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4-Nonylphenol Increased NO Synthesis via a Non-genomic Action in GH3 Cells

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뇌하수체 세포인 GH3세포에서 non-genomic action을 통한 Nonylphenol의 nitric oxide 증진효과

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요 약

본 연구는 환경호르몬(endocrine disruptors)으로 분류되었으며, 에스트로젠 화합물의 특성을 지닌 4-Nonylphenol (NP)이 설치류 pituitary 세포 중 성장호르몬을 분비하는 GH3 세포의 Nitric oxide (NO)을 증가시키는 작용기전을 규명코자 수행되었다. 먼저 GH3 세포에 NP 처리 농도에 따른 NO의 생성을 측정한 결과 NP 처리농도 의존적으로 증가시켰다. 이러한 NO의 증가가 genomic action인지를 확인하기 위해 GH3 세포의 NO를 증가시키는 효소인 neuronal oxide synthase의 단백질량을 측정한 결과 GH3 세포에서 NP에 의한 nNOS의 단백질의 변화는 없었다. 에스트로젠 화합물인 NP가 에스트로젠 리셉터 (ER)와의 관계를 조사하기 위해 ER 억제제 (ICI 168,780)를 처리한 경우 NP에 의해 증가한 NO가 감소하였다. 또한 유전자 전사억제제인 actinomycin D 및 단백질 발현 억제제인 cycloheximide을 처리한 경우는 NP에 의한 NO 증가억제효과가 없었다. 이러한 결과를 종합해 볼 때 GH3 세포에서 NP는 ER을 매개한 non-genomic action에 의해 NO를 증가키는 것으로 사료된다.

Key words : Nonylphenol, GH3, Estrogen, Nitric oxide

INTRODUCTION

Environmental chemicals that disrupt endocrine function have been linked to adverse effects on the reproductive system in wildlife and humans (Colborn *et al.*, 1993). 4-Nonylphenol (NP) is a degradation product of a widely used non-ionic surfactant group, alkylphenol polyethoxylates, which are found mainly as an intermediate in the chemical manufacturing industry. NP is used for example to label tax-favored light fuel oil, as a preservative agent in the tanning industry, and in pesticide and cosmetic formulation, etc (Nimrod and Benson, 1996). Environmental estrogens are a class of natural and synthetic compounds, which can mimic the function or activity of endogenous estrogen 17β-estradiol (E2). NP has been shown to possess estrogenic properties (White *et al.*,

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1994; Laws *et al.*, 2000; Gutendorf *et al.*, 2001). Therefore, NP is known an "endocrine disrupter" that has a significant influence on sexual and reproductive development (Sharpe *et al.*, 1995). Human exposure to environmental compounds with estrogenic activity and their potential effects on human health is the subject of an ongoing scientific debate (Muller *et al.*, 1995). NP was reported to cause reproductive toxicity and affect cellular development in rats and mice (Hossaini *et al.*, 2001). However, the mechanism by which NP causes these adverse effects is unclear. There is a general consensus that NP is a weak estrogen, requiring 2000~5000-fold higher concentrations than 17-estradiol (E2) to stimulate prolactin release from GH3 cells (Steinmetz *et al.*, 1997) and stimulate cell proliferation and induction of progesterone receptor in breast cancer MCF-7 cells (Krishnan *et al.*, 1993). Although the effects of NP are weak as compared with those of E2 ,even minor disturbances in endocrine activity, particularly during critical stages of fetal and/or neonatal development, are known to lead to major, long-lasting, and permanent changes in the adult. There are various in vitro assays for xenoestrogens with multiple estrogen-responsive endpoints including cell proliferation, gene/protein expression, enzyme activity, and reporter gene activity in cells transiently transfected with a promoter reporter construct. Because the relative potencies of xenoestrogens are assay-dependent, multiple reliable and practical bioassays must be utilized. It has been known for several decades that environmental estrogens including NP produced weak responses in uterotropic assays (Laws *et al.*, 2000). In the uterus, early events occur within minutes to a few hours after exposure to estrogen and include increases in vascular permeability, water imbibition, increases in organ wet weight, and induction of protooncogenes (Weisz and Bresciani, 1988; Loose-Mitchell *et al.*, 1988). It has been known that upregulation of nitric oxide (NO) synthesis contributes to uterine edema caused by E2 (Chaves *et al.*, 1993). These observations suggest that NO might be involved in uterotropic actions of environmental estrogens. E2-induced up-regulation of neuronal NO synthase (nNOS) mRNA and NOS activity has been reported in cell culture (Hishikawa *et al.*, 1995; MacRitchie *et al.*, 1997) and in vivo (Weiner *et al.*, 1994), although it has been reported as inconclusive in some cases (Cho *et al.*, 1999). Although phytoestrogens may affect NOS activity, this effect still remains to be clarified (Chen *et al.*, 1999b; Lemos *et al.*, 1999). Since NO is also involved in reproductive processes such as fertilization (Heck *et al.*, 1994), implantation (Purcell *et al.*, 1999) and ovulation (Ellman *et al.*, 1993), inappropriate NO synthesis causes reproductive disorders (Drazen *et al.*, 1999; Sengoku *et al.*, 2001). However, there is no simple and reproducible assay to evaluate the effect of environmental chemicals on NO synthesis. In the present study, we investigated the effects of NP on NO synthesis using the murine murine pituitary cell line, GH3. The involvement of ER in this process was also investigated using the ER antagonist, ICI 182.780. We provide evidence to support the NP induced NO via a non-genomic action in GH3 cells.

