RO-1561 reaction oven, Jasco NP pak RL reducer column (35 mm × 4.6 mm *id.*, Jasco, Tokyo, Japan), Shimadzu CTO-10Avp column oven, and Jasco FP-1520S fluorescence detector. The NP pak RL reducer column was packed with alumina (<150 μm of particle size) coated with platinum (Pt).

Samples of 3,6-DNBeP fractions were dissolved in 85% ethanol, and 0.2 ml of each solution was injected into a Wakosil PAHs column (5 μm particle size, 250 mm × 4.6 mm *id.*, Wako Pure Chemical, Osaka, Japan) as a stationary phase, connected with the NP pak RL reducer column, continuously. After separation from interfering peaks with the Wakosil PAHs column, 3,6-DNBeP was reduced to 3,6-diaminobenzo[*e*]pyrene (DABeP) by on-line reduction using the NP pak RL column at 80°C to be detected using the fluorescence detector. Elution was carried out with 95% methanol at a flow rate of 0.7 ml/min. The detection excitation and emission wavelengths were 319 and 443 nm, respectively. Authentic 3,6-DNBeP as a standard was also dissolved in 85% ethanol injected at more than three doses into a column in order to draw calibration curves. The calibration curves were drawn with the peak heights of 3,6-DABeP on the chromatograms. HPLC procedure was carried out at 30°C.

HPLC Analysis of 1,3-, 1,6-, and 1,8-DNP Isomers—— 1,3-, 1,6-, and 1,8-DNP isomers were analyzed as described previously.[26–28] Samples of DNP fractions were dissolved in 50% ethanol, and 0.2 ml of each solution was injected into a Cosmosil $5C_{18}$-AR-II column (5 μm particle size, 250 mm × 4.6 mm *id.*, Nacalai Tesque) as the stationary phase, and connected with the NP pak RL reducer column, continuously. Three DNP isomers were separated using the Cosmosil $5C_{18}$-AR-II column and reduced to the corresponding diaminopyrene (DAP) isomers by on-line reduction using the NP pak RL column at 80°C to detect the fluorescence. Elution was carried out with 85% methanol at a flow rate of 0.7 ml/min. The detection excitation and emission wavelengths were 375 and 450 nm, respectively. Authentic DNP isomers were dissolved in 50% ethanol, and 0.2 ml of each solution was injected at more than three doses into a column in order to draw calibration curves. The calibration curves were drawn with the peak heights of 1,3-, 1,6-, and 1,8-DAP isomers on the chromatograms. HPLC procedure was carried out at 30°C.

Quality Assurance—— The relative standard division ($n = 4$) of 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP isomers was less than 2.2%. Under the analytical conditions described above, 3,6-DNBeP and DNP isomers standards were injected into the columns and the calibration graphs showed good linearity ($r^2 > 0.9999$) in the range of 0.2–200 pg.

3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP isomers standards 500 pg were applied on silica gel, COSMOSIL $5C_{18}$-MS-II and Luna 5 μ Phenyl-Hexyl columns. After elutes were collected, amounts of 3,6-DNBeP and DNP isomers in the elutes were compared to each standard. The recoveries of 3,6-DNBeP and three DNP isomers on each column were shown in Table 1. The recoveries were enough high in more than 92%, perpetually.

When the extracts were applied on the silica gel columns, each extract was divided to three aliquot

Table 1. Recoveries and Retention Times of 3,6-DNBeP and Three DNP Isomers on Each Purification Step

Step	Column	Compound	Retention time (min)	Recovery (%)
1	Silica gel column	3,6-DNBeP	—[a]	93
		1,3-DNP	—[a]	98
		1,6-DNP	—[a]	98
		1,8-DNP	—[a]	100
2	Cosmosil $5C_{18}$-MS-II	3,6-DNBeP	32.1[b]	98
		1,3-DNP	19.5[b]	98
		1,6-DNP	17.5[b]	98
		1,8-DNP	16.8[b]	99
3	Luna 5 μ Phenyl-Hexyl	3,6-DNBeP	25.3[c]	100
		1,3-DNP	27.3[d]	92
		1,6-DNP	21.5[d]	94
		1,8-DNP	22.9[d]	93

a) 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP isomers were eluted in toluene fraction. *b*) Elution was carried out with 70% acetonitrile at a flow rate of 0.7 ml/min. *c*) Elution was carried out with 90% methanol at a flow rate of 0.7ml/min. *d*) Elution was carried out with 85% methanol at a flow rate of 0.7 ml/min.

in order to confirm the accuracy. Quantification results were shown as "mean value ± standard division" (n = 3).

Mutagenicity Test of Surface Soil—The extracts of surface soils were dissolved in dimethyl sulfoxide and assayed by the preincubation method[29] using *S.* typhimurium TA98 without S9 mix. The mutagenic potencies of samples were calculated from linear portions of the dose-response curves obtained with three or four doses and duplicated plates at each dose. The slope of the dose-response curves was adapted as the mutagenic potency. When the samples induced two-fold increases over the average of spontaneous revertants and showed well-behaved concentration-response patterns, the samples were judged positive. The mutagenic potency of 3,6-DNBeP used to calculate the contribution ratio was 1.357 revertants/pg.

