Original Paper

Lacidipine Attenuates Apoptosis via a Caspase-3 Dependent Pathway in Human Kidney Cells

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Key Words

Lacidipine • Apoptosis • ATP depletion and recovery • Human kidney cells

Abstract

Background: Acute kidney injury (AKI) is common in hospitalised patients and has a poor prognosis. Therefore, new therapeutic strategies are anticipated. Lacidipine, a novel third-generation dihydropyridine calcium channel blocker, has been demonstrated effective for hypertension. However, its potential effect on renal injury remains unknown. In the present study, an *in vitro* model of renal ischemia reperfusion (I/R) injury was used to investigate the protective effect and underlying mechanisms of lacidipine on human kidney cell (HKC) apoptosis. **Methods:** HKCs were subjected to adenosine triphosphate (ATP) depletion and recovery (0.01 μ M AA, depletion for 2 h and recovery for 30 min), with or without lacidipine (1 μ M and 10 μ M, 24 h), then cell viability and apoptosis were determined using the cell counting kit-8 (CCK-8) assay and Annexin V flow cytometry. The expression of Bcl-2, Bax, and cytochrome c (cyt c) was examined by western blot. **Results:** Antimycin A (AA) was found to induce apoptosis of HKCs. The proportion of early apoptosis and activity of caspase-3 peaked at 30 min after ATP depletion and recovery and were attenuated by lacidipine. The expression of cyt c and Bax was decreased, while that of Bcl-2 was increased significantly in lacidipine treated group. **Conclusion:** We conclude that lacidipine protects HKCs against apoptosis induced by ATP depletion and recovery by regulating the caspase-3 pathway.

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Introduction

Acute kidney injury (AKI) is characterised by a rapid reduction in kidney function resulting in a failure to maintain fluid, electrolyte and acid-base homeostasis in clinical practice. Recently, AKI has been paid more attention because of its high morbidity and mortality [1]. The incidence of AKI in adults and children has been increasing over the past decades. Ischemic hypoxic renal injury is the leading cause of AKI [2], and renal ischemia/reperfusion (I/R) injury is one of the main causes of ischemic AKI. In children with complex systemic disease who survive AKI episodes, the high risk for chronic kidney disease is quite evident [3]. Cellular hypoxic damage, mainly induced by renal I/R, occurs in human kidney cells (HKCs), with necrosis and apoptosis of HKCs being the underlying pathophysiological mechanisms. Therefore, apoptosis of HKCs is an important outcome of renal I/R [4, 5].

The principal of treatment of AKI are to remove its causes and maintain homeostasis. Results from double-blind, randomized controlled studies have shown that the nephroprotective effects of low-dose dopamine and loop diuretics were ineffective in reducing the incidence of AKI [6, 7]. Besides, no specific drug-based intervention has proved to be protective after hemodynamic redevelopment and removal of nephrotoxins [8]. Thus, to our knowledge, there is no established pharmacotherapeutic procedure for AKI.

Lacidipine is a novel third-generation dihydropyridine calcium channel blocker that could effectively lower blood pressure and reduce damage to the heart, brain and kidneys [9-11]. However, the effect of lacidipine on AKI is unknown, and the molecular mechanism of lacidipine in conferring protection against kidney damage is unclear. It has been suggested that lacidipine exerts an antioxidant effect and could protect endothelial cells from free-radical injury [12]. Therefore, we hypothesize that lacidipine exerts therapeutic effects against I/R through an anti-apoptotic mechanism.

In this study, we confirmed that adenosine triphosphate (ATP) depletion and recovery induce HKC apoptosis, using an *in vitro* model mimicking the cell injury caused by I/R. We also investigated whether lacidipine can protect HKCs from apoptosis under conditions of ATP depletion and recovery and determined the underlying mechanism.

