Endocrine, *vol. 22, no. 3, 275–284, December 2003* 0969–711X/03/22:275–284/$25.00 © 2003 by Humana Press Inc. All rights of any nature whatsoever reserved.

The Food Contaminants Bisphenol A and 4-Nonylphenol Act as Agonists for Estrogen Receptor α in MCF7 Breast Cancer Cells

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Xenoestrogens are chemically distinct industrial products potentially able to disrupt the endocrine system by mimicking the action of endogenous steroid hormones. Among such compounds, the ubiquitous environmental contaminants bisphenol A (BPA) and 4-nonylphenol (NPH) may promote adverse effects in humans triggering estrogenic signals in target tissues. Following a research program on human exposure to endocrine disruptors, we found contamination of fresh food by BPA and NPH. More important, these contaminants were found to display estrogen-like activity using as a model system the estrogen-dependent MCF7 breast cancer cells (MCF7wt); its variant named MCF7SH, which is hormone-independent but still ERα-positive, and the steroid receptor–negative human cervical carcinoma HeLa cells. In transfection experiments BPA and NPH activated in a direct manner the endogenous ERα in MCF7wt and MCF7SH cells, as the antiestrogen hydroxytamoxifen was able to reverse both responses. Moreover, only the hormone-binding domains of ERα and ERβ expressed by chimeric proteins in HeLa cells were sufficient to elicit the transcriptional activity upon BPA and NPH treatments. Transfecting the same cell line with ERα mutants, both contaminants triggered an estrogen-like response. These transactivation properties were interestingly supported in MCF7wt cells by the autoregulation of ERα which was assessed by RT-PCR for the mRNA evaluation and by immunoblotting and immunocytochemistry for the determination of protein levels. The ability of BPA and NPH to modulate gene expression was further confirmed by the upregulation of an estrogen target gene like pS2. As a biological counterpart, concentrations of xenoestrogens eliciting transcriptional activity were able to stimulate the proliferation of MCF7wt and MCFSH cells. Only NPH at a dose likely too high to be of any physiological relevance induced a severe cytotoxicity in an ERα-independent manner as ascertained in HeLa cells. The estrogenic effects of such industrial agents together with an increasing widespread human exposure should be taken into account for the potential influence also on hormone-dependent breast cancer disease.

Key Words: Bisphenol A; 4-nonylphenol; estrogens; estrogen receptor; MCF7 breast cancer cells.

Introduction

The endocrine disrupting chemicals (EDCs) are exogenous agents that interfere with the synthesis, secretion, transport, action, or elimination of natural hormones, which sustain development, reproduction, and behavior *(1–3)*. After ingestion and/or absorption the EDCs can alter endocrine functions through a variety of mechanisms including nuclear receptor binding and activation *(4,5)*.

Among these chemicals, bisphenol A (BPA), largely used in polycarbonate plastics as well as polystyrene resins, and 4-nonylphenol (NPH), a derived product of nonionic surfactants such as NPH ethoxylates, are both ubiquitously present in the environment and resistant to degradation *(6–10)*. Microgram amounts of BPA and NPH have been detected in liquid from canned vegetables *(7)*, in different commercially available foodstuffs *(9)*, and even in the saliva of patients treated with dental sealants *(10)*. The exposure to these xenoestrogens may lead to important biological activities in estrogen-sensitive tissues *(11,12)*, even though they do not share structural homology with estradiol (E2) (Fig. 1) and exhibit a lower potency in estrogen receptor (ER) α and ERβ binding affinity with respect to the natural ligand E2 *(1,13,14)*.

The hormone-dependent transcriptional activity of ERα is mediated by the constitutive activation function-1 (AF-1) located within the amino-terminus and the ligand-dependent AF-2 in the hormone-binding domain (HBD) *(15)*, which includes the highly conserved amphipathic α-helix *(16,17)*.

Received August 18, 2003; Revised September 23, 2003; Accepted October 6, 2003.

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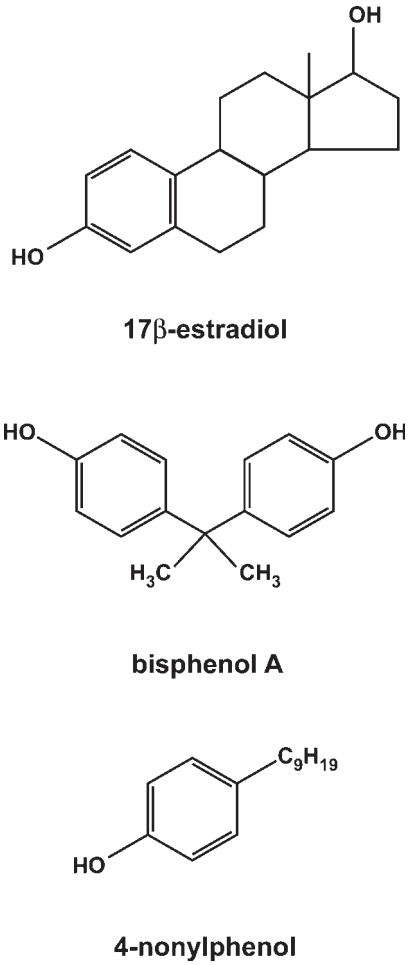

Fig. 1. Chemical structures of 17β-estradiol, bisphenol A, and 4-nonylphenol.

