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Exposure of Environmental Estrogenic Compound Nonylphenol to Noble Rats Alters Cell-Cycle Kinetics in the Mammary Gland

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p-Nonylphenol is an environmental estrogen-like chemical. Nonylphenol has previously been shown to mimic the actions of estrogen both in vivo and in vitro. In the present study, we have examined the effects of an environmental estrogenic chemical, nonylphenol, on the proliferative activity, cell-cycle kinetics, and differentiation of the mammary gland of female Noble rats. The exposure of animals to two different doses of nonylphenol (0.01 and 7.1 mg/24 h) significantly increased proliferation of the mammary epithelial cells. Both labeling index and growth fraction were increased by nonylphenol treatment. Labeling index and growth fraction were 24 and 38%, respectively, for a low dose of nonylphenol; and 32 and 67%, respectively, for a high dose of nonylphenol, compared to 18 and 35%, respectively, of controls. In addition, nonylphenol exposure altered cell-cycle kinetics. Both low and high doses of nonylphenol significantly increased the conversion of mammary epithelial cells from G0 to G1 and S-phase cells by 2.2- and 2.6-fold, and by 11- and 4-fold, respectively, compared to that of controls. Differentiation measured by the degree of lobular maturation revealed that the conversion of immature structures to mature structures was significantly increased in response to nonylphenol exposure compared to that of control. Based on the previously reported estrogenic activity of an equivalent dose of nonylphenol to that of DES (0.01 mg/d), a calculated theoretical dose of the order of 10^5- to 10^6-fold higher of nonylphenol will be required to produce the same biological effects as DES. However, the data of this study showed that exposure of 0.01 mg/d of nonylphenol produced profound effect on cell proliferation in the mammary gland. The weak estrogenic activity of nonylphenol does not explain its profound effect on cell proliferation observed in this study. Perturbation of cell cycle is considered as a risk factor for the development of cancer. Changes in proliferation and cell cycle have been shown to lead to genetic instability, ultimately resulting in cell transformation. Our results indicated an increase in labeling index and growth fraction, and a perturbation in cell-cycle kinetics from nonylphenol exposure. Perturbation of cell cycle in response to nonylphenol exposure may produce adverse effects in the mammary glands of the Noble rats.

Key Words: Nonylphenol; perturbation; cell cycle.

Introduction

Nonylphenol is an alkylphenolic compound used in the preparation of lubricating oil additives, plasticizers, resins, detergents, and surface-active agents (Soto et al., 1991; White et al., 1994). Recently, nonylphenol has been shown to possess estrogen-like properties (Soto et al., 1991). Nonylphenol exposure to human estrogen-sensitive MCF-7 breast cancer cells produced an increase in cell proliferation and changes in the level of progesterone receptor. Nonylphenol also triggered mitotic activity in rat endometrium (Soto et al., 1991). Indication of changes in mitotic activity in the rat endometrium is considered an effect of estrogen action (Soto et al., 1991). Although the proliferative effect of nonylphenol in established estrogen-sensitive MCF-7 cells in culture has been reported (Soto et al., 1991), whether exposure of an environmental estrogen-like chemical, nonylphenol, to animals also affects the growth and development of the mammary gland is not clear.

Recently, environmental estrogens have been implicated to be involved in mammary carcinogenesis of some mammals (Duax and Weeks, 1980; Hunter and Kelsey, 1993; Wolff et al., 1993). For example, it has been reported that American Caucasian women with the highest DDT (an estrogenic chemical) exposure had four times the risk of breast cancer compared to women with the least exposure (Hunter and Kelsey, 1993; Wolff et al., 1993). A second epidemiological study also observed a positive association (although not statistically significant) between DDT and breast cancer risk in Caucasian and African-American

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women (Krieger et al., 1994). There are also reports showing elevated levels of organochlorine compounds in breast cancer tissues (Falck et al., 1992). In contrast, there are some studies that suggest that it is not plausible that industrial estrogen-like chemicals contribute to increased incidence of breast cancer (reviewed by Safe, 1994). Despite these controversies, there is general agreement that an appropriate hormonal milieu is required for proliferation of the epithelial cells of the mammary gland, and human populations are continually exposed to a wide variety of environmental and dietary estrogen-like chemicals. Environmental estrogen-like chemicals that may influence the growth and development of the mammary gland have not been carefully studied. Regardless of whether there is a relationship between environmental estrogen-like chemicals and breast cancer in humans as judged by the premise of the presence of an environmental estrogen-like chemical in breast cancer tissue of some patients, it is important to determine if exposure of environmental estrogen-like chemicals influences the normal growth and development of the mammary gland of animals. Therefore, investigation of the influence of an environmental estrogen-like chemical, nonylphenol, on the normal growth and development of the mammary gland of rats is the subject of this study.

In the present study, we have investigated the effect of in vivo exposure of the environmental estrogen-like chemical, nonylphenol, on cellular growth and differentiation, and on cell-cycle kinetics in the individual components of the mammary gland of Noble rats. We have used Noble rats as a model in this study because:

1. Estrogen treatment of female Noble rats for 11–12 mo induces almost 80–90% of tumors;
2. Estrogen acts as both an initiating and promoting agent; and
3. Growth of the tumor is estrogen-dependent (Cutts, 1964).