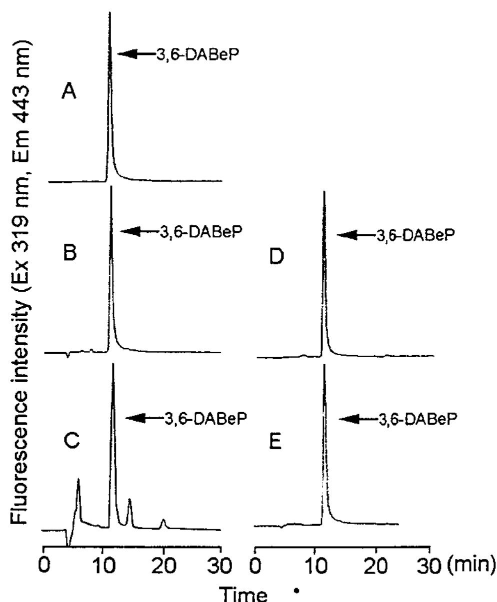

Fig. 3. Chromatograms of 3,6-bNBeP

Typical chromatograms of authentic 3,6-DNBeP (A), and 3,6-DNBeP in surface soil (B), airborne particles (C), diesel particles (D), and incinerator dusts (E). After purification with the silica gel column and two HPLC columns, 3,6-DNBeP injected into the Wakosil PAHs column. 3,6-DNBeP was reduced by on-line reduction with the NP pak RL, and detected as 3,6-DABeP by a fluorescence detector. The chromatograms were monitored with the excitation and emission wavelengths of 319 and 443 nm, respectively. Because 3,6-DNBeP in each extract was purified efficiently by the three-step purification, few interfering peaks were observed nearby the peaks of 3,6-DABeP.

RESULTS

Determination of 3,6-DNBeP in Surface Soil and Airborne Particles Using Fluorescence Detection

Surface soil was collected in Kyoto and Osaka prefectures (the Kinki region), Aichi prefecture (the Chukyo region), and Saitama and Kanagawa prefectures (the Kanto region). Since 3,6-DNBeP was detected in Kyoto, Izumiotsu, Nagoya, and Hekinan cities in the previous study,[14, 17] sampling was carried out at their environs. Airborne particles were collected in Aichi prefecture (the Chukyo region) since 3,6-DNBeP was detected in the surface soil collected in Aichi prefecture.[14] Surface soil and airborne particles were extracted with methanol. After clean-up using silica gel column chromatography and HPLC, 3,6-DNBeP fractions from extracts of surface soil and airborne particles were analyzed by HPLC coupled with on-line reduction and a fluorescence detector. Typical chromatograms of authentic 3,6-DNBeP and 3,6-DNBeP in surface soil and airborne particles are shown in Fig. 3A, B, and C, respectively. 3,6-DNBeP was reduced using the NP pak RL column to be detected as 3,6-DABeP at a retention time of 11.4 min on the chromatograms. Table 2 shows the amounts of 3,6-DNBeP detected in surface soils and the contribution ratios of 3,6-DNBeP to the mutagenicity of surface soil. 3,6-DNBeP was detected in all surface soil samples in the range of 8–975 pg/g of soil, and the mutagenic contribution ratios were quite high in the range of 4.5–75.1%. Table 3 shows the amounts of 3,6-DNBeP detected in airborne particles collected in Nagoya city. 3,6-DNBeP was detected in all airborne particles collected in three sampling sites, Nagoya city in the range of 19–76 fg/m^{3}after the correction by recoveries.

Particle-size-determination of 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP Isomers in Airborne Particles Collected in Wako City

3,6-DNBeP was detected in every particle size, *i.e.* <1.1, 1.1–2.0, 2.0–3.3, 3.3–7, and >7 μm, collected in Wako city in the range of 188–530 fg/m^3, and the total amount was 1433 fg/m^3 (Table 4). The highest particle-size-distribution in airborne particles of 3,6-DNBeP was observed in 2.0–3.3 μm (530 fg/m^3, 37.0%). The second and third highest distributions were observed in 3.3–7.0 μm (273 fg/m^3, 19.1%) and >7 μm (244 fg/m^3, 17.0%) sized particles, respectively.

Typical chromatograms of authentic three DNP isomers and those in airborne particles are shown in Fig. 4A and B, respectively. Peaks of 1,3-, 1,6-, and 1,8-DNP isomers, detected as corresponding DAP isomers, were observed at retention times of 18.5,

Table 2. Amount of 3,6-DNBeP and Mutagenicity in Surface Soil

	Sampling site	Sampling date	Amount[a] MV ± S.D. (pg/g of soil)	Mutagenicity[b] (revertants/g of soil)	Contribution[c] (%)
	Kinki region				
	Kyoto prefecture				
	Kyoto city				
1	Sakyo ward	18 Nov. 2006	29 ± 1	124	31.7
2	Kita ward	18 Nov. 2006	97 ± 29	479	25.7
3	Ukyo ward	18 Nov. 2006	270 ± 40	1840	19.9
4	Kamigyo ward	21 Dec. 2007	20 ± 1	98	27.7
5	Nakagyo ward	18 Nov. 2006	46 ± 14	401	15.6
6	Shimogyo ward	18 Nov. 2006	8 ± 5	203	5.3
7	Higashiyama ward	5 Apr. 2008	25 ± 3	460	7.4
8	Yamashina ward	5 Apr. 2008	671 ± 115	1210	75.3
9	Minami ward-1[d]	25 Dec. 2004	355 ± 17	2900	16.6
10	Minami ward-2[d]	25 Dec. 2004	347 ± 81	2860	16.5
11	Fushimi ward	18 Nov. 2006	44 ± 7	194	30.8
12	Nishikyo ward	18 Nov. 2006	60 ± 9	591	13.8
	Osaka prefecture				
13	Takaishi city	19 Nov. 2005	149 ± 21	743	27.2
14	Izumiotsu city-1	19 Nov. 2005	975 ± 266	6370	20.8
15	Izumiotsu city-2	19 Nov. 2005	72 ± 23	625	15.6
16	Izumiotsu city-3	19 Nov. 2005	30 ± 12	913	4.5
17	Izumiotsu city-4	19 Nov. 2005	54 ± 1	501	14.6
18	Kishiwada city	19 Nov. 2005	181 ± 14	627	39.2
	Chukyo region				
	Aichi prefecture				
	Nagoya city				
19	Minato ward	8 Dec. 2005	93 ± 7	447	28.2
20	Minami ward-1	8 Dec. 2005	187 ± 11	669	37.9
21	Minami ward-2	8 Dec. 2005	313 ± 108	913	46.5
22	Takahama city	25 Nov. 2005	25 ± 1	155	21.9
23	Handa city	25 Nov. 2005	43 ± 9	354	16.5
	Kanto region				
	Saitama prefecture				
24	Wako city	30 Nov. 2005	34 ± 5	211	21.9
	Kanagawa prefecture				
	Kawasaki city				
25	Nakahara ward	8 Dec. 2005	51 ± 7	401	17.3
26	Kawasaki ward	2 Apr. 2005	82 ± 28	1100	10.1