MATERIALS AND METHODS

Reagents

Chemicals and cell culture materials were obtained from the following sources: NP, dimethyl sulfoxide (DMSO), 17-estradiol (E2), actinomycin D, and cycloheximide were purchased from Sigma Chemical (Sigma, Korea). ICI 182,780 was purchased from Tocris (Ballwin, MO). Dulbecco's modified Eagle's medium (DMEM) without phenol red, α-minimum essential medium (α-MEM), fetal calf serum (FCS), penicillin, and streptomycin were purchased from GIBCO BRL (Grand Island, NY). Monoclonal antibody to neuronal NOS (nNOS) were purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibody to estrogen receptor-α (ER-α) and polyclonal antibody to ER-β were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Anti-rabbit IgG conjugated to horseradish peroxidase and

anti-mouse IgG conjugated to horseradish peroxidase were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). E2, NP, and ICI 182,780 were dissolved in DMSO.

Cell culture

GH3 cells from the American Type Culture Collection were cultured at 37°C in a CO_2 incubator that provided a humidified environment of 95% air and 5% CO_2.

Drug treatments and sample preparation

GH3 cells were seeded in 24-well plates (5×10^5 cells/ml) containing of growth medium. After 12 hr incubation, the medium was changed to a steroid-free medium of the same volume comprised of phenol red free DMEM containing 10% charcoal-treated FCS, 100 units/ml penicillin, and 100 mg/ml streptomycin (treatment medium). After another 12 hr, the medium was changed to two-fifths volume of fresh treatment medium containing test compound. Cells were harvested by treatment with trypsin-EDTA, washed twice with ice-cold PBS, sonicated on ice in 50 mM Tris/HCl buffer (pH 8) containing 150 mM NaCl, 3 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 0.5 mM pepstatin, and 2 mg/ml aprotinin. Samples in triplication were harvested from three to six independent cultures. Each assay was performed using samples from at least three independent cultures.

Measurement of nitrite and nitrate

After incubating for 24 h, NO synthesis was determined by assaying the culture supernatants for nitrite, the stable reaction product of NO with molecular oxygen, using Griess reagent as described previously (Choi *et al.*, 2001).

Western blot

Samples (40 μg of protein) were analyzed with the use of SDS-polyacrylamide gel electrophoresis (7% gel). The gels were blotted onto a nitrocellulose membrane, blocked with 0.2 mg/ml thimerosal in blocking buffer and probed with anti-nNOS monoclonal antibody (1 : 2000), anti-ER-α polyclonal antibody (1 : 2000), or anti-ER-β polyclonal antibody (1 : 2000). Anti-mouse IgG or anti-rabbit IgG conjugated to horseradish peroxidase (1 : 10,000) was used as a secondary antibody. Amersham ECL reagent (Amersham International, Buckinghamshire, England) and Hyperfilm ECL (Amersham, Arlington Heights, IL) was used to detect the peroxidase conjugate as described by the manufacturer

Statistical analysis

All experiments were repeated at least three times. Student's *t* test was used to assess the statistical significance of differences. A confidence level of < 0.05 was considered significant.