Materials and Methods

Reagents and antibodies

Antimycin A (AA) was purchased from Sigma (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS). Lacidipine was provided by Sanchine (Harbin, China), which was dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO did not exceed 0.1%. Cell counting kit-8 (CCK-8), bicinchoninic acid (BCA) detection kit and anti-caspase-3 antibodies were purchased from Beyotime (Jiangsu, China). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Billerica, MA, USA). Enhanced chemiluminescence (ECL) kit was provided by Thermo Fisher Scientific (Rockford, IL, USA). Rabbit anti-Bax, Bcl-2 and cyt-c antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti- β -Actin antibody was purchased from Beijing Zhongshan Biotech Co. (Beijing, China). Goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Rockland (Gilbertsville, PA, USA). Annexin V-FITC and propidium iodide (Annexin V-FITC/PI) was provided by Biosea Corporation (Beijing, China).

Cell culture

HKCs were obtained from Cell Resource Center, IBMS, CAMS/PUMC (CRC/PUMC, China) and grown in Dulbecco's Modified Eagle's medium/F12 (DMEM/F12, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37°C with 5% CO₂ in a humidified atmosphere. The cells used in these experiments were in their logarithmic phase of growth. The *in vitro* model of HKC apoptosis was established using AA. After designated doses of AA were added in the medium, the cells were incubated for 2h, after which the culture medium was changed to the regular medium. The cells had been

pre-incubated with designated concentrations of lacidipine for 24 h before the model of apoptosis was established.

Cell proliferation assay

HKCs were seeded in 96-well plates at a density of 7500 cells per well with 200 μ l of complete culture medium. They were incubated in DMEM/F12 with 10% FBS for 24 h. After equal durations of treatment with AA (final concentration, 0.0001 μ M to 10 μ M) and lacidipine (final concentration, 0.01 μ M to 100 μ M), the cells were trypsinized and their viability determined using CCK-8. After chemical stimulation, the supernatant was removed, and 200 μ l of DMEM/F12 medium containing 20 μ l CCK-8 was added to each well, followed by further incubation for 3 h at 37°C. The culture plates were shaken for 10 min, and the optical density (OD) values were read at 450 nm. For each sample, six parallel experimental groups were used to assess cell viability.

Flow cytometric analysis

For this analysis, 10^6 cells were incubated with 0.01 μ M AA. The culture medium was then changed to the regular medium at different times. Two concentrations of lacidipine were added to the HKC 24 h before the model of apoptosis established. The prepared cells were washed twice with cold PBS and resuspended in 500 μ l of binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM CaCl_2) at a concentration of 1×10^6 cells/ml. Then, 5 μ l of AnnexinV-FITC and 10 μ l of 20 μ g/ml PI were added to the cells, which were analyzed with a flow cytometer (Beckman, S. Kraemer Boulevard Brea, CA, USA). Viable cells were negative for both PI and Annexin V; apoptotic cells were positive for Annexin V and negative for PI, and late-apoptotic dead cells showed both Annexin V and PI positivity. Non-viable cells, which underwent necrosis, were positive for PI and negative for Annexin V.

Assay of caspase-3 activity

Caspase-3 activity was measured using the caspase-3 assay kit according to the manufacturer's protocol. After treatment with AA and lacidipine, the cells were lysed with 50 μ l of chilled cell lysis buffer, and 50 μ l of 2 $\times$ reaction buffer and 5 μ l of the appropriate conjugated substrate at a concentration of 1 mM were added to each lysate. The mixture was incubated in a water bath at 37 °C for 1 h, and the absorbance was measured using a microtiter plate reader at a test wavelength of 405 nm.

Western blot analysis

HKCs treated with lacidipine (1 μ M or 10 μ M for 24 h) and AA (0.01 μ M for 2 h, with 30 min recovery) were suspended in lysate buffer. Protein concentrations were assayed with the BCA detection kit, and samples were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). These samples were transferred into PVDF membranes. Then, membranes were blocked with Tris buffered saline (TBS) with 0.05% Tween-20 containing 5% nonfat dry milk for 1 h and then incubated at 4°C overnight with appropriate primary antibodies (β -actin, 1:800; Bax, 1:1000; Bcl-2, 1:300; cyt-c, 1:300). Membranes were washed with TBST, incubated with appropriate secondary antibodies (1:4000) for 1 h. Signal strength was measured using an ECL kit and the relative photographic density quantified using an Odyssey Fc Imaging System. Protein levels were normalized to β -actin.