In the absence of agonist, ERα is sequestered within the nuclei of target cells in an inactive form *(18,19)*. The interaction with ligands induces receptor conformational changes that allow the formation of an AF-2 hydrophobic pocket and the recruitment of coactivators *(16,20–22)*. Different classes of compounds exert estrogen-like effects or display antiestrogenic properties in target tissues on the basis of resultant orientations in the HBD that have a profound impact on ERα pharmacology and ultimately on transcription machinery *(1,23)*. Among the synthetic ER modulators (SERMs), antiestrogens such as tamoxifen and raloxifene exhibit antagonistic action in the mammary gland *(24,25)* but agonistic activity on the endometrium and bone, respectively *(26,27)*. On the contrary, faslodex (ICI 182,780) acts in an antiestrogenic fashion in all tissues analyzed *(28–30)*. The natural estrogen-like compounds named phytoestrogens are considered potentially able to exert a protective role against the development of hormone-dependent tumors *(31,32)*, although they bind and activate ERα, which in turn may trigger proliferative effects in breast cancer cells

Table 1
Quantitative Evaluation (mg/kg)
of Bisphenol A (BPA) and 4-Nonylphenol (NPH) in Fresh Food[a]

Food	BPA	NPH
Apple	nd	nd
Cherry	0.86 ± 0.06	0.14 ± 0.02
Courgette	0.51 ± 0.04	0.23 ± 0.03
Cucumber	nd	nd
Eggplant	0.25 ± 0.02	0.21 ± 0.04
Fennel	nd	nd
Green bean	nd	0.27 ± 0.03
Lettuce	nd	nd
Medlar	0.47 ± 0.02	0.56 ± 0.04
Orange	1.11 ± 0.09	0.37 ± 0.01
Peach	0.47 ± 0.03	0.12 ± 0.01
Pepper	0.94 ± 0.07	0.45 ± 0.06
Strawberry	nd	1.2 ± 0.11
Tomato	0.91 ± 0.08	0.47 ± 0.03

[a]Values (mean ± SD) represent analysis of at least three different samples of each food. nd = no detection.

(33). It has been largely demonstrated that xenoestrogens are responsible for an estrogen-like activity both in vitro and in vivo *(12,34–45)*. Of note, in previous studies BPA and NPH promoted the proliferation of hormone-dependent breast cancer cells *(39,46,47)*, but recently *(48)* BPA was reported to strongly reduce the rate of apoptosis without effects on cell proliferation.

In the present work we show that BPA and NPH contaminate fresh food as ascertained following a research program on human exposure to endocrine disruptors. Moreover, we provide insight into the agonistic activity of BPA and NPH for ERs demonstrating their ability (1) to transactivate the endogenous ERα in estrogen-dependent MCF7wt breast cancer cells as well as in its hormone-independent variant MCF7SH *(49)*, (2) to transactivate ERα and ERβ expressed in human cervical carcinoma HeLa cells, (3) to modulate ERα target genes, and (4) to promote growth stimulatory effects in the two sublines of MCF7 cells.

Results

Bisphenol A and 4-Nonylphenol Contaminate Fresh Food

Table 1 shows a quantitative evaluation of xenoestrogens in randomly selected fresh food samples from an area of southern Italy. The levels of BPA and NPH ranged from 0.25 to 1.11 mg/kg and from 0.12 to 1.2 mg/kg, respectively. The retention times in the extracts were 25.32 min for BPA and 18.71 min for NPH (Fig. 2), overlapping those of standards (25.28 min and 18.73 min, respectively), which also exactly matched the mass spectra obtained from food samples. In order to definitively confirm the detection of BPA

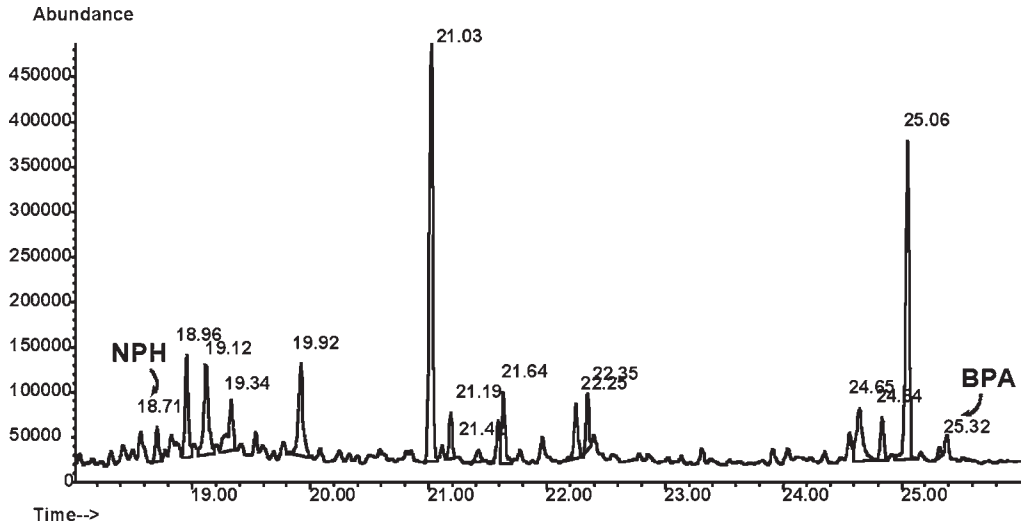

Fig. 2. Representative expanded GC/MS chromatogram showing the detection of BPA and NPH from a sample of eggplant extract.

and NPH, we used as a model system a positive sample of eggplant shown in Fig. 2. After extraction, it was evaporated to dryness and 0.5 mL from solutions of 5 mg/L of both standards were added. The sample diluted to 1 mL with methanol was analyzed by GC/MS. The chromatogram obtained showed an evident increase of the intensities of BPA and NPH peaks with respect to those obtained without the addition of standards. These increases were related to the intensity of the peak corresponding to the pesticide Vinclozolin present in the chromatogram at retention time 21.42 min. The ratio between the peak intensity of BPA and Vinclozolin increased from 1.0 to 1.4, while that between NPH and Vinclozolin from 2.8 to 4.3.