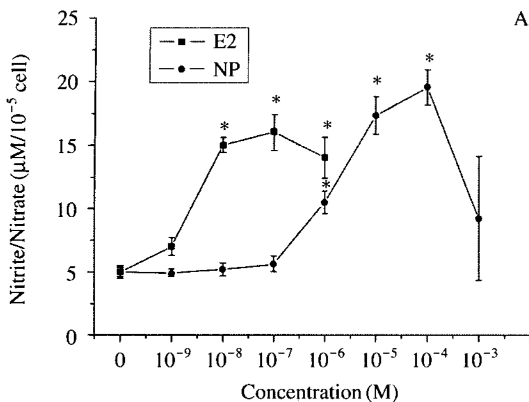
RESULTS

NP induces dose-dependent NO synthesis in GH3 cells

No statistically significant accumulation of nitrite and nitrate was not detected in the culture medium of untreated GH3 cells within 18 h. Treatment with NP increased the levels of nitrite and nitrate in a dose-dependent manner (Fig. 1A). The highest dose of NP (1 μM) did not increase nitrite and nitrate significantly. However, increase of nitrite and nitrate was statistically significant when normalized by cell viability. A selective inhibitor of NOS, L-NAME (1 μM), suppressed this increase by treatment with NP (10 μM) (Fig. 1B).

nNOS and ER-α protein is expressed in GH3 cells

Western blot analysis exhibited immunoreactivities to nNOS in untreated GH3 cells. Treatment with NP (10 μM) for 18 h did not induce nNOS. No significant increase in nNOS expression level was observed



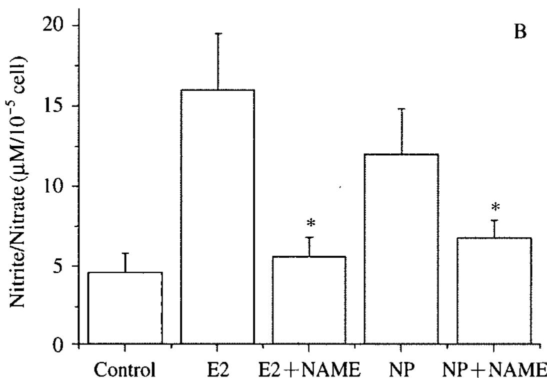


Fig. 1. Effects of NP on NO production in GH3 cells. (A) NP or E2 induces dose-dependent NO synthesis in GH3 cells. (B) NOS inhibitor, L-NAME (1 μM), reduces NP-(10 μM) or E2-(10 nM) induced NO production in GH3 cells. Three experiments were conducted for this determination. *, denotes statistical significance $p < 0.05$)

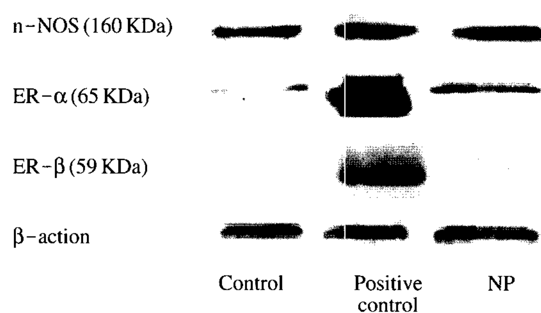


Fig. 2. Expression of NOS isoforms or ER in GH3 cells by NP (10 μMM). Positive control for nNOS, ER-α and ER-β was mouse placenta homogenate, lipopolysaccharide, human endometrial carcinoma cell line HEC-1 cell lysate, respectively.

