Enhancing Effect of the Endocrine Disruptor *para*-Nonylphenol on the Generation of Reactive Oxygen Species in Human Blood Neutrophils

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Although *para*-nonylphenol (NP) is known as an endocrine disruptor, the immunologic effect of NP has been poorly analyzed. We found that NP from 5 to 50 μM caused a dose-dependent stimulatory effect on the generation of reactive oxygen species (ROS) in human blood neutrophils, which was measured by using a chemiluminescence reagent, luminol. Furthermore, ROS-scavenging enzymes such as catalase and superoxide dismutase and antioxidative agents α-tocopherol and β-carotene showed strong preventive effects on NP-induced ROS generation. To analyze the biochemical mechanism of NP-induced ROS generation in human neutrophils, we investigated the effects of different types of metabolic inhibitor for the activation pathways of ROS generation in the cells. Reduced nicotinamide adenine dinucleaotide phosphate (NADPH)-dependent oxidase inhibitor, diphenyl iodonium chloride and the myeloperoxidase inhibitor sodium azide (NaN_3) showed remarkable inhibitory effects on ROS generation induced by NP, but an inhibitor against mitochondrial respiratory function, potassium cyanide (KCN), did not exhibit significant effect. Furthermore, the phosphatidylinositol-3 (PI3) kinase inhibitor wortmannin and the tyrosine kinase inhibitor protein phosphorylation inhibitor 1 (PP1) caused strong suppression against NP-induced ROS generation. The selective protein kinase C inhibitor Ro-32-0432, p38 MAP kinase inhibitor SB 203580, and ERK MAP kinase inhibitor PD 98059 also showed significant suppressive effects on NP-induced ROS generation. These results suggest that NP causes an enhancing effect on ROS generation in human blood neutrophils through the activation of signal transduction pathways associated with the respiratory burst function in these cells. Additionally, to examine *in vivo* effects of NP, we also analyzed the effects of NP itself and the synergistic effects of NP and a typical inflammatory agent, opsonized zymosan, on human whole blood including neutrophils. *Key words:* human blood, neutrophils, *p*-nonylphenol, protein kinase cascade, ROS generation, signal transduction pathways. *Environ Health Perspect* 112:553–556 (2004). doi:10.1289/ehp.6584 available via *http://dx.doi.org/* [Online 8 January 2004]

Alkylphenols are widely used as components in chemically synthesized products such as plastics, detergents, and other formulated products (Nimrod and Benson 1996). They are degraded to free *para*-nonylphenol (NP) in seawater or sewage sludge (Ekeland et al. 1993; Giger et al. 1984), and it accumulates in plants and animals in the aquatic environment (Ahel et al. 1993). Furthermore, NP leaks from autoclaved plastic and increases the cell growth and progesterone receptor expression of mammary tumor cells (Soto et al. 1991), and stimulates the production of the estrogenic protein vitellogenin in fish hepatocytes (Jobling and Sumpters 1993). NP also enhances the uterine DNA and protein synthesis in immature female rats (Lee and Lee 1996) and disrupts the gonad development in neonatal rats (Nagao et al. 2000). These findings evoked a new understanding of this chemical as an environmental endocrine disruptor (Colborn et al. 1996). However, the effects of NP on the functions of human immunocompetent cells have been poorly analyzed.

On the other hand, some previous reports have indicated that estrogenic agents cause free-radical–mediated effects on various physiologic functions. For example, a synthetic estrogenic agent, diethylstilbestrol (DES), is oxidized to DES quinone to yield reactive oxygen species (ROS) by hydroperoxidase activity of lipoxygenase (Nunez-Delicado et al. 1997). Furthermore, NP has been shown to cause a strong suppressive effect on cell growth and cellular respiration of yeast cells, and these effects were associated with NP-induced ROS generation (Okai et al. 2000). These findings seem to suggest a possible relationship between NP-induced pleiotropic effects and ROS generation. In the present study, we analyzed the effect of NP on ROS-producible immunocompetent cells, especially neutrophils in human venous blood. Neutrophils are highly differentiated cells that produce ROS by various chemical and biologic stimulants. Additionally, because the biochemical mechanism of oxidative bursts in human neutrophils has been considerably analyzed to date, it may be easier to analyze the biochemical mechanism of NP effects on these cells compared with other cells. We detected that NP enhanced the respiratory burst function through the activation of signal transduction pathways in these cells.

