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Inhibition of autophagy aggravated 4-nitrophenol-induced oxidative stress and apoptosis in NHPrE1 human normal prostate epithelial progenitor cells

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ABSTRACT

4-Nitrophenol (PNP), a well-established human carcinogen, has been proven to have detrimental effects on reproductive system of male rats in previous studies. The molecular mechanisms involved PNP-induced damage remain to be established. Autophagy can exert protective effects on various cytotoxic factors that induce injury. In the present study, we aim to investigate whether autophagy is induced by PNP and the function of autophagy in PNP-induced injury in NHPrE1, a normal human prostate epithelial progenitor cell line. Our results indicate that PNP induced oxidative stress as evidenced by increased MDA levels and decreased activity of SOD and GSH-Px. PNP also increased apoptosis of NHPrE1 cells as evidenced by western blot and Hoechst 33258 staining and activated autophagy in NHPrE1 cells detected by RT-PCR and western blot. Inhibition of autophagy by 3-MA further increased PNP-induced oxidative stress and apoptosis of NHPrE1 cells. We also found that PNP-induced apoptosis was suppressed by N-acetylcysteine, suggesting oxidative stress may play an important role in PNP cytotoxicity. Furthermore, phosphorylation of mTOR protein was inhibited by PNP, indicating that PNP might induce autophagy in NHPrE1 cells via inhibiting mTOR pathway. In conclusion, these results suggest that activation of autophagy should play a protective role in PNP-induced oxidative stress and apoptosis of NHPrE1 cells, which might be mediated through mTOR pathway.

1. Introduction

4-Nitrophenol (PNP) is an important intermediate in the synthesis of organic compounds and widely used in the manufacture of pesticides, fungicides, paints, dyes, leather preservative and drugs, which can easily become anthropogenic pollutants and pose a threat to the environment and human public health (Bielska and Szymanowski, 2004). PNP has been found commonly in industrial wastewaters, pesticide residue and diesel exhaust particles (Bhushan et al., 2000; Mori et al., 2003). PNP is a major metabolite of some organophosphorus insecticides, such as parathion, fenitrothion, and methyl parathion (Kim et al., 2006). Consequently, the uncontrolled use of these insecticides increases PNP bioaccumulation (Abu-Qare et al., 2001). Moreover, since acetaminophen, which is used as a popular analgesic and antipyretic in many pharmaceutical formulations, is synthesized by PNP, the presence of PNP as an impurity in pharmaceuticals is of concern (Eichenbaum et al., 2009).

Due to the high-volume of production, application and potential toxicity, PNP has been rated as a priority pollutant and recommended to restrict its concentration in natural water below 10 ng/L by US Environmental Protection Agency (Eckenfelder, 1989). PNP is known to resist biodegradation and therefore can accumulate and may magnify from lower to higher tropic levels in organisms (Banik

Abbreviations: PNP, 4-nitrophenol; DMEM, Dulbecco's modified Eagles medium; FBS, fetal bovine serum; ITS, insulin-transferrin-selenium-X; BPE, bovine pituitary extract; EGF, epidermal growth factor; 3-MA, 3-methyladenine; LC3, light chain 3; mTOR, mammalian target of rapamycin; SEM, standard error of the mean; NHPrE1, a human normal prostate epithelial progenitor cell line.

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et al., 2008). The possibility of entering the food chain is not to be ignored, since besides surface water and sediments, PNP had been detected in fish (Lam et al., 2013). It has been detected in human urine likely due to exposure to organophosphorus pesticides (Hryhorczuk et al., 2002). PNP has an adverse impact on some fundamental biological processes, such as endocrine system (Ahmed et al., 2015; Hass et al., 2012; Li et al., 2006, 2009; Taneda et al., 2004). In addition, PNP can induced oxidative stress, apoptosis of germ cells (Mi et al., 2010, 2013).

The prostate is an important gonadal organ, which has the functions of internal and external secretion. The presence of exogenous hormones could disrupt the functional integrity of this function (Sweeney et al., 2015). Chronic exposure of humans or rodents to exogenous hormones leads to increased risk of prostate cancer (Saffarini et al., 2015). Dietary and environmental factors are gradually known to be involved in the etiology of this disease accompanied with the increasing prevalence of prostate cancer (Venkateswaran and Klotz, 2010). Our previous *in vivo* studies showed that PNP induced hormone disturbance (Zhang et al., 2013), oxidative stress and apoptosis of germ cells (Zhang et al., 2015, 2016). The effects of PNP on prostate function needs to be addressed.