Findings of this study demonstrated that nonylphenol exposure to animals accelerated the growth of mammary glands and perturbed epithelial cell-cycle kinetics in the mammary gland.

Materials and Methods

Chemicals

Nonylphenol was purchased from Aldrich Chemical Co., Milwaukee, WI. Methyl green was purchased from Sigma Chemical Co., St. Louis, MO. Alzet osmotic minipumps, model 2002 was purchased from Alza Corporation, Palo Alto, CA. The PCNA monoclonal antibody (MAb), PCIO Clone, used for immunohistochemical staining, was purchased from Signet Labs., Dedham, MA.

Treatment

Female Noble rats 4–5-wk-old from Frederick Research Laboratory, Frederick, MD, were acclimatized for 1 wk prior to treatment. Animals were divided into two treatment groups and one control group. Nonylphenol was dissolved in DMSO and loaded into alzet osmotic minipumps (model 2002), with a mean pumping rate of 0.48 μL/h and a mean fill volume of 228 μL. The alzet minipumps were implanted sc in the dorsal side of the cervical region of the rat. One treated group received a daily dose of 7.12 mg/24 h of nonylphenol (high dose) for 11 d. Another treatment group received a daily dose of 0.01 mg/24 h of nonylphenol (low dose) for 11 d, whereas the control group received the vehicle (DMSO). After 11 d of treatment, the rats were euthanized and necropsied. A midline incision was made, and both skin and mammary tissue were rapidly pulled away from the peritoneal wall. The abdominal mammary glands were dissected from the skin, and processed for PCNA staining or whole mounts.

Mammary Gland Differentiation

Gland differentiation/growth was examined in the methyl green-stained sections of whole mounts as described previously (Colerangle and Roy, 1995a,b; Holland and Roy, 1995). Terminal ducts (TDs), Terminal end buds (TEBs), and lobules (Lob 1, Lob 2, and Lob 3) were quantified to determine the degree of differentiation or growth. An intraocular calibrated grid was used to divide each mammary section into four areas using the lymph node as a reference point. Thee of the four areas were consistently analyzed in each section. TDs, i.e., terminal mammary structures with diameters between 50 and 100 μm; TEBs, i.e., structures in diameters >100 μm; and gradation of Lobs 1, 2, and 3 based on both size and number of alveolar buds were estimated (Russo and Russo, 1987). Lobules containing up to 11 alveolar buds were considered Lob 1, 12–47 alveolar buds/lobule were Lob 2, and Lob 3 contained 48–81 alveolar buds/lobule (Russo and Russo, 1987). The number of TDs, TEBs, and lobules counted per three areas of 16 mm^2/section were recorded.

Cell Proliferation Assay

Immunohistochemical analysis for reactivity to anti-proliferating cell nuclear antigen (PCNA) MAb (PC10 clone) was used to measure cell proliferation as described previously (Colerangle and Roy, 1995a,b; Holland and Roy, 1995). The immunohistochemical staining procedure yielded consistent staining patterns, which allowed epithelial cells in S. G1, and G0 phases of the cell cycle to be identified. Positive PCNA staining was categorized as either G1 or S phase. Cells in the G0 or quiescent phase expressed no detectable staining. Positive PCNA staining with patchy to uniform, light-brown nuclear staining without cytoplasmic staining as G1 and with uniform dark brown to black nuclear staining without cytoplasmic staining as S phase were considered (Foley et al., 1993). All cells counted were epithelial cells. Myoepithelial cells, fibroblasts, and adipocytes were excluded. The total number of cells in three 16-mm^{-2} areas of each gland was determined. Similarly, the number of cells per structure was determined within the same area to give an estimation of total cell pro-

Table 1
Influence of Nonylphenol on Differentiation and Cell Growth[a]

Treatment group	Average no. of structures/16 mm²					Total structures
	TD	TEB	L1	L2	L3	
Control	10 ± 1.1	11 ± 1.1	12 ± 0.8	5 ± 0.6	2 ± 0.7	40 ± 2.8
Nonylphenol (0.01 mg/d)	5 ± 0.7*	7 ± 0.5*	15 ± 0.9	9 ± 0.3*	5 ± 0.4*	41 ± 0.3*
Nonylphenol (7.12 mg/d)	6 ± 0.4*	12 ± 0.7	17 ± 0.1*	17 ± 0.3*	9 ± 0.3*	61 +0.8*
	Total no. of cells, proliferating + nonproliferating/16 mm²					Total, TD + TEB + L1 + L2 + L3
	TD	TEB	L1	L2	L3	
Control	136 ± 3.1	242 ± 1.2	660 ± 7.9	645 ± 3.6	372 ± 2.4	2055 ± 6.3
Nonylphenol (0.01 mg/d)	100 ± 2.8*	223 ± 2.4*	1158 ± 9.1*	1449 ± 6.1*	1170 ± 2.5*	4100 ± 3.1*
Nonylphenol (7.12 mg/d)	252 ± 3.9*	1080 ± 3.1*	2558 ± 2.3*	1597 ± 8.2*	1174 ± 6.1*	8068 ± 1.3*

