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Nonylphenol acts on prostate adenocarcinoma cells via estrogen molecular pathways

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ABSTRACT

Estrogens play a role in the patho-physiology of the prostate. In the present work we studied the effects of nonylphenol (NP), a xenoestrogen, on human adenocarcinoma prostate cells (LNCaP). In order to understand molecular and cellular involvement, we observed the effects on cell cycle and we investigated the expression and the cellular localization of estrogen receptors and gene expression of cyclin D1, ki-67, c-myc, IL-8, IL-1β. We performed the same experiments with 17β-estradiol (E2), the most abundant estrogen circulating in nonpregnant humans in order to compare these two different substances. We demonstrated the ability of 1×10^{-10} M NP to induce proliferation of LNCaP, S-phase progression, increase of ERα expression and its translocation from the cytoplasm to the nucleus. Moreover, we observed an up-regulation of key target genes involved in cell cycle and inflammation process. Particularly, after NP treatment, IL-8 and IL-1β mRNA levels are increased more than 50% indicating a major NP involvement in inflammation processes than E2. These data suggest the proliferative effects of NP on prostate adenocarcinoma cells and highlight some aspects of molecular pathways involved in prostate responses to NP.

1. Introduction

Prostate development is influenced by the levels of circulating androgens and estrogens (Prins and Korach, 2008). In recent years, there are several data, from *in vitro* and *in vivo* studies which demonstrate the pivotal role of estrogens in prostate physiology, exerting both protective and deleterious effects (Ho et al., 2011). When estrogen levels (particularly 17β-estradiol and estrone) increase and conversely androgen levels decrease, prostate cells reprogram their cell cycle, resulting in an increased proliferation, which in turn lead to an aberrant growth of the gland (Ho et al., 2011). As final results, the prostate undergoes in benign prostatic hyperplasia and prostate cancer (Prins and Korach, 2008). Different confounding factors are associated with human prostate cancer risks such as family history, age, hormones, environmental compounds, unknown etiological factors and antiretroviral therapy (Bostwick et al., 2004; Hentrich and Pfister, 2017). The increase in the number of cases of prostate cancer, especially in industrialized countries suggest an association with the exposure to environmental pollutants (Sweeney et al., 2015).

The substances with estrogens mimicking actions are called xenoestrogens (De Falco et al., 2015). The most studied xenoestrogens are Bisphenol A and alkylphenols, which include Octylphenol and Nonylphenol (NP). These compounds are widely used in plastics formulation as non ionic surfactants, in agricultural products, in personal care products and therefore found as contaminants in rivers, lakes, seas, groundwater and sediments (Asimakopoulos et al., 2012; Careghini et al., 2015). They can act on different molecular targets and humans are exposed through skin absorption, inhalation and ingestion of contaminated food and water (Nappi et al., 2016). Xenoestrogens have been reported to bind both estrogen receptor α (ERα) and β (ERβ) and may enhance the processes of estrogen-related cancers (Hwang et al., 2013; In et al., 2015; Park and Choi, 2013). In particular, NP is able to inhibit adenocarcinoma gastric cell proliferation inducing apoptosis in a time and dose dependent manner (Manente et al., 2011). Regarding the prostate, epidemiological studies as well as *in vitro* and animal studies suggest the relationship between EDC exposure like triclosan and benzophenone-1 and prostate cancer (Hess-Wilson and Knudsen, 2006; Kim et al., 2015; Koutros et al., 2013). We recently demonstrated

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tha NP induces cell proliferation of human non tumorigenic prostate cells, affecting estrogen related gene expression and ERα cellular localization (Forte et al., 2016). Given this background, in this work we evaluated the effects of NP, comparing to the endogenous hormone 17β-estradiol (E2) on human prostate adenocarcinoma cells (LNCaP). These cells represent a useful *in vitro* model of prostate and they are often used for the study of exogenous and endogenous compound action on prostate since they are hormone responsive and express all the prostate specific markers (Horoszewicz et al., 1983). We analyzed the effects on proliferation, cell cycle, localization of ERs and genetic expression of key target genes (cyclin D1, ki-67, c-myc, IL-8, IL-1β) involved in the normal and abnormal growth of prostate.

2. Materials and methods

2.1. Cell culture

LNCaP cells (CRL-1740™ American Type Culture Collection, Manassan, VA) were grown in RPMI 1640 (Gibco, Invitrogen), supplemented with 10% FBS, 2 mM glutamine, 1× non essential amminoacid, 1× penicillin/streptomycin, 10 μg/mL gentamycin (Euroclone) at 37 °C, 5% CO_2 in an humidified incubator. When 70% confluent, cells were enzymatically detached with trypsin-EDTA (Euroclone) and seeded in a new cell culture flask. Medium was changed twice a week. Cells were used from passage 9 to 20.

2.2. Chemicals

Nonylphenol (NP; 290858, technical grade, Sigma Aldrich) and 17β-estradiol (E2, E8875, HPLC purity > 98%, Sigma Aldrich) were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO) and dissolved in DMSO (Invitrogen Carlsbad, CA). Then, NP and E2 were diluted in RPMI 1640 red-phenol free at the concentrations used for the experiments. Control cells were treated with vehicle (DMSO 0,01%).

