Resveratrol alleviates the cytotoxicity induced by the radiocontrast agent, ioxitalamate, by reducing the production of reactive oxygen species in HK-2 human renal proximal tubule epithelial cells in vitro

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Abstract. Radiocontrast-induced nephropathy (RIN) is one of the leading causes of hospital-acquired acute kidney injury (AKI). The clinical strategies currently available for the prevention of RIN are insufficient. In this study, we aimed to determine whether resveratrol, a polyphenol phytoalexin, can be used to prevent RIN. For this purpose, in vitro experiments were performed using a human renal proximal tubule epithelial cell line (HK-2 cells). Following treatment for 48 h, the highly toxic radiocontrast agent, ioxitalamate, exerted cytotoxic effects on the HK-2 cells in a concentration-dependent manner, as shown by MTT assay. The half maximal inhibitory concentration (IC50) was found to be approximately 30 mg/ml. Flow cytometry also revealed a marked increase in the number of apoptotic cells following exposure to ioxitalamate. In addition, the number of necrotic, but not necroptotic cells was increased. However, treatment with resveratrol (12.5 µM) for 48 h significantly alleviated ioxitalamate-induced cytotoxicity, by reducing cytosolic DNA fragmentation, increasing the expression of the anti-apoptotic protein, Bcl-2 (B-cell lymphoma 2), and survivin, activating caspase-3, preventing autophagic death and suppressing the production of reactive oxygen species (ROS). Resveratrol also suppressed the ioxitalamate-induced formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage. N-acetylcysteine (NAC), a ROS scavenger commonly used to prevent RIN, also reduced ioxitalamate-induced cytotoxicity, but at a high concentration of 1 mM. Sirtuin (SIRT)1 and SIRT3 were not found to play a role in these effects. Overall, our findings suggest that resveratrol may prove to be an effective adjuvant therapy for the prevention of RIN.

Introduction

Radiocontrast agents are a type of medical contrast medium which are used when performing computed-tomography or angiography to improve visibility and observe vessels and changes in tissues more clearly. These agents are associated with adverse effects, mainly radiocontrast-induced nephropathy (RIN), which is one of the leading causes of hospital-acquired acute kidney injury (AKI), and accounts for approximately 10% of all causes (2). Some patients may suffer from stage 3 AKI and may thus require dialysis (3,4). Once RIN has developed, the mortality rate increases significantly (4). At least three mechanisms, renal vasoconstriction, increased oxidative stress and direct renal tubular toxicity are known to be involved in the pathophysiology of RIN (5). Clinically, the standard prophylaxis for RIN is intravenous hydration and/or the oral administration of N-acetylcysteine (NAC). Intravenous hydration is mainly used to maintain renal perfusion in order to overcome radiocontrast-induced renal vasoconstriction. However, hydration is contraindicated for some cases, such as congestive heart failure and chronic kidney disease. The antioxidant agent, NAC, acts as free radical scavenger. However, certain meta-analyses and randomized trials have demonstrated...
variable and inconsistent outcomes (6,7). Therefore, the development of a novel strategy for the prevention of RIN is required.

Resveratrol (3,5,4’-trihydroxy-trans-stilbene) is a natural polyphenolic compound found in several plants (e.g., grape skins)(8). The renoprotective effects of resveratrol, particularly those regarding diabetic nephropathy, have been attributed to its antioxidant and anti-inflammatory effects (9). Thus, the aim of the present study was to investigate the protective effects of resveratrol against toxicity induced by the radiocontrast agent, ioxitalamate, in human renal proximal tubule epithelial cells in vitro.

