Formation of DNA Damaging Product from Light-Irradiated Nonylphenol

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(Received November 18, 2005; Accepted November 29, 2005; Published online December 2, 2005)

Nonylphenol (NP) is widely distributed in the environment as a microbial metabolite of nonylphenol polyethoxylates, which are commercially important surfactants. Although there are concerns regarding the estrogenic activity of NP and its effects on wildlife, this paper reports a new detrimental effect for NP; oxidative DNA damage. After UV or sunlight irradiation, several NP photoproducts were detected by high performance liquid chromatography (HPLC). Two HPLC fractions induced formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in calf thymus DNA. Based on gas chromatography/mass spectrometry and UV/visible spectrometry, one fraction was confirmed to contain 4-nonylcatechol. The other active compound remains unidentified. Non-irradiated NP did not exhibit any detectable 8-oxodG formation. These results suggest that NP induces oxidative DNA damage following environmental light exposure. This DNA damaging activity is a new adverse effect of NP and, in addition to estrogenic activity, should be investigated further and taken into consideration during NP risk assessment.

Key words —— carcinogenesis, alkylcatechol, risk assessment, ring hydroxylation, 8-oxodG, sunlight exposure

INTRODUCTION

Nonylphenol (NP) is widely distributed in the environment as a microbial metabolite of nonylphenol polyethoxylates, which are commercially important surfactants. The concentrations of NP in river water range from 0.01 to 2.87 μg/l, those in indoor air were found to be 125 ± 150 ng/m^3 (house) or 95.2 ± 108 ng/m^3 (office building), and those in outdoor air were 7.5 ± 10.4 ng/m^3.[1,2] In 2001, NP was listed as an endocrine disruptor by the Japanese Ministry of the Environment.

In addition to microbial degradation, photodegradation is an important pathway for the disappearance of environmental NP.[3,4] Several groups have carried out photocatalytic degradation of environmental NP using Fe(III), hydrogen peroxide and TiO_2.[5,6] These are useful methods to remove NP from the environment. However, the photoproducts of environmental chemicals such as alkylphenol polyethoxylates, ethylbenzene and phthalates have adverse effects.[7–9] Therefore, NP is also likely to induce adverse effects following light irradiation. We thus investigated the NP photoproducts formed by UV or sunlight irradiation and their abilities to cause oxidative DNA damage. We identified active NP photoproducts by high performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS). In addition, we measured the potential of NP to form 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a characteristic oxidative product of DNA, using an HPLC equipped with an electrochemical detector (ECD).

MATERIALS AND METHODS

Chemicals —— 4-*n*-Nonylphenol (NP), $FeCl_3 \cdot 6H_2O$ and NADH were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bis(trimethylsilyl)trifluoroacetamide (BSTFA), 2′-deoxyguanosine monohydrate (dG), 8-hydroxy-2′-deoxyguanosine and calf thymus DNA were from Sigma Chemical (St. Louis, MO, U.S.A.). Nuclease P_1 was from Yamasa Shoyu Co. (Choshi, Japan). Calf intestine alkaline phosphatase (CIP) was from Roche Diagnostics (Mannheim, Germany). All other chemi-

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cals used were of guaranteed reagent grade.

Light Irradiation of Nonylphenol —— NP was dissolved in ethanol-water (1 : 1). Light irradiation of 10 mM NP (1 ml) was carried out in a quartz cuvette sealed with a teflon stopper using artificial UV light from a Hg arc (254 nm) or sunlight. UV irradiation was carried out for 2 hr using a UV crosslinker (0.194 J/min, Atto Corp., Tokyo, Japan). For sunlight irradiation, the quartz cuvette was held in a bright location for 8 hr. After irradiation, NP solutions were evaporated, dissolved in 200 μl of methanol and subjected to HPLC.

HPLC and GC/MS Analyses of Photoproducts —— For HPLC analysis, irradiated NP solution was applied to an HPLC system (LC-VP, Shimadzu, Kyoto, Japan) equipped with a diode array detector (SPD-M10A, Shimadzu) and a Develosil packed column (4.6 i.d. $\times$ 250 mm, Nomura Chemical Co., Ltd., Aichi, Japan). The column was eluted with 80%(v/v) methanol/water containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min at 40°C. UV traces were obtained by monitoring at 215 nm. Light-irradiated NP was fractionated every 30 sec, and fractions were evaporated and their DNA oxidation activities were surveyed.

For GC/MS analysis, photoproducts were isolated by HPLC, evaporated and incubated for 30 min at 60°C in BSTFA before analysis. Samples were injected into a gas chromatograph (HP 6890 GC System Plus, Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with a mass spectrometer (JMS-700 MStation, JEOL, Tokyo, Japan) that used electron impact ionization at 70 eV. Helium at a flow rate of 1 ml/min was used as a carrier gas. Temperature of the injector, interface and ion source was 250°C. The temperature program for the DB-1 column (0.25 mm i.d. $\times$ 30 m, film thickness 0.25 μm, J&W Scientific, Folsom, CA, U.S.A.) was as follows: 70°C (2 min isothermal), 70–280°C (10°C/min) and 280°C (2 min isothermal).