Statistical Analysis

The results are presented as the means $\pm$ standard deviation (SD). The data were analyzed by one-way ANOVA using SPSS17.0. The Student t-test was used to analyse the differences between the groups, which were considered statistically significant at $p < 0.05$.

Results

Effect of AA on the viability of HKCs

In order to assess the cytotoxic effect of AA on HKCs, the cells were cultured with AA at final concentrations ranging from 0.0001 μ M to 10 μ M for 2 h, then CCK-8 assays were tested.

Fig. 1. Effects of lacidipine, antimycin A (AA) and ATP depletion and recovery on the viability of human kidney cells (HKCs) and caspase-3 activity. (A): Effect of AA on the viability of HKCs. The cells were treated with ATP depletion buffer (control, containing 0.0001 μM to 10 μM AA) for 2 h. Values are means ± SD; n=6. ***vs control, p < 0.001. Y-axes mean optical density (OD) values. (B): Effect of lacidipine on the viability of HKCs. The cells were treated with lacidipine buffer (control, containing 0.01 μM to 100 μM lacidipine) for 24 h. Values are means ± SD; n=6. ***vs control, p < 0.001. Y-axes mean OD values. (C): Effect of ATP depletion and recovery on Caspase-3 activity in HKCs. The cells were treated with ATP depletion (AA 0.01 μM for 2 h) and recovery for 0 (A), 15 (B), 30 (C), 60 (D), 120 (E) min (recovery for 0 min, recovery for 15 min, recovery for 30 min, recovery for 60 min, recovery for 120 min). The mixture was incubated in a water bath at 37 °C for 1 h, and the absorbance was measured by microtiter plate reader at a test wavelength of 405 nm. The standard curve is $y=415.0x-21.06$, $R^2=0.999$. Values are means ± SD; n=3. ***vs control, p < 0.001. (D): Effect of lacidipine on Caspase-3 activity in injured HKCs. The cells were treated with lacidipine (1 μM, 10 μM) for 24 h, after which apoptosis model was established. Values are means ± SD; n=3. ***vs control, p < 0.001, # VS 1 μM, p < 0.05.

Cells cultured in AA-free media were used as the control. The inhibition of HKC proliferation caused by AA was assessed. As shown in Fig. 1A, *in vitro* HKCs growth was inhibited by AA in a concentration-dependent manner. Significant differences were found in viability reduction from the 0.01 μM AA group to 10 μM AA group (p < 0.05). In subsequent experiments, 0.01 μM AA was used.

Effect of lacidipine on the viability of HKCs

HKCs were cultured with lacidipine at final concentrations ranging from 0.01 μM to 100 μM for 24 h, and their viability was assessed. As shown in Fig. 1B, lacidipine inhibited HKCs proliferation *in vitro* in a concentration-dependent manner. OD values decreased gradually from 1 μM to 100 μM (p < 0.05). Therefore, 1 μM and 10 μM lacidipine were selected in subsequent experiments.

Fig. 2. Flow cytometric analysis of apoptosis rates of HKCs treated with AA (proportion of early apoptosis cells %). The cells were treated with AA 0.01 μ M for 2 h and recovery for 0, 15, 30, 60, 120 min (recovery for 0 min, recovery for 15 min, recovery for 30 min, recovery for 60 min, recovery for 120 min). Then the cells were trypsinized and stained with Annexin V and PI. The proportion of early apoptotic cells was different. (F): the proportion of early apoptosis cells induced by ATP depletion for 2 h and recovery for different times. The highest rate of early apoptosis is that at the ATP depletion for 2 h and recovery for 30 min. The results were presented as the means $\pm$ SD; n=3.*vs control, p < 0.05.

