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p-Cresol mediates autophagic cell death in renal proximal tubular cells

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HIGHLIGHTS

- *p*-Cresol (PC), a uremic toxin, decreases proliferation of renal proximal tubular cells (RPTC).
- PC induces autophagy in RPTC through c-Jun activation mediated p62 accumulation.
- PC promotes autophagic cell death in RPTC *via* caspase 8, followed by caspase 3 activation, which participates significantly to cell apoptosis.

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ABSTRACT

Higher serum level of *p*-cresol (PC) in chronic kidney disease (CKD) patients has been linked with CKD progression. The toxic effect of PC on diverse cells has been reported by prior studies, except for renal tubular cells. Both autophagy and apoptosis contribute to renal tubular cell death, yet evidence of its response to PC is limited and their crosstalk is still unclear. Autophagy is an important cellular process involved in toxin-induced cell death. Renal tubular cell death in tubular injury is thought to be one of the key events causing the progression of CKD. Thus, we treated rat (NRK-52E) and human (HRPTEC) renal proximal tubular cells (RPTC) with PC and found the cell proliferation was significantly decreased. Cell apoptosis was significantly increased and accompanied with the activation of autophagy as evidenced by increases in LC3-II, beclin 1 and Atg 4. We also found an increase of p62 by c-Jun activation. p62 accumulation could mediate the activation of caspase 8-dependent cell apoptosis. Conversely, knockdown of p62 by siRNA of p62 had the opposite effect by arresting LC3-II accumulation and promoting increasing cell viability. We conclude that PC triggered autophagic RPTC death *via* JNK-mediated p62 accumulation and then activated caspase 8-dependent cell death pathway. PC can be considered as one of the key events causing progression of CKD, which might affect drug disposition in CKD cases.

1. Introduction

Current available therapy is not uniformly successful in reversing, stabilizing, or preventing renal injury *via* diabetic or chronic kidney disease (CKD) (Decleves and Sharma, 2010). Diverse mechanisms (Lopez-Novo et al., 2010) have been proposed to explain the common pathway of CKD progression, such as intraglomerular hypertension, renal fibrosis, inflammation, cytokine imbalance, genetics and endogenous or exogenous toxins (Motojima et al., 1991).

CKD with renal failure is characterized by the retention of solutes normally excreted in urine. Such retained solutes, called uremic toxins (Cohen et al., 2007; Vanholder et al., 2008) render organ biochemical or physiological dysfunction. Various mechanisms contribute to the toxic effect of uremic toxin. *p*-Cresol (PC), an end product of protein metabolism (Curtius et al., 1976), is the prototype member of a larger group of protein-bound uremic toxins, as well as a metabolite of phenylalanine and phenol from diet. In clinical situations, serum PC level predicts cardiovascular mortality in dialysis patients (Meijers et al., 2008). PC has also been found to exert various *in vitro* toxic effects on cells in previous reports. It hinders the response of activated polymorphonuclears

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(Carvalho et al., 2011; Vanholder et al., 1995), inhibits platelet activating factor synthesis (Wratten et al., 1999) by human adherent monocytes, decreases endothelial cell response (Dou et al., 2002) to inflammatory cytokines, inhibits endothelial cell proliferation and repair (Dou et al., 2004), has direct toxic effect on rat liver cells by inhibiting mitochondrial respiration (Kitagawa, 2001) and induces disruption of gap junctions of cardiomyocytes (Peng et al., 2012, 2013). The role of PC in pathophysiology of renal tubular cell decrease remains unexplored.

Morphologically, apoptosis of renal tubular cells was found in kidney diseases (Devarajan, 2006; Garcia-Sanchez et al., 2010). Apoptosis is usually a response to cell microenvironment, contributing to renal parenchymal cell loss in the course of acute kidney injury (AKI) and CKD (Gobe, 2009; Sanz et al., 2008). In general, causes of renal tubular cell apoptosis include cell ischemic injury, toxins or oxidant stress, ligand-receptor interaction by FasL/Fas TNFα/TNFα receptor or angiotension II/angiotension II receptor, and change of default pathway by growth factor deficiency, loss of cell-matrix adhesion or loss of cell–cell adhesion (Sanz et al., 2008).

The role of autophagy in cell homeostasis is well recognized as a process that is considered as an alternative cell death mechanism, termed Type II programmed cell death (Baehrecke, 2005). Autophagy may engage in a complex relationship with apoptosis, processes concomitantly activating or acting in antagonism as a function of cell type, stimulus and/or environment (Eisenberg-Lerner et al., 2009; Ferraro and Cecconi, 2007; Fimia and Piacentini, 2010). Recent studies have revealed that p62/SQSTM1 (Moscat and Diaz-Meco, 2009), as sequestosome 1, plays a critical role in the formation of cytoplasmic proteinaceous inclusion. P62 acts as a signaling hub through its ability to recruit and oligomerize important signaling molecules in cytosolic speckles to control cell survival and apoptosis. Caspase 8 was also recently found to interact with p62 and initiate apoptotic cell death (Sohn et al., 2005).