a) Amounts were corrected by the recovery of each purification step. *b*) Mutagenicity of 3,6-DNBeP was tested in *S. typhimurium* TA98 without mammalian metabolic system (S9 mix). *c*) The mutagenic contribution ratios of 3,6-DNBeP to surface soils were calculated as 1.357 revertants/pg of 3,6-DNBeP. It is supposed that mutagens in each sample don't interact. *d*) The data were reported in the previous study.[17]

13.3, and 16.0 min, respectively. Table 4 shows the particle-size-distribution of three DNP isomers in airborne particles collected in Wako City. 1,3-, 1,6-, and 1,8-DNP isomers were detected in the range of 5–60, 8–34, and 1–20 fg/m^3, respectively, and the total amounts of the three DNP isomers were 93, 76, and 58 fg/m^3, respectively. The highest particle-size-distribution of 1,3-, 1,6-, and 1,8-DNP isomers was observed in <1.1 μm (60, 34, and 20 fg/m^3, and 64.5%, 44.7%, and 34.5%, respectively).

Determination of 3,6-DNBeP in Diesel Particles and Incinerator Dusts Using Fluorescence Detection

Diesel particles extracts SRM 1975, purchased from National Institute of Standards and Technology, is the extract of particle collected from a diesel engine used for industrial forklift.[23] Diesel parti-

cles No.1 was collected from a diesel engine used for general automobiles. Incinerator dusts No.1–4 were bottom ash, and JSAC0511 was fly ash.[24] The chloroform extracts of diesel particles and incinerator dusts were analyzed by HPLC coupled with online reduction and a fluorescence detector after purification. Typical chromatograms of 3,6-DNBeP in diesel particles and incinerator dusts are shown in Fig. 3D and F, respectively. Peaks of 3,6-DNBeP, detected as 3,6-DABeP, were observed at a retention time of 11.4 min without any interfering peaks nearby.

3,6-DNBeP was detected in all samples analyzed in the ranges of 29–90 ng/g of diesel particle and 3–5900 pg/g of incinerator dust. Tables 5 and 6 show the amounts of 3,6-DNBeP detected in diesel particles and incinerator dusts, respectively.

Determination of 1,3-, 1,6-, and 1,8-DNP Isomers in Diesel Particles and Incinerator Dusts with Fluorescence Detection

Typical chromatograms of three DNP isomers in diesel particles and incinerators dusts are shown in Fig. 4 C and D, respectively. Peaks of 1,3-, 1,6-, and 1,8-DNP isomers, detected as corresponding DAP, were observed at retention times of 18.5, 13.3, and 16.0 min, respectively. Three DNP isomers were purified using the silica gel column and two HPLC columns. Tables 5 and 6 show the amounts of three DNP isomers detected in diesel particles and incinerator dusts. 1,3-, 1,6-, and 1,8-DNP isomers were detected at the amounts of 4, 5, and 5 ng/g of diesel particle, respectively, and detected in the range of ND–5, ND–5, and ND–5 pg/g of incinerator dust, respectively.

Table 3. Amount of 3,6-DNBeP in Airborne Particles Collected in Nagoya City

Sampling site	Sampling date	Amount[a] (fg/m³ of airborne) MV ± S.D.
Chukyo region		
Aichi prefecture		
Nagoya city-1	27 Jan. 2005	76 ± 5
Nagoya city-2	31 Jan. 2005	48 ± 13
Nagoya city-3	1 Feb. 2005	19 ± 6

a) Amounts were corrected by the recovery of each purification step.

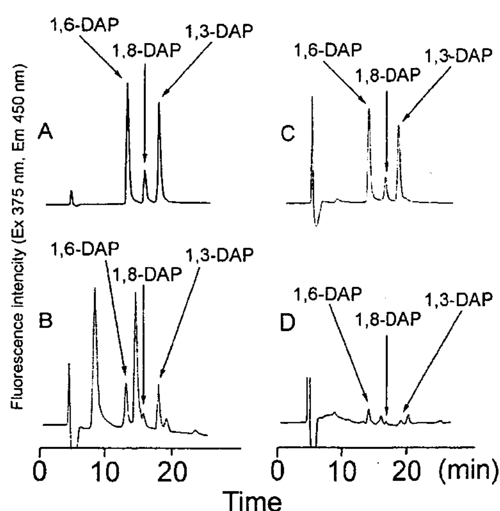

Fig. 4. Chromatograms of 1,3-, 1,6-, and 1,8-DNP isomers

Typical chromatograms of authentic 1,3-, 1,6-, and 1,8-DNP isomers (A), and 1,3-, 1,6-, and 1,8-DNP isomers in airborne particles (B), diesel particles (C), and incinerator dusts (D). After purification with the silica gel column and two HPLC columns, 1,3-, 1,6-, and 1,8-DNP isomers were injected into the Cosmosil $5C_{18}$-AR-II column. Three DNP isomers were reduced by on-line reduction with the NP pak RL, and detected as 1,3-, 1,6-, and 1,8-DAP isomers by a fluorescence detector. The chromatograms were monitored with excitation and emission wavelengths of 375 and 450 nm, respectively.