2.3. Treatment

LNCaP were treated with NP and E2 from 1×10^{-6} M to 1×10^{-12} M for 48 h in order to perform the MTT assay. MTT assay allowed us to set compounds concentration to use for the subsequent experiments. Immunofluorescence was performed after 2 h and 6 h of exposure with 1×10^{-10} M NP and 1×10^{-9} M E2. FACS, Western Blot and RT-qPCR were carried out after 48 h of exposure to 1×10^{-10} M NP and 1×10^{-9} M E2.

2.4. MTT assay

NP and E2 effects on cell viability were evaluated through MTT assay. LNCaP were seeded at a density of 1×10^4/well in 96 multiwell and starved (FBS 1%) for 24 h and exposed for 48 h to NP and E2 (1×10^{-6} M to 1×10^{-12} M). Briefly, 10 μL of MTT were added to each well. After 4 h 37 °C 5% CO_2 of incubation, a solution of isopropanol and DMSO (1:1) was added in order to dissolve the crystal of formazan produced in each well (Perna et al., 2018). Then, the solution was read at 570 nm using a microplate reader. The value of absorbance is proportional with the number of living cells. Each MTT assay was performed in triplicate.

2.5. Fluorescence-activated cell sorting (FACS) analysis

Fluorescence-activated cell sorting (FACS) analysis was used to evaluate the distribution of LNCaP cells in cell cycle phases, analyzing the content of DNA after 48 h of treatment with 1×10^{-10} M NP and 1×10^{-9} M E2. In order to synchronize cells in G0/G1 phase, after 1% FBS starvation, LNCaP were washed with PBS, centrifuged at 800 g for 5min and fixed in 70% ethanol. Then, pellet was resuspended in 100 μg/ml RNAse for 30 min at 37 °C. After, 20 μg/ml propidium iodide was added to each sample at 4 °C for 30 min in the dark. FACScan™ flow cytometry system (Becton Dickinson, San Jose, CA) was used to analyze cell distribution. For each sample, 5×10^4 events were analyzed and percentage distribution in each phase of cell cycle was calculated (Perna et al., 2017). Each experiment was performed in triplicate.

2.6. RNA extraction and RT-qPCR

Expression levels mRNA of estrogen target, proliferation, and inflammation genes were analyzed using real-time PCR (Esposito et al., 2015). Total RNA from control and treated LNCaP with 1×10^{-10} M NP and 1×10^{-9} M E2 for 48 h, was extracted using Trizol (Life Technologies). After purification of genomic DNA with TURBO DNA-free™ Kit (Ambion, Life Technologies), the total amount of RNA was quantified with a NanoDrop spectrophotometer. cDNAs were synthesized from 1 μg RNA using the High Capacity cDNA Reverse Transcriptase (Life Technologies) and quantitative PCR was performed by using the 7500 Real-Time PCR System and SYBR® Select Master Mix 2× assay (Applied Biosystem). All primers used were designed according to the sequences published on GenBank using Primer Express software version 3.0. The amount of target cDNA was calculated by comparative threshold (Ct) method and expressed by means of the $2^{-\Delta\Delta Ct}$ method using hypoxanthine phosphoribosyltransferase 1 (HPTR1) as housekeeping gene (Speranza et al., 2015). See Table 1 for primer details.

2.7. Protein extraction and Western blot analysis

For protein extraction LNCaP cells were seeded in 10 cm cell dishes. After 48 h of treatment with 1×10^{-10} M NP and 1×10^{-9} M E2, confluent control and treated cell dishes were putted on ice for 10 min and washed with ice cold PBS. Then, PBS-EDTA was added, cells were scraped, collected and centrifuged for 5 min at 4000 g at 4 °C. Pellets obtained were resuspended for 30 min with RIPA lysis buffer containing protease and phosphatase inhibitors cocktail (Santa Cruz). Homogenates were centrifuged at 12,000 g for 20 min and total protein amounts were defined using BCA protein assay reagent kit (PIERCE). For each sample, 50 μg of protein was boiled for 5 min in SDS buffer [50 mMTris-HCl (pH 6.8), 2 g 100 mL^{-1}SDS, 10% (v/v) glycerol, 0.1 g 100 mL^{-1}Bromophenolblue], separated on 10% SDS-PAGE and transferred to a PVDF membrane for blotting (Trans-Blot® Semi-Dry Transfer Cell, Biorad) (Zizza et al., 2018). The membranes were incubated for

Table 1
Details of primers used for RT-qPCR.

