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4-Nonylphenol induces apoptosis, autophagy and necrosis in Sertoli cells: Involvement of ROS-mediated AMPK/AKT-mTOR and JNK pathways

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ABSTRACT

The xenoestrogen 4-nonylphenol (NP) induces reproductive dysfunction of male rats, but the fundamental mechanism of this phenomenon is largely unexplored. Sertoli cells (SCs) are pivotal for spermatogenesis and male fertility. The involvement of autophagy in NP-induced apoptotic and necrotic death of SCs was investigated. In this study, 24-h exposure of SCs to 20–30 μM NP decreased cell viability, caused G2/M arrest, triggered $\Delta\Psi$m loss, increased ROS production and induced caspase-dependent apoptsis, necrosis as well as autophagosome formation. NP-induced autophagy was confirmed by monodansylcadaverine-staining and LC3-I/LC3-II conversion. Furthermore, NP up-regulated the ^{Thr172}p-AMPK/AMPK and $^{Thr183/185}$p-JNK/JNK ratios. This was followed by the down-regulation of ^{Ser473}p-Akt/Akt, ^{Thr1462}p-TSC2/TSC2, ^{Ser2448}p-mTOR/mTOR, ^{Thr389}p-p70S6K/p70S6K and $^{Thr37/45}$p-4EBP1/4EBP1. Intriguingly, NP-induced apoptosis, autophagy and necrosis could be inhibited through blocking ROS generation by *N*-acetylcysteine. Autophagy inhibitor 3-MA enhanced NP-induced apoptosis and necrosis. Moreover, The activation of AMPK/mTOR/p70s6k/4EBP1 and JNK signalling pathways induced by NP could be efficiently reversed by pretreatment of *N*-acetylcysteine or 3-MA. Collectively, our findings provide the first evidence that NP promotes apoptosis, autophagy and necrosis simultaneously in SCs and that this process may involve ROS-dependent JNK- and Akt/AMPK/mTOR pathways. Modulation of autophagy induced by NP may serve as a survival mechanism against apoptosis and necrosis.

1. Introduction

4-Nonylphenol (NP) is ubiquitous in aquatic and terrestrial ecosystems and is the product of the biological breakdown of alkylphenol polyethoxylates and non-ionic surfactants that are widely used in commercial and domestic applications (McAdam et al., 2011). In China, NP concentrations of 30.05–288.75 μg/L have been measured in untreated surface waters from reservoirs and rivers that supply water to local waterworks (Jin et al., 2014). Mean exposure to NP was estimated to be 520 ng/kg body weight/day in Chinese adults (Niu et al., 2015). Moreover, the geometric mean concentration of urinary NP was 15.92 μg/g in children and students aged from 3 to 24 years old in Guangzhou City (Li et al., 2013b). NP tends to bio-accumulate in the body tissues and organs due to its lipophilic properties (De Falco et al., 2014). NP has the capacity to bind to estrogen receptors within target cells to mimic the action of endogenous estrogens. This capacity potentially disturbs the endocrine and reproductive systems (Liu et al., 2014). Despite the relatively low concentration of NP detected in natural water, drinking water and food, NP affects human health. In fact, there is growing concern and worldwide debate regarding its association with male reproductive disorders.

The effect of xeno-oestrogen NP on male reproductive systems has been investigated in both *in vitro* and *in vivo* models. Results from rodent studies suggested detrimental effects of NP on male reproductive function. These effects include testicular apoptosis,

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seminiferous tubule degeneration, reduction in the number of testicular germ cells and SCs, sperm abnormality, and decreases in sperm quality, counts and survival (Ponzo and Silvia, 2013). NP has also been found to induce apoptosis of various cell lines *in vitro* (Ying et al., 2012), in particular, testicular SCs (Choi et al., 2014; Liu et al., 2014). Recently, several studies have established NP-induced apoptosis through mechanisms that may be initiated by increased endoplasmic reticulum stress and reactive oxygen species (ROS) generation (Choi et al., 2014; Hu et al., 2014). NP-induced ROS targeted COX-2, Akt and Fas/FasL (Manente et al., 2011), or activated MAPK signaling (Liu et al., 2014). SCs act as nurse cells and provide nutrients to foster germ cells during mammalian spermatogenesis (Liu et al., 2014). Since SCs have been found to be targets of NP (Monsees et al., 2000), we suppose that NP-induced apoptosis of SCs is regulated by ROS-mediated signalling. Cell growth is tightly regulated by cell-cycle checkpoints. Although two reports have noted that NP exhibits cytotoxic effects through decreasing cell viability via cell-cycle arrest at the G2/M phase (Kudo et al., 2004; Qi et al., 2013), whether NP-induced suppression of Sertoli cell proliferation was due to specific block in the G2/M phase remains unclear.