Table 4. Particle-size-distribution of 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP Isomers in Airborne Particles Collected in Wako City

Particle size (μm)	Amounts[a] (fg/m³ of airborne)			
	3,6-DNBeP MV ± S.D.	1,3-DNP MV ± S.D.	1,6-DNP MV ± S.D.	1,8-DNP MV ± S.D.
> 7	244 ± 7	6 ± 3	12 ± 5	14 ± 7
3.3–7	273 ± 134	10 ± 2	9 ± 1	8 ± 2
2.0–3.3	530 ± 55	5 ± 1	13 ± 1	15 ± 0
1.1–2.0	198 ± 100	12 ± 6	8 ± 10	1 ± 0
< 1.1	188 ± 40	60 ± 7	34 ± 10	20 ± 1
total	1433	93	76	58

a) Amounts were corrected by the recovery of each purification step.

Table 5. Amounts of 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP isomers in diesel particle

	Amounts[a] (ng/g of diesel particle)			
	3,6-DNBeP MV ± S.D.	1,3-DNP MV ± S.D.	1,6-DNP MV ± S.D.	1,8-DNP MV ± S.D.
No. 1	29 ± 7	4 ± 3	5 ± 4	5 ± 4
SRM1975	90 ± 7	—[b]	—[b]	—[b]

a) Amounts were corrected by the recovery of each purification step. *b*) — : Not analyzed.

Table 6. Amounts of 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP Isomers in Incinerator Dust

	Amounts[a] (pg/g of incinerator dust)			
	3,6-DNBeP MV ± S.D.	1,3-DNP MV ± S.D.	1,6-DNP MV ± S.D.	1,8-DNP MV ± S.D.
Bottom Ash				
No. 1	5900 ± 1200	5 ± 6	5 ± 5	2 ± 0
No. 2	164 ± 3	2 ± 1	3 ± 1	5 ± 2
No. 3	15 ± 3	ND[b]	ND[b]	ND[b]
No. 4	3 ± 0	ND[b]	ND[b]	ND[b]
Fly Ash				
JSAC 0511	51 ± 10	3 ± 0	4 ± 1	ND

a) Amounts were corrected by the recovery of each purification step. *b*) ND : Not detected.

DISCUSSIONS

3,6-DNBeP was detected in all surface soil samples collected in three metropolitan areas in Japan. Moreover, 3,6-DNBeP was detected in all surface soil samples collected in 11 wards of Kyoto city. These results indicated that the three metropolitan areas in Japan were contaminated with 3,6-DNBeP, and that the contamination was not restricted in particular sites. The amounts of 3,6-DNBeP in surface soil were 8–975 pg/g of soil, and the contribution ratio of 3,6-DNBeP to the mutagenicity of surface soil toward *S.* typhimurium TA98 without S9 mix was in the range of 4.5–75.1%. The mean value of the mutagenic contribution ratio of 3,6-DNBeP was quite high at 17.3%. Significant differences were not observed in the mutagenic contribution ratios between the three metropolitan areas. The correlation between the mutagenicity of surface soil (X pivot) and amounts of 3,6-DNBeP in surface soil (Y pivot) is shown in Fig. 5. The slope was 0.1468 and the coefficient of correlation was quite high at 0.8653. These results suggested that there was a positive correlation between the mutagenicity of surface soil and the amounts of 3,6-DNBeP in surface soil, and that 3,6-DNBeP was a major mutagen in surface soil.

3,6-DNBeP was detected in airborne particles collected at three sampling sites in Nagoya city,

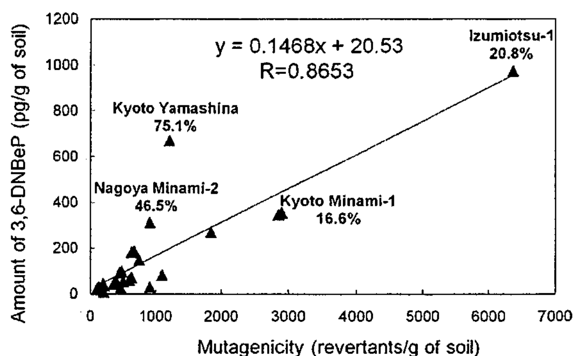

Fig. 5. Correlation between the Amount of 3,6-DNBeP in Surface Soil and Mutagenicity of Surface Soil Collected in Three Metropolitan Areas Toward *S.* typhimurium TA98 without S9 Mix

The slope was 0.1468, and the coefficient of correlation was high in 0.8653. The positive correlation between the amount of 3,6-DNBeP in surface soil and mutagenicity of surface soil was determined from this correlation graph. Representative sampling sites and their mutagenic contribution ratios (%) are noted in the graph.

Aichi prefecture (the Chukyo region). The detection of 3,6-DNBeP in airborne particles collected in Kyoto and Osaka prefectures (the Kinki region), and Tokyo prefecture (the Kanto region) was reported previously.[17] These results suggested that 3,6-DNBeP was widely distributed in the air in these three metropolitan areas in Japan.