Gene	*Forward*	*Reverse*
Cyclin D1	5′-CGTGGCCTCTAAGATGAAGGA-3′	5′CGGTGTAGATGCACAAGCTTCTC-3′
Ki67	5′-CCCGTGGGAGACGTGGTA-3′	5′-TTCCCGTGACGCTTCCA-3′
p53	5′-TCTGTCCCTTCCCAGAAAACC-3′	5′-CAAGAAGCCCAGAAACGGAAA 3′
c-myc	5′-AGGGTCAAGTTGGACAGTGTCA-3′	5′- TGGTGCATTTTCGGTTGTTG-3′
IL-8	5′-CTGGCCGTGGCTCTCTTG-3′	5′-CTTGGCAAAACTGCACCTTCA-3′
IL-1β	5′-ACGATGCACCTGTACGATCACT-3′	5′-CACCAAGCTTTTTTGCTGTGAGT-3′
HPTR1	5′-GACTTTGCTTTCCTTGGTCAGGCA-3′	5′- ACAATCCGCCCAAAGGGAACTGA-3′

1 h with blocking buffer (TBS, 0.05% Tween-20 and 5% milk) at room temperature and after blocking were incubated overnight at 4 °C with primary antibodies diluted in TBS-T containing 2% milk. Primary antibodies used were: rabbit polyclonal anti-human ERα (1:200, Santa Cruz – sc544), rabbit polyclonal anti-human ERβ (1:200, Santa Cruz sc-8974), rabbit polyclonal Cyclin D1 (1:200, Abcam – ab 74646) and rabbit polyclonal anti-human β-actin (1:200, Santa Cruz sc-7210). The day after, the membranes were washed four times for 10 min in TBS, 0.05% Tween-20 and incubated with secondary antibody for 1 h. Secondary antibody used was goat anti-rabbit IgG (HRP) (1:3000, Abcam ab-6721) and it was diluted in TBS-T containing 2% milk. After incubation, the membranes were washed again four times for 10 min and specific protein bands were detected with chemiluminescence using the C-DiGitChemiluminescent Western Blot Scanner (LI-COR). Western blots were analyzed using Image Studio Software to determine optical density (OD) of the bands. The OD reading was normalized to β-actin to account for variations in loading. Western blots were performed as previously reported in Di Lorenzo et al. (2018) and all experiments were performed in triplicates.

2.8. Immunofluorescence

LNCaP cells were cultured in 4-well chamber slides (Sarstedt, Nürnbrecht, Germany) at a density of 5×10^4 cells overnight. After 24 h 1% FBS starvation, cells were treated with 1×10^{-10} M NP and 1×10^{-9} M E2 for 2 h and 6 h. Then, cells were fixed with ice cold methanol for 10 min, washed with PBS, permeabilized with 0,4% Triton in PBS for 10min and blocked with 5% normal goat serum (NGS) for 30min. Then, cells were incubated overnight at 4 °C with anti-human ERα antibody (Santa Cruz SC-544) or anti-human ERβ antibody (Santa Cruz SC-8974), diluted 1:100 in 1% NGS. For detection of ERα and ERβ, Alexa Fluor 488 (diluted 1:200 in 1% NGS) was used for 1 h at 37 °C in the dark. Cell nuclei were stained for 3min with 1 μg/ml Höechst and the images were taken on an Axioskop (Carl Zeiss) 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 experiments were performed for each experimental conditions and the fields were randomly chosen (Mazzarella et al., 2017).

2.9. Statistical analysis

Statistical analysis was performed by Graph Pad Prism 5 software. Data are expressed as mean values ± SEM for the indicated number of independent determinations. The statistical significance was calculated by the Student's t-test for FACS analysis. For the MTT assay, migration assay, Western blot and qPCR analyses the one way ANOVA with Bonferroni's multiple comparison test was performed and differences were considered statistically significant when the P values was at least < 0.05. All of the experiments were performed in triplicate and repeated at least three times.

3. Results

3.1. MTT assay

We assessed the effects of NP and E2 on LNCaP proliferation after 48 h of exposure. NP induced a significant increase of LNCaP cell proliferation at 10^{-10} M (Fig. 1a). E2 stimulated LNCaP proliferation at 10^{-9} M (Fig. 1b). No effects were observed neither at the highest concentrations (from 10^{-6} M to 10^{-8} M) used neither at the lowest (from 10^{-10} M to 10^{-12} M). For this reason, we chose these two concentrations to perform all the other experiments.