Bisphenol A and 4-Nonylphenol Activate the Endogenous ERα

The food contamination by BPA and NPH prompted us to evaluate their estrogenic properties on two sublines of MCF7 breast cancer cells. Figure 3 shows the results obtained with the MCF7wt and its variant MCFSH, both containing exclusively ERα and no ERβ as judged by RT-PCR (data not shown). MCF7SH cells exhibit the unique property of proliferating in the absence of hormone, although still ERα-dependent and sensitive to antiestrogens *(49)*. In these cells the reporter gene XETL responds to E2 with remarkable sensitivity and efficacy *(50)*, with 10 n*M* of hormone resulting as the half-maximal stimulation (Fig. 2A). Of note, BPA and NPH are strong activators of ERα in MCF7SH, although at higher doses and with reduced efficacies with respect to E2 (Fig. 3A) as also observed in MCF7wt cells (Fig. 3B). Besides, in both MCF7 cell lines the antiestrogen OHT abolishes the signals induced by these chemicals, suggesting that a direct ERα-mediated mechanism is involved in transcriptional activity (Fig. 3C).

Transcriptional Activation of ERα and ERβ by Bisphenol A and 4-Nonylphenol in a Heterologous System

To provide evidence that transactivation of ERα by BPA and NPH is not related to the specificity of a cell type and to examine the response of ERβ, we turned to a completely heterologous system. Chimeric proteins consisting of the heterologous DNA-binding domain of the yeast transcription factor Gal4 and the ERα or ERβ HBDs respond to E2, BPA, and NPH in a transient expression assay in HeLa cells (Fig. 4A). These results demonstrate that the HBD of each ER isoform is sufficient to elicit a response and that both xenoestrogens are AF2 agonists. Moreover, we assessed the response of ERα HBD mutants by using Gal4 fusion proteins (Fig. 4B). The two point mutants L525A and G521R that require considerably higher E2 concentrations for activation *(51)* fail to respond to 1 μ*M* BPA and NPH. The AF2 mutant M543/L544A does not respond to either E2 or both chemicals, while the mutant ΔF lacking the F-domain at the C-terminal of HBD core, displays a strong response to E2, BPA, and NPH. Hence, the two contaminants trigger a transcriptional activity like E2 either in the presence of mutated hormone-binding pocket of ERα or with the truncated receptor ΔF, which suggests a modulatory role of this domain on receptor transactivation by agonists.

Bisphenol A and 4-Nonylphenol Down-Regulate ERα mRNA and Protein Levels

It is well known that E2 down-regulates the levels of ERα in breast cancer cell lines through both an increased turnover of the E2-activated ERα protein as well as the reduced transcription rate of its own gene (*52*, and references therein). Therefore, the repression of ERα protein levels might

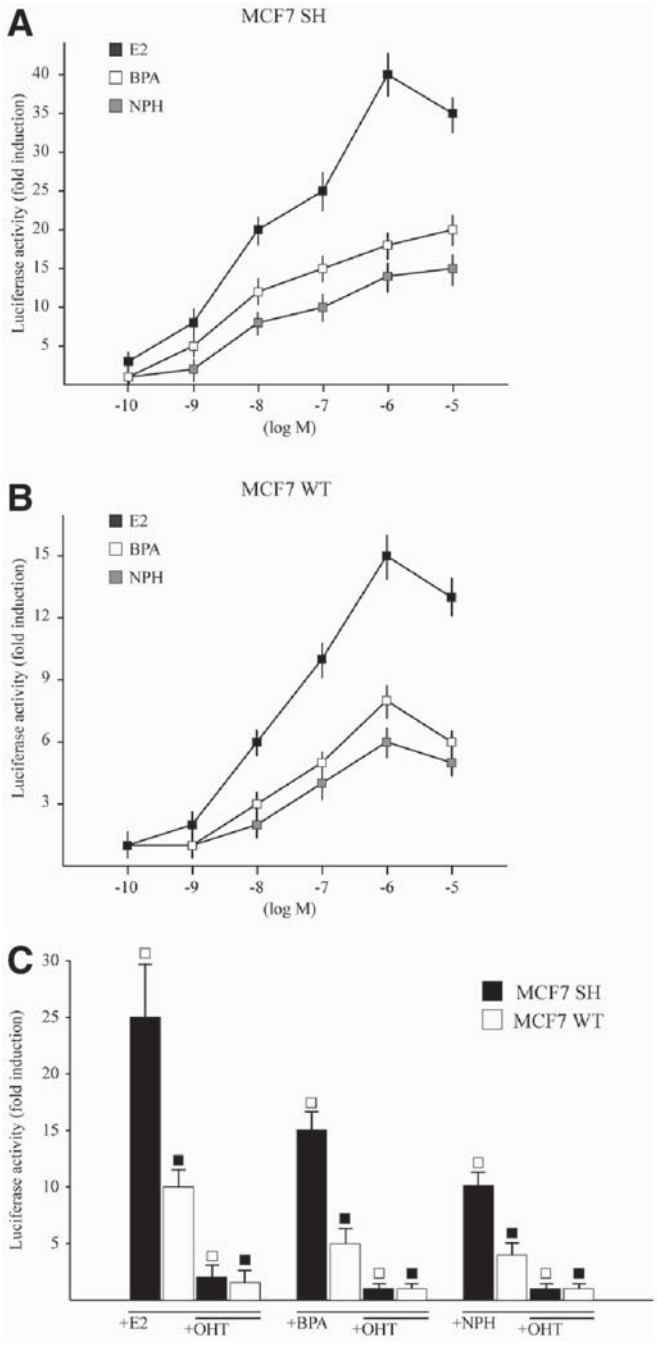

Fig. 3. BPA and NPH activate endogenous ERα. The indicated human breast cancer cell lines (**A**, MCF7SH; **B**, MCF7wt) were transfected with the luciferase reporter plasmid XETL (carrying firefly luciferase sequences under the control of an estrogen response element upstream of the thymidine kinase promoter), and treated with increasing concentrations (logarithmic scale) of E2, BPA, and NPH. Luciferase activities were standardized to the expression of renilla used as an internal transfection control, and reported as the ratio of induced activity to activity in absence of ligand. Values of cells receiving vehicle was set as onefold induction, upon which the results of treatments were calculated. (**C**) Activation by xenoestrogens is mediated by ERα. Transfected MCF7SH and MCF7wt cells were treated with 100 n*M* of E2, BPA, and NPH with or without 10 μ*M* of the antiestrogen hydroxytamoxifen (OHT). Each data point represents the mean of triplicate samples of a representative experiment. ☐■ $p < 0.05$.