Detection of apoptosis in AA-treated HKCs

HKCs treated with AA (0.01 μ M, 2 h) followed by recovery (0, 15, 30, 60, 120 min) showed significant changes in Annexin V/PI staining. The percentages of early apoptotic cells are $0.27\% \pm 0.06\%$, $2.27\% \pm 0.21\%$, $4.57\% \pm 0.61\%$, $1.537\% \pm 0.23\%$, $0.377\% \pm 0.21\%$, respectively. As shown in Fig. 2F, the 15-, 30- and 60- min groups showed an increased proportion of early apoptotic cells (p < 0.05) compared to the control. The highest proportion of apoptosis ($4.57\% \pm 0.61\%$) occurred with ATP depletion for 2 h and recovery for 30-min (p < 0.05). Consistent with this observation, the activity of caspase-3 changed. As shown in Fig. 1C, the caspase-3 activity of the 30-min group was the highest (p < 0.05). Taken together, the results indicated that the model was well established (Fig. 1C and Fig. 2), and ATP depletion for 2 h and recovery for 30 min were selected to explore the influence of lacidipine on apoptosis of HKCs.

Lacidipine prevents AA-induced HKC apoptosis

The proportion of early apoptotic cells and caspase-3 activity were analysed after HKCs were treated with lacidipine (1 μ M and 10 μ M, 24 h) and AA (0.01 μ M for 2 h and 30-min recovery). As shown in Fig. 3D, compared with the control group ($3.83\% \pm 0.35\%$), the lacidipine test groups showed a lower proportion of early apoptotic cells (the percentages of 1 μ M and 10 μ M groups are $1.47\% \pm 0.35\%$ and $0.30\% \pm 0.10\%$, p < 0.05). Furthermore, caspase-3 activity was significantly reduced in the lacidipine-treated cells compared with the untreated group (p < 0.05, Fig. 1D). Additionally, the protective effect of lacidipine on damaged cells was found to be concentration dependent.

Fig. 3. Flow cytometric analysis of AA-induced HKCs apoptosis prevented by lacidipine. The cells were treated with lacidipine (1 μ M, 10 μ M) for 24 h before apoptosis model was established. The cells were trypsinized and stained with Annexin V and PI. (A): ATP depletion for 2 h and recovery for 30 min without lacidipine. (B) The cells pre-treated with 1 μ M lacidipine for 24h and ATP depletion for 2 h and recovery for 30 min. (C): The cells pre-treated with 10 μ M lacidipine for 24 h and ATP depletion for 2 h and recovery for 30 min. The results were presented as the means $\pm$ SD; n=3. ***vs control, p <0.001, #vs 1 μ M, p <0.05.

Effect of lacidipine on activation of apoptotic pathways

On the basis of the caspase-3 activity at different time-points following ATP depletion and recovery, the mitochondrial pathway of apoptosis was assessed and key factors, including cyt c, Bax, Bcl-2, were detected by western blot. In order to analyse the role of mitochondria and cyt c dependency, the expression of cyt c was analysed. Lacidipine decreased the expression of cyt c of injured cells following ATP depletion and recovery (Fig. 4A, p <0.05).

In addition, we determined whether the change of cyt c level was mediated by alterations in expression of the anti-apoptotic protein, Bcl-2 and pro-apoptotic protein Bax. Significant changes were found in Bcl-2 and Bax protein levels between the injured cells group and lacidipine-treatment group. Lacidipine significantly increased the expression of the Bcl-2 protein (Fig. 4B, p <0.05). In contrast, the expression of the Bax protein decreased in cells treated with lacidipine (Fig. 4C, p <0.05).

