Irbesartan attenuates contrast media-induced NRK-52E cells apoptosis

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Abstract

Background: Radiocontrast nephropathy (RCN) is a major complication after radiographic examination. The precise mechanisms underlying RCN are not well understood. Renal tubular cell apoptosis is a feature of RCN, but hyperosmolality cannot fully explain the cytotoxicity of contrast media. There is accumulating evidence that reactive oxygen species (ROS) is involved in the pathophysiology of RCN, whereas the correlation between oxidative stress and contrast media-induced cell apoptosis is not clear. We hypothesized that ROS mediated apoptosis in renal tubular cells exposed to contrast media. Irbesartan, a selective AT1 receptor antagonist has been demonstrated an antioxidative effect. The present study was designed to determine whether irbesartan attenuated the contrast media-induced renal tubular cell apoptosis.

Methods: NRK-52E cells were exposed to increasing concentration (25, 50, 100, 150 mg iodine mL$^{-1}$, 335, 384, 420, 521 mOsm kg$^{-1}$) of ioversol (a non-ionic contrast media) for 1 h or incubated in ioversol (100 mg iodine mL$^{-1}$, 420 mOsm kg$^{-1}$) for 15 min, 30 min, 60 min, 120 min, 240 min, respectively. Mannitol with the same osmolality as ioversol (420 mOsm kg$^{-1}$) also treated NRK-52E cells for 1 h. In separate experiment, irbesartan (0.01, 0.1, 1 mmol L$^{-1}$) was added 1 h before incubation with ioversol (100 mg iodine mL$^{-1}$, 420 mOsm kg$^{-1}$) for 1 h. Apoptosis was determined by Hoechst staining and flow cytometry with annexinV-FITC and propidium iodide. The intracellular formation of ROS was detected by confocal microscopy with fluorescent probe CM-H2DCFDA. Bax and bcl-2 mRNA expression were determined by reverse transcription–polymerase chain reaction (RT–PCR).

Results: Ioversol induced NRK-52E cells apoptosis in a concentration- and time-dependent manner. The intracellular ROS generation was greatly increased following ioversol stimulus. Furthermore, ioversol induced a decrease in the expression for bcl-2 mRNA and an increase for bax mRNA. Irbesartan attenuated the ioversol-induced apoptosis in NRK-52E cells in a dose-dependent manner, in which the protective effect of irbesartan was dependent on decreasing intracellular ROS formation. In addition, irbesartan reversed the ioversol-induced increase in bax mRNA and decrease in bcl-2 mRNA.

Conclusion: Ioversol induced NRK-52E cells apoptosis in a concentration- and time-dependent manner via an increase in oxidative stress and subsequent to the increase in mRNA expression for bax and reduction in bcl-2 mRNA. Irbesartan attenuated the ioversol-induced apoptosis in NRK-52E cells by reducing oxidative stress and reversing the enhancement of bax mRNA and the reduction in bcl-2 mRNA.

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1. Introduction

Radiocontrast nephropathy (RCN) is a major complication after radiographic examination and is reported to be the third most common cause of hospital-acquired acute renal failure [1]. Risk factors for RCN include pre-existing renal insufficiency, diabetes, congestive heart failure, age over 75 years, hypercholesterolemia [2] and concomitant use of nonsteroidal antiinflammatory drugs. Although numerous trials have been proposed to provide prophylactic approaches against RCN, the results remain a matter of debate. Until recently, only intravenous hydration is generally accepted to prevent RCN [3].

The precise mechanism underlying RCN is not fully understood, the decrease in renal blood flow [4] and direct toxic action on renal tubular cells [5] have been considered to be involved in the pathogenesis of RCN. Recently, several clinical studies have demonstrated that antioxidant agents such as N-acetylcysteine (NAC) [6] and ascorbic acid [7] rather than agents that improve the renal blood flow can produce a significant effect to prevent RCN. Furthermore, an increased
level of 15-isoprostane F2t, a specific marker of oxidative stress was observed after radiographic examination in patients with renal insufficiency [8]. These results have suggested that reactive oxygen species (ROS)-mediated renal tubular cell injury plays an important role in the development of RCN.

Renal tubular cells apoptosis is a feature of RCN [9,10], which was due to the hyperosmolality of contrast media (CM) previously [11,12]. However, Yano et al. [13] recently showed that low-osmolar CM also induced early stage of cell apoptosis. On the other hand, the mannitol solution did not induce significant cell injury. This finding has indicated that hyperosmolality cannot totally explain the mechanism of CM-induced cell apoptosis. The degree of nephrotoxicity of a CM might well be associated more with its physicochemical characteristics than with its osmolality.