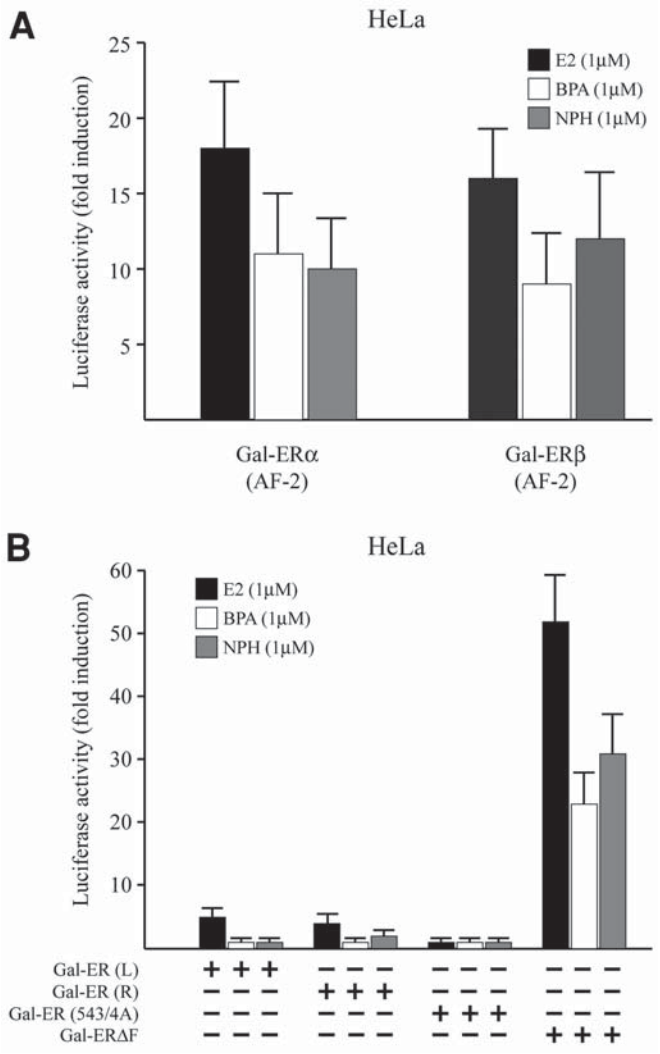

Fig. 4. BPA and NPH are ERα and ERβ agonists in a heterologous system. (**A**) Fusion proteins consisting of an HBD and the Gal4 DBD are activated by BPA and NPH in transfected HeLa cells. (**B**) An intact hormone binding pocket and AF2 are required for activation of Gal4-ERα HBD chimeras by BPA and NPH; Gal-ER(L), Gal-ER(R), and Gal-ER(543/4A) are chimeras with the HBD point mutants L525A, G521R, and M543A-L544A, respectively; Gal-ERΔF lacks the F-domain. The data of panels **A** and **B** are from the same experiment and each data point represents the mean of triplicate samples of a representative experiment.

be considered an additional hallmark of receptor activation by an agonist. Thus, we evaluated whether the levels of ERα are also sensitive to BPA and NPH in MCF7wt cells. The mRNA levels were compared with semiquantitative RT-PCR and standardized using the mRNA levels of the housekeeping gene *36B4*. The exposure to these chemicals for 24 h clearly down-regulates ERα mRNA (Fig. 5A,B) as well as protein content (Fig. 5C,D), although with less efficacy compared to E2. In order to provide further evidence that BPA and NPH are able to autoregulate ERα, we performed an immunocytochemical staining in MCF7wt cells after 2 h of treatment (Fig. 6). Interestingly, the basal immunoreactiv-