Therefore, the purpose of this study was to decipher the effect of PC on RPTC cells and to investigate whether PC has the capability to trigger both autophagic and apoptotic cell death and crosstalk.

2. Methods

2.1. Reagents and antibodies

All chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). The PC stock solution, from Supelco (Bellefonte, PA, USA), was diluted in 100% methanol to different concentrations (Cohen et al., 2007): 0.98 (in normal subjects), 1.95, 15.63 (CKD cases), 31.25 (dialysis patients), 62.5, 125, 250, and 500 μg/ml. Cells in each trial were treated with varying PC concentrations or equal volume of methanol as control. Anti-Atg4, anti-beclin-1, anti-LC3B-I and-II, anti-GRP78 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), anti-caspase 8 antibody from Millipore Corp. (Bedford, MA, USA), anti-p62 and anti-caspase 8 (p18) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-NaPi-IIa antibody from Barits Biotechnologie (Regensburg, Germany), and anti-GRP94 antibody from GeuTex Inc. (Irvine, CA, USA).

2.2. Cell culture, characterization of renal proximal tubular cell

Rat renal proximal tubular cell line (NRK-52E) was purchased from American Type Culture Collection (ATCC) and human renal proximal tubular epithelial cells (HRPTECs) from Cambrex BioScience, Inc. (Walkersville, MD, USA). NRK-52E cells were grown in Dulbecco's modified Eagle medium (DMEM), containing 2 mM non-essential amino acids and 5% FBS. HRPTECs were cultured in DMEM with equal volume of F-12 nutrient mixture, 1% sodium pyruvate, 2 mM L-glutamine, 5 μg/ml insulin, 5 μg/ml transferring and 20% FBS. Cells were maintained at 37 °C in humidified atmosphere of 5% CO_2 in air, cultured for three days and medium completely replaced every three days. Characterizations of renal proximal tubular cells of NRK-52E and HRPTEC were examined from the expression of mRNA and protein levels of NaPi-IIa co-transporter. Sodium-phosphate transport was measured by radiolabeled phosphate uptake. Cells were incubated in transport medium consisting of (in mM) 137 NaCl or 137 tetramethylammonium chloride (TMA-Cl), 5.4 KCl, 2.8 $CaCl_2$, 1.2 $MgSO_4$, and 0.1 KH_2PO_4. Phosphate uptake was initiated by the addition of [^{32}P] orthophosphate to the transport medium. After 10 minutes at room temperature, cells were washed 3 times with ice-cold fresh medium in which sodium chloride was substituted with TMA-Cl and 0.5 mM sodium arsenate was added. After the uptake measurements were completed, the cells were solubilized in 0.5% Triton X-100 for 90 minutes at room temperature and analyzed with liquid scintillation spectroscopy.

2.3. Cell proliferative assay

To test the effect of PC on cell proliferation, cells were synchronized at G1/S boundary and either the synchronized NRK-52E cells or HRPTECs were released to grow with different concentration of PC using BrdU incorporation assay. Double thymidine block was used for synchronization at the G1/S boundary. Cells were incubated overnight in serum-free medium containing 2.5 mmol/l of thymidine, changed to medium supplemented with 10% FBS, and incubated for 7 h and then in thymidine-containing medium overnight. Cell proliferation was measured using a commercially available kit (BD Pharmingen, San Jose, CA, USA). Cells cultured in 96-well plates were treated with 10 mg/ml of the protein preparations for 24, 48 and 72 h. The volume of the culture medium was 100 μl. Ninety minutes before the pre-determined time periods, cells were incubated with 10 μl of bromodeoxyuridine (BrdU) labelling solution. After incubating at 37 °C for 90 min, the culture medium was removed and the cells were treated with Fixdenat for 15 min. The Fixdenat was removed and the cells were incubated with anti-POD labelling solution and incubated at room temperature for 60 min. After three washes using the BrdU wash solution, the color was developed using the substrate and read using a microplate reader at a wavelength of 370 nm with a reference wavelength of 492 nm.

2.4. Apoptosis assays by flow cytometry

Apoptosis and necrosis were determined by flow cytometry using a commercially available apoptosis detection kit (BD PharMingen, San Jose, CA, USA). Briefly, after the appropriate treatment periods, non-adherent cells were pelleted and added to trypsinized and pelleted adherent cells. The cells were resuspended in 100 ml of binding buffer containing fluorescein isothiocyanate (FITC)–annexinV and propidium iodide and incubated at room temperature for 15 min. After the incubation period, 300 ml of binding buffer was added and the cells were analyzed by FACS scan flow-cytometer (FC500, Beckman Coulter, Fullerton, CA, USA), acquiring 10,000 events (Tsai et al., 2014).

2.5. Evaluation of apoptosis by microscopy

Cells were cultured overnight and treated with different PC concentrations for 24 h. Incubated nuclei were stained with 5 mg/ml Hoechst 33342 (obtained from Molecular Probes, Eugene, OR, USA) to label DNA fragments. Cells were observed and representative photomicrographs of HRPTECs were obtained using