[a] Female Noble rats were treated with DMSO, which served as control, low dose (0.01 mg/d), and high dose (7.12 mg/d) of nonylphenol for 11 d. The average number of structures (i.e., TD = terminal duct; TEB = terminal end bud; L1 = lobule type 1; L2 = lobule type 2; and L3 = lobule type 3) and total number of cells in the individual structures of the mammary gland present in three 16-mm² areas of each section of the abdominal gland stained with methyl green were counted. Each value is a mean ± standard deviation from 6 experiments. The value of p was calculated by comparing the effects of low- and high-dose nonylphenol treatment to control (*$p < 0.05$).

liferation (G1 + S). intestinal sections were used as positive and negative controls. The negative control was not incubated with the PCNA MAb and, thus, did not show any staining for proliferating cells. Labeling index (LI) and growth fraction were generated for all experimental groups. LI was calculated by dividing cells exhibiting an S phase by the total number of cells (Colerangle and Roy, 1995a,b). An estimation of cells active in the cell cycle was quantified by dividing the G1 + S cells by the total number of cells counted to generate the growth fraction (GF) (Colerangle and Roy, 1995a,b).

Statistical Analysis

The data generated for each treatment group were tested for statistical significance. A two-tailed, one-way ANOVA was used to compare data from each group.

Results

Analysis of mammary glands from untreated and nonylphenol-treated animals indicated an increase in proliferating cells and a perturbation of cell-cycle kinetics from nonylphenol exposure. The results of the differentiation and the proliferation are shown in Tables 1–3 and Figs. 1–4.

Influence of Nonylphenol on the Growth of the Mammary Gland

To test whether nonylphenol exposure to female Noble rats influenced the growth of mammary glands, sections of whole mounts of the mammary glands were stained with methyl green, and structures were analyzed microscopically (Fig. 1). It was observed that a significant increase in Lob 1, Lob 2, and Lob 3 occurred after high dose nonylphenol treatment (Table 1). Treatment with low-dose nonylphenol did not show any significant increase in structures over control, except for its effect on Lobs 2 and 3. As seen in Table 1, the control group had mainly TDs and TEBs (53%) compared to lobules (47%). Although the TDs and TEBs were relatively less (29% for a low dose and 30% for a high dose) in the nonylphenol-treated groups compared to control, a 1.5-fold increase (71% for a low dose) and 1.49-fold increase (70% for a high dose) in lobules occurred after nonylphenol treatment. Only Lobs 2 and 3 were significantly increased by nonylphenol exposure ($p < 0.05$) compared to control.

Table 1 shows cell growth in all structures of the mammary gland in response to nonylphenol exposure. The number of cells in three 16-mm² areas of the section of whole mammary gland in the control group was 2055 compared to 4100 and 8068 in animals exposed to 0.01 mg/d (low dose) and 7.1 mg/d (high dose) doses of nonylphenol, respectively. Low-dose nonylphenol treatment significantly increased the number of cells in Lobs 1, 2, and 3 compared to controls. A high dose of nonylphenol treatment significantly increased the number of cells in the TEBs as well as Lobs 1, 2, and 3. This increment in cells numbed was significant ($p < 0.05$) compared to that of the control group. Overall, low-dose nonylphenol treatment induced a twofold increase in cell numbers over control, whereas high-dose nonylphenol treatment induced a fourfold increase in cell numbers over controls (Table 1).

Table 2
Influence of Nonylphenol on Cell-Cycle Kinetic Constants in the Mammary Gland of Noble Rats[a]

Compound	Estrogenic activity	Proliferative effect	T_{pot}, h (h)	L1, %	GF, %	Total no. of cells/16 mm^2
Control	Not applicable	100%	55–65	18	31	2055 ± 11
Nonylphenol (0.01 mg/d)	0.00001–0.000003 (Soto et al., 1991; White et al., 1994)	200%	57–63	24	38	4100 ± 3
Nonylphenol (7.12 mg/d)	0.0071 (Soto et al., 1991; White et al., 1994)	400%	50–55	32	67	8068 ± 1
DES (0.01 mg/d)	1.0	600%	22–24	71	158	11,781 ± 6

[a] Female Noble rats were treated with DMSO, which served as control, low dose (0.01 mg/d), and high dose (7.12 mg/d) of nonylphenol or DES (0.01 mg/d) for 11 d. Proliferating cells in three 16-mm^2 areas of each section of mammary gland from 6 animals/experimental group were analyzed by PCNA method (Colerangle and Roy, 1995a). Labeling index, growth fraction, and T_{pot} were calculated as described in the Materials and Methods section.