In the airborne particles collected in Wako

city, the highest level of 3,6-DNBeP was observed in 2.0–3.3 μm sized particles, while those of the three DNP isomers were observed in <1.1 μm sized particles. However, the amount of 3,6-DNBeP (188 fg/m^3) in particles sized <1.1 μm was 3–9 times greater than those of 1,3-, 1,6-, and 1,8-DNP isomers (60, 34, and 20 fg/m^3, respectively) in this study. It is known that particles with a diameter >5 μm tend to be filtered out in the nose for the most part, and that those <1–2 μm in diameter are deposited predominantly in the alveolar regions of lung.[30] The amount of 3,6-DNBeP that shall be deposited in alveolar tissue was thought to be much greater than those of the three DNP isomers.

3,6-DNBeP was detected in both diesel particles in the range of 29–90 ng/g of diesel particle. SRM 1975 and diesel particles sample No.1 were collected from diesel engines used for industrial forklifts and general motor vehicles, respectively. These results suggested that diesel engines were one of the sources of 3,6-DNBeP, and that 3,6-DNBeP was produced by the combustion of various diesel engines used for industry and general transportation to be emitted into air and surface soil.

3,6-DNBeP was detected in all analyzed incinerator dusts in the range of 3–5900 pg/g of incinerator dust. 3,6-DNBeP was detected not only in the bottom ash of incinerator dusts but also in fly ash, which was emitted into the air directly, like diesel particles. These results suggested that 3,6-DNBeP was produced by combustion in incinerators, and emitted into air and surface soil. The five incinerator dusts analyzed in this study were collected from incinerators that had different combustion scales and temperatures. The differences between incinerators may affect the production of 3,6-DNBeP in the incinerator dusts.

NPAHs are known to be produced by the combustion of diesel engines and incinerators. The amounts of 3,6-DNBeP in diesel engine particles and incinerator dusts were higher than those of 1,3-, 1,6-, and 1,8-DNP isomers. These results suggested that 3,6-DNBeP was more easily produced by combustion in diesel engines and incinerators rather than DNP isomers. Some NPAHs are reported to be produced from PAHs and NO_X by photochemical reactions with ultraviolet rays[31] or PAHs and N_2O_5 without photoradiation[32] in the atmosphere. Because benzo[*e*]pyrene (BeP) is detected in ambient air,[33] the formation of 3,6-DNBeP from BeP and NO_X with or without photoradiation might be another source of 3,6-DNBeP in the environment.

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Genotoxicity of 3,6-dinitrobenzo[*e*]pyrene, a novel mutagen in ambient air and surface soil, in mammalian cells *in vitro* and *in vivo*

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3,6-Dinitrobenzo[*e*]pyrene (3,6-DNBeP), newly identified in airborne particles and surface soil, is a potent mutagen in *Salmonella typhimurium*. The present study investigated the genotoxic potency of 3,6-DNBeP *in vitro* and *in vivo* using mammalian cell strains (Chinese hamster CHL/IU and human HepG2) and ICR mice, respectively. In the *hprt* gene mutation assay using HepG2 cells, the spontaneous mutant frequency was 61.1 per 10^5 clonable cells, which increased to 229 per 10^5 clonable cells after treatment with 1.0 μg/ml (3 μM) 3,6-DNBeP. Notably, in HepG2 cells with increased *N*-acetyltransferase 2 activity, the mutant frequency increased to 648 per 10^5 clonable cells by treatment of 1.0 μg/ml (3 μM) 3,6-DNBeP. The sister chromatid exchange frequency increased approximately three times the control level in HepG2 cells treated with 3,6-DNBeP at a concentration of 1.0 μg/ml (3 μM). In HepG2 and CHL/IU cells, the frequency of the cells with micronuclei was 0.9 and 1.2%, and the frequencies increased to 2.3 and 7.6% after 1.0 μg/ml (3 μM) 3,6-DNBeP-treatment, respectively. The H2AX phosphorylation level increased 8-fold compared with the background level with 1.0 μg/ml (3 μM) 3,6-DNBeP-treatment in HepG2 cells. Moreover, the comet assay showed that 3,6-DNBeP produced DNA damage in the cells of liver, kidney, lung and bone marrow in ICR mice 3 h after intraperitoneal injection at 40 mg/kg (0.12 mmol/kg) body weight. These data indicate that 3,6-DNBeP is genotoxic to mammalian cells *in vitro* and *in vivo*.

Introduction

A large number of aromatic compounds are emitted into the atmosphere from various anthropogenic sources. Epidemiological studies have shown the association of air pollution trends and the incidence of lung cancer (1–6). Recently, 3,6-dinitrobenzo[*e*]pyrene (3,6-DNBeP, Figure 1) was identified as a novel mutagenic nitropolycyclic aromatic hydrocarbon (nitro-PAH) in airborne particles and surface soil, on which atmospheric compounds are thought to descend and accumulate, in residential areas in Japan (7–9). The concentration of 3,6-DNBeP in airborne particles and surface soil was in the ranges of 137–1238 fg/m^3 and 347–5007 pg/g of soil, respectively (8). 3,6-DNBeP is highly mutagenic in *Salmonella typhimurium* TA98 in the absence of the mammalian metabolic system (S9 mix), inducing 285 000 revertants/nmol in TA98 (7), and this potency was similar to those of 1,6- and 1,8-dinitropyrenes, which are the most potent mutagens reported to date (10).

Nitro-PAHs are well known to bind to DNA after metabolic activation by cellular enzymes. As a result of nitroreduction, nitro-PAHs form *N*-hydroxyarylamines, which are also reactive metabolic intermediates of aromatic amines (11). *N*-Hydroxyarylamines can be further activated by *O*-esterification (11). These reactive derivatives can bind covalently to DNA bases. In mammalian cells, the nitroreduction is mediated by cytochrome P450 and cytosolic nitroreductases, such as xanthine oxidase, aldehyde oxidase or DT-diaphorase (12). The esterification is catalyzed by acetyltransferase [(*N*-acetyltransferase (NAT)] and sulphotransferase (12). PAH–DNA adducts are considered to lead to mutation, chromosome aberration and cancer.