As we known, ROS have been implicated in several biologic responses including apoptosis [14]. Oxidative stress is linked to the activation of program for apoptosis in several cell lines. In diabetic nephropathy, it has been confirmed that apoptosis induced by hyperglycemia is mediated by ROS in both human tubular cells [15] and mesangial cells [16]. However, the correlation between ROS and cell apoptosis induced by CM is uncertain. We hypothesized that ROS mediated the CM-induced renal tubular cell apoptosis.

Irbesartan, a selective AT1 receptor antagonist with higher bioavailability, lower plasma protein binding, and a longer half-life than losartan and valsartan, has been demonstrated to reduce oxidative stress in diabetic kidney [17,18]. A recent report also indicated that irbesartan was associated with a significant reduction in plasma levels of 8-isoprostanone, a marker of oxidative stress in patients with metabolic syndrome. Furthermore, the antioxidative effect of irbesartan seemed to be independent of its antihypertensive properties [19]. This finding gives further support to the antioxidant capacity of irbesartan. Therefore, we assumed that irbesartan might attenuate the oxidative stress in renal tubular cells induced by CM, thereby yielding a protective effect against cell apoptosis.

In the present study, we evaluated the intracellular ROS production in the NRK-52E cell line incubated with CM. Moreover, we wish to elucidate the possible relation between oxidative stress and apoptosis in cell mechanism of RCN in vitro. We also first investigated whether the administration of irbesartan was able to attenuate the CM-induced cell injury.

2. Materials and methods

2.1. Regents

Ioversol (Optiray 320), a nonionic iodinated contrast agent with a stock solution of 702 mmol L−1 and 320 mg iodine mL−1, was obtained from Mallinckrodt Inc. (St. Louis, MO, USA). Fluorescent probe 5-(and-6) chloro-methyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) was obtained from Molecular Probes (Eugene, OR, USA). Irbesartan was kindly donated from Jiangsu Hengrui Medicine Co. Ltd. (Jiangsu, China).

2.2. Cell cultures

NRK-52E cell stocks (a renal tubule epithelium cell line from normal rat) were obtained from American Type Culture Collection (CRL-1571; Manassas, VA, USA). Cells were grown in 25-cm² cant-necked, vent-cap, uncoated flasks (Becton Dickinson, Oxnard, CA, USA) and propagated in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) plus 100 U mL−1 penicillin and 100 μg mL−1 streptomycin (Sigma) in an atmosphere of 5% CO2 in air at 37°C. Medium was replaced every 3 days. Cells were subcultured when the cell monolayer reached 80% confluence.

2.3. Hoechst 33258 stains

NRK-52E cells were seeded on sterile cover glasses placed in the six-well plates at a density of 1.0 × 10⁶ cells cm⁻² and cultured at 37°C for 24 h. After synchronization in serum-free culture medium, cells were exposed to ioversol (100 mg iodine mL⁻¹, 420 mOsm kg⁻¹) for 1 h. Subsequently, cells were fixed, washed twice with phosphate-buffer saline (PBS) and stained with Hoechst 33258 staining solution according to the manufacturer’s instructions (Beyotime, Jiangsu, China). Stained nuclei were observed under a confocal microscope (Leica, Germany). Similar staining procedures were performed with control cells.

2.4. Annexin V and propidium iodide stains

AnnexinV and propidium iodide (PI) stains were performed by using AnnexinV-FITC Apoptosis Detection Kit (BD Co. Ltd., USA). NRK-52E cells were cultured for 48 h on 6 cm Petridish (Becton Dickinson, Oxnard, CA, USA) and incubated in serum-free culture medium for 12 h. Cells were exposed to ioversol (25–150 mg iodine mL⁻¹, 335–521 mOsm kg⁻¹) and to mannitol with the same osmolality as ioversol (420 mOsm kg⁻¹) for 1 h at 37°C. Other NRK-52E cells were incubated in ioversol (100 mg iodine mL⁻¹, 420 mOsm kg⁻¹) for 15 min, 30 min, 60 min, 120 min, 240 min, respectively. In separate experiment, irbesartan (0.01, 0.1, 1 μmol L⁻¹) was added 1 h before incubation with ioversol (100 mg iodine mL⁻¹, 420 mOsm kg⁻¹) for 1 h. Cells were trypsinized, centrifuged and washed twice with ice-cold PBS. After the last wash, cells were resuspended using 1 × binding buffer and the cell concentration was adjusted to 1 × 10⁶ mL⁻¹. 100 μL of cell suspension was taken and added into 5 mL centrifuge tube, followed by adding 5 μL annexin V-FITC and 10 μL PI. Cells were incubated in the dark at room temperature for 15 min and resuspend in 400 μL 1 × binding buffer. Finally, cells were analyzed by the EPICS ALTRAII flow cytometer (Beckman, USA) with an excitation wavelength at 488 nm. Ten thousand cells were collected for every sample. All experiments were repeated three times. The criteria for early and late apoptotic cells are annexin V positive, PI negative and annexin V positive, PI positive, respectively.
2.5. Visualization of intracellular reactive oxygen species