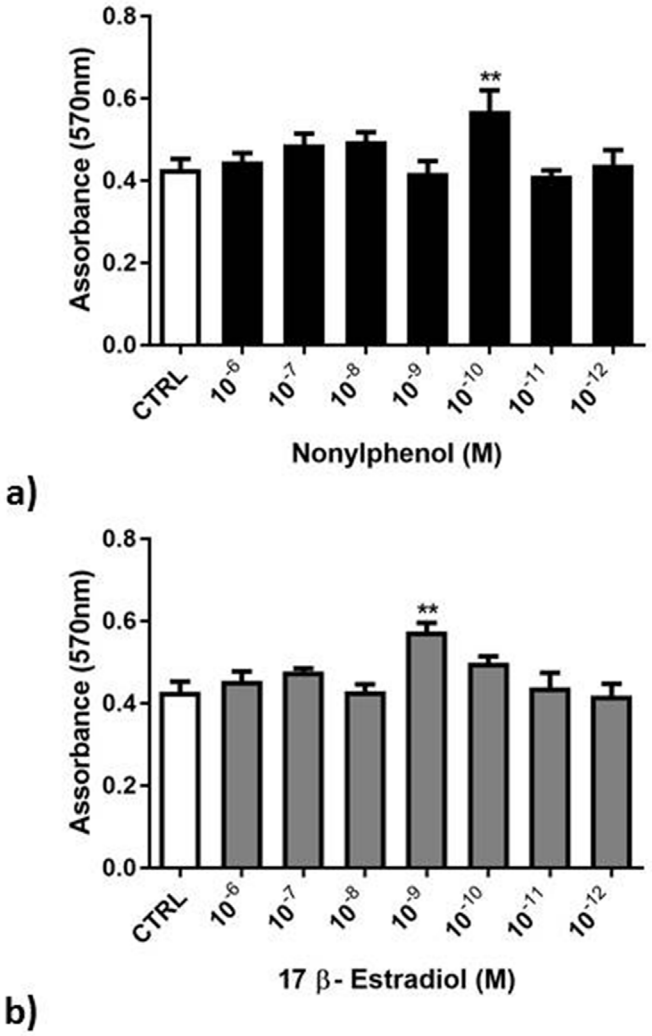

Fig. 1. MTT assay of 48 h exposure to 17β-estradiol (E2) and nonylphenol (NP). NP stimulates LNCaP proliferation with a significant effect at 1×10^{-10} M (**a**). E2 increases LNCaP proliferation at 1×10^{-9} M (**b**). Control cells were treated with vehicle (0,01% DMSO). Data represent the mean ± SE of three independent experiments (**p < 0,01, in the comparison with the control*).

3.2. FACS analysis

Cell cycle analysis was performed with FACS using propidium iodide staining after 48 h of treatment. As shown in Fig. 2 both 1×10^{-10} M NP (Fig. 2c) and 1×10^{-9} M E2 (Fig. 2b) increased cell distribution in S phase and decreased cell distribution in G0/G1 phase compared to control cells. Comparing both treatments (Fig. 2d), we didn't observe differences in cell distribution between E2 and NP treated cells.

3.3. RT-qPCR

We studied the expression of genes involved in cell cycle regulation as well as in inflammation (Fig. 3 a-f) signaling pathways after the treatment with 1×10^{-10} M NP and 1×10^{-9} M E2. NP increased mRNA levels of Ki67 (Fig. 3a) and CYCD1 (Fig. 3b) of two and one fold respectively while decreased p53 gene expression of 20% (Fig. 3c). E2 LNCaP treated cells shown an up-regulation of one fold of CYCD1 (Figs. 3b) and 20% of c-Myc (Fig. 3d), and a down-regulation of 50% of p53 (Fig. 3c). Regarding inflammation genes, only NP showed a significant effect, increasing mRNA levels of IL-8 (Fig. 3e) and IL-1β

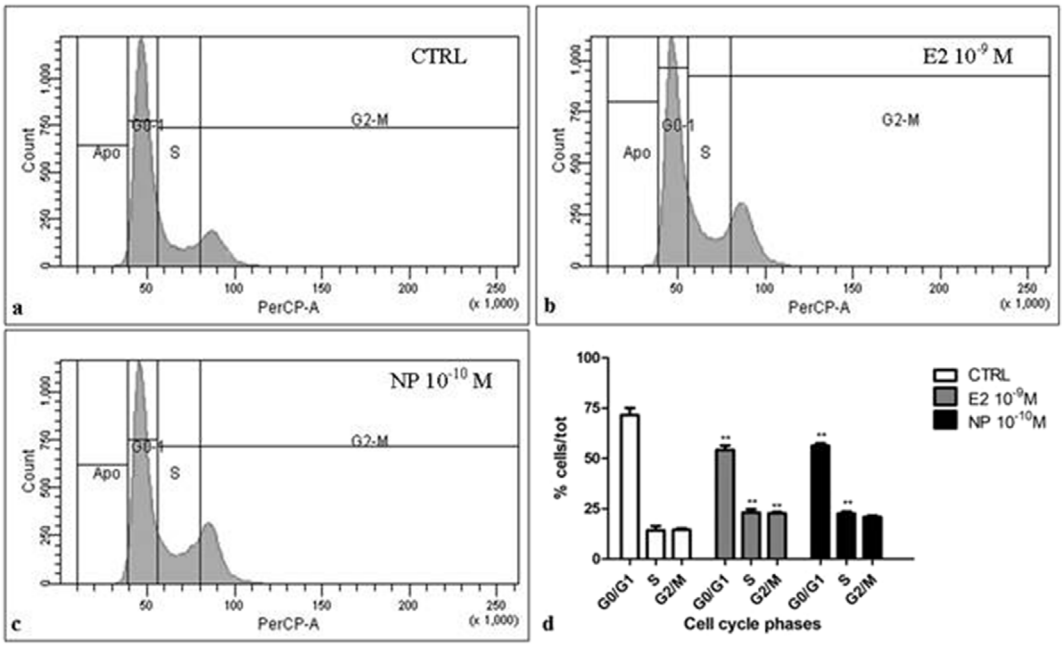

Fig. 2. Cell cycle analysis of 48 h exposure to 17β-estradiol (E2) and nonylphenol (NP). Rappresentativehistograms (**a**, **b**, **c**) and relative data summary of the cell cycle distribution (**d**). 1×10^{-9} M E2 (**b**) and 1×10^{-10} M NP (**c**) increase cells in phase S, consequently decreasing G0/G1 cell accumulation. Control cells were treated with vehicle (0,01% DMSO). Data represent the mean ± SE of three independent experiments (*$p < 0,01$, in the comparison with the control*).