Materials and Methods

Reagents. Luminol sodium salt was purchased from Wako Pure Chemical Company (Osaka, Japan). NP was purchased from Kanto Chemicals Company (Tokyo, Japan). Diphenyl iodonium chloride (DPI), wortmannin, protein phosphorylation inhibitor 1 (PP1), Ro-32-0432, SB 203580, and PD 98059 were purchased from Calbiochem Company (La Jolla, CA, USA). Sodium heparin solution was purchased from Aventis Pharma Company (Tokyo, Japan). Bovine liver catalase, Cu/Zn-superoxide dismutase (SOD), α-tocopherol, β-carotene, and zymosan were purchased from Sigma-Aldrich Japan Company (Tokyo, Japan). Other chemicals for experiments were purchased from Sigma-Aldrich Japan Company except for specific chemicals described in the text.

Preparation of human blood neutrophils. Human venous blood samples were obtained from nonsmoking healthy male donors with 0.1% sodium heparin. Isolation of neutrophils was carried out as follows. Human venous blood was obtained with 3.8% citrate and centrifuged using Mono-poly Resolving Medium (Dai-nihon Pharmaceutical Co., Osaka, Japan) and Krebs-Ringer phosphate solution (KRP, pH 7.4). The cell layer of neutrophils was recovered and washed two times with KRP by centrifugation, and the isolated neutrophils were kept in ice until use for the experiment. The viabilities of neutrophils used in ROS generation experiments were > 95%.

Assay for ROS generation in human neutrophils and whole blood. Assay for ROS generation was carried out according to the method of Imada et al. (1999). Human neutrophils (1×10^5 cells) or whole blood were incubated with 50 mM phosphate-buffered saline (pH 7.4) in the presence of 1 mM luminol sodium salt at a final volume of 500 μL.

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After incubation of the cell suspension mixture at 37°C for 5 min, the reaction was started by adding different concentrations of NP and further incubated for 30 min at 37°C. For the analysis of the biochemical mechanism of NP-induced respiratory burst, various metabolic inhibitors were added to human blood cells before exposure of NP to the cells and preincubated for 15 min at 37°C until the addition of NP. The chemiluminescence (CHL) intensity of the cell mixture was recorded continuously during the incubation for 30 min at 37°C using Luminescence Reader BLR-201 (Aloka, Tokyo, Japan). One unit of CHL intensity (count per minute) corresponds to the photons released from luciferin and luciferase system catalyzing 1×10^{-16} moles of adenosine 5′-triphosphate.

Statistical analysis. The statistical comparison between control and sample-treated experiments was carried out using Student's *t*-test. A *p*-value < 0.05 was considered significantly different.

Table 1. Enhancing effect of NP on ROS generation in human blood neutrophils.

NP concentration	ROS generation [CHL intensity (cpm × 10^3)]
Control (methanol alone)	ND
5 μM	0.86 ± 0.19
10 μM	3.48 ± 0.58
20 μM	22.39 ± 1.95
50 μM	80.55 ± 6.09

ND, not detected. Results are shown as mean ± SD of NP-induced ROS-generating activities using the isolated neutrophils derived from three different donors.

Results

When various amounts of NP were exogenously added to human neutrophils, a luminol-dependent CHL was generated in a dose-dependent manner from 5 to 50 μM (Table 1), which reflects enhancing activity of NP for the generation of ROS. Generally, various types of ROS, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), are generated by ROS-producing cells. To analyze ROS generated by NP, we examined the effects of ROS-scavenging enzymes such as bovine SOD and catalase on NP-induced ROS generation. As shown in Table 2, they showed the strong preventive effects on NP-induced ROS generation at 100 and 500 units/mL. This result indicates that NP causes the generation of ROS, including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Additionally, typical antioxidative agents α-tocopherol and β-carotene exhibited strong preventive effects on NP-dependent ROS generation at 20 and 100 μM. This result also suggests that NP causes the generation of ROS in human blood neutrophils.