Intracellular ROS production was measured by the method of Chen et al. [20]. In brief, cover glasses of confluent cells preincubated with or without irbesartan (0.01, 0.1, 1 mmol L$^{-1}$) followed by further incubation in ioversol (100 mg iodine mL$^{-1}$, 420 mOs/m kg$^{-1}$) were washed with PBS and incubated in the dark for 40 min in the presence of 10 μmol L$^{-1}$ of CM-H$_2$DCFDA at 37 °C. Cells were washed in Hank’s balanced salt solution (HBSS) three times. ROS generation was detected by a confocal microscope (Leica, Germany) as a result of the oxidation of DCFH (excitation, 504 nm; emission, 529 nm). To avoid photo-oxidation of DCF, the fluorescence images were collected using a single rapid scan and identical settings were used for all samples. Fluorescence data are expressed as percent increase over untreated samples.

2.6. Reverse transcription–polymerase chain reaction (RT–PCR)

The mRNA was isolated from NRK-52E cells using RNA-Solv Reagent (Omega Bio-tek, USA). The mRNA was eluted with the elution buffer and quantified from the absorbance at 260 nm. First-strand cDNA was synthesized from 3 μg of total RNA with M-MuLV reverse transcriptase (Toyobo Co. Ltd.) and Oligo(dt)$^{18}$ (0.5 μg μL$^{-1}$). Reverse transcription was performed at 42 °C for 60 min followed by incubation at 70 °C for 10 min. The final 50 μL PCR reaction contained cDNA, 10 pmol μL$^{-1}$ of each oligonucleotide primer, 10 mmol L$^{-1}$ of deoxynucleoside triphosphate (dNTP) and 1 U μL$^{-1}$ of Taq DNApolymerase (Toyobo Co. Ltd.). Amplification was carried out according to the following temperature profile: 94 °C, 45 s; 56 °C, 1 min; and 72 °C, 45 s. At the end of 30 cycles, the reaction was prolonged for 10 min at 72 °C. The primers for bcl-2 and bax were designed based on the sequences described by Haikun L et al. [21].The sequences of the primers were as follows: for Bcl-2: 5′-ACTTGTGCCAGATGTCCAGTCCAG-3′ (sense) and 5′-GTTCAAGTCTCATCCACAG-3′ (antisense); for bax: 5′-GGAGGAAATCCAGTCCAG-3′ (sense) and 5′-TGCAGAGATGGTCTGAC-3′ (antisense); for GAPDH: 5′-ATGGTCTACATGTTCCAGA-3′ (sense) and 5′-TCAGATCCAAACGGATACA-3′ (antisense). The primers were purchased from SBS Gene Technology Co. Ltd. (Shanghai, China). The PCR products were subjected to electrophoresis on 1.5% agarose gel and the DNA was visualized by staining with ethidium bromide under ultraviolet irradiation. The intensities of the PCR products were semiquantitatively densitometrically by using Alpha Image 2200 (Alpha Innotech Corporation, Sanleandro, CA, USA). The ratio of bcl-2 or bax mRNA to GAPDH mRNA were regarded as the relative concentration of bcl-2 and bax.

2.7. Statistics analysis

All data are expressed as means ± S.D. One-way ANOVA with post-hoc test was used to assess the statistical significance of differences. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Protective effect of irbesartan against ioversol-induced apoptosis in NRK-52E cells

NRK-52E cells incubated with ioversol as well as control cells were stained with Hoechst 33258, respectively. Chromatin in a few nuclei begins to condense amidst NRK-52E cells incubated with ioversol for 1 h indicating very early stage of apoptosis. However, almost no apoptotic nuclei were observed in control cells (Fig. 1).