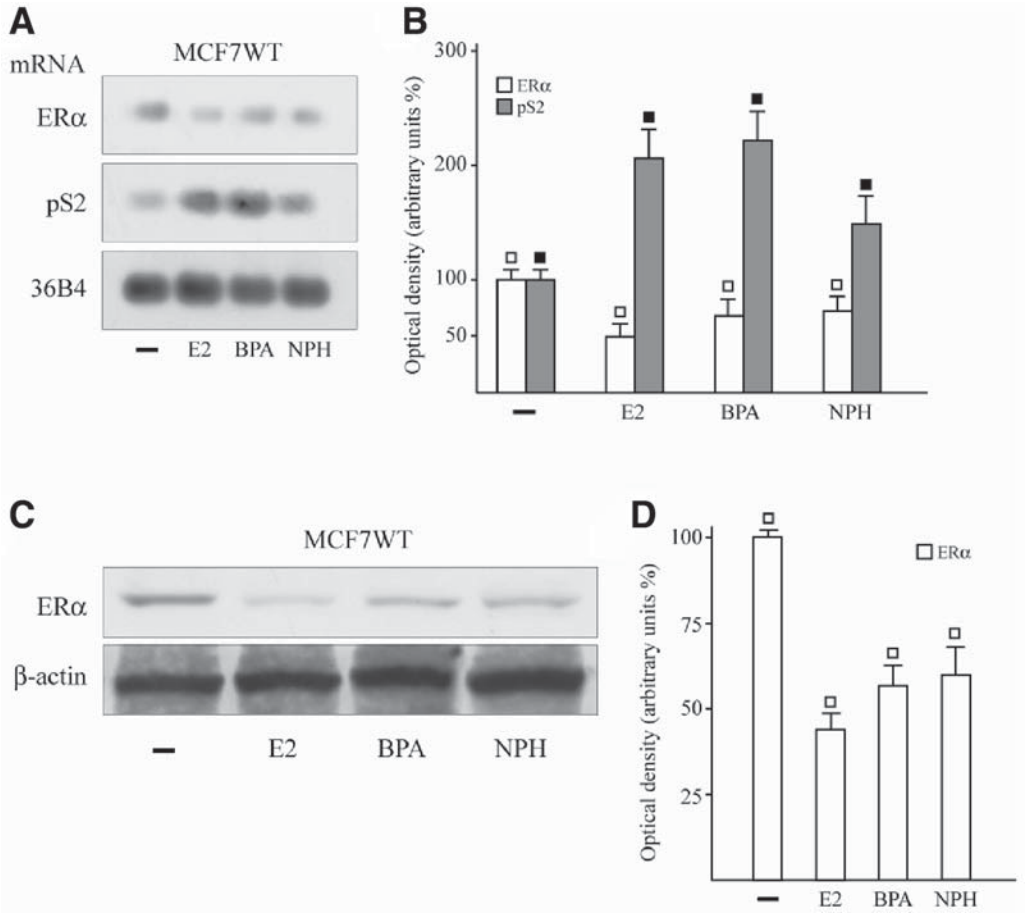

Fig. 5. Effects of BPA and NPH on ERα mRNA and protein levels and on pS2. (**A**) Semiquantitative RT-PCR of ERα and pS2 mRNA. MCFwt cells were stimulated for 24 h with 1 μ*M* of ligands; 36B4 mRNA levels were determined as a control. (**B**) Quantitative representation of data of two independent experiments including that of panel **A** after densitometry and correction for 36B4 expression. (**C**) Immunoblot of ERα from MCF7wt cells treated with 1 μ*M* of ligands for 24 h. (**D**) Quantitative representation of data of two independent experiments including that of panel **C** after densitometry. β-actin serves as loading control. □■ $p < 0.05$.

ity of ERα detected exclusively in the nuclear compartment (Fig. 6, panel A) was clearly reduced by the addition of either E2, BPA, and NPH (Fig. 6, panels B and D). In contrast, no signals were observed either replacing the anti-ER antibody by irrelevant rabbit IgG (small squares in Fig. 5) or using the primary antibody preabsorbed with an excess of receptor protein (data not shown).

Bisphenol A and 4-Nonylphenol Up-regulate pS2 mRNA

ERα is a ligand-dependent transcription factor that regulates gene expression binding to nuclear proteins such as SP1/SP3 *(53,54)* and interacting with DNA sequences termed estrogen response elements (ERE) located within the regulatory regions of target genes like pS2. Hence, we investigated whether in MCF7wt cells the expression of pS2 is sensitive to BPA and NPH. The mRNA levels were compared with semiquantitative RT-PCR and standardized using the mRNA levels of the housekeeping gene *36B4*. It is interesting to note that a 24-h exposure to both BPA and NPH up-regulates the mRNA of pS2, overlapping the expression induced by E2 (Fig. 5A,B).

Bisphenol A and 4-Nonylphenol Stimulate the Proliferation of MCF7 Breast Cancer Cells

Having determined that BPA and NPH are able either to activate ERs or to modulate the expression of ERα target genes, we evaluated a more complex physiological response such as cell proliferation. MCF7wt and MCF7SH cells were treated with BPA and NPH for 5 d, the next day they were counted and compared with untreated cells. Notably, the two chemicals induce the proliferation of both MCF7 sublines at a concentration range consistent with their affinity for ERα (Fig. 7A) *(13,14)*. Cell growth can be inhibited by the antiestrogen OHT (Fig. 7B), confirming that an ERα-mediated mechanism is involved in the proliferation effects. NPH addition at 100 μ*M* promotes a severe drop in MCF7 cell numbers as a consequence of massive cytotoxicity (Fig. 7A). The ER-negative and estrogen-insensitive HeLa cells are