Table 3
Influence of Nonylphenol on Cell Proliferation[a]

Treatment group	Average no. of proliferating cells/structure					Total, TD + TEB + L1 + L2 + L3
	TD	TEB	L1	L2	L3	
Control	70 ± 0.9	87 ± 1.1	206 ± 2.5	207 ± 2.5	156 ± 0.7	726 ± 2.3
Nonylphenol (0.01 mg/d)	81 ± 1.2	136 ± 2.7*	468 ± 0.9*	443 ± 3.7*	443 ± 0.9*	1571 ± 1.1*
Nonylphenol (7.12 mg/d)	197 ± 3.1*	934 ± 8.1*	2199 ± 7.2*	1323 ± 7.2*	760 ± 5.4*	5413 ± 1.9*

[a] Female Noble rats were treated with DMSO, which served as control, low dose (0.01 mg/d), and high dose (7.12 mg/d) of nonylphenol for 11 d. The average number of PCNA positive proliferating cells (G1 + S) in the individual structures (i.e., TD = terminal duct; TEB = terminal end bud; L1 = lobule type 1; L2 = lobule type 2; and L3 = lobule type 3) of the mammary gland present in three 16-mm^2 areas of each section of the mammary gland were counted. Each value is a mean ± SD from 6 experiments.The value of p was calculated by comparing the effects of low- and high-dose nonylphenol treatment to control (*$p < 0.05$).

Cell Proliferation and Cell-Cycle Kinetics

To understand nonylphenol-mediated increase in cell growth compared to that of untreated animal mammary glands, we examined the effects on the rate of cell proliferation in response to nonylphenol exposure. The rate of cell proliferation was examined by estimating the labeling indices and growth fractions. The control group had a labeling index of 18%, indicating that actively proliferating cells totaled 18% of the overall cell population. The nonylphenol-treated groups had relatively higher labeling indices, 24 and 32% for the low- and high-dose nonylphenol-treated groups, respectively (Table 2). Analysis of the growth fraction revealed that only 35% of the total number of cells counted in the control group was active in the cell cycle. In the nonylphenol-treated groups, 38 and 67% of the cells were actively cycling cells in the low- and high-dose treated groups, respectively (Table 2). It is concluded that total cell proliferation was increased significantly in all structures of the nonylphenol-treated groups compared to that of control (Table 2).

To understand the nonylphenol-mediated increase in proliferative activity in the individual components of the mammary gland, we examined alterations in the cell cycle in response to low and high doses of nonylphenol exposure compared to controls (Fig. 2). Figures 3 and 4 show changes in the number of cells in the S and G1 phase/16-mm^2 whole-mount sections of mammary gland from all treatment groups. The cells in S phase were significantly increased in all mammary structures by high-dose treatment compared to the effect of low-dose treatment and controls. The effect of low-dose nonylphenol treatment was also significant in the TEBs and Lobs 1, 2, and 3 compared to controls. High-dose nonylphenol treatment induced a sevenfold increase in S-phase cells compared to control, whereas the low-dose nonylphenol treatment induced a 2.6-fold increase in S-phase cells. A comparison of the effects of high- and low-

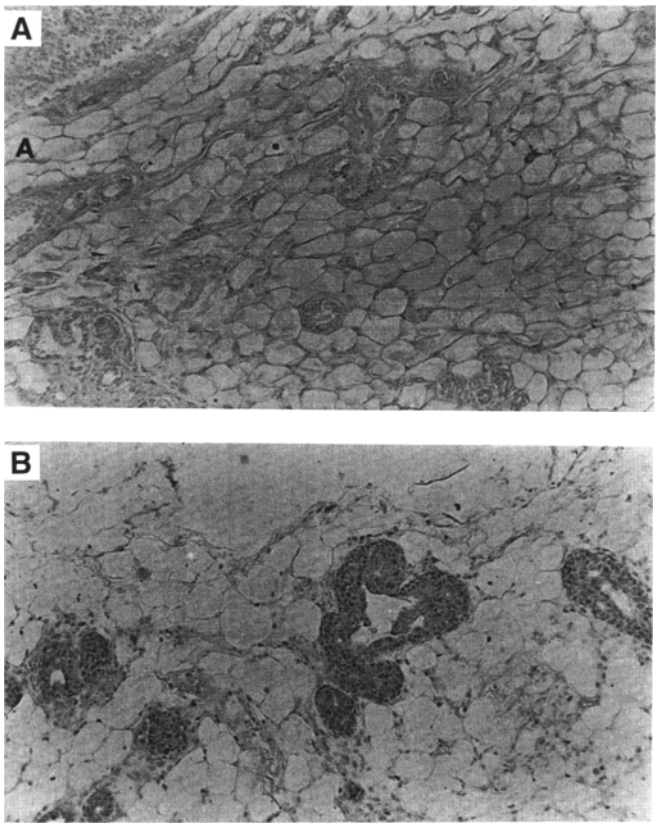

Fig. 1. Representative photomicrographs showing the influence of nonylphenol exposure to female Noble rats on mammary gland differentiation. Female Noble rats were treated with 7.1 mg nonylphenol/24 h **(B)** or vehicle **(A)** sc for 11 d. Mammary gland differentiation was examined in the sections of the abdominal gland after staining with 0.1% methyl green (×20).

