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# Nonylphenol effects on human prostate non tumorigenic cells

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ABSTRACT

Nonylphenol (NP) is an industrial chemical with estrogenic activity both *in vivo* and *in vitro*; estrogens play a critical role in the development of prostate and may be the cause of some pathological states, including cancer. In this study we examined the effects of NP on human prostate non tumorigenic epithelial cells (PNT1A) investigating on cell proliferation, interaction with estrogen receptors (ERs) and gene expression of genes involved in prostate diseases. We found that NP affects cell proliferation at $10^{-6}$ M, promoting a cytoplasm-nucleus translocation of ERα and not ERβ, like the natural estrogen 17β-estradiol (E2). Moreover, we showed that NP enhances gene expression of key regulators of cell cycle. Estrogen selective antagonist ICI182780 in part reverted the observed effects of NP. These results confirm the estrogenic activity of NP and suggest that other transduction pathways may be involved in NP action on prostate.



## 1. Introduction

Nonylphenol (NP) is generated by the degradation of nonylphenol ethoxilates (NPEs). NPEs are chemicals widely used as non ionic surfactants in the manufacture of rubber and plastic for domestic, industrial and agricultural products (Fiege et al., 2000; Langford, 2002; Vazquez-Duhalt et al., 2005). Due to its high hydrophobicity and low solubility NP accumulates in several environmental matrices, such as seas, rivers, soils, groundwaters and sediments, in a range between $10^{-13}$ to $10^{-6}$ M (Berryman et al., 2004; Careghini et al., 2015; Vazquez-Duhalt et al., 2005). It was also found as a contaminant of food and drinking water (Gyllenhammar et al., 2012; Maggioni et al., 2013; Soares et al., 2008). Human exposure to NP may occur by inhalation, cutaneous absorption and ingestion of contaminated food or water (Guenther et al., 2002; Soto et al., 1991). In this regard, NP was found in human amniotic fluid, urine and plasma samples, breast milk, fetal cord serum, placenta and maternal blood, with levels in these tissues generally varying from $10^{-10}$ to $10^{-9}$ M (Calafat et al., 2005; Huang et al., 2014). Hovewer, in some cases, concentrations of NP have been reported to be much higher in human samples. In this regards in breast milk of healthy Italian women, Ademollo et al. (Ademollo et al., 2008) detected about $10^{-7}$ M of NP as well as in urine and in plasma of textile and housekeeping workers were found the same NP levels (Chen et al., 2005). Instead, in maternal cord blood, Chen et al. (Chen et al., 2008) found a concentration of NP of about $10^{-6}$ M.

NP belongs to the subclass of endocrine disrupting chemicals (EDCs) that mimic the endogenous estrogens, called xenoestrogens (Falconer et al., 2006; Wozniak et al., 2005), that also includes dioxins, polychlorinated biphenyls, hexachlorocyclohexane, octylphenol and bisphenol A (Kuo et al., 2012; Forte et al., 2016). Estrogenic activity of NP has been reported both *in vitro* (de Weert et al., 2008; Soto et al., 1991; White et al., 1994) and *in vivo*, in reproductive and in non reproductive tissues, such as brain (Blom et al., 1998; Laws et al., 2000; Nagel et al., 1999; ter Veld et al., 2008; Xia et al., 2008; De Falco et al., 2014, 2015) and it has been shown that NP interacts with estrogen receptors (ERs), competing with the natural estrogen 17β-estradiol (E2) (Bechi et al., 2006; Kwack et al., 2002; White et al., 1994), although with less specificity (Bechi et al., 2010; Blom et al., 1998; Nagel et al., 1999).

Estrogens predominantly bind two nuclear receptors: the estrogen receptor alpha (ERα) and the estrogen receptor beta

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(ERβ). Both ERα and ERβ bind to the active form of estrogen E2, with similar affinities (Siewit et al., 2010). ERs mediated estrogen signaling in reproductive tissues but also in non-reproductive tissues as the brain, lungs, colon, prostate and cardiovascular system (Shanle and Xu, 2011). In the cell, E2 is able to activate both genomic and non-genomic responses. In the genomic pathway, E2 mediates target gene regulation through binding directly DNA at estrogen response elements (EREs) or indirectly through transcription factors like Sp1 or AP-1 (Kushner et al., 2000; Saville et al., 2000); the non-genomic pathway is not mediated by ERα or ERβ but through the G-protein coupled receptor, GPR30, that localizes in the plasma membrane activating rapid responses such as increased levels of c-AMP (Filardo et al., 2007; Lin et al., 2009; Wang et al., 2010).

Several studies have suggested the role of estrogens in normal and aberrant growth of prostate, alone or in synergy with androgens (Ho et al., 2011) and epidemiological and experimental studies underline a relationship between estrogens/xenoestrogens and pathogenesis of prostate cancer (PCa) (Bostwick et al., 2004; Ho et al., 2006). Neonatal treatment with Bisphenol A (BPA), a well-known xenoestrogen, was reported to induce high-grade prostatic intraepithelial neoplasia in Sprague-Dawley rats (Ho et al., 2006) and to increase cell proliferation of urogenital sinus epithelium (UGE) in the primary prostatic ducts of CD1 mice (Timms et al., 2005). BPA was also found to increase the number of basal epithelial cells in the adult prostate of BALB/c mice (Ogura et al., 2007). Recently, Tarapore et al. (2014) found in prostate cancer patients high BPA urinary levels compared to non prostate cancer patients.