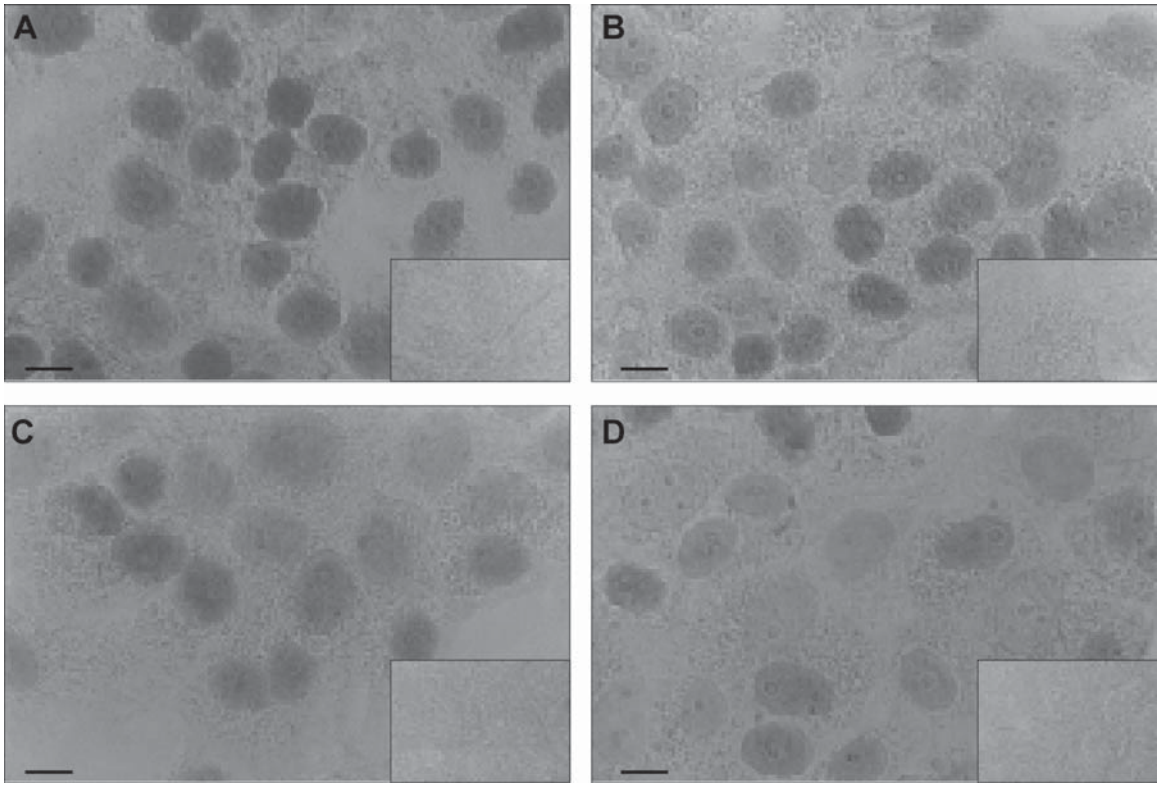

Fig. 6. Immunostaining of ERα in MCF7wt. Cells were treated with vehicle (**A**) or 1 μ*M* E2 (**B**), BPA (**C**), and NPH (**D**) for 2 h. No immunodetection was observed replacing the anti-ERα antibody with an irrelevant rabbit IgG (insets). Each experiment is representative of at least 10 tests. Bar, 5 μm.

not stimulated by xenoestrogen treatments but are inhibited only by the highest dose of NPH (Fig. 7A).

Discussion

In the present study we have demonstrated the contamination of fresh food by the industrial products BPA and NPH. Moreover, we have provided new data indicating that both xenoestrogens studied act as agonists for the ERs since (1) they activate endogenous ERα in two sublines of breast cancer cells, (2) they down-regulate ERα mRNA and protein levels, (3) they up-regulate the mRNA of the estrogen target gene *pS2*, (4) they activate the ligand-dependent AF-2 transactivation functions of ERα and ERβ in the context of chimeric proteins with the Gal4 DNA binding domain, and (5) this activity depends on an intact hormone binding pocket as AF-2 mutants exhibit different responses, and (6) they induce growth stimulatory effects on MCF7 cells as a result of the aforementioned biological properties.

In recent years, structure-activity relationship (SAR) studies have been performed for steroidal *(55)* and nonsteroidal *(56)* estrogens to focus on chemical characteristics dictating the binding affinity for ERs. Computer-based tools have also enabled the development of quantitative SAR models to identify three-dimensional steric and electrostatic molecular features responsible for estrogenic activity (*1*, and references therein). Indeed, the complex ER pharmacology has been solved by crystallographic models demonstrating that a large number of ligands may induce receptor conformational changes driving the transcriptional machinery toward differential gene modulation in target cells (*22*,*57*, and references therein). Of note, the binding and transactivation properties of agonists have been shown to impact on the stability of ERα. For instance, the receptor half-life of approx 4–5 h in the absence of ligands upon introduction of E2 the half-life decreases through a proteosome-mediated degradation process, which consequently regulates the response to an activating agent (*58*, and references therein). Given the biological relevance of consecutive events such as receptor activation and degradation, which are directly correlated with the ligand-induced transcriptional activity, we have performed a consistent assay designed to elucidate the pharmacological estrogenic attributes of BPA and NPH.

In transfection assays, which monitor the activation of endogenous ERα in MCF7 as well as both ER isoforms in HeLa cells, the two chemicals are strong transcriptional in-

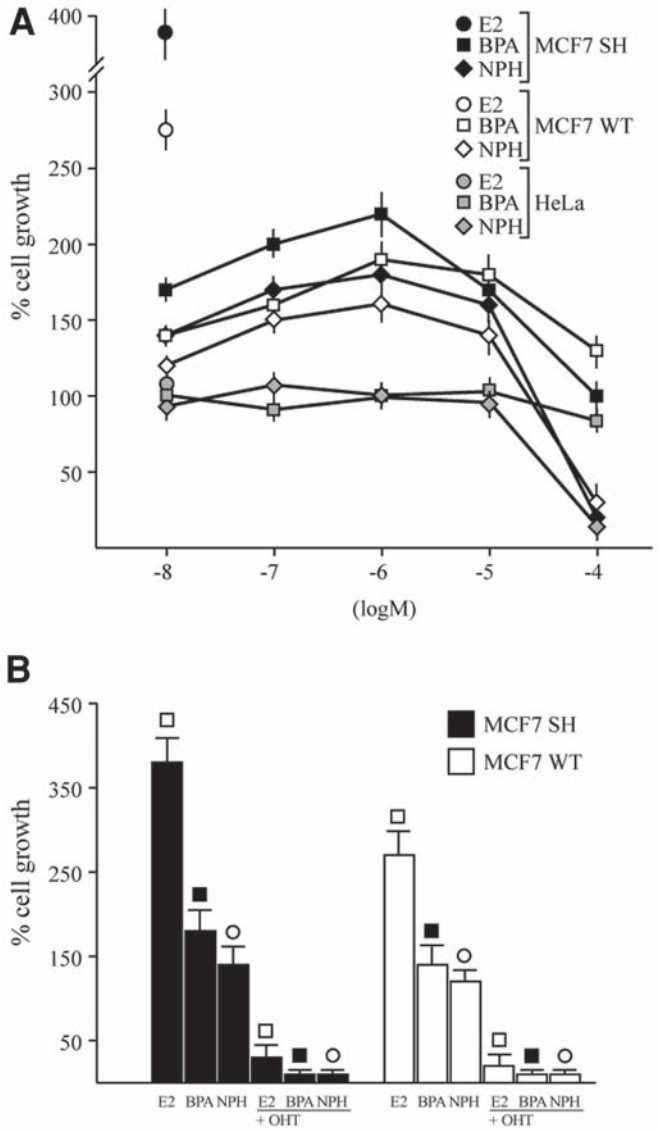

Fig. 7. BPA and NPH stimulate the proliferation of breast cancer cells. (**A**) Both xenoestrogens stimulate MCF7wt and MCF7SH cell growth, only NPH is cytotoxic at very high concentration, even for HeLa cells. Low numbers of cells were seeded in 24-multiwell plates, treated with 10 n*M* of E2 or increasing concentrations (logarithmic scale) of both xenoestrogens, and counted on d 6 as described in *Material and Methods*. Cell numbers are expressed as a percentage of number of cells treated with vehicle alone. (**B**) Proliferative effects are ERα-mediated. Proliferation of MCF7wt and MCF7SH cells was assayed as above except that cells were treated with 100 n*M* E2 and xenoestrogens plus 10 μ*M* OHT. Each data point is the average of several independent experiments. □■ $p < 0.05$.