(Fig. 3f) more than 50%.

3.4. Western blot analysis

Western blot analysis was performed in order to evaluate the expression of estrogen receptors and cell cycle proteins. Results showed the presence of ERα (66 KDa), ERβ (56 KDa), Cyclin D1 (33 KDa) both in control and treated LNCaP cells (Fig. 4a). The densitometric analysis revealed higher levels of ERα both with NP and E2 treatment (Fig. 4b). NP treatment did not interfere with ERβ protein expression (Fig. 4c), instead E2 significantly enhanced its expression (Fig. 4c). Moreover, both NP and E2 enhanced Cyclin D1 protein expression (Fig. 4d).

3.5. Immunofluorescence

ERα and ERβ cellular localizazion were studied with fluorescence microscopy after two different time of treatment: 2 h and 6 h. In LNCaP control cells, ERα appears to be mostly localized in the cytoplasm, with

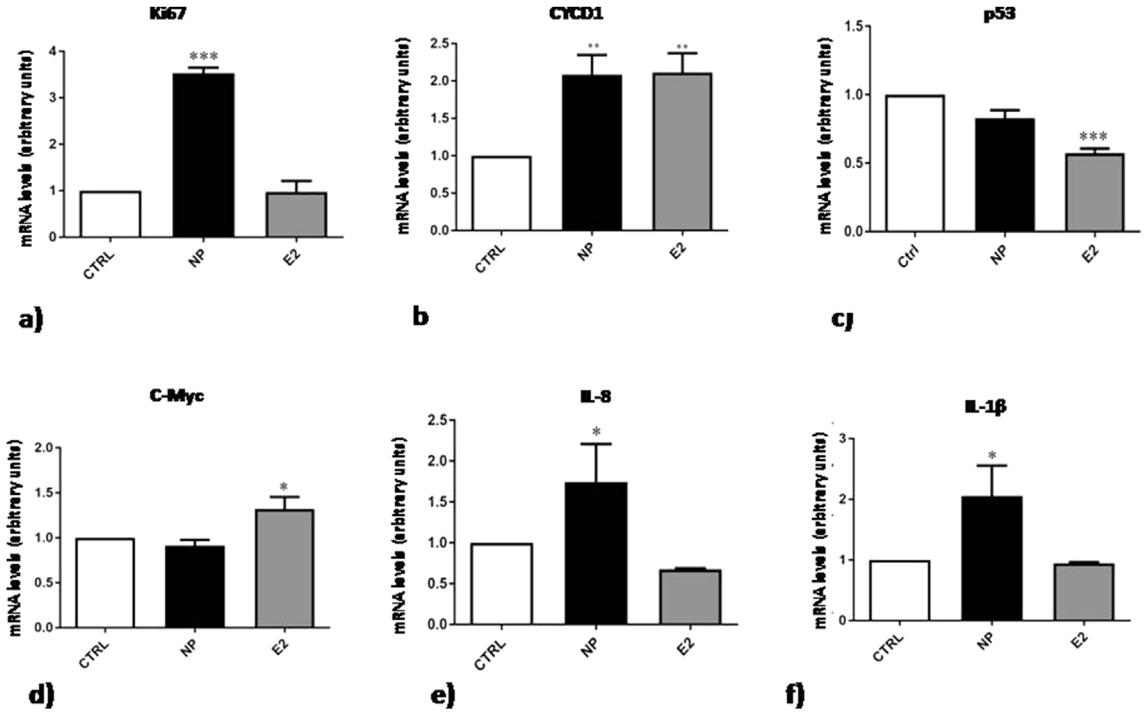

Fig. 3. qPCR analysis of genes involved in cell cycle regulation and inflammation processes (a–f). To note the different actions on gene expression of nonylphenol (NP) and 17β-estradiol (E2). Look the test for details. (*$p < 0,05$; **$p < 0,01$; ***$p < 0,001$, *in the comparison with the control*).