Analysis of 8-oxodG Formation in Calf Thymus DNA —— Calf thymus DNA (50 μM/base) was incubated with HPLC fractions of light-irradiated NP plus 20 μM $CuCl_2$ and 100 μM NADH for 1 hr at 37°C. After ethanol precipitation, DNA was digested to nucleosides with nuclease P_1 and CIP, and the amount of 8-oxodG was then measured using an HPLC equipped with an ECD, as described previously.[10]

Identification of Catechol Derivatives —— In order to detect catechol derivatives, UV/visible (UV/VIS) spectral change of the photoproduct by $FeCl_3$ was determined.[11] Evaporated HPLC fractions were dissolved in 20 μl of methanol. The solution (5 μl) was added to 100 μl of 100 mM acetate buffer (pH 5.0) containing 250 μM $FeCl_3$. After 60 sec, the UV/VIS spectrum (200 to 900 nm) was recorded on a spectrophotometer (model 228A, Hitachi, Tokyo, Japan).

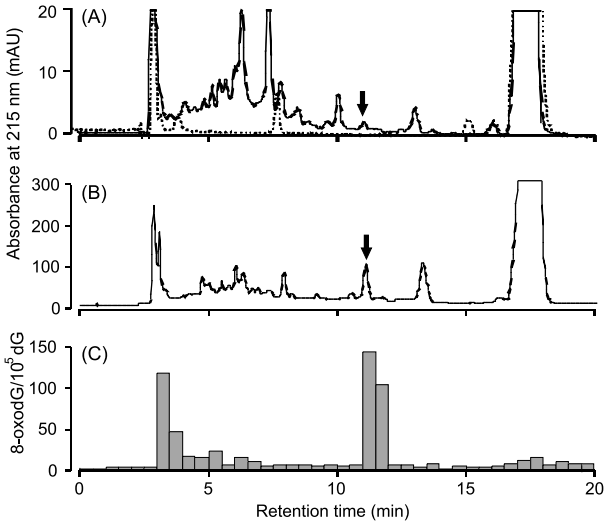

Fig. 1. HPLC Chromatograms of Light-Irradiated Nonylphenol (A and B), and 8-oxodG Formation by Each HPLC Fraction (C)

(A) Sunlight-irradiated NP (solid line) and non-irradiated NP (broken line). (B) UV-irradiated NP. (C) Oxidative DNA damaging activities of UV-irradiated NP (fractionated each 30 sec). Arrows indicate the photoproduct able to form 8-oxodG in calf thymus DNA.

RESULTS

Identification of DNA Damaging Photoproducts of Nonylphenol

NP solution was irradiated with either sunlight or UV for 8 or 2 hr, respectively, as described in MATERIALS AND METHODS. Chromatograms for sunlight- or UV-irradiated NP are shown in Fig. 1A and 1B. Several photoproducts were seen on both chromatograms. UV-irradiated NP solution was fractionated every 30 sec by HPLC, and the 8-oxodG formation activity of each fraction was estimated by HPLC-ECD, as described in MATERIALS AND METHODS. Fractions containing the products eluted at 2.8–3.0 and 11.1 min showed prominent 8-oxodG formation, while the fraction containing NP (retention time, 17.3 min) exhibited no detectable 8-oxodG formation (Fig. 1C). These results demonstrate that NP photoproducts induce oxidative DNA damage.

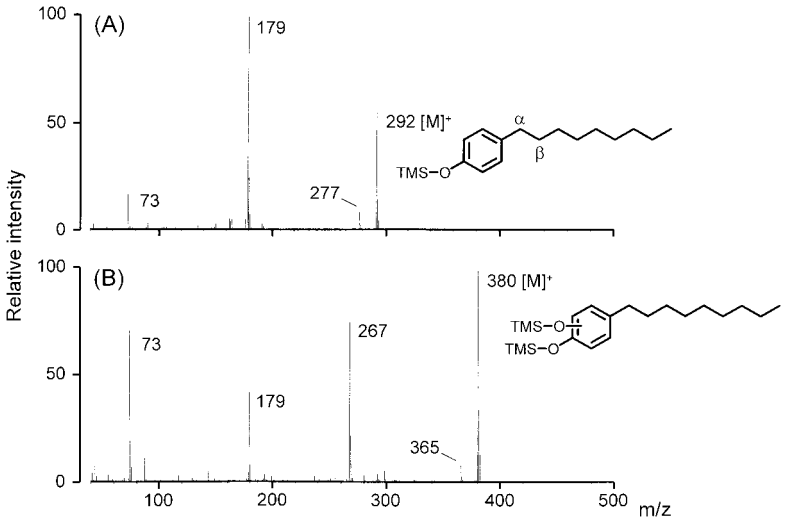

Fig. 2. EI/MS Spectra of (A) Trimethylsilylated Nonylphenol and (B) Trimethylsilylated Photoproduct
NP and photoproduct were incubated with BSTFA at 60°C for 30 min and trimethylsilyl derivatives were detected by GC/MS.