Despite the relationship between estrogen and prostate, the precise functions of the two ER subtypes in this gland remain unclear; several authors have reported differential expression patterns of the two receptors between the epithelial and stromal compartment of the prostate, with ERα localized predominantly in the stroma and ERβ in the epithelium (Fixemer et al., 2003; Leav et al., 2001; Tsurusaki et al., 2003; Weihua and Warner, 2002).

Considered this background and given the human exposure to EDCs, the estrogen-like action of NP is conceivable to influence the normal growth of prostate and to be the cause of some pathological states of this gland, affecting the male reproductive functions. Thus, in this study we evaluated the effects of NP on the proliferation of human non tumorigenic prostate cells (PNT1A), which is responsive to sex hormones (Stephen et al., 2004), the cellular localization of ERα and ERβ after exposure to NP and gene expression of genes involved in pathological states of the prostate such as *cyclin D*, *Ki67*, *p53* and *IL1-β*. We performed the same experiments treating cells with the natural estrogen E2 and with the selective antagonist of estrogen receptors ICI 182,780 (Osborne et al., 2004). This study aims to facilitate the understanding of the mechanisms by which xenoestrogens and estrogens may exert their activity on prostate.

## 2. Material and methods

### 2.1. Cell culture

PNT1A cells (a human prostate cell line established by immortalization of adult prostate epithelial cells) were obtained from the European Collection of Cell Culture (ECACC Salisbury, UK). PNT1A cells were grown in red phenol free RPMI-1640 medium (LONZA, Basel, Switzerland), supplemented with 10% dextran-coated charcoal fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 2 mM L-glutamine and antibiotics (100 U/mL penicillin/streptomycin, 10 μg/mL gentamicin) in a humidified incubator at 37 °C and 5% $CO_2$. When confluent, the cells were detached enzymatically with trypsin-ethylenediamine tetra-acetic acid and subcultured into a new cell culture flasks. The medium was replaced every 2 days. Cells were used for experiments between passages 5–20.

### 2.2. Chemicals

Nonylphenol (NP), 17β-Estradiol (E2) and selective estrogen antagonist ICI 182,780 (ICI) were purchased from Sigma-Aldrich (Sigma Aldrich, St. Louis, MO) and were dissolved in DMSO (Invitrogen Carlsbad, CA). NP, E2 and ICI were diluted with culture medium at final concentrations from $10^{-12}$ to $10^{-6}$ M for NP and E2 and $10^{-5}$ M for ICI. In all the experiments with the inhibitor, ICI was added 1 h prior to start treatments. Final concentration of DMSO in the medium did not exceed 0.01%.

### 2.3. MTT assay

The effects of NP or E2 on PNT1A cells proliferation was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-3,5 diphenyl tetrazolium bromide (MTT) test (Sigma Aldrich, St. Louis, MO). Cells were seeded in 200 μL of growth medium ($5 \times 10^4$ cells/well) in 96-well plates and hormone deprived (1% FBS) for 24 h. Then, NP or E2 was added after dilution to an appropriate concentration (from $10^{-12}$ M to $10^{-6}$ M), with or without $10^{-5}$ M ICI. Control cells were treated with vehicle (DMSO 0.01%). The test was performed for 24 h of incubation. After the incubation period, 10 μL of a MTT solution was added to each well. After 4 h of 37 °C incubation, the culture medium was gently aspirated and replaced by 100 μL of DMSO/isopropanol (1:1) in order to dissolve the formazan crystals. The absorbance of the solubilized dye, which correlates with the number of living cells, was measured with a microplate reader at 570 nm. The test was performed in triplicate.

### 2.4. Fluorescence microscopy

PNT1A cells were seeded in 4-well chamber slide (Sarstedt, Nürnbrecht, Germany) overnight at a density of $5 \times 10^4$/well. After 24 h serum starvation (1% FBS), cells were incubated with $10^{-6}$ M NP or $10^{-6}$ M E2, with or without $10^{-5}$ M ICI for four different times: 15 min, 1 h, 2 h and 6 h. Control group was treated only with vehicle (DMSO 0.01%). Control and treated cells were fixed with methanol for 10 min at RT, permeabilized with 0.25% Triton X-100 for 10 min, washed in PBS, and blocked in 5% normal goat serum (NGS) for 1 h at RT. Then cells were subjected to immunofluorescence protocol using a mouse monoclonal anti-human ERα (Santa Cruz Biotechnology, Santa Cruz, CA, Cat. sc-8005) and a mouse monoclonal anti- human ERβ antibodies (Santa Cruz, Cat. sc-373853), diluted 1:100 in 1% NGS for 24 h at 4 °C. For detection of ERα and ERβ, secondary goat anti-mouse Alex Fluor 488 (Cat. A11001, Invitrogen, Carlsbad, CA), dilution 1:200 in 1% NGS was used. Cell nuclei were counterstained with 0.1 μg/mL Höechst (Invitrogen, Carlsbad, CA, Cat. H3570). Negative control for ERα and ERβ was performed by avoiding incubation with the primary antibodies (Supplementary data Fig. S1). Fluorescent images were taken on an Axioskop (Carl Zeiss, Milano, Italy) epifluorescence microscope using a 40× objective. Axiocam MRc5 and the acquisition software Axiovision 4.7 (Carl Zeiss) were used to capture the images in different channels (Alexa Fluor 488, Höechst 33258). Three independent immunofluorescence experiments were performed for each experimental conditions and different fields were randomly chosen for data analysis. Then, images were processed with the Image J software (developed by Wayne Rasband, National Institutes of Health, USA).