ducers with the following rank order of efficacy (fold induction): E2 > BPA > NPH. Considering that the activity of xenoestrogens in reporter gene systems could be dependent on the chosen response element *(59)*, our results obtained with different assay models may collectively support the transcriptional properties of the compounds analyzed. Besides, the up-regulation of pS2 expression contributes to a reliable assessment of xenoestrogen action in target DNA response elements despite a lower binding affinity for ERs *(1)*.

ERα is distributed in a reticular manner within the nuclei in the absence of agonists *(60)* and the differences in the susceptibility to degradation of an ERα-ligand complex is regulated at least in part by receptor subcellular localization *(56,61)*. Thus, in order to evaluate the effect of short- (2 h) and long- (24 h) term xenoestrogen treatment, we followed the pattern of ERα in MCF7wt by immunocytochemistry and immunoblot analysis, respectively. In both assays, BPA and NPH qualitatively behave like the physiological ligand E2, as the xenoestrogen-activated receptor was recognized as a complex to be processed by the degradation machinery, adding a further mechanism by which these environmental contaminants may have an impact on ERα pharmacology.

In agreement with a recent study *(62)*, our results indicate that BPA and NPH stimulate the proliferation of MCF7 cells through an ERα-mediated mechanism without effects like inhibition of apoptosis as reported by other authors *(48)*. The biological efficacy was particularly evident with the MCF7 variant cell line MCF7SH, which is hypersensitive to E2 and responds very well to several ER agonists *(50)*. BPA and NPH at a concentration of 10 n*M* induce reporter gene expression 12- and 8-fold, respectively, and can already stimulate cell proliferation. Because long-term estrogen-deprived breast tumor cells, for which MCF7SH can be considered a model, are extremely sensitive to E2 *(49,63)*, these findings suggest that even very low levels of such industrial products could be involved in the growth of certain breast tumors.

Human exposure to BPA and NPH was estimated to be in the microgram range *(7,9,10)*, although epidemiological studies are still lacking to critically evaluate and establish the consequences of these relevant doses. In addition, it would be interesting to evaluate the relationship occurring among the detoxification of xenoestrogens *(64)*, the metabolic generation of potent metabolites *(65,66)*, and the hormone-dependent breast cancer disease.

Materials and Methods

Reagents

E2, BPA, NPH and the antiestrogen 4-hydroxytamoxifen (OHT) *(25)* were purchased from Sigma.

Gas Chromatography/Mass Spectrometry Analysis

Envi-18, Envi-carb, and aminopropyl Sep-pak cartridges were purchased from Supelco. Solvents were purified, dried, and distilled by standard procedures. Gas chromatography/mass spectrometry (GC/MS) analysis was performed by HP 5890 A series II chromatograph fitted with HP 5972 A mass spectrometer, using a 30 m cross-linked 5% PHMes-

iloxane capillary column (0.25 mm internal diameter and a 0.25 μm film thickness). The mass detector operated in the electron impact ionization mode (EIMS) with an electron energy of 70 eV. The injection conditions were splitless (1 min). In all GC/MS analyses the temperature program was the following: initial temperature 60°C for 2 min, then raised 7°C/min up to 280°C and held for 10 min. Fruits and vegetables were randomly purchased from greengroceries in southern Italy. Each representative sample was prepared collecting different parts of the same food and then subjected to the following solid-phase extraction procedure: 100 g of material were homogenized with 100 mL acetonitrile (e.g., Omni-mixer, half-speed, 5 min); 20 mL of acetonitrile top layer were eluted through a preconditioned solid-phase extraction Envi-18 tube (C-18 tube contains an octadecyl phase bonded to a silica support that removes nonpolar interferences from polar sample matrix); sodium sulfate was added to the recovered solution to remove water; and finally the sample was filtered and concentrated to 0.5 mL. The sample was transfered to a system of two cartridges: Envi-carb and aminopropyl Sep-pak. The aminopropyl cartridge was placed behind the Envi-carb. (This step removes polar interferences and offers a consistent recovery for a wide range of organic contaminants.) The analytes were eluted with 20 mL acetonitrile/toluene (3:1). Samples were concentrated to 1 mL using a rotary evaporator. 2X 10 mL acetone was added to carry out the solvent exchange. The final extract was diluted to 1 mL with methanol and analyzed by GC/MS. The quantitative evaluation was performed comparing the areas of peaks of analytes to those obtained from standard solutions of BPA (50 mg/L) and NPH (30 mg/L).