Next, to analyze the biochemical mechanism of NP-induced ROS generation in human neutrophils, we investigated the effects of different types of metabolic inhibitor against the activation pathways of respiratory burst. Because nicotinamide adenine dinucleaotide phosphate (NADPH)-dependent oxidase is known as a primary ROS-generating enzyme in respiratory burst of neutrophils (Scha'afi and Molski 1988), DPI was treated before the exposure of NP to the cells as an inhibitor for NADPH-oxidase (Cross and Jones 1986). As indicated in Table 2, 20 and 50 μM DPI showed strong suppression against ROS generation in human blood induced by 20 μM NP. As an inhibitor for another ROS-generating enzyme myeloperoxidase, which links to the NADPH oxidase reaction and catalyzes the reaction from hydrogen peroxide to hypochlorite (HOCl), sodium azide (NaN_3) caused a remarkable suppressive effect on ROS generation at 5 and 10 mM. However, another type inhibitor against mitochondrial respiratory function, potassium cyanide (KCN), did not show significant inhibition against NP-induced ROS generation at 50 and 200 μM. These results suggest that the primary action of NP is the activation of ROS-generating enzyme system in human neutrophils including NADPH oxidase and myeloperoxidase, but that the mitochondrial respiratory chain system is not substantially responsible for the NP-induced ROS generation in the cells.

Previously, it was elucidated that ROS-generation of neutrophils depends on the signal transduction pathways associated with the activation of NADPH oxidase function, especially various protein kinase cascades (Downey et al. 1998; Dudley et al. 1995; Sue-a-quan et al. 1997; Torres et al. 1993; Wilkinson et al. 1993). We analyzed the effect of various types of protein kinase inhibitor on NP-induced ROS generation in human neutrophils. As shown in Table 3, the phosphatidylinositol-3 (PI3) kinase inhibitor wortmannin showed strong inhibitory effects on NP-induced ROS generation at 20 and 60 nM, and the tyrosine kinase inhibitor PP1 exhibited a dose-dependent suppression at 0.5 and 2 μM. A selective protein kinase C inhibitor, Ro-32-0432, also caused strong suppressive effects on NP-induced ROS generation at 20 and 100 nM. To analyze upstream regulations of these kinase reactions, we examined the effects of SB 203580 (p38 MAP kinase inhibitor) and PD 98059 (ERK MAP kinase inhibitor). They exhibited significant inhibitory effects

Table 2. Effects of ROS-scavenging enzymes, antioxidants, and metabolic inhibitors on NP-induced ROS generation in human blood neutrophils.

ROS generation	Relative activity [CHL intensity (cpm × 10^3)]	Percent
NP alone (20 μM)	23.12 ± 1.08	100
+ SOD (500 U/mL)	5.08 ± 0.65 ($p < 0.001$)	22.0
+ SOD (100 U/mL)	12.23 ± 2.04 ($p < 0.01$)	55.7
+ Catalase (500 U/mL)	3.72 ± 0.50 ($p < 0.001$)	16.1
+ Catalase (100 U/mL)	10.59 ± 0.86 ($p < 0.01$)	45.8
+ α-Tocopherol (100 μM)	0.45 ± 0.12 ($p < 0.001$)	1.9
+ α-Tocopherol (20 μM)	1.90 ± 0.33 ($p < 0.001$)	8.2
+ β-Carotene (100 μM)	0.60 ± 0.09 ($p < 0.001$)	2.6
+ β-Carotene (20 μM)	4.02 ± 0.26 ($p < 0.001$)	17.4
+ DPI (50 μM)	0.20 ± 0.05 ($p < 0.001$)	0.9
+ DPI (20 μM)	0.49 ± 0.03 ($p < 0.001$)	2.1
+ NaN_3 (10 mM)	0.55 ± 0.10 ($p < 0.001$)	2.4
+ NaN_3 (5 mM)	0.86 ± 0.07 ($p < 0.001$)	3.7
+ KCN (200 μM)	20.46 ± 0.95 ($p < 0.100$)	88.5
+ KCN (50 μM)	21.73 ± 1.54 ($p < 0.500$)	94.0

Results show mean ± SD of the maximal CHL intensities of triplicate assays. All the results with ROS-scavenging enzymes, antioxidants, or metabolic inhibitors are statistically different compared with the control result of NP alone except for the result of KCN in analysis by Student's *t*-test.