Materials and methods

Cell and cell culture. The human renal proximal tubule epithelial cell line, HK-2, immortalized by transduction with human papilloma virus 16 (HPV-16) E6/E7 genes, was purchased from the Bioresource Collection and Research Center (BCRC), Hsin-Chu, Taiwan. The HK-2 cells were maintained in keratinocyte-serum-free medium (KSFM) supplemented with 5 ng/ml recombinant epidermal growth factor and 40 ng/ml bovine pituitary extract (Invitrogen, Carlsbad, CA, USA), and cultured in 5% CO₂ at 37°C in a humidified incubator, as previously described (10).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The HK-2 cells were seeded in 96-well plates at 1x10⁴ cells/well and used to evaluate cell viability following treatment with ioxitalamate (Telebrix™; Guerbet, Paris, France) and/or resveratrol (Tocris Bioscience, Minneapolis, MN, USA), NAC (Sigma-Aldrich, St. Louis, MO, USA), necrostatin-1 (Nec-1) (BioVision, Inc., Milpitas, CA, USA), sirolimus and everolimus (both from LC Laboratories, Woburn, MA, USA), and EX-527 and SRT-1720 (both from Selleck Chemicals, Houston, TX, USA). The HK-2 cells were incubated with complete medium at 37°C overnight first, then treated with the above-mentioned agent(s) for 48 h. Subsequently, MTT solution (Sigma-Aldrich) was added to each well followed by incubation for 4 h. The supernatant was then removed, and 100 μl of dimethyl sulfoxide (DMSO) (J.T. Baker Chemical Co., Phillipsburg, NJ, USA) was added to each well. The absorbance at 565 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader and cell viability was calculated according to the following formula: cell viability = (absorbance of the experimental group)/(absorbance of reference group) x100. The reference group was treated with an equal volume of phosphate-buffered saline (PBS) as the control.

Flow cytometry. Flow cytometry was performed using the Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Cell death resulting from either apoptosis or necrosis following treatment with ioxitalamate was analyzed by flow cytometry (BD Biosciences) with conventional protocols (11). The Cyto-ID® autophagy detection kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA) was used for the staining of autophagic vacuoles, as previously described (12).

DNA damage assay. To evaluate DNA fragmentation in HK-2 cells, the cell death detection ELISA Plus kit (Roche, Mannheim, Germany) was used. The HK-2 cells were seeded in 12-well plates at 1x10⁴ cells/well. The supernatant of the culture medium and cytoplasmic fraction was collected following exposure to ioxitalamate and/or resveratrol for 48 h. To determine the occurrence of oxidative DNA damage, the OxiSelect™ Oxidative DNA Damage ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) was used for the detection and quantification of 8-hydroxy-2’-deoxyguanosine (8-OHdG).

Western blot analysis. The HK-2 cells were homogenized in cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA), and the protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Conventional procedures were followed, as previously described (13). Primary antibodies were purchased from Cell Signaling Technology, Inc. [anti-cleaved caspase-3 (9661), anti-survivin (#2808) and anti-LC3B (#2775)], GeneTex [Irvine, CA, USA; anti-B-cell lymphoma 2 (Bcl-2; GTX127958), anti-cleaved caspase-3 (GTX61962), anti-sirtuin (SIRT)1, anti-sirtuin 3 (SIRT3; GTX89507)]. The expression of α-tubulin (sc-8305) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or GAPDH (GTX100118) or β-actin (GTX100118) and Genetex (Irvine, CA, USA) was used as the internal standard. To quantify the changes in protein expression, the levels of intensity were calculated as follows: (immunoreactive intensity of the target protein)/(immuno-reactive intensity of internal control) using NIH software (ImageJ v1.40).