Discussion

AKI is a common clinical complication characterised by an abrupt decrease in the glomerular filtration rate. Despite renal replacement therapy, the mortality associated with AKI remains high [13]. To date, no specific therapy is available to improve the clinical outcome of this condition, and therapy mostly relies on nutritional and supportive care [14, 15]. Therefore, new therapeutic strategies for patients with AKI are necessary. Interventions for inhibiting apoptosis have the potential to minimize renal dysfunction and accelerate recovery after AKI. The present study has two major findings: (1) lacidipine is effective in attenuating cell apoptosis following I/R-induced HKC injury, and (2) the protective mechanism of lacidipine is related to caspase-3 pathway.

To determine the mechanism by which lacidipine protects against HKC apoptosis, we used an *in vitro* cell culture model to mimic I/R. The main advantage of this model was

Fig. 4. Effects of lacidipine on activation of apoptotic pathways in apoptotic HKCs. The cells were pre-treated with lacidipine (1 μ M, 10 μ M) for 24 h and treated with ATP depletion for 2 h and recovery for 30 min. (A, B): Cytochrome *c* level was detected by western blot. The results showed cyt *c* level was decreased in lacidipine treated groups. (C, D): Bcl-2 was detected by western blot. Lacidipine regulated the expression of Bcl-2 in apoptotic HKCs. The results showed that the protein level of Bcl-2 was increased in lacidipine treated groups. (E, F): Bax was detected by western blot. Lacidipine regulated the expression of Bax in apoptotic HKCs. The results showed that the protein level of Bax was decreased. Each graph represents means $\pm$ SD; n=3. *vs control, $p < 0.05$, **vs control, $p < 0.01$. Y-axes mean cyt *c* relative level in HKCs, Bcl-2 relative level in HKCs, Bax relative level, respectively.

that the complexity of an *in vivo* model was eliminated. AA, an inhibitor of complex III of the electron-transport chain, can inhibit succinate oxidase, nicotinamide adenine dinucleotide health oxidase, and mitochondrial electron transport between cytochromes *b* and *c* [16]. The inhibition of electron transport causes a collapse of the proton gradient across the mitochondrial inner membrane, thereby breaking down the transmembrane potential [16, 17]. Since AA acts on mitochondria, apoptosis induced by AA has been reported in some studies [18, 19]. While earlier studies employed different concentrations of AA [20–22], we applied a concentration of AA suited to the subsequent experiments involving CCK-8. Cells undergoing early apoptosis are usually characterized by phosphatidylserine exposure on the outer leaflet side of the plasma membrane. Based on their Annexin V-affinity, apoptotic cells can be distinguished from Annexin V-negative living cells using cytometric procedures. Further, the double labelling assay with Annexin V and PI allows a distinction between necrotic or late apoptotic (Annexin V+/PI+) and early apoptotic cells (Annexin V+/PI-). As shown in Fig. 2, pilot experiments indicated that AA can induce apoptosis of HKCs, a finding that corresponds with that of another study [4]. ATP depletion for 2 h and recovery for 30 min with 0.01 μ M AA resulted in HKC apoptosis.

It has been generally accepted that apoptosis of HKC contributes to ischemic renal dysfunction [23, 24]. This conclusion is based on reports using multiple approaches (caspase-3 activity, cyt *c* release, Bax activation, loss of intact Bcl-2, etc.) that have shown apoptosis is a consequence of ischemia [25]. Different anti-apoptotic treatments reduce apoptosis and improve renal function after ischemia, as evidenced by studies *in vivo* [26, 27] and *in vitro* [28, 29]. Ca^{2+} influx plays a crucial role in apoptosis [30]. Ca^{2+} overload via the reverse

