucol in a whole animal model. However, further studies should address whether anisodamine and probucol are protective against toxicity induced by contrast medium of different osmolality and viscosity as well as the potential use in humans to prevent CIN.

Conclusions

Our data suggested that anisodamine and probucol attenuated renal injury in diabetic CIN rats and these beneficial effects are mainly mediated via anti-oxidation and anti-apoptotic effects. Thus, anisodamine and probucol may represent potential drugs for prevention of dia-
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Disclosure of conflict of interest

None.

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