Because 3,6-DNBeP is a novel substance detected as a major mutagenic constituent in surface soil, no other biological activity of 3,6-DNBeP has been reported except for mutagenicity in *S. typhimurium* strains (7). As 3,6-DNBeP is an extremely potent bacterial mutagen, other biological activities of 3,6-DNBeP, including metabolism, mutagenicity and carcinogenicity, should be elucidated. The purpose of the present study was to assess the genotoxicity of 3,6-DNBeP *in vitro* and *in vivo* using mammalian cell strains and mice, respectively.

Materials and methods

Chemicals

3,6-DNBeP (CAS 847862-64-0) was prepared as described previously (7). Purity was >99%. Dimethyl sulphoxide (DMSO) was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade and obtained from Sigma-Aldrich Japan (Tokyo, Japan).

Cell culture

Human hepatoma HepG2 cells obtained from RIKEN Cell Bank (Wako, Japan) were cultured in RPMI1640 medium (Sigma-Aldrich Japan) supplemented with 10% foetal bovine serum (JRH Biosciences, Lenexa, KS, USA) in a 5% CO_2 atmosphere at 37°C. The Chinese hamster CHL/IU cell strain was obtained from Dainippon Sumitomo Pharma Co., Ltd (Osaka, Japan). CHL/IU cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 10% heat-inactivated foetal calf serum (Hana-Nesco Bio Co. Ltd, Tokyo, Japan) in a humidified atmosphere with 5% CO_2 at 37°C.

To measure cell viability, cells cultured in a 96-well microtitre plate were exposed to various concentrations of the chemicals, living cells were stained with 50 μg/ml of neutral red (Wako Chemical Co., Osaka, Japan), fixed with 1% formalin containing 1% $CaCl_2$ for 1 h, and neutral red was extracted with 50% ethanol containing 50% acetic acid. OD_{540} was measured using a Microplate Reader Model 680 (Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was calculated as the ratio of the OD_{540} of chemical-exposed cell against that of solvent-exposed control cells. Exposure of HepG2 cells to 3,6-DNBeP was carried out under conditions where cell viability was >95%.

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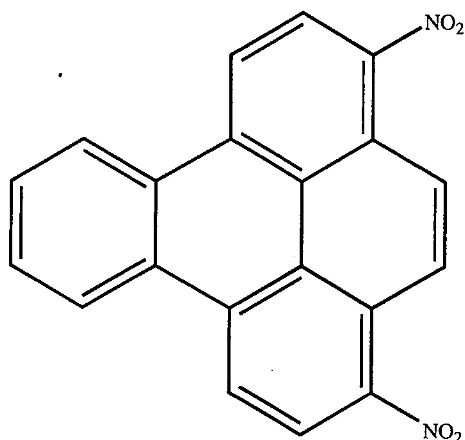

Fig. 1. Chemical structure of 3,6-DNBeP.

Construction of HepG2-expressing human N-acetyltransferases

Plasmids carrying human *N*-acetyltransferase (hNAT) 1 and hNAT2 coding sequence (CDS), pNAT1 and pNAT2 (13), respectively, were a kind gift from Dr D. M. Grant (The Hospital for Sick Children, Toronto, Ontario, Canada). Construction of the expression plasmids was carried out by inserting these cDNA fragments into the multicloning site of pcDNA3 (Invitrogen, Carlsbad, CA, USA). Briefly, pNAT1 and pNAT2 were digested with EcoRI and SalI, and DNA fragments containing the hNATs CDSs were separated by agarose gel electrophoresis. The fragments were sub-cloned between the EcoRI and XhoI sites of pcDNA3. The resultant plasmids, designated as pcDNA3/hNAT1 and pcDNA3/hNAT2, were linearized by PvuI digestion and introduced into HepG2 cells by electroporation (PDS, Madison, WI, USA). Stable integrants were selected in medium containing 1 mg/ml Geneticin (Invitrogen). Several Geneticin-resistant clones were chosen randomly and used for further study.

Real-time polymerase chain reaction (PCR) was carried out to confirm expression of the integrated genes. Briefly, total RNA was extracted from the integrant cells using an RNeasy Mini kit (QIAGEN K.K., Tokyo, Japan). The extracted RNA was treated with DNaseI (Invitrogen) to eliminate genomic DNA contamination. The amount of total RNA per sample was measured using an ND-1000 UV/VIS Spectrophotometer (NanoDrop Technology, Wilmington, DE, USA). After repurification of the RNA using an RNeasy Mini kit, a reverse transcription reaction was performed using ExScript RT-PCR kit with random six-mer primers (Takara Bio Inc., Shiga, Japan). The mRNA expression levels of hNAT1 and hNAT2 in the integrants were measured by real-time reverse transcriptase (RT)–PCR [SmartCycler V2.0 (Cepheid, Sunnyvale, CA, USA)] using the following primers, 5′-AGTTCAAGACTCTGAGTGAGGAAGA-3′ and 5′-TTGGGCACAAGCTTTCTCTG-3′ for hNAT1 and 5′-CACTGAG-GAAGAGGTTGAAG-3′ and 5′-ATCCATCACCAGGTTTGG-3′ for hNAT2. Amplification was performed with an initial incubation at 95°C for 10 sec, followed by 30 cycles of 95°C for 5 sec and 60°C for 20 sec using an SYBR Premix ExTaq Perfect Real Time kit (Takara Bio Inc.). Various amounts (from 20 to 0.02 pg with 10-fold intervals) of pcDNA3/hNAT1 or pcDNA3/hNAT2 were used as templates to generate standard curves for quantification, and total RNA was assumed to be equivalent to 40 pg/cell. In these conditions, the quantification limit was 1.0 copy/cell. The absence of amplification of non-specific PCR products was confirmed by gel electrophoresis.