Statistical analysis. Data were analyzed using the Student’s t-test, Mann-Whitney U test, or one-way analysis of variance (ANOVA) based on individual data, and are presented as the means ± SEM (standard error of the mean). In all cases, a value of p<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxic effects of ioxitalamate on HK-2 cells determined by MTT assay. Cell viability following 48 h of treatment with ioxitalamate is shown in Fig. 1. Cell viability was 80.2±2.2, 64.2±2.8, 51.8±2.5, 38.9±3.8 and 31.0±3.5% following treatment with 10, 20, 30, 40 and 50 mg/dl of ioxitalamate, respectively (n=8 experiments). Ioxitalamate induced significant cell cytotoxicity...
Resveratrol alleviates cytotoxicity induced by ioxitalamate in HK-2 cells. Cell viability following 48 h of treatment with ioxitalamate (30 mg/ml) in combination with resveratrol is shown in Fig. 3. Resveratrol alone did not influence the viability of the HK-2 cells. Ioxitalamate induced significant cytotoxicity in a concentration-dependent manner (p<0.001, as shown by one-way ANOVA, n=5 experiments). However, treatment with resveratrol at 12.5 and 25 µM increased cell viability from 53.7±2.8 to 66.8±2.1 and 73.2±3.2%, respectively (p=0.006 and 0.002, respectively). The difference in cell viability between treatment with 12.5 and 25 µM of resveratrol did not reach statistical significance (p=0.14). Therefore, resveratrol at 12.5 µM was selected for use in subsequent experiments.

Apoptosis and autophagic death induced by ioxitalamate are suppressed by resveratrol. A significant increase in cytosolic DNA fragmentation, indicative of apoptosis, was observed following treatment with ioxitalamate (30 mg/ml) compared to the controls (p=0.008, n=5 experiments). Treatment with resveratrol (12.5 µM) significantly suppressed ioxitalamate-induced apoptosis (p=0.008; Fig. 4A). A significant increase in DNA fragmentation in the culture medium, indicative of necrosis, was also observed following treatment with ioxitalamate compared to the controls (p=0.016; Fig. 4B). However, the difference in DNA fragmentation in the culture medium of the cells treated with ioxitalamate plus resveratrol and those treated with ioxitalamate alone did not reach statistical significance (p=0.584; Fig. 4B).

The activation of caspase-3 plays an important role in apoptosis (16). The expression of cleaved caspase-3, the active form of caspase-3 (both 17 and 19 kDa) was markedly upregulated following treatment with ioxitalamate for 24 h (p=0.01, n=3 experiments) and 48 h (p=0.03) . However, treatment with resveratrol significantly attenuated this increase (p=0.0002 and 0.0004 at 24 and 48 h, respectively) (Fig. 4C and D). Resveratrol also alleviated the ioxitalamate-induced activation of LC3B-II, a marker of autophagy converted from LC3B-I (p=0.0002 and 0.04 at 24 and 48 h, respectively; n=3 experiments) (Fig. 4E and F).

Changes in the expression of anti-apoptotic proteins following treatment with ioxitalamate and/or resveratrol. Anti-apoptotic proteins [e.g., survivin (17), Bcl-2 (18), cIAP (11), Bcl-xL (19)] play important roles in the death of renal tubule epithelial cells. Following treatment with ioxitalamate for 48 h, the expression of Bcl-2 significantly decreased (p=0.005, n=3 experiments); however, resveratrol significantly reversed this decrease in Bcl-2 expression (p=0.01; Fig. 5A and B). The expression of survivin was significantly downregulated following treatment with ioxitalamate for 24 and 48 h (p=0.008 and 0.0001, respectively; n=3 experiments); however, resveratrol reversed this decrease in survivin expression at 48 h of treatment (p=0.0004) (Fig. 5A and C). The expression levels of cIAP1, cIAP2 and Bcl-xL were not altered following treatment with ioxitalamate and/or resveratrol (Fig. 5A).