mode of $\text{Na}^+/\text{Ca}^{2+}$ exchange plays an important role in the pathogenesis of I/R-induced renal injury [31]. Reducing the influx of Ca^{2+} can, therefore, effectively prevent apoptosis. Several studies show that calcium channel antagonists have favorable impact on different cells. For example, amlodipine pretreatment can counteract angiotensin II-triggered endothelial apoptosis through the upregulation of the Bcl-2/Bax ratio and downregulation of the LOX-1 expression [32]. Nifedipine and amlodipine play a renoprotective role on gentamicin-induced renal tubular toxicity through its antioxidant properties and suppressing apoptosis [33]. Moreover, calcium channel antagonists plus angiotensin receptor blocker protect cardiac myocytes from apoptosis mediated mitochondrial pathway [34]. It has been manifested that the beneficial impact of diltiazem on cyclosporin A treated kidney transplant recipients is not through its direct cytoprotective effect on renal tubular cells but through improving the glomerular hemodynamics, protective effect on endothelium and enhancing immunosuppression [35]. Lacidipine is a third generation vasoselective lipophilic dihydropyridine calcium channel blockade with anti-atherosclerotic [36] and anti-adhesion [11] properties. It has been previously shown that lacidipine is one of several substances with powerful antioxidative effects [37, 38]. Lacidipine is usually used as an antihypertensive agent. Lacidipine pretreatment has also been shown to prevent cyclosporine-induced nephrotoxicity [39]. Nevertheless, whether lacidipine could be used for the treatment of AKI remained unknown. We therefore hypothesized that: (1) lacidipine prevents HKC apoptosis from I/R and (2) the mechanism underlying apoptosis attenuation is related to Bcl-2, Bax regulation and the change of cyt *c* level.

Apoptosis can be triggered by a variety of stressors such as inflammatory cytokines, ischemia, oxidative stress, etc. Apoptotic mechanisms are complex, with factors affecting a number of pathways. Among the multiple mediators of the complex process of apoptosis, the caspase enzyme cascade plays a central role. The caspase family is an important initiator and effector of apoptosis [24, 40]. Caspase-3 is one of the crucial enzymes among cysteine proteases that exist as inactive zymogens. The intrinsic pathway initiates apoptosis through the release of cyt *c* into the cytosol, and results in activation of caspase-3. The activation of caspase-3 results in the degradation and deactivation of important proteins. Caspase-3 activation is observed during the course of apoptosis induced by triptolide in human proximal tubular cell [41]. The release of cyt *c* from the mitochondria is regulated by various proteins including the anti-apoptotic protein, Bcl-2, and the pro-apoptotic Bax protein [42, 43].

It seems to be possible to alleviate AKI by inhibiting caspase-3-mediated cell apoptosis [23]. In the present study, caspase-3 activity was reduced by lacidipine treatment. The extent of early apoptosis was also decreased by lacidipine treatment, as measured by flow cytometric. As shown in Fig. 3, these findings indicated that lacidipine is capable of alleviating the cytotoxicity associated with the injury. Our *in vitro* results correlated with the results of an *in vivo* study [39]. This study certified that lacidipine has a protective effect on the kidney that is independent of its blood-pressure lowering effect. We observed that apoptosis attenuation by lacidipine follows ATP depletion and recovery via the caspase-3 mediated mechanism. As shown in Fig. 4, Bcl-2 protein levels increased and Bax protein levels decreased in the lacidipine group, corresponding with the reduction in cyt *c* levels. Lacidipine was effective in upregulating Bcl-2 and downregulating Bax protein expression, and influencing the expression of cyt *c*. Lacidipine may exert its advantage by antioxidant properties, suppressing apoptosis mediated mitochondrial pathway, improving the glomerular hemodynamics and protective effect on endothelium. These findings support the hypothesis that lacidipine prevents apoptosis in HKCs, injured by ATP depletion and recovery, through regulation of the intrinsic apoptotic pathway. Interestingly, lacidipine decreased caspase-3 activity in a concentration-dependent manner (Fig. 1), but its effect on the expression of cyt *c* and the Bcl-2 and Bax proteins differed. The levels of these proteins were decreased by lacidipine, but there were no differences between the 1 μM and 10 μM groups (Fig. 4). Further studies are required to better understand kidney-protection mechanism of lacidipine.

In summary, our data suggest that lacidipine treatment could protect HKCs against I/R injury by inhibiting protein expression of Bax and *cyt c* and increasing the Bcl-2. Therefore, lacidipine might be a novel and potent therapeutic option for AKI.

Conflict of Interest

None declared.

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