Enzyme assays were also carried out to confirm elevated activities of NAT in the integrants. The cells, grown to confluent in a dish (100 mm in diameter), were washed with phosphate-buffered saline (PBS) and scraped into a microfuge tube. The cells were harvested by centrifugation at 1000 × *g* for 1 min and re-suspended in 100 μl of 0.25 M Tris–HCl buffer (pH 7.5) containing 1 mM dithiothreitol. The cells were disrupted by three cycles of freezing and thawing. Then, the suspension was centrifuged at 10 000 × *g* for 10 min, and the supernatant was used for the enzyme assays. Isoniazid NAT activity and *p*-aminobenzoic acid (PABA) NAT activity were measured by the methods of Hein *et al.* (14) and Weber and King (15), respectively. The protein concentration was determined using a Modified Lowry Protein Assay kit (Pierce, Rockford, IL, USA). Each of the desired clones for hNAT1 and hNAT2 expression were chosen and designated as HepG2/NAT1 and HepG2/NAT2, respectively.

Hypoxanthine-guanine phosphribosyltransferase mutation assay

One day before the experiment, 75-cm^2 flasks were inoculated with 2 × 10^6 cells. Then, the cells were treated with the indicated concentrations of 3,6-DNBeP for 24 h in medium containing 10% serum. After 24 h, the treatment medium was removed and replaced by fresh complete medium. Then, cells were cultured for 2 weeks to allow phenotypic expression. At the end of the incubation period, 5 × 10^5 cells were plated into each of ten 100-mm cell culture dishes per experimental group in selective medium containing 30 μM 6-thioguanine (6-TG; Nacalai Tesque). Cells from each culture dish were also plated at 1 × 10^4 cells into each of ten 100-mm dishes in the absence of 6-TG. After 2 weeks of incubation, colonies were counted and colony-forming efficiency (CE) was calculated. The mutation frequency was calculated as (CE in the presence of 6-TG) ÷ (CE in the absence of 6-TG). Two-way analysis of variance (ANOVA) was carried out using Microsoft Excel 2004 for Mac software in order to show effect of cell type.

Sister chromatid exchange test

Cells were treated with the indicated concentrations of 3,6-DNBeP in medium containing 10% serum and 10 μg/ml 5-bromodeoxyuridine (BrdU; Sigma-Aldrich Japan) for 48 h. Colcemid (Nacalai Tesque) was added for 4 h at a concentration of 60 ng/ml before fixation. Cells were trypsinized and centrifuged, re-suspended in 0.075 M KCl and incubated for 15 min. The cells were fixed three times in methanol:glacial acetic acid (3:1). The cell solution was dropped onto slides in Metaphase Spreader HANABI (AD Science Technology, Funabashi, Japan). The slides were soaked in 50 μg/ml Hoechst #33258 (Sigma-Aldrich, St. Louis, MO, USA), covered with 0.01 M sodium phosphate buffer (pH 7.6) and cover glasses and irradiated with black light (365 nm) for 3 h. Subsequently, the slides were stained with 6% Giemsa (Merck KGaA, Darmstadt, Germany) in 0.06 M sodium phosphate buffer (pH 6.4) for 10 min. Sister chromatid exchanges (SCEs) were scored using a microscope.

Detection of γH2AX foci

The cells were seeded on a slide glass at a density of 10^6 cells/ml. The cells were exposed to 3,6-DNBeP at a concentration of 1 μg/ml (3 μM) for 1 h. 3,6-DNBeP were dissolved in DMSO and diluted to the above concentration with serum-free culture medium. Cells grown on slide glasses were washed with PBS solution and incubated with serum-free medium containing the chemicals for 1 h. After exposure, the cells were grown in medium for 1 h and fixed with ice-cold methanol for 20 min. Methanol-fixed cells on slide glasses were rinsed with ice-cold PBS three times and immersed in PBS containing 0.5% Triton X-100 for 30 min. The cells were rinsed with ice-cold PBS three times and kept in PBS containing 5% bovine serum albumin for 18 h at room temperature. Cells were then rinsed once in PBS and treated with a rabbit anti-phospho-H2AX antibody (4 μg/ml in PBS) (Trevigen 4411-PC-100) for 1 h at room temperature. The cells were rinsed with ice-cold PBS three times and treated with a second antibody, goat Alexa Fluor 488 anti-rabbit IgG (Molecular Probe A11034) (1/1000 diluted in PBS), for 1 h. After the cells were rinsed with ice-cold PBS three times, they were soaked in Vectorshield mounting solution containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector H-1200) and overlaid with a cover glass.

Fluorescent nuclei were observed using ×100 magnification lenses of a fluorescence microscope (Olympus BX-URA2), and images were recorded using a cooled CCD camera (Keyence VB-7000) in a dark room. DAPI-stained nuclei fluoresced blue and γH2AX foci fluoresced green. The blue and green areas of each nucleus were measured by an image analyzing system (Keyence VH-H1A5). To represent the relative level of H2AX phosphorylation of each nucleus, the value of the green area was divided by the blue area.