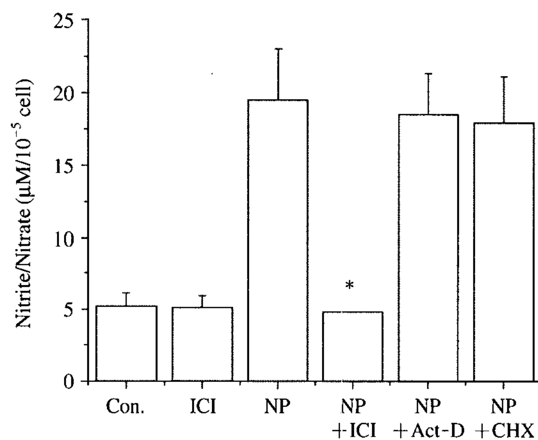


Fig. 3. Effects of ICI 182,780, actinomycin-D (Act-D), or cycloheximide (CHX) on NP-induced NO synthesis in GH3 cells. GH3 cells were pre-treated with ICI 182,780 (ICI), Act-D, or CHX for 30 min before 18-hr treatment with NP. Three experiments were conducted for this determination. *, denotes statistical significance $p < 0.05$)

within the 18 h of NP treatment (Fig. 2). Western blot analysis also demonstrated the existence of ER-α protein in cell lysate of untreated GH3 cells. However, ER-β protein was not detected in GH3 cells by antibodies that we obtained (Fig. 2). As the results, NP only increased NO secretion via ER-α in GH3 cells.

ER antagonist suppresses NO synthesis induced by NP

ICI 182,780 (10 μM), a complete antagonist of ER-α and ER-β, suppressed both E2-induced (data not shown) and NP-induced NO synthesis in GH3 (Fig. 3). These results suggest that NP induced NO synthesis in GH3 cells is likely regulated by ER-α.

We investigated whether or not NP stimulates NO synthesis through a genomic mechanism. Pretreatment of the cells for 30 min with a gene transcription inhibitor, actinomycin D (1 μg/ml), caused no signifi-

cant change in NP-induced NO synthesis (Fig. 3). Furthermore, pretreatment of the cells for 30 min with a protein synthesis inhibitor, cycloheximide (40 μM) had no significant effects on NP-induced NO synthesis.

DISCUSSION

In the present study, we evaluated the potency of NP on NO synthesis in pituitary cells for the first time and our results demonstrated that NP was approximately 100~1000-fold less potent than E2. Because NO is known to play important roles in physiological and pathological phenomena in various organs including the reproductive organs (Purcell *et al.*, 1999; Ellman *et al.*, 1993), the stimulating effects of NP on NO synthesis in vitro and in vivo merit further investigation. We demonstrated that ER was involved in both E2-and NP-induced NO synthesis in GH3 cells. NP has been reported to interact with both ER-α and ER-β (Hiroi *et al.*, 1999). Our Western blot analysis suggests that ER-α might be the main type of ER in untreated as well as NP-stimulated GH3 cells derived from the rat pituitary. However, ER-β, which might be at less than an immunodetectable level, may also be involved. In the present study, we observed no significant increase in the nNOS protein level of GH3 cells treated for 18 h with E2 or NP as compared with untreated cells. Furthermore, the activation of nNOS by treatment with E2 or NP was not inhibited by actinomycin D or cycloheximide. These results indicate that NOS activation in our cell model system is a non-genomic action. Chen *et al.* (1999a) also reported that activation of nNOS by short-term treatment with E2 was non-genomic. However, binding of environmental estrogens to estrogen receptors (ER) are known to initiate transcription of ER-regulated genes in vitro and in vivo (Hyder *et al.*, 1999). Cho *et al.* (1999) reported that nanomolar concentrations of E2 causes an nNOS-related decrease in paracellular permeability of human umbilical vein endothelial cells while micromolar concentrations of E2 causes an iNOS-related increase in permeability. Although we could not detect immunoreactivities of iNOS protein in untreated, E2-treated, or NP-treated GH3 cells, further investigation is necessary on the activation of iNOS by NP. These results suggest that NP may not be merely mimicking a weak estrogen but exhibiting a distinct mechanism of action at the ER in some cases. Because actions of environmental estrogens are extremely complex, various assays should be performed. Evaluation of their potency on NO synthesis is a novel point of view in biological actions of environmental estrogens.

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