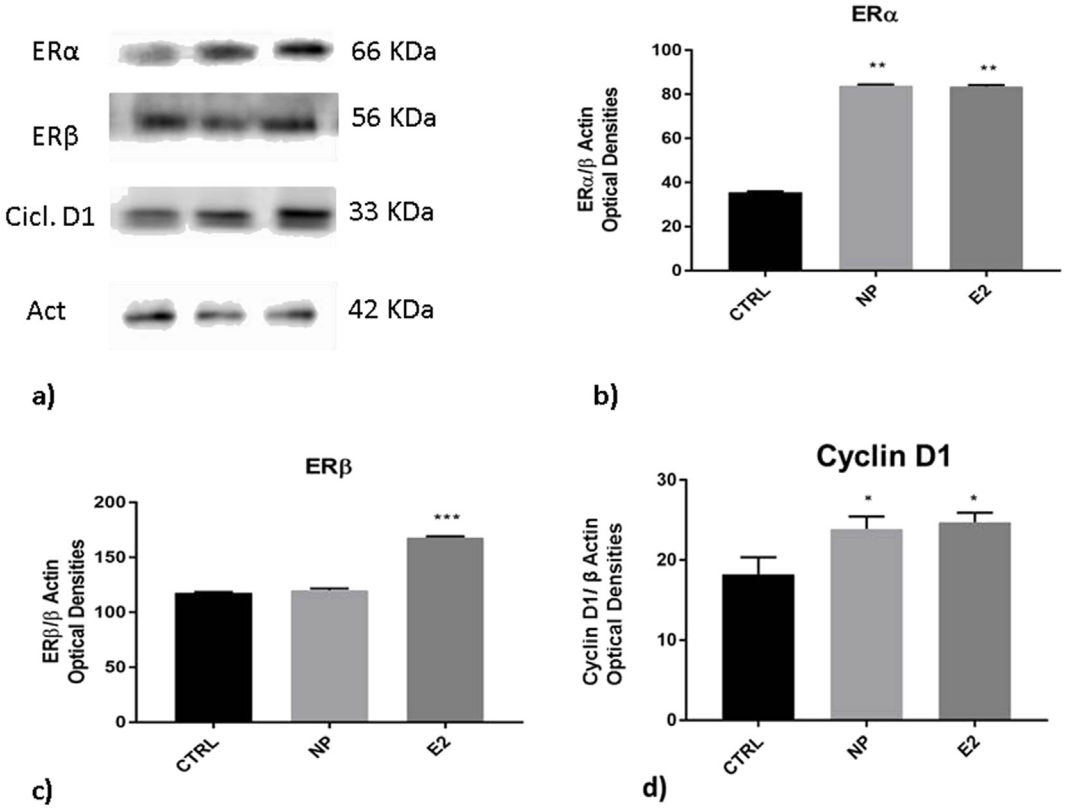

Fig. 4. Western blot analysis. The graphs represented the optical density (O.D.) ratio of ERα (b), ERβ (c), Ciclyn D1 (d) normalized on β actin. . (*$p < 0{,}005$; **$p < 0{,}01$; ***$p < 0{,}001$ in the comparison with the control).

no evident fluorescent signal in cell nucleus (Fig. 5). In treated cells, E2 induced a nuclear localization of ERα at both time considered, without any localization in the cytoplasm. In contrast, NP didn't translocate ERα to the nucleus after 2 h of treatment, but act on ERα translocation only after 6 h of treatment (Fig. 5). In control cells, ERβ is predominantly located in the cytoplasm (Fig. 6). NeitherE2 nor NP acted on ERβ translocation which remained cytoplasmatic at both times of treatment.

4. Discussion

Estrogenic chemicals can increase normal and abnormal growth of the human prostate (Hu et al., 2011; Leung et al., 2010; Nelles et al., 2011). Thus, we decide to investigate the molecular pathways involved in the action of nonylphenol (NP) on LNCaP prostate cells. In parallel, we performed the same experiments with the estrogen 17β-estradiol (E2) because one explanation of the association between prostate cancer and inflammation is the estrogen like action of different EDCs. In rats, neonatal treatment with BPA was reported to induce prostatic intraepithelial neoplasia (Ho et al., 2006; Prins et al., 2011) and to increase cell proliferation in the primary prostatic ducts of mice (Timms et al., 2005). Similarly, neonatal and developmental exposure to estradiol and BPA was shown to induce prostate carcinogenesis in rats and to target human progenitor prostate stem cells (Ho et al., 2006; Prins et al., 2014, 2011). Recently, Tarapore et al. (2014) found in prostate cancer patients significant urinary levels of BPA compared to non prostate cancer patients. In the same study, they demonstrated that BPA is able to induce centrosome abnomarmalities and neoplastic transformation in several prostate cell lines. Moreover, BPA was found to induce prostate cells migration in LNCaP cells (Derouiche et al., 2013). In a previous study we have already demonstrated NP ability to enhance PNT1A cells proliferation and act like 17β-estradiol (Forte et al., 2016). In the present study, after 48 h of exposure, NP and E2, respectively at 1×10^{-10} M and 1×10^{-9} M, enhanced LNCaP proliferation and cell progression in S phase of cell cycle. In order to understand the molecular events involved in the increase of proliferation and to investigate the effects of proliferation on cell physiology, through qPCR analysis, we studied changing in gene expression of genes involved in proliferation and inflammation pathways. The most effects on gene expression were the up-regulation of Ki67, CYCD1, IL-8 and IL1β. All of the four genes resulted up-regulated in prostate cancer (Dey et al., 2013; Sfanos and De Marzo, 2012). In particular, IL-8 is considered as prognostic markers of prostate cancer (Araki et al., 2007). Up-regulation of Ki67, CYCD1, IL-8 and IL-1β after NP treatment, may be responsible for the observed increase in cell proliferation and S phase cell distribution. Gene expression after E2 treatment does not completely overlap with NP treatment. Moreover, E2 didn't affect gene expression of IL-8 and IL-1β, compared to NP-treated LNCaP cells. Probably, NP, plus than E2, evokes an inflammation process in LNCaP cells. Then, to highlight a possible estrogen receptors involvement, we studied their expression and cellular localization. Differently from E2, NP induced a strong increase only in ERα expression and it did not interfere with ERβ expression. In order to understand a possible activation of ER pathway, we have also observed their cell localization since ER cytoplasm to nucleus traslocation suggest ER activation. NP and E2 act only on ERα cytoplasm to nucleus translocation, although with different times, 2 h for E2 and 6 h for NP. Some data reported the role of ERα in the proliferation and carcinogenesis of prostate cells, while ERβ seems to play a protective role for the gland, inducing apoptosis (Hartman et al., 2012). So the involvement of ERα after NP treatment suggest that this substance is able to increase cell response and to promote cell proliferation, according to MTT and FACS assays. Moreover, we evaluated cyclin D1 protein expression since it is an estrogen response target and promotes G1/S phase transition of cell cycle (Kastan and Bartek, 2004). We have observed that both NP and E2 enhanced cyclin D1 protein expression. Thus, our data are in agreement with the demonstrated proliferation caused by NP and E2 and suggest