Micronucleus test

3,6-DNBeP was dissolved in DMSO, of which the final concentration was adjusted to 1% in the culture medium. Cells were treated with 3,6-DNBeP at the indicated concentrations in medium without serum for 1 h. After removing 3,6-DNBeP, the cells were cultured for a further 24 h. Then, the cells were trypsinized, counted and centrifuged. Growth inhibition was calculated using the following formula: growth rate = (the number of treated cells) ÷ (the number of non-treated cells). The cells were re-suspended in 0.075 M KCl and incubated for 5 min. Then, the cells were fixed three times in methanol:glacial acetic acid (3:1) and washed with methanol containing 1% acetic acid. Finally, the cells were re-suspended in methanol containing 1% acetic acid. The cell solution was dropped onto slides and the nuclei were stained by mounting in 40 μg/ml acridine orange (Nacalai Tesque) solution. The stained cells were observed immediately by fluorescence microscopy using a B-excitation mirror unit. The number of cells with micronuclei was recorded based on observation of 1000 interphase cells. The data were compared statistically with the corresponding solvent control using a chi-square test.

Comet assay

Seven-week-old male ICR mice were supplied from Japan SLC (Hamamatsu, Japan) and used for the experiments at 8 weeks old. Five mice were assigned randomly to each group. 3,6-DNBeP was suspended in olive oil and injected

intraperitoneally (i.p.) once to mice at doses of 40 and 80 mg/kg (0.12 and 0.23 mmol/kg) body weight. In the negative and positive control groups, olive oil and ethyl methansulphonate [EMS; 160 mg/kg (1.3 mmol/kg) body weight], which was dissolved in physiological saline, were injected i.p., respectively.

The alkaline comet assay was performed in accordance with a modification of the method of Tice *et al.* (16). Liver, kidney, lung and bone marrow were removed 3 h after injection. Each organ, except for bone marrow, was minced, suspended in chilled buffer (pH 7.5, containing 0.075 M KCl and 0.03 M sodium EDTA) and homogenized gently using a Dounce-type homogenizer. One hundred microlitres of normal-melting-point agarose were layered as the first layer on a glass slide and then 50 µl of low-melting-point agarose containing ~10 000 nuclei was layered on the first layer. Finally, 100 µl of normal-melting-point agarose was layered onto the second layer. The slides were immersed in ice-cold lysing solution (pH 10, containing 2.5 M NaCl, 100 mM sodium EDTA, 10 mM Tris–HCl, 1% sodium *N*-lauryl sarcosinate, 10% DMSO and 1% Triton X-100) for 60 min. The slides were then placed on a horizontal gel electrophoresis platform and covered with chilled alkaline solution (containing 300 mM NaOH, 1 mM sodium EDTA) for 20 min to allow for the unwinding of DNA and the expression of alkali-labile sites. The nuclei were electrophoresed at 25 V for 20 min. After electrophoresis, specimens were rinsed twice with 400 mM Tris–HCl (pH 7.5) to neutralize excess alkaline solution, stained with 50 µl of ethidium bromide solution, and covered with a cover slip. One hundred nuclei were examined from each organ of animals using a fluorescence microscope (×800) equipped with a CCD camera. The tail moment of the DNA was measured using Comet Assay software (Kinetic Imaging Ltd, Liverpool, UK).

Student's *t*-test was used to test the difference in the DNA tail moment in the Comet assay between the treated and control groups; a *P*-value lower than 0.05 was considered to be statistically significant.

Results

Construction of HepG2-expressing hNATs

Human hepatoma HepG2 cells exhibit the activities of various phase I and phase II enzymes that play a crucial role in the metabolic activation of Nitro-PAHs (17). However, there are several reports on low activity of NATs in HepG2 cells (17–19). Therefore, HepG2 cells were generated with increased activity of NATs by introducing expression vectors with hNAT1 or hNAT2 cDNAs. Table I shows the levels of NAT mRNAs measured by real-time RT-PCR and the enzyme activity measured using PABA. The isoniazid NAT activity was too weak to quantify (data not shown). HepG2 cells in which hNAT1 or hNAT2 were integrated (HepG2/NAT1 and HepG2/NAT2, respectively) clearly showed higher levels of NAT1 and NAT2 mRNA than the control cells (HepG2/pcDNA). Moreover, HepG2/NAT1 and HepG2/NAT2 exhibited significantly higher NAT activities with PABA than control HepG2 cells ($P < 0.05$). The activities of HepG2/NAT1 and HepG2/NAT2 were 30.6 and 20.4 nmol/min/mg protein, respectively, and that of control HepG2 cells was 4.88 nmol/min/mg protein (Table I).

Table I. mRNA level and enzyme activity of human NATs in HepG2 and its derivatives

Cell type	mRNA (copy/cell)		NAT activity (nmol/min/mg protein)[a]
	NAT1	NAT2	
HepG2	NA	NA	4.88 ± 2.30
HepG2/pcDNA	<1.0[b]	<1.0[b]	8.47 ± 1.54
HepG2/NAT1	58.2	NA	30.6 ± 9.88*
HepG2/NAT2	NA	47.3	20.4 ± 3.71*

NA, not assayed.
[a]Mean ± SD of at least three independent experiments.
[b]Under quantification limit.
*$P < 0.05$ (versus HepG2/pcDNA).

HPRT mutation assay

As shown in Figure 2, the exposure of control HepG2 cells to 1.0 µg/ml (3 µM) 3,6-DNBeP for 24 h resulted in the induction of *hprt* mutations. In control HepG2 cells, the background mutation frequency was 6.11×10^{-4} (61.1 mutants per 10^5 clonable cells), and the frequency increased significantly ($P < 0.05$) to 2.29×10^{-3} after treatment with 1.0 µg/ml (3 µM) 3,6-DNBeP. Notably, in HepG2/NAT2 cells, the mutation frequency increased significantly ($P < 0.05$) to 6.16×10^{-3} and 6.48×10^{-3} after treatment of 0.6 and 1.0 µg/ml (1.8 and 3 µM) 3,6-DNBeP, respectively, and the background frequency was