Autophagy is an evolutionarily conserved process and initiated in the formation of double-membrane autophagosomes, which enfold cytoplasm and organelles and then merge with lysosomes, thus degrading the enveloped nonessential cellular components to generate energy and to build new proteins and membranes for cellular metabolism (Rabinowitz and White, 2010). Autophagy plays an important role in cell growth, survival and differentiation (Moreau et al., 2011; Noboru and Beth, 2010). Autophagy protects cells from various cytotoxic stimuli such as oxidative stress through degrading damaged organelles (Mizushima and Komatsu, 2011). Increasing the level of autophagy can alleviate apoptosis of cells induced by serum deprivation (Ding et al., 2010a), while the inhibition of autophagy can aggravate the apoptosis induced by environmental toxins (Ni et al., 2012; Duan et al., 2016). Nevertheless, when excessive autophagy is induced, autophagy is also determined as another programmed cell death, autophagic cell death, which is different from apoptotic cell death or apoptosis (Fulda and Kogel, 2015). Therefore, the appropriate regulation of autophagy is helpful for improving the defense of cells to adverse stress.

In the present study, we investigated whether autophagy is induced by PNP and the function of autophagy in PNP-induced injury in NHPrE1, a normal human prostate epithelial progenitor cell line (Jiang et al., 2010).

2. Materials and methods

2.1. Cell culture

NHPrE1 cells were cultured in 50/50 Dulbecco's modified Eagles medium (DMEM)/F12 (Gibco) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Gibco), 1% insulin-transferrin-selenium-X (ITS) (Gibco), 0.4% bovine pituitary extract (BPE) (Hammond Cell Tech), and 10 ng/ml epidermal growth factor (EGF) (Sigma) with 1% penicillin (100 U/ml) and streptomycin (100 mg/ml) (Gibco), similar to a previously described formulation (Jiang et al., 2010), at 37 °C in an incubator with humidified atmosphere of 5% CO_2.

2.2. Reagent and antibodies

PNP monomer dry crystals ($C_6H_5NO_3$, >99.9% purity, CAS 100-02-7) were purchased from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). 3-Methyladenine (3-MA), ammonium chloride (NH_4Cl) and N-acetylcysteine (NAC) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Cleaved-caspase-3, Beclin-1, microtubule-associated protein 1 light chain 3 (LC3), p62, mammalian target of rapamycin (mTOR) and p-mTOR were purchased from Cell Signaling Technologies (Beverly, MA, USA). Cell counting kit-8 (CCK8), Hoechst 33258, secondary antibodies and polyclonal antibodies against β-actin and Tublin were purchased from Beyotime Biotechnology (Beyotime, Jiangsu, China). RNA extraction Kit, RNA reverse transcription Kit and SYBR-Green Master mixture were from Takara company.

2.3. RNA extraction, reverse transcription (RT) and quantitative PCR

NHPrE1 cells were plated in 6-well plates and harvested at indicated time. Total RNA was extracted from the cells using the RNeasy Mini kit® (Takara) according to the manufacturer's instructions. The concentrations and purity of the isolated total RNA were determined spectrophotometrically at 260 and 280 nm with a Nanodrop® 8000 (Thermo Fisher Scientific). The total RNA (1 μg) was reverse transcribed to cDNA with an Omniscript® Reverse Transcription kit (Takara) with Oligo-dT primers (Takara), according to the manufacturer's protocol. The target fragments were quantified by real-time PCR using a QuantiTect™SYBR Green® PCR Kit (Takara) with 100 ng of the cDNA template. Each sample was tested in duplicate. The gene expression data were normalized to β-actin expression. The specific primer sets, designed using Primer 5 Plus software, are shown in Table 1. For quantification of real-time PCR results the threshold cycle Ct was determined for each reaction. Ct values for each gene of interest were normalized to the housekeeping gene; PCR amplification efficiencies were taken into account by amplifying various amounts of target cDNA for each reaction. Normalized values were used to calculate the degree of induction or inhibition expressed as a “fold difference” compared to normalized control values. Therefore, all data were statistically analyzed as “fold induction” between treated and control.

2.4. Protein extraction and western blotting

Proteins were extracted with a protein extraction kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The proteins were measured with the BCA Protein Assay Protocol and subsequent quantifications of LC3, Belin-1, p62, Cleaved-caspase-3, mTOR and p-mTOR proteins were determined by western blot analysis. Total protein (50 μg) was loaded in each lane of 8–15% PAGE with a MiniProtean Tetra System (BioRad; Hercules, CA, USA) using Precision Plus Protein molecular weight standards (BioRad). The proteins were transferred to nitrocellulose membranes. Membranes were incubated with fat free milk powder for one hour at RT to block non-specific binding. Primary antibodies were incubated for overnight at 4 °C. Membranes were washed and incubated with HRP-labeled goat anti-rabbit IgG as a secondary antibody, followed by visualization using enhanced chemiluminescence detection reagents (Thermo, OH192985). Bands were scanned in a computer, and their relative intensities were determined by densitometry using Scion Image v. 4.0.2 (Scion Corporation, Frederick, USA). Negative controls for western blots were membranes incubated with the appropriate pre-absorbed primary antibody or with blocking solution without primary antibodies. The relative levels of proteins were normalized to β-actin and Tublin. Densitometry was performed with Image J software.

2.5. Biochemical assay

NHPrE1 cells were prepared in 24-well plates and treated with PNP, then the culture solution was collected and centrifugated. The