Ioversol induced NRK-52E cells apoptosis in a concentration- and time-dependant manner. When the concentration of ioversol within 100 mg iodine mL$^{-1}$, cell percent in early stage of apoptosis was dramatically higher than that of late stage of apoptosis. However, when the concentration of ioversol reached to 150 mg iodine mL$^{-1}$, we observed an absolutely opposite result of comparison between early and late apoptotic cells (Fig. 2). To evaluate the effect of irbesartan on ioversol-induced cell apoptosis, the presence of apoptotic cells preincubated with or without irbesartan was further confirmed by flow cytometry. As shown in Fig. 3, irbesartan attenuated the ioversol-induced apoptosis in NRK-52E cells in a dose-dependent manner. On the other hand, a similar change in osmolality with ioversol (420 mOs/m kg$^{-1}$) obtained by the use of mannitol failed to induce apoptotic changes.

3.2. Effect of irbesartan on the production of ROS in NRK-52E cells incubated with ioversol

The exposure of NRK-52E cells to ioversol (100 mg iodine mL$^{-1}$, 420 mOs/m kg$^{-1}$) associated with a significant increase of intracellular ROS generation. Irbesartan (0.01, 0.1, 1 mmol L$^{-1}$) showed a dose-responsive suppression on the increase of intracellular ROS induced by ioversol (Fig. 4).

3.3. Effect of irbesartan on changes in mRNA expression for bcl-2 and bax in NRK-52E cells induced by ioversol

Ioversol decreased the expression for bcl-2 mRNA with a concomitant increase in bax mRNA expression. Irbesartan (0.1, 1 mmol L$^{-1}$) almost completely reversed the ioversol-induced decrease in bcl-2 mRNA and increase in bax mRNA (Fig. 5).

4. Discussion

The osmolality of CM was considered to play an important role in the pathogenesis of RCN. Low-osmolar non-ionic CM (osmolality 600–850 mOs/m kg$^{-1}$) have long been known to have fewer direct cytotoxic effects compared to high-osmolar ionic CM (osmolality 1500–1800 mOs/m kg$^{-1}$ [22,23]). This led to the generally accepted concept that the osmolality contributed to the nephrotoxicity of CM. In order to diminish the frequency of RCN, low-osmolar CMs were recommended in
place of high-osmolar CMs. However, the clinical results are worse than expected. In at-risk patients undergoing diagnostic/interventional angiography, the incidence of RCN is still relatively high despite the use of modern CM with a reduced toxicity [24]. Although the low-osmolar CMs have reduced the complication rate, hyperosmolality do not fully explain the pathogenesis of RCN.

Experimental study centers on the different mechanism of nephrotoxicity of different types of CM. It was concluded that the direct cytotoxic effects of CM molecules contribute to their cytotoxic effects besides hyperosmolality [25]. Ioversol, a non-ionic monomer iodinated CM with osmolality in the range of 502–792 mOsm kg\(^{-1}\) and viscosity in the range of 3.0–9.0 cPs at 37°C [26], has been increasingly used for diagnostic and treatment purpose on account of its low-osmolality. Yano et al. [13] have shown that ioversol induce apoptosis in a porcine renal tubular cell line (LLC-PK1 cells).

In the present study, we focused on the direct toxic action of ioversol in NRK-52E cells. We found that ioversol induced NRK-52E cells apoptosis in a concentration- and time-dependent manner determined by Hoechst staining and flow cytometry with annexin V and PI double stains. It was presented that early stage of apoptotic cells were exceeded by late stage of apoptotic cells when the concentration of ioversol increased up to 150 mg iodine mL\(^{-1}\) while the former was in dominance when the concentration of ioversol was within 100 mg iodine mL\(^{-1}\). Our findings suggest that the osmolality plays a partial effect on cell injury, but it is not a major factor. Hyperosmolality could not account for the mechanism of cell apoptosis since hyper-osmolar mannitol solution did not mimic the action of ioversol. In fact, the so-called low-osmolar CM still have an increased osmolality compared with plasma. A systematic review [27] has recently revealed that factors other than osmolality such as viscosity or direct molecular toxicity play a significant role in the pathogenesis of RCN, at least for agents with osmolarities of 800 mOsm kg\(^{-1}\) or less. Based on the results of this study, we analyzed that the cytotoxicity of ioversol might be explained by a combination of osmolality and molecular properties.
Fig. 3. Protective effect of irbesartan against ioversol-induced apoptosis in NRK-52E cells. a: control; b: cells were exposed to ioversol (100 mg iodine mL$^{-1}$, 420 mOsm kg$^{-1}$) for 1 h; c: cells were exposed to mannitol with the same osmolality as ioversol (420 mOsm kg$^{-1}$) for 1 h; d–f: irbesartan (0.01, 0.1, 1 mM) was added 1 h before ioversol treatment. (A) a–f: the cell apoptosis was assessed by flow cytometer with annexin V–FITC and propidium iodide and represented in the scatterplot. (B) a–f: data show the means ± S.D. (N=3). $p<0.01^*$ compared to early apoptotic cells in group b. $p<0.01^#$ compared to late apoptotic cells in group b.