Resveratrol alleviates ioxitalamate-induced ROS generation without SIRT1 and SIRT3 activation. The protective effects of resveratrol are mediated through at least two mechanisms, direct antioxidant activity and SIRT1 activation (8). ROS, indicated by green fluorescence from 2',7'-dichlorodihydro-

Figure 1. Cytotoxic effects of ioxitalamate on HK-2 cells. The viability of HK-2 cells was measured by MTT assay following treatment with ioxitalamate for 48 h. ***p<0.001.
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Ioxitalamate-induced cell death patterns in HK-2 cells. Following 48 h of treatment with 30 mg/ml ioxitalamate, cell death patterns were determined by various methods. (A) PI and Annexin V staining of HK-2 cells was detected by flow cytometry. Ratio of (B) Annexin V-positive and (C) PI-positive cells by flow cytometry. (D) Representative western blots showing the expression levels of receptor-interacting protein kinase 3 (RIP3), a marker of necroptosis, examined following treatment with ioxitalamate for 24 and 48 h. (E) Compared to treatment with ioxitalamate alone, changes in cell viability were determined following 48 h of treatment with ioxitalamate plus necroptosis inhibitor necrostatin-1 (Nec-1) by MTT assay. (F) Autophagic flux by Cyto-ID® dye was measured by flow cytometry. Immuno-fluorescent distribution shown by the solid and dashed line represents treatment with ioxitalamate and the control, respectively. (G) Compared to treatment with ioxitalamate alone, cell viability following 48 h of treatment with ioxitalamate plus the autophagy inducers, sirolimus or everolimus, was measured by MTT assay. *p<0.05, **p<0.01 and ***p<0.005.

Figure 3. Resveratrol alleviates ioxitalamate-induced cytotoxicity in HK-2 cells. Changes in the viability of HK-2 cells following 48 h of treatment with ioxitalamate (30 mg/ml) plus resveratrol at 6.25, 12.5 or 25 µM were measured by MTT assay. *p<0.01 and **p<0.005.

ROS (fluorescein (DCF)), were abundantly generated following treatment with ioxitalamate for 48 h. Resveratrol suppressed the production of ROS induced by ioxitalamate (Fig. 6A). DNA fragmentation may result from oxidative stress (20). Ioxitalamate significantly induced oxidative DNA damage, as shown by the detection of the formation of 8-OHdG (p=0.0002, n=4 experiments; Fig. 6B). However, resveratrol significantly suppressed the ioxitalamate-induced formation of 8-OHdG (p=0.01; Fig. 6B). We also determined the therapeutic effects of the ROS scavenger, NAC. NAC also significantly reduced ioxitalamate-induced cytotoxicity in the HK-2 cells (p=0.001, as shown by one-way ANOVA, n=3 experiments) (Fig. 6C). However, a high concentration of NAC (1 mM) was required to increase cell viability form 52.4±3.7 to 70.2±3.3%.

Furthermore, we examined the expression of SIRT1, which is associated with reno-protection in AKI (21). The expression of SIRT1 and phospho-SIRT1 was not altered following treatment with ioxitalamate and/or resveratrol (Fig. 7A). Resveratrol is also known as a SIRT3 activator (22). Cisplatin-induced tubular damage may result from the downregulation of SIRT3 (23). In our study, SIRT3 was upregulated following treatment with
ioxitalamate. Following treatment with resveratrol, SIRT3 expression was increased even further. However, there was no difference observed in the expression of SIRT3 between the cells treated with ioxitalamate alone and those treated with ioxitalamate plus resveratrol (Fig. 7A). No changes in cell viability were observed following treatment with the SIRT1 inhibitor, EX-527 (p=0.123 in one-way ANOVA, n=3 experiments). By contrast, the SIRT1 activator, SRT-1720, reduced cell viability compared to ioxitalamate treatment (p<0.001, as shown by one-way ANOVA, n=3 experiments), particularly at a high concentration (p=0.002 and 0.00002 at 2,500 and 5,000 nM, respectively) (Fig. 7B). There are no available specific SIRT3 activators and inhibitors. These results indicated that activation of SIRT1 and SIRT3 played no roles in these effects.