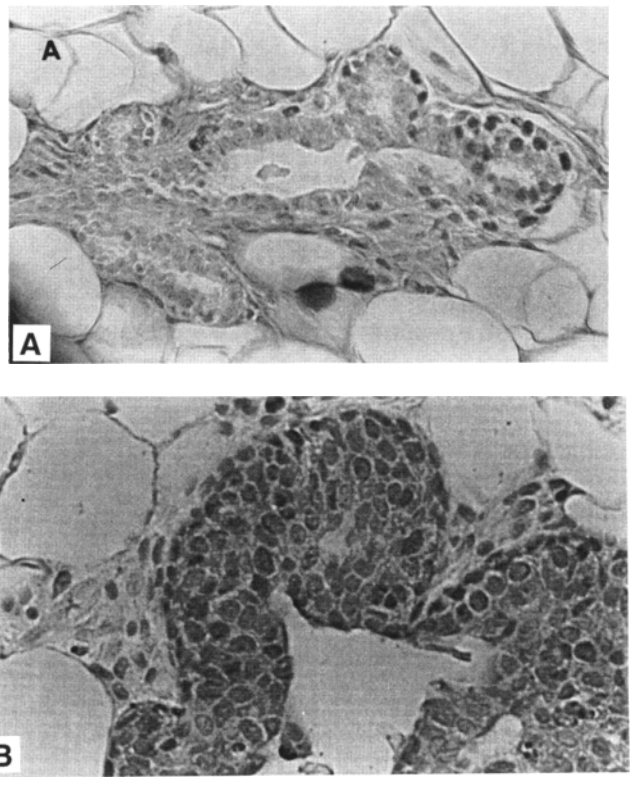

Fig. 2. Representative photomicrographs showing the influence of nonylphenol on cell proliferation in the mammary gland of female Noble rats. Female noble rats were treated with 7.1 mg nonylphenol/d sc for 11 d **(B)** or vehicle **(A)**. Cell proliferation was examined by immunohistochemical detection using PCNA antibody (Colerangle and Roy, 1995a,b) (×400).

dose nonylphenol treatment revealed that high-dose nonylphenol treatment induced a 2.6-fold increase in S-phase cells compared to low-dose nonylphenol treatment ($p < 0.05$) (Fig. 3). Similarly, cells in G1 phase portrayed a similar trend. A significant increase in total G1-phase cells was observed in all mammary structure, except for the TDs and TEBs of the low-dose-treated group. The high-dose-treated group showed an 11-fold increased in G1-phase cells ($p < 0.05$) over control, whereas the low-dose-treated group showed only a 2.2-fold increase in G1-phase cells ($p < 0.05$) over the control. A comparison of the effects of high-dose to low-dose nonylphenol treatment revealed a significant increase in G1-phase cells, 4.8-fold ($p < 0.05$), by high-dose treatment (Fig. 4).

Estimation of cells in G1 +S phase across each treatment group revealed a similar trend. Although the control group had a total of 726 G1 + S-phase cells/16 mm^2, the nonylphenol-treated groups had a total of 1571 (2.2-fold increase) and 5413 (7.5-fold increase) in G1 + S-phase cells/16 mm^2 for the low- and high-dose-treated groups, respectively (Table 3). The cells in G2 and M phases were very few. Therefore, we did not analyze G2- and M-phase cells.

Correlation Between Previously Reported Estrogenic Activity of Nonylphenol and Changes in Proliferative Activity in the Mammary Gland in Response to Exposure of Nonylphenol

Based on previous in vitro studies by Soto et al. (1991) and White et al. (1994) and our in vivo studies (Colerangle and Roy, 1995a), the estrogenic activity of different doses of nonylphenol used in this study would be 10^{-5} to 10^{-6}-fold assuming the estrogenic activity of DES, the more potent estrogenic compound, to be 1. Administration of DES (0.01 mg/d) to female Noble rats for 11 d produced a sixfold increase in proliferative activity compared to controls (Table 2), which is in agreement with our recent report (Colerangle and Roy, 1995a). The potential doubling time (T_{pot}), i.e., time required for cells numbers to double was drastically reduced from 55–65 h (control) to 22–24 h after DES treatment (Colerangle and Roy, 1995b). The exposure of a 10μg/d dose of nonylphenol, which should be estrogenically 10^5–10^6 less potent than the same dose of DES, produced a 200% increase in proliferative activity compared to controls, whereas an equivalent dose of DES produced a 600% increase in the proliferative activity over the controls (Table 2). Similarly, the labeling index and growth fraction of an equivalent dose of nonylphenol were about