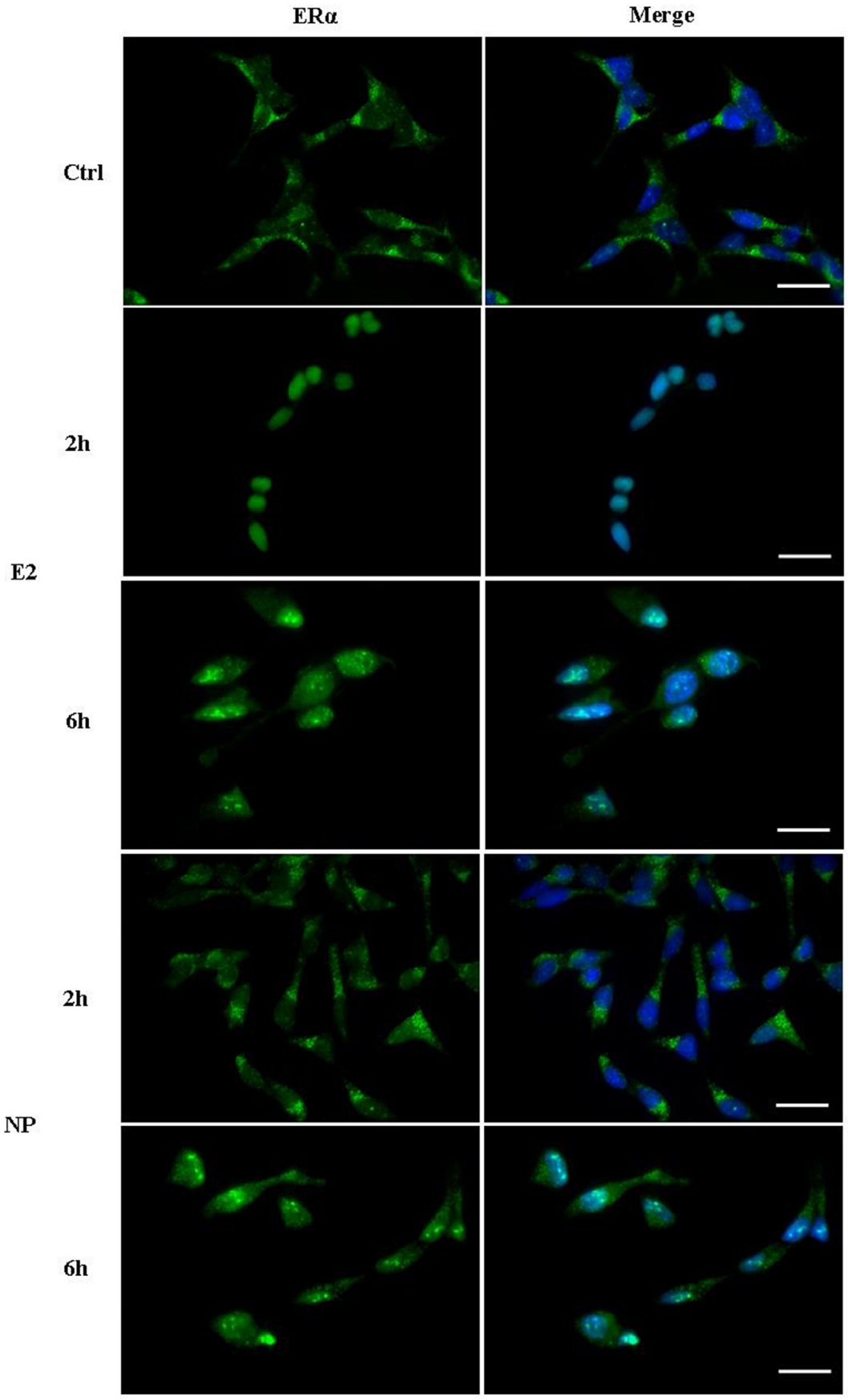

Fig. 5. ERα localization in LNCaP cells treated with17β-estradiol (E2) and nonylphenol (NP). ERα appears to be localized in the cytoplasm in control cells. E2 induced ERα translocation at both time considered (2 h, 6 h). NP induced a switch cytoplasm-nucleus after 6 h. LNCaP were plated in chamber slide under hormone deprived conditions. ERα (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence. Scale bar 10 μm.