Cell apoptosis and autophagy are two major morphologically distinctive forms of programmed cell death (PCD) that play a significant role in the development and control of male reproductive functions (Bustamante-Marin et al., 2012). Autophagy is induced in response to diverse stress stimuli that ultimately result in apoptosis (Hamacher-Brady and Brady, 2015). Ample evidence demonstrated that oxidative stress, including ROS production, can induce either apoptosis or autophagy, or both, depending on the cellular content (Suzuki et al., 2015). Whereas no study to date has investigated the ability of the NP-induced over-expression of ROS to stimulate an autophagic response, not to mention the crosstalk between apoptosis and autophagy. Although many questions remain unanswered regarding mechanisms underlying autophagy induction and the role of autophagy, recent evidence confirms that autophagy can be activated by JNK (Xu et al., 2014), and that the mammalian target of rapamycin (mTOR) serves as a master regulator of autophagy induction (Li et al., 2015). Moreover, a well-established upstream regulator of mTOR is Akt signalling which has been shown to inhibit autophagy or control apoptosis (Sun et al., 2010; Buck Louis et al., 2014). Recent studies have shown that AMPK/mTOR signalling plays an important role in regulating autophagy (Sun et al., 2010; Wu et al., 2011). It is highly probable that the AMPK/Akt/mTOR pathways and JNK signaling cascade are involved in NP-induced PCD in SCs, but this probability has not yet been proved by reported evidence.

This study explores the cytotoxic effects of NP in SCs, and for the first time, examines whether NP causes autophagy in SCs. In addition, it investigates the molecular mechanisms underlying NP-induced apoptotic and autophagic death in SCs. More importantly, it uses ROS scavenger *N*-acetyl-cysteine (NAC) and the autophagy inhibitor 3-methyladenine (3-MA) to verify the underlying mechanisms of the NP-induced apoptosis and autophagy respectively. It provides the novel evidence supporting the important role of ROS-mediated signalling and the involvement of AMPK/Akt/mTOR and JNK pathways in NP-induced PCD.

2. Materials and methods

All experimental protocols using animals and cell samples were approved by Wuhan Institutes for Biological Science, Chinese Academy of Sciences. All methods were carried out in accordance with the approved guidelines.

2.1. Reagents and antibodies

NP (mixture of isomers; CAS no. 84852-15-3; empirical formula $C_{15}H_{24}O$; molecular meight 220.35) with 99% analytical standard was purchased from ACROS Organics (Leicestershire, UK). The purity of NP used in this study was 99%. Monodansylcadaverine (MDC), NAC and 3-MA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trypsin was purchased from Amresco (Solon, OH, USA). Type I collagenase was purchased from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Evergreen Biological Engineering Company (China). Dulbecco's modified Eagle's medium (DMEM) containing a high concentration of glucose was purchased from Hyclone Company (Logan, UT, USA). Cell counting kit-8 (CCK8) was purchased from Dojindo Laboratories (Kumamoto, Japan). The Annexin V-FITC apoptosis detection kit was purchased from KeyGEN Biotech (Nanjing, China). ROS assay kit, 5,50,6,60-tetrachloro-1,10,3,30-tetraethylben zimidazolcarbocyanine iodide (JC-1), cell cycle assay kit, penicillin–streptomycin solution, BCA protein assay kit, Western and IP cytolysis were purchased from Beyotime Company of Biotechnology (Shanghai, China). Rabbit monoclonal antibody against pro-caspase-3 and rabbit polyclonal antibody against cyclin A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit monoclonal antibodies against cytochrome c, Apaf-1, cleaved-caspase-3, Bim, Bad, Bax, Bcl-2, Mcl-1, p21, cyclin D1, cyclin B1, LC3, Beclin-1, AMPK, p-AMPK^{Thr172}, JNK, p-JNK $^{Thr183/Thr185}$, Akt, p-Akt^{Ser473}, TSC2, p-TSC2^{Thr1462}, mTOR, p-mTOR^{ser2448}, 4EBP1, p-4EBP1$^{Thr37/Thr45}$, p70S6K, p-p70S6K^{Thr389} and rabbit polyclonal antibody against p27^{Kip1} were purchased from Cell Signalling Technology (Cambridge, MA, USA). Secondary antibody (horseradish peroxidase-labelled goat anti-rabbit) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.2. Isolation and culture of rat Sertoli cells and cell treatments

Sprague–Dawley rats were purchased from the animal laboratory of Tongji Medical College (Wuhan, China). In each experiment, three 18–20-day-old male rats were randomly chosen from the same nest to isolate SCs, as at this stage the majority of the cells inside the seminiferous tubules in the rat testes are SCs. The primary culture of SCs was prepared using previously described sequential enzymatic procedures (Song et al., 2011). The final SCs suspension was supplemented with 20% FBS and seeded in a culture bottle in a humidified atmosphere of 95% air–5% CO_2 at 35 °C. The DMEM medium containing 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin was renewed every 2 days.

NP was dissolved in DMSO as stock solution and diluted with DMEM medium to the indicated concentrations (0, 10, 20 and 30 μM) before usage. The final DMSO concentration in the medium was not more than 1‰ (v/v), which did not influence the viability of SCs. The blank control and solvent control SCs were cultured with no DMSO and 1‰ DMSO respectively.

2.3. Cell viability and morphology

Cell viability was determined using CCK-8 assay. In brief, SCs (2–4×10^3 per well) were plated in 96-well microplates. After growing for 48 h, the cells were treated with indicated concentrations of NP for 24-h. The CCK-8 solution (10 μL) was added to each well for an additional 2-h. Finally, sample absorbance at 450 nm (650 nm as reference wavelength) was measured by using the ELISA reader (Bio-Rad instrument Group, Hercules, CA). Cell viability was expressed as the percentage of viable cells, assuming that the absorbance of the untreated cells was 100%. Cell viability