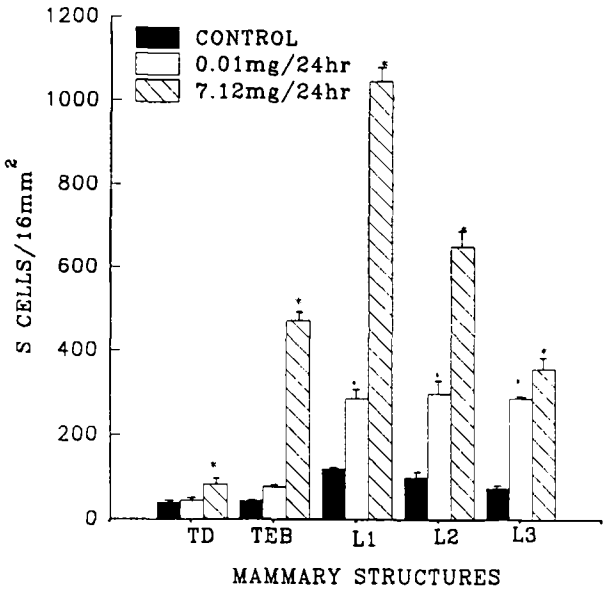

Fig. 3. Influence of nonylphenol on S cells in the individual structures of the mammary gland of Noble rats. Female Noble rats were treated with DMSO, which served as control, low dose (0.01 mg/d), and high dose (7.12 mg/d) of nonylphenol for 11 d. The total number of S cells in the individual structures (i.e., TD = terminal duct; TEB = terminal end bud; L1 = lobule type 1; L2 = lobule type 2; and L3 = lobule type 3) of the mammary gland present in three 16-mm^2 areas of each section of the abdominal gland stained with methyl green were counted. Each value is a mean ± SD from 6 experiments.The value of p was calculated by comparing the effect of low- and high-dose nonylphenol treatment on S cells to control ($*p < 0.05$).

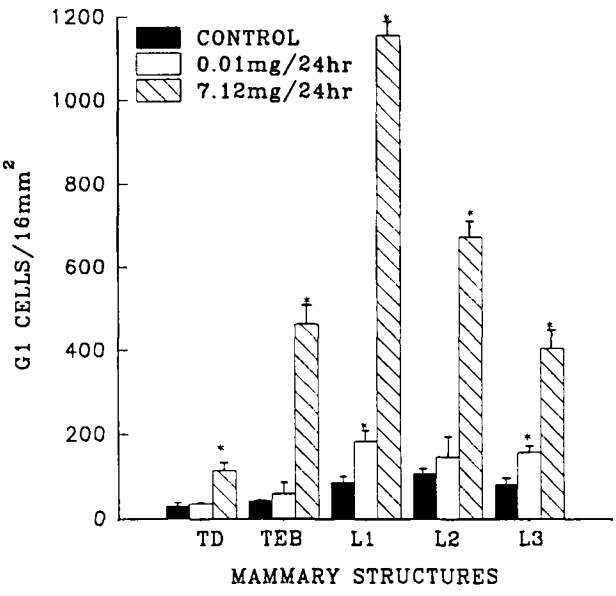

Fig. 4. Influence of nonylphenol on G1 cells in the individual structures of the mammary gland of Noble rats. Female Noble rats were treated with DMSO, which served as control, low dose (0.01 mg/d), and high dose (7.12 mg/d) of nonylphenol for 11 d. The total member of G1 cells in the individual structures (i.e., TD = terminal duct; TEB = terminal end bud; L1 = lobule type 1; L2 = lobule type 2; and L3 = lobule type 3) of the mammary gland present in three 16-mm^2 areas of each section of the abdominal gland were counted. Each value is a mean ± SD from 6 experiments.The value of p was calculated by comparing the effect of low- or high-dose nonylphenol treatment on G1 cells to control ($*p < 0.05$).

24 and 38%, respectively, compared to 71 and 158%, respectively, of DES. Based on these findings, we administered a higher dose of nonylphenol (7.12 mg/d), which should be estrogenically 10^2-fold less active than the dose of DES (0.01 mg/d). The proliferative activity of this dose of nonylphenol was 400% compared to controls. Similarly, the labeling index and growth fraction of the 7.12 mg/d dose of nonylphenol were 32 and 67%, respectively, compared to 71 and 158%, respectively, of DES (Table 2). Based on these data, it appears that despite the relatively low estrogenic activity of nonylphenol, its effect on the proliferative activity in the epithelial cells of the mammary gland of Noble rats is profound.

Discussion

We demonstrate, for the first time, that exposure of Noble rats to nonylphenol increases the proliferative activity and alters cell-cycle kinetics of epitlhelial cells of the mammary gland. Both low and high doses of nonylphenol significantly increased the conversion of mammary epithelial cells from G0- to G1- and S-phase cells by 2.2- and 2.6-fold, and by 11- and 4-fold, respectively, compared to that of controls. Increased cell proliferation and alteration in cell-cycle kinetics are considered important factors for the development of genetic instability (Prestin-Martin et al., 1990). For example, cell proliferation may allow mitotic recombination to occur, which may result in more profound changes than those of a single mutation (Prestin-Martin et al., 1990). Damage to DNA, such as incorporation of a wrong nucleotide, can occur during DNA synthesis. Damaged DNA is considered to be repaired during the G1 phase of the cell cycle (Cohn et al., 1978). Nonylphenol alone is capable of producing telomeric associations and chromosomal aberrations (Banerjee and Roy, 1996). As observed in this study, the massive burst in the synthesis phase of the cell cycle in response to the exposure of nonylphenol coupled with a decrease in DNA repair may allow DNA damage, acquired during synthesis, to accumulate and fix the genetic instability. These events may produce genetic instability, which could be a factor in the development of adverse effects in the mammary gland. It remains to be confirmed, however, that any one of these individual events occurs in direct response to nonylphenol exposure. Since we have established here that indeed nonylphenol exposure can perturb the cell cycle, now we will seek to understand whether perturbations of the cell cycle predispose the rat mammary gland to develop genetic instability. Such studies would provide insights regarding how environmental or ovarian estrogen may contribute to genetic instability.