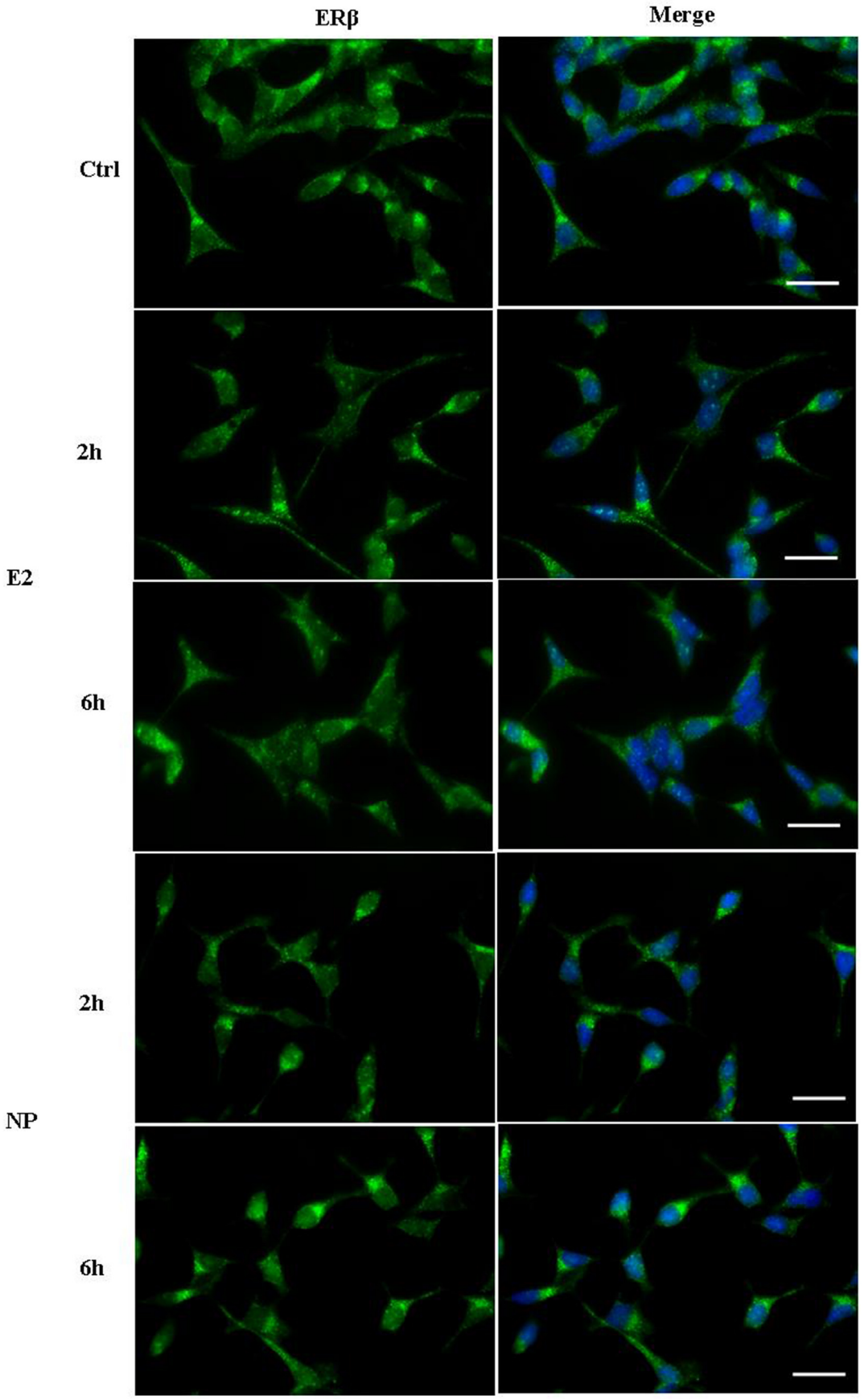

Fig. 6. ERβ localization in LNCaP cells treated with17β-estradiol (E2) and nonylphenol (NP). Treatment didn't affect cellular localization of ERβ, that was localized in the cytoplasm. LNCaP were plated in chamber slide under hormone deprived conditions. ERβ (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence. Scale bar 10 μm.

the estrogen-like action of NP, probably mediated via ERα. In addition, ERα is not the only pathway that mediate the effect of NP. In conclusion, we can summarize our results in some points: 1) estrogens and xenoestrogens stimulate cell proliferation, phase S progression of LNCaP, that may be a risk factors for prostate cancer; 2) ERα switch in the nucleus after NP and E2 exposure, increasing gene expression of target genes; 3) NP probably not only acts with a mechanism dependent from estrogen signaling pathways.

"Competing financial interests"

The authors declare they have no actual or potential competing financial interests.

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