Table 3. Effects of various protein kinase inhibitors on NP-induced ROS generation in human blood neutrophils.

ROS generation	Relative activity [CHL Intensity (cpm × 10^3)]	Percent
20 μM NP alone	22.68 ± 1.53	100
+ Wortmannin (60 nM)	3.89 ± 0.25 ($p < 0.001$)	17.1
+ Wortmannin (20 nM)	10.61 ± 2.03 ($p < 0.01$)	46.8
+ PP1 (2 μM)	1.94 ± 0.08 ($p < 0.001$)	8.6
+ PP1 (0.5 μM)	5.22 ± 0.92 ($p < 0.001$)	23.0
+ Ro-32–0432 (100 nM)	1.50 ± 0.20 ($p < 0.001$)	6.6
+ Ro-32–0432 (20 nM)	4.72 ± 0.38 ($p < 0.001$)	20.8
+ SB 203580 (10 μM)	3.95 ± 0.62 ($p < 0.001$)	17.4
+ SB 203580 (2 μM)	10.93 ± 1.88 ($p < 0.01$)	48.2
+ PD 98059 (10 μM)	8.62 ± 1.05 ($p < 0.01$)	38.0
+ PD 98059 (2 μM)	14.70 ± 1.36 ($p < 0.02$)	64.8

Results are expressed as mean ± SD of the maximal CHL intensities in triplicate assays. All results with protein kinase inhibitors are statistically different from the control result of NP alone in analysis by Student's *t*-test.

on ROS generation at 2 and 10 μM, respectively (Table 3). These results indicate that NP enhances NADPH oxidase-dependent ROS generation in human neutrophils through the activation of signal transduction pathways including various protein kinase cascades.

In addition, to examine the *in vivo* effect of NP on neutrophils, we also analyzed the effect of NP on fresh human whole blood, including neutrophils. Although the whole blood did not apparently respond to 5–25 μM NP, they showed weak but significant ROS generation from 50 to 500 μM NP in a dose-dependent manner (Table 4). Furthermore, we analyzed the effects of the same antioxidative substances and metabolic inhibitors mentioned above on NP-induced ROS generation in whole blood, and similar results were observed compared with those of the isolated neutrophils (data not shown).

As another important *in vivo* possibility, we examined whether lower concentrations of NP express priming or synergistic effects with other immuno-enhancing agents. As a preliminary experiment, when human whole blood samples were exposed with lower concentrations of NP, significant enhancements of ROS generation were observed in the presence of a low concentration of a typical inflammatory stimulant, opsonized zymosan (Table 5). We are analyzing the details of the synergistic effects of NP and various inflammatory agents as a subsequent study.

Discussion

As described above, various estrogenic effects of NP have been previously reported in *in vitro* and *in vivo* studies. However, the molecular or biochemical mechanism of pleiotropic effects of NP as an endocrine disruptor has been poorly analyzed. The possibility of estrogenic actions comes from reports indicating that estrogens and their metabolites cause free-radical–mediated effects on various physiologic functions. The synthetic estrogen DES is oxidized to DES quinone to yield ROS by hydroperoxidase activity of lipooxygenase, which might be associated with the toxic effect of this synthetic estrogen (Nunez-Delicado et al. 1997). Furthermore, estrogenic hormones also caused the destruction of metabolizing enzymes such as cytochrome P450 in hamster kidney cells via reactive estrogen metabolites and lipid hydroperoxide, which might be related to kidney carcinogenesis (Roy and Liehr 1992). These findings imply a strong relationship between estrogenic agents and ROS-induced cytotoxic effects. However, the effects of NP on ROS generation have been analyzed in only a few studies. For example, NP has been shown to cause the formation of hydroxyl radical in rat brain striatum, and its effect was suppressed by tamoxifen (Obata and Kubota 2000).

Furthermore, Okai et al. (2000) indicated that NP exhibited suppressive effects on cell growth and cellular respiration of yeast cells as a simple eukaryotic cell model, which was associated with ROS generation induced by NP. In the present study, we clearly showed that NP caused an enhancing effect on ROS generation in human neutrophils through the activation of signal transduction pathways including the protein kinase cascade.