The exact mechanism by which nonylphenol might have induced cell proliferation and altered the cell cycle is not clear. A range of alkylphenols with an alkyl group in the *para-* (or forth) position on the phenol ring were able to stimulate the growth of MCF-7 cells (Soto et al., 1992). The alkylphenols are mitogenic presumably as a result of their ability to bind to estrogen receptors (Soto et al., 1992; White et al., 1994). Alkylphenols have been shown to be estrogenic in fish, avian, and mammalian cells and they mimic the effects of 17β-estradiol by binding to the estrogen receptor (White et al., 1994). The actions of alkylphenols were inhibited by estrogen antagonists (White et al., 1994). Moreover, the observation that the mutant receptor G-525R, which is defective in estrogen binding (Danielian et al., 1993), is also insensitive to alkylphenols suggests that alkylphenols interact with a similar region of the hormone- binding domain as does 17β-estradiol. In spite of the low binding activity of alkylphenols, it is striking that alkyl–phenols are able to stimulate a number of biological responses, such as massive burst of proliferative activity as observed in this study and specific gene transcription to the same extent as 17β-estradiol itself (White et al., 1994). These findings suggest that both transcriptional activation functions, TAF-1 and TAF-2, are functional when an alkylphenol is bound to the receptor. This is supported by the previously reported observation comparing the activity of the wild-type receptor with that of the deletion mutant MOR 121-599 (White et al., 1994). In chicken embryo fibroblast (CEF) cells, the TAF-2 activity exhibited by the deletion mutant receptor was induced by an alkylphenol as well as by 17β-estradiol, and maximum transcriptional activation by the wild-type receptor, which depends on TAF-1, was also induced similarly by either ligand (White et al., 1994). It seems remarkable that a molecule as structurally different from 17β-estradiol as alkylphenol is able to mimic the action of the natural hormone in inducing fail transcriptional activity of the receptor (White et al., 1994). Estrogen-like chemicals have previously been shown to influence ovarian and pituitary hormone release. Therefore, we cannot exclude the possibility of the involvement of both peptide and steroid hormones in nonylphenol- induced proliferative acitivity in the mammary gland (Safe, 1994). Also we do not rule out the possibility of involvement of other pathway(s), such as growth factors and growth-regulating proto-oncogens, for the effects observed in the mammary gland in response to nonylphenol exposure.

Alkylphenols are widely used as plastic additives, surfactants, in industrial detergents, and in other formulated products, such as paints, herbicides/pesticides, thoughout the world (Soto et al., 1991, 1992; White et al., 1994). Nonylphenol has been shown to leach from plastic used in food processing and wrapping (Gilbert et al., 1992). Presently, there is no direct evidence that the estrogenic activity possessed by this group of chemicals might be responsible for any deleterious effect in any species. However, the widespread use of alkylphenols and the persistence of their degradation products in the environment coupled with the concern about inadvertent exposure of humans and wildlife to "estrogens" raise considerable disquiet. Tlhe relatively low estrogenic activity of nonylphenol does not rule out its potential toxicity after chronic exposure to animals or human beings. Fish in the Detroit River's Trenton channel, near a chemical plant manufacturing alkylphenols, were reported to contain 40 μg of *p-tert*-pentylphenol/g of fat tissue, a concentration higher than the ones found in the river sediment (Shiraishi et al., 1989) . The *p-tert*-pentylphenol concentration in carp adipose tissue was comparable to the alkylphenol concentration eliciting maximal cell proliferation in MCF-7 cells. This compound causes vaginal cornification in ovariectomized rats (Dodds and Lawson, 1938) and interacts with estrogen receptors (Mueller and Kim, 1978) . The bioaccumulative properties of alkylphenols (Shiraishi et al., 1989) parallel those of chlordecone, which is sequestered in liver and adipose tissue, eliciting considerable estrogenic activity in spite of its low potency when compared to E_2 (Hammond et al., 1979; Egle et al., 1978). Reproductive effects, such as oligospermia and sterility, were reported in workers exposed to chlordecone (Cohn et al., 1978). Estrogenic effects of alkylphenol and chlordecone in MCF-7 cells occur at comparable doses (Soto et al., 1991). Based on nonylphenol's weak estrogenic activity, a calculated theoretical dose on the order 10^5- to 10^6-fold higher of nonylphenol will be required to produce the same biological effects as DES (0.01 mg/d). According to the weak estrogenic concept, exposure of 0.01 mg/d dose of nonylphenol should not have produced any biological effects in the mammary gland of animals. In contrast, exposure of a 0.01 mg/d dose of nonylphenol produced a profound effect on proliferative activity in the epithelial cells of mammary gland. The weak estrogenic activity of nonylphenol does not explain its profound effect on cell proliferation as observed in this study. A recent report also indicated a substantially higher activity for related alkylphenols in vivo than prior reports in vitro and was much more active relative to DES when measured in vivo (Nagel et al., 1995). These findings suggest that the exposure of alkylphenols may pose hazards to humans and other animals when target cells *in situ* become exposed to these levels of alkylphenols.