Oxidative stress is considered to be involved in the development of RCN, because antioxidant-mediated protection on renal function has been demonstrated in vivo [6–8,28]. However, there is also controversial conclusion that CM toxicity can be dissociated from tubular cell oxidative stress since CM do not increase tubular malondialdehyde (MDA) content [29]. But to our knowledge, there is little experimental evidence on ROS formation at the tubular cell level directly. In the present study, we visualized
intracellular ROS in renal tubular cells by confocal microscopy with fluorescent probe CM-H2DCFDA. This method excludes the limitation of oxidative products MDA on the direct detection of intracellular ROS because of its short half-life and the high reactivity [30]. Data presented here showed that increased intracellular ROS formation was observed in renal tubular cells exposing to ioversol for 1 h. It suggested that ioversol induce ROS accumulation in renal tubular cells as early as 1 h. A major limitation is that the initial timing of ROS generation induced by ioversol is unknown.

Irbesartan, a selective AT1 receptor antagonist, was firstly used as a potentially prophylactic agent to prevent direct toxic action of CM on renal tubular cells in vitro. Our study showed that irbesartan reversed NRK-52E cell apoptosis induced by ioversol. And the protective effect of irbesartan was dose-dependent. These results indicate that irbesartan have antioxidant effect, which may account for the prevention against cell apoptosis induced by CM. Although the antioxidant effect has been observed, mechanism of the protective effect is still unclear, but it does not appear to be mediated through the AT1 receptor because protection in vivo was dissociated from the antihypertensive effects. This vivo experiments even state that angiotensin II receptor blockers (ARBs) could modulator the activity of NADH/NADPH oxidase, which help normalize superoxide production in vessels, thereby yielding an antioxidant-like effect [19]. In cell level, the mechanism needs further study.
Fig. 5. Effect of irbesartan on ioversol-induced changes in the mRNA expression for bcl-2 and bax in NRK-52E cells. A: control; B: cells were exposed to ioversol (100 mg iodine mL^{-1}, 420 mOsm kg^{-1}) for 1 h; C–E: irbesartan (0.01, 0.1, 1 mM) was added 1 h before ioversol treatment. The mRNA was isolated and mRNAs for bcl-2 and bax were measured by reverse transcription–polymerase chain reaction (RT–PCR). The intensities of PCR products were determined by densitometric analysis. Histograms show the relative concentration of mRNAs for bcl-2 and bax to GAPDH mRNA. Data represent the means±S.D. (N=3). *p<0.05 compared to cells incubated in ioversol in the absence of irbesartan.

The up-regulation of bax, an apoptosis-facilitatory protein and down-regulation of bcl-2, an apoptosis-inhibitory protein, depolarize mitochondrial membrane to stimulate the release of cytochrome c [31]. And cytochrome c release and caspase activation are critical events in triggering oxidant-induced apoptosis [32]. In the present study, ioversol markedly increased the expression for bax mRNA while reducing bcl-2 mRNA. Notably, the enhancement of bax mRNA and the reduction in bcl-2 mRNA induced by ioversol were reversed by irbesartan in dose-dependent manner. Therefore, it is likely that the site of action of irbesartan in attenuating ioversol-induced renal tubular cell apoptosis is upstream to the expression for bcl-2 and bax.

There are two major limitations underlying these renal tubular cell studies. Firstly, the incubation period of 1 h is potentially too short for RCM cytotoxicity to develop, so the frequency of apoptosis is quite low especially for late apoptosis and changes in chromatin structure are not significant. Secondly, mannitol used as a hyperosmotic control in our study could inhibit ROS generation, and it may be the reason why hyper-osmolar mannitol solution did not induce evident cell apoptosis as contrast media except for difference in physicochemical properties.

5. Conclusion

In summary, ioversol induced NRK-52E cells apoptosis in a concentration- and time-dependant manner via an increase in oxidative stress and subsequent to the increase in mRNA expression for bax and reduction in bcl-2 mRNA. The mechanism of cell injury was not only dependent on the osmolality of ioversol, it might be associated with its molecular properties. Irbesartan attenuated the ioversol-induced apoptosis in NRK-52E cells by reducing oxidative stress and reversing the enhancement of bax mRNA and the reduction in bcl-2 mRNA.

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