In addition, the relationship between ROS generation and the activation of immunocompetent cells by NP has been poorly analyzed. Recently, Yamashita et al. (2002) reported that several endocrine disruptors, including NP, enhanced the proliferative responses of murine spleen cells in the presence and absence of lipopolysaccharide or concanavalin A, and they also stimulated the cytokine and antibody production of the same cells. Interestingly, an older study showed that early activation of rat T-lymphocytes with concanavalin A caused luminol-dependent CHL generation, although the mechanism of the CHL generation was not analyzed (Wrogemann et al. 1978). As a possible hypothesis, early activation of proliferation and functions of immunocompetent cells such as neutrophils and lymphocytes by NP may be associated with ROS generation.

Possibly, some of the NP-dependent pleiotropic effects may be explained by ROS-generating activity of NP, but the mode of action of NP might express in different ways in different cell types or different physiologic states. For example, NP caused strong suppression against cell growth and oxygen consumption with ROS generation in actively growing yeast cells under aerobic culture condition (Okai et al. 2000), but the stationary phase yeast cells under anaerobic condition were much more resistant to NP than are actively growing cells (data not shown). Interestingly, although the respiratory chain system of abundant mitochondria in the growing wild-type yeast cells is actively working, the mutant yeast deficient in mitochondrial function showed the resistant property of cell growth to NP compared with wild-type yeast (Okai et al. 2000). This result suggests that one of the major target sites of NP in the growing yeast cells is the mitochondria.

In contrast, neutrophils are highly differentiated cells to generate ROS, and a mitochondrial function inhibitor, KCN, did not show significant effects on NP-induced ROS generation (Table 2), which seems to be responsible for the insufficient development of mitochondria. In these cells, ROS-generating enzymes such as NADPH-dependent oxidase and myeloperoxidase are highly expressed, and the inhibitors against these ROS-generating enzymes exhibited remarkable suppression against NP-induced ROS generation (Table 2). These results indicate that NP-induced pleiotropic effects associated with ROS generation may depend on the cell types or the physiologic state of the cells.

Generally, in ROS-producing cells such as neutrophils, superoxide anion is generated by NADPH-dependent oxidase from molecular oxygen and dismutated to hydrogen peroxide by SOD, which can be converted to the more active radical, hydroxyl radical, by Haber-Weiss or Fenton reactions, and hydrogen peroxide is further metabolized to hypochlorite by myeloperoxidase (Imlay and Linn 1988). As shown in Table 2, typical inhibitors against NADPH oxidase and myeloperoxidase caused strong suppression against NP-induced CHL generation in human neutrophils. Furthermore, luminol-dependent CHL generation in human neutrophils by NP was considerably prevented by ROS-scavenging enzymes such as SOD and catalase (Table 2). In addition, when we examined the effect of NP on CHL generation by using another CHL reagent, L-012, which reflects hydroxyl radical and hypochlorite (Imada et al. 1999), significant L-012–dependent CHL generation was

Table 4. Enhancing effect of NP on ROS generation in human whole blood.

Concentration of NP	ROS generation [CHL intensity (cpm × 10^3)]
Control (methanol alone)	ND
5 μM	ND
10 μM	ND
25 μM	ND
50 μM	0.06 ± 0.02
100 μM	0.18 ± 0.05
250 μM	0.41 ± 0.12
500 μM	1.85 ± 0.37

ND, not detected. Results represent mean ± SD of the maximal CHL intensities in triplicate assays using human whole blood derived from three different donors that contained approximately 1 × 10^5 neutrophils in the assay mixture.

Table 5. Synergistic effect of NP and opsonized zymosan (OZ) on ROS generation in human whole blood.

	ROS generation [CHL intensity (cpm × 10^3)]	
Concentration of NP	–OZ	+OZ (3 μg/mL)
Control (methanol alone)	ND	0.15 ± 0.05
5 μM	ND	0.37 ± 0.10 ($p < 0.05$)
10 μM	ND	0.67 ± 0.18 ($p < 0.02$)
25 μM	ND	0.98 ± 0.26 ($p < 0.02$)
50 μM	0.05 ± 0.01	2.23 ± 0.38 ($p < 0.01$)

Results were expressed as mean ± SD of the maximal CHL intensities of triplicate assays. Results with NP (5–50 μM) and OZ are statistically different from the result with OZ alone in analysis by Student's *t*-test.

observed (data not shown). These results suggest that NP induces the generation of superoxide anion, hydrogen peroxide, hydroxyl radicals, and hypochlorite in neutrophils.