In summary, our findings are significant because we demonstrate for the first time that exposure of Noble rats to an environmental estrogen-like chemical, nonylphenol, alters the cell cycle of the epithelial cells of the mammary gland. Our results may also provide markers for evaluating similarities and differences of early changes in the growth and development of the mammary gland in response to the exposure of environmental estrogen-like chemicals to animals and humans on the basis of proliferative effect and the degree of perturbation of cell-cycle kinetics.

References

Banerjee, S. K. and Roy, D. (1996). *Proc. Am. Assoc. Cancer Res.* **37,** in press.

Cohn, W. J., Boylan, J. J., Blanke, R. V., Farris, M. W., Howell, J. R., and Guzelian, P. S. (1978). *N. Engl. J. Med.* **298,** 243–248.

Colerangle, J. and Roy, D. (1995a). *Cancer Lett.* **94,** 55–63.

Colerangle, J. and Roy, D. (1995b). *Int. J. Oncol.* **7,** 1361–1366.

Cutts, J. H. (1964) *Cancer Res.* **24,** 1124–1130.

Danielian, P. S., White, R., Hoare, S. A., Fawell, S. A., and Parker, M. G. (1993). *Mol. Endocrinol.* **7,** 232–240.

Dodds, C. E. and Lawson, W. (1938). *Proc. Royal Soc. (Lond.)* **B125,** 222–232.

Duax, W. L. and Weeks, C. M. (1980). In: *Estrogens in the environment* (McLachlan, J. A. (ed.). Elsevier/North Holland: New York, pp. 11–30).

Egle, J. L., Fernandez, S. B., Guzelian, P. S., and Borzelleca, J. F. (1978). *Drug Metab. Disp.* **6,** 91–95.

Falck, F., Ricci, A., Wolff, M. S., Godbold, J., and Deckers, P. (1992). *Arch. Environ. Health* **47,** 143–146.

Foley, J., Ton, T., Maronport, R., Butterworth, B., and Goldsworthly, T. L. (1993). *Environ. Health Perspect.* **101,** 199–205.

Gilbert, M. A., Shepherd, M. K., Startin, J. R., and Walwork, M. A. (1992). *J. Chomatgr.* **237,** 249–261.

Hammond, B., Katzenellenbogen, B. S., Krauthammer, N., and McConnel, J. (1979). *Proc. Natl. Acad. Sci. USA* **76,** 6641–6654.

Holland, M. and Roy, D. (1995). *Carcinogen* **16(8),** 1955–1961.

Hunter, D. J. and Kelsey, K. T. (1993). *J. Natl. Cancer Inst.* **85,** 598–599.

Krieger, N., Wolff, M. S., Hiatt, R. A., Rivera, M., Vogelman, J., and Orentreich, N. (1994). *J. Natl. Cancer Inst.* **86,** 589–599.

Mueller, G. and Kim, U. H. (1978). *Endocrinology* **102,** 1429–1435.

Nagel, S. C., Thayer, K. A., Boechler, M., Dhar, M., Morellini, F., Taylor, J. A., vom Saal, F. S., and Welshons, W. V. (1995). *Proc. Soc. Reprod.* **28,** 96.

Prestin-Martin, S., Pike, M. C., Ross, R. K., Jones, P. A., and Henderson, B. E. (1990). *Cancer Res.* **50,** 7415–7421.

Russo, J. and Russo, I. H. (1987). *Lab. Invest.* **57,** 112–137.

Safe, S. (1994). *Environ. Health. Persp.* **103,** 346–351.

Shiraishi, H., Carter, D. S., and Hites, R. A. (1989). *Biomed. Environ. Mass. Spectrom.* **18,** 478–483.

Soto, M. A., Justicia, H., Wray, J. W., and Sonnenschein, C. (1991). *Environ. Health Perspect.* **92,** 167–173.

Soto, A. M., Lin, T. M., Justicia, H., Silvia, R. M., and Sonnenschein, C. (1992). In: *Advances in modern environmental toxicology*, vol. 21. Colborn, T. and Clement, C. (eds.). Princeton Scientific: Princeton, pp. 295–309.

White, R., Jobling, S., Hoare, S. A., Sumpter, J. P., and Parker, M. G. (1994). *Endocrinology* **135,** 175–182.

Wolff, M. S., Toniolo, P. G., Lee, P. W., Rivera, M., and Dubin, M. (1993). *J. Natl. Cancer Inst.* **85,** 648–652.