Plasmids

Firefly luciferase reporter plasmids used were XETL *(67)* for the ERs and GK1 *(68)* for the Gal4 fusion proteins. The reporter plasmid XETL carries firefly luciferase sequences under the control of an estrogen-response element upstream of the thymidine kinase promoter. As an internal transfection control, we cotransfected the plasmid pRL-CMV (Promega) that expresses the renilla luciferase enzymatically distinguishable from firefly luciferase by the strong cytomegalovirus enhancer/promoter. Gal4 chimeras Gal-ERα, Gal-ER(R), Gal-ER(L), Gal-ER(543/4A), Gal-ER(ΔF), and Gal-ERβ were expressed from plasmids GAL93.ER(G), GAL93.ER(R), GAL93.ER(L), GAL93.ER(ML543/4AA), GAL93.ER(ΔF), and GAL93.ERβ, respectively. They were constructed by transferring the coding sequences for the hormone-binding domain (HBD) of ERα (amino acids 282–595) from HEG0 *(12)*, pCMVhERG521R *(69)*, pCMVhERL525A *(69)*, a PCR-mutagenized intermediate with the point mutations M543A-L544A, a PCR fragment lacking the coding sequences for the F domain and for the ERβ HBD (C-terminal 287 amino acids) from plasmid pCMV5-hERbeta (a gift from J.-Å. Gustafsson), respectively, into the mammalian expression vector pSCTEVGal93 *(70)*.

Cell Culture

Wild-type human breast cancer MCF7 (MCF7wt) cells were a gift from E. Surmacz (Philadelphia, PA, USA). MCF7wt and HeLa cells were maintained in DMEM without phenol red supplemented with 10% FCS. The variant cell line MCF7SH *(49)* was maintained in DMEM without phenol red supplemented with 5% charcoal-stripped (CS) FCS. MCF7wt cells to be processed for immunoblot, immunostaining, or RT-PCR assays were switched to DMEM supplemented with 5% CS-FCS 4 d before treatments.

Transfections and Luciferase Assays

Cells were transferred into 24-well plates with 500 μL of regular growth medium/well the day before transfection. The medium was replaced with DMEM lacking phenol red as well as serum on the day of transfection, which was performed using the Fugene6 Reagent as recommended by the manufacturer (Roche Diagnostics) with a mixture containing 0.5 μg of reporter plasmid, 5 ng of pRL-CMV, and 0.1 μg of effector plasmid where applicable. After 6 h the medium was replaced again with serum-free DMEM lacking phenol red, ligands were added at this point, and cells incubated for 20–24 h. Luciferase activity was then measured with the Dual Luciferase Kit (Promega) according to the manufacturer's recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the *Renilla* luciferase activity. Luciferase activity of cells receiving vehicle was set as one-fold induction, upon which the results of treatments were calculated.

RT-PCR

The evaluation of gene expression was performed by semiquantitative RT-PCR as we have previously described *(70)*. For ERα, the estrogen-inducible trefoil factor pS2 *(71)*, and the human acidic ribosomal phosphoprotein 36B4 used as an internal control *(72)*, the primers were: 5'GGAG ACATGAGAGCTGCCA3' (ERα forward) and 5'CCAGC AGCATGTCGAAGATC3' (ERα reverse), 5'TTCTATCC TAATACCATCGACG3' (pS2 forward) and 5'TTTGAGT AGTCAAAGTCAGAGC3' (pS2 reverse), 5'CTCAACAT CTCCCCCTTCTC3' (36B4 forward) and 5'CAAATCCC ATATCCTCGTCC3' (36B4 reverse) to yield products of 438 bp, 210 bp, and 408 bp with 20, 15, and 15 PCR cycles, respectively, as we confirmed sequencing these probes.

Immunoblotting

MCF7wt cells were grown in 10-cm dishes and exposed to ligands for 24 h before lysis in 500 μL of 50 m*M* HEPES (pH 7.5), 150 m*M* NaCl, 1.5 m*M* $MgCl_2$, 1 m*M* EGTA, 10% glycerol, 1% Triton X-100, a mixture of protease inhibitors (Aprotinin, PMSF), and Na-orthovanadate. Equal amounts of total protein were resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, probed with the antibodies F-10 against ERα and

β-actin (Santa Cruz, CA), and revealed using the ECL System (Amersham) *(73)*.

Immunocytochemical Staining

Cultured MCF7wt were fixed in fresh paraformaldehyde (PFA) (2% for 30 min). After PFA removal, hydrogen peroxide (3% in methanol for 30 min) was used to inhibit endogenous peroxidase activity. Cells were then incubated with normal horse serum (10% for 30 min) to block the nonspecific binding sites. Immunocytochemical staining was performed using as the primary antibody a mouse monoclonal IgG generated against the human ERα (F-10, Santa Cruz Biotechnology, CA) (1:50 overnight at 4°C). A biotinylated horse–anti-mouse IgG (1:600 for 60 min at room temperature) was applied as the secondary antibody (Vector Laboratories, Burlingame, CA). Subsequently, the amplification of avidin-biotin-horseradish peroxidase complex was carried out (ABC complex/HRP) (Vector Laboratories, Burlingame, CA) (1:100 for 30 min. at room temperature) and 3-3'-diaminobenzidine tetrachloride dihydrate (Vector Laboratories, Burlingame, CA) was used as a detection system. Cells were rinsed after each step with Tris-buffered saline (0.05 *M* Tris-HCl plus 0.15 *M* NaCl, pH 7.6) containing 0.05% Triton-X100 (TBS-T). In control experiments, cells were processed replacing the primary antibody with mouse serum (Dako S.p.A., Milan, Italy) or using a primary antibody preabsorbed (48 h at 4°C) with an excess of purified ERα protein (M-Medical, Florence, Italy).

Proliferation Assays

For quantitative proliferation assays 1×10^4 cells were seeded in 24-well plates in regular growth medium. Cells were washed extensively once they had attached and were further incubated in medium without serum for 24 h. On the second day, the medium of MCF7wt and HeLa cells was changed and supplemented with 5% CS-FCS, and the medium of MCF7SH was supplemented with 2.5% CS-FCS. Ligands were added at this point; thereafter, medium was changed every day (with ligands). On d 6, cells were trypsinized and counted in a hemocytometer.

Statistical Analysis

Statistical analysis was performed using ANOVA followed by Newman–Keuls testing to determine differences in means. p values <0.05 were considered significant.

Acknowledgments

This work was supported by "Lega Italiana per la lotta contro i tumori."

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