Furthermore, we analyzed the contribution of reactive nitrogen species such as nitric oxide and peroxynitrite to NP-induced CHL generation in human neutrophils. NO is generated by inducible NO synthetase (iNOS) from arginine, and NO and hydrogen peroxide make peroxynitrite, and then it reacts with luminol, which causes CHL generation (Kikuchi et al. 1993). When human neutrophils were pretreated with a specific iNOS inhibitor (L-NAME) before the exposure of NP, no significant effects were observed on luminol-dependent CHL generation induced by NP (data not shown). This result implies that reactive nitrogen species such as NO and peroxynitrite do not contribute substantially to luminol-dependent CHL generation induced by NP under our experimental condition.

To consider *in vivo* effects of NP on ROS generation, we examined NP effects on human whole blood including neutrophils. We found the dose-dependent ROS generation in whole blood induced by NP, albeit their dose concentrations were relatively high compared with those of neutrophils alone (Tables 1 and 4). In a separate experiment, typical neutrophil stimulants such as opsonized zymosan, *N*-formyl-methionyl-leucyl-phenylalanine, and phorbol myristate acetate showed much higher ROS generation in human neutrophils than in whole blood. Effective concentrations of these stimulants for human neutrophils were much lower than those for whole blood (data not shown). Therefore, the difference in NP effects on neutrophils and whole blood is the same as the results of these typical stimulants. The difference of effective NP concentrations for responsibilities of whole blood and neutrophils seems to be associated with the presence of various soluble molecules, cellular components, and antioxidants in whole blood, which bind to neutralize its effect or reduce the effect of NP.

We also detected the synergistic effect of lower concentrations of NP and a typical inflammatory agent, opsonized zymosan, on ROS generation in whole blood (Table 5). We recently detected the similar synergistic effect of lower concentrations of NP and other inflammatory agents (data not shown). Interestingly, in these experiments, NP has a priming activity for ROS generation in whole blood; specifically, when low concentrations of these stimulants did not cause significant ROS generation by themselves, the pretreatment of low concentrations of NP caused considerable ROS generation similar to that induced by the same low concentrations of stimulants. These results indicate that low concentrations of NP raised the sensitivity of whole blood to respond efficiently to neutrophil stimulants. Thus, although NP concentrations to exhibit significant ROS generation in whole blood were relatively high, it seems more important that low concentrations of NP are able to cause priming or synergistic effects on whole blood with low concentrations of other neutrophil stimulants.

Generally, defensive activities of ROS against pathogenic microorganisms may be involved in tumor-cell death, but they are also responsible for tissue damage through the attack to important cellular biomolecules such as nucleic acids, proteins, and lipids (Weiss 1989), which are related to chronic diseases such as cancer and cardiovascular and inflammatory diseases (Halliwell 1997; Sahn 1991). In a recent report, NP had a promoting effect on rat lung carcinogenesis (Seike et al. 2003). Although the biochemical mechanism of the tumor-promoting effect of NP was not analyzed in that study, NP-induced ROS generation in lung neutrophils may be responsible for the tumor-promoting effect.

On the other hand, a previous report indicated that some sewage sludges contained high concentrations of NP, from 1.3 mmol/kg to 4.7 mmol/kg (Ekeland et al. 1993). NP is also accumulated to a considerable degree in various plants and animals living in aquatic environments, and their bioconcentration factors in food chains of the ecologic system have shown very divergent values (13- to 1,000-fold; Ahel et al. 1993). Judging from our present results, if we intake these bioorganisms as foods, these concentrations of NP seem to cause possible toxic effects on the body through ROS generation of immunocompetent cells, including blood neutrophils.

Another important finding in our present study is that NP-induced ROS generation in human blood neutrophils can be prevented by ROS-scavenging enzymes or antioxidants (Table 2). This result implies that NP-induced toxic effects on the body can be prevented by the ingestion of high amounts of these preventive substances.

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