Identification of Active Photoproduct

The active photoproduct (retention time, 11.1 min) was isolated by HPLC. After evaporation, the residual photoproduct was trimethylsilylated with BSTFA and subjected to GC/MS. Mass spectra for NP-TMS and photoproduct-TMS are shown in Fig. 2. The molecular ion mass of NP-TMS was *m*/*z* 292 and the fragment ion masses were *m*/*z* 277 (M-CH_3) and 179 (M-C_8H_{17}) (Fig. 2A). The molecular ion mass of the photoproduct-TMS was *m*/*z* 380, which was 88 units larger than that of NP-TMS (Fig. 2B). This mass difference was estimated to be the sum of TMS and an oxygen atom. Therefore, the photoproduct is believed to be oxidized NP. The fragment ion masses of the photoproduct were *m*/*z* 365 (M-CH_3), 267 (M-C_8H_{17}) and 179 (M-$C_{11}H_{25}Si$). [M-C_8H_{17}]$^+$ would result from nonyl group cleavage between the α- and β-positions (see Fig. 2). The fragment lacking the nonyl group (*m*/*z* 267) had the expected mass difference (88 units) when compared with NP (*m*/*z* 179), thus suggesting that the oxygen atom is incorporated as a hydroxyl group in the benzene ring of the photoproduct.

In order to characterize the position of the hydroxyl group, the UV/VIS spectral change based on catechol derivative-iron chelation was determined (Fig. 3). Fe(III)-catechol chelate showed a broad spectrum. No absorption at around 600–700 nm was observed for the mixture of acetate buffer and Fe(III) (Fig. 3A and 3B). The photoproduct itself exhibited low absorption at around 600–700 nm (Fig. 3C). In the presence of Fe(III) and the photoproduct, the absorption based on the catechol-iron chelation[11] was observed (Fig. 3D). These results strongly suggest that the photoproduct is a catechol.

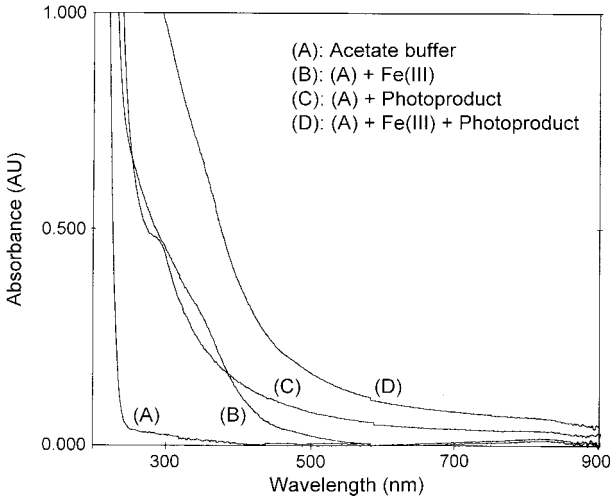

Fig. 3. UV-VIS Spectral Change in Photoproduct in the Presence of Fe(III)
(A), 100 mM acetate buffer (pH 5.0); (B), buffer + Fe(III); (C), buffer + photoproduct; (D), buffer + Fe(III) + photoproduct. Spectral change between (C) and (D) indicates the interaction of photoproduct with Fe(III).

DISCUSSION

After UV or sunlight irradiation of NP, two active photoproducts able to oxidize DNA were detected (Fig. 1). Based on GC/MS analysis, the pho-

toproduct eluted at 11.1 min on HPLC analysis was confirmed to be ring-hydroxylated NP (Fig. 2). Moreover, the photoproduct was able to chelate iron. These results suggest that the photoproduct is 4-nonylcatechol (4-NC). The active photoproduct eluted around 2.5–3.0 min is more hydrophilic than 4-NC by the retention time. We propose that the photoproduct is probably formed by hydroxylation of 4-NC on benzene ring or alkyl chain.

Catechol derivatives such as catechol, 4-methylcatechol and 4-ethylcatechol were shown to induce oxidative DNA damage in the presence of Cu(II).[12–15] These compounds reduce O_2 to superoxide by auto-oxidation, and superoxide then reduces Cu(II) to Cu(I). The hydrogen oxide generated from the superoxide forms a complex with Cu(I) and oxidizes DNA specifically at guanine bases. The basal mechanism for DNA damage by 4-NC would be the same as with these compounds.

Many researchers have investigated the estrogenic activity of NP. This study reveals that NP induces oxidative DNA damage after light irradiation. Our new finding could be related to the report[16] that NP enhanced the rat lung carcinogenesis together with genistein. Further investigation of novel adverse effects of NP, in addition to known activities, should therefore be performed.

Acknowledgements This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Scientific Frontier Research Project of Meijo University. We would also like to thank the Academic Research Institute of Meijo University for their financial support.

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