Discussion

The present study demonstrated that the radiocontrast agent, ioxitalamate, exerted cytotoxic effects on HK-2 human renal proximal tubule epithelial cells in vitro. Resveratrol alleviated...
Resveratrol alleviates ioxitalamate-induced renal tubular toxicity

Ioxitalamate-induced oxidative stress and apoptosis by reversing the downregulation in Bcl-2 and survivin expression. The proximal tubule is particularly vulnerable to extensive damage along the segments by a number of toxins, including radiocontrast agents (24). Ioxitalamate has been reported to exert more potent cytotoxic effects than other radiocontrast agents in an in vitro study using the porcine proximal tubular epithelial cell line, LLC-PK1 (25). Ioxitalamate is a high-osmolar ionic monomer radiocontrast agent. In a previous study, iodine alone and hyperosmolar mannitol solution did not elicit significant cytotoxicity on human embryonic kidney (HEK 293) cells, porcine proximal renal tubular (LLC-PK1) and Madin-Darby...
canine kidney (MDCK) distal tubular renal cells in vitro (26). However, the HK-2 human renal proximal tubule epithelial cell line is a more suitable model than LLC-PK1 for in vitro toxicity studies to determine drug-induced nephrotoxicity in humans (27). To the best of our knowledge, the investigation of RIN (not ioxitalamate) using HK-2 cells has only been reported in one study to date (28). Therefore, the use of ioxitalamate to examine the therapeutic effects of resveratrol on HK-2 cells is novel.

Not only apoptosis, but also necrosis is induced as a result of cellular damage induced by radiocontrast agents (29). However, no necrosis was noted in the LLC-PK1 in vitro model of RIN (30). Again, the HK-2 in vitro model may be more compatible with the in vivo condition of RIN. In a previous study, necroptosis occurred in a murine ischemia/reperfusion injury-based approach of iomeprol-induced nephropathy, and Nec-1 prevented RIN (31). Liang et al reported that necroptosis contributed to damage to HK-2 cells in an ATP-depleted model (32). However, necroptosis was not observed in this study, and the necroptosis inhibitor, Nec-1, did not reduce ioxitalamate-induced cytotoxicity. If possible, contrast agent-induced necroptosis should be confirmed in human specimens. Besides, perhaps different stimuli cause different patterns of death, even in the same cells. Perhaps the preventive effects of resveratrol against ioxitalamate-induced damage are not sufficient as resveratrol did not prevent the ioxitalamate-induced necrosis. Currently, the most toxic radiocontrast agent, ioxitalamate, is not often used for intravenous infusion. The protective effects of resveratrol, particularly in combination with standard hydration therapy, against the nephrotoxicity induced by other radiocontrast agents should be investigated in the future.

The inhibitor of apoptosis protein, survivin, plays an important role in the regulation of cellular division and survival, particularly in embryonic and cancer cells. Lechler et al examined the expression of survivin in human proximal tubular cells, particularly at the apical membrane (33). In a previous study, the upregulation of survivin expression in renal proximal tubule epithelial cells resulted in the functional and structural recovery of the kidneys from AKI (17). In AKI, the expression of Bcl-2 in proximal renal tubular cells is also decreased, and any management which aims to increase Bcl-2 expression reduces the apoptosis of renal proximal tubule epithelial cells and improves renal function (34). The changes in the immunoreactivity of survivin were more prominent than those of Bcl-2 in our study. This suggests that survivin plays a more important role in the ioxitalamate-induced apoptosis of HK-2 cells. To the best of our knowledge, to date, no agent has been reported to increase both Bcl-2 and survivin expression in AKI. The reverse effects on both Bcl-2 and survivin expression may explain the prominent anti-apoptotic effects of resveratrol in our study. In cancer cells, resveratrol has been shown to induce apoptosis through the downregulation of Bcl-2 (35) and survivin (36). It is possible that resveratrol exerts antioxidant effects in normal cells and pro-oxidant effects in cancer cells. One explanation is the ‘Warburg effect’ (37). The glycolytic pathway is not the primary resource of energy generation and the accumulation of lactate develops in normal cells, promoting physiological pH, to maintain DNA integrity with copper bound to DNA bases hidden in a tight conformation. Resveratrol cannot easily access the protected copper ions and therefore cannot chelate and/or reduce the metal ion in the normal DNA configuration. In cancer cells, the primary energy generating mechanism is the glycolytic pathway that leads to the accumulation of lactate due to the hypoxic environment. Such a low pH may induce base rotation in DNA, particularly copper bound N7 guanine. Resveratrol then attacks exposed copper, becomes oxidized itself through a copper peroxide mechanism to induce DNA strand breakage in cancer cells (38).

Autophagy, so-called type II cell death, was triggered by RIN in our study. The cell death-promoting role of autophagy has also been shown in renal tubular cells (39). However, autophagy plays a protective role in cisplatin-induced renal tubular damage (40). Autophagy is a double-edged sword (41). The discrepancy among these studies is generally believed to be due to the different experimental conditions used. Autophagy can be either protective or detrimental in renal tubule cells, depending on the different types of injury (15). In this study, ioxitalamate-induced autophagy played a pro-apoptotic role, as the autophagy inducers, sirolimus and everolimus, potentiated ioxitalamate-induced cytotoxicity. By contrast, sirolimus has been shown to alleviate cisplatin induced nephropathy (42). Sirolimus and everolimus bind to FKBP12 and inhibit mTOR, a key regulator of cell proliferation, growth, apoptosis and survival in response to growth factors and cytokines (43). For
example, sirolimus has been shown to inhibit the growth-factor-induced proliferation of renal proximal tubule cells and promote apoptosis by blocking the survival effects of the same growth factors mediated by the inhibition of p70S6k (44). Clinically, the deterioration of renal function has been observed in some patients using mTOR inhibitors (45). To determine the definite role of autophagy in ioxitalamate-induced cytotoxicity, specific genetic approaches, such as the knockdown or transfection of related targets should be used.

Ioxitalamate induces renal tubule cell apoptosis via ROS production (46). The reno-protective effects of resveratrol through antioxidant activity have been also confirmed (8). Thus, it is not surprising that resveratrol alleviated ioxitalamate-induced ROS production in HK-2 cells. To date, the administration of NAC is still the standard treatment used to prevent RIN. Liu et al evaluated 9 randomized trials which suggested that NAC helps to prevent the decline in renal function induced by RIN (47). However, not all of the trials were consistent with the protective effects of NAC. The meta-analysis by Nallamothu et al included 20 trials with 2,195 patients who met the inclusion criteria, but the results were not significant as regards the benefits in patients treated with NAC (48). Perhaps the dosage of NAC used is not sufficient to promote the antioxidant effects in the renal proximal tubule in these clinical studies. Zhang et al demonstrated the cytoprotective effects of NAC at 1 mM against ROS-induced cytotoxicity in HK-2 cells (49), and we found that NAC alleviated ioxitalamate-induced cytotoxicity at the same concentration. Compared to NAC, resveratrol was a robust scavenger of ROS in our study.

Resveratrol is a SIRT activator. However, the expression of SIRT1 was not altered following treatment with 12.5 µM resveratrol in the present study. It is reasonable that resveratrol only activates SIRT1 effectively at a concentration >200 µM (50). In addition, no changes in cell viability were observed following treatment with the SIRT1 inhibitor, EX-527, suggesting that SIRT1 does not play a role in ioxitalamate-induced cytotoxicity in HK-2 cells. Beyond the anti-apoptotic effects of SIRT1 via the loss of Ac K382 mark on p53, SIRT1 may also upregulate SIRT3 expression in our study. Additional studies are needed to determine the role of SIRT3 in RIN should be conducted through specific genetic approaches of knockdown or transfection in the future.

In conclusion, the reno-protective effects of resveratrol against ioxitalamate-induced nephropathy was demonstrated by a decrease in ROS production and the suppression of apoptosis in HK-2 human renal proximal tubule epithelial cells. Further in vivo investigations of the therapeutic effects of resveratrol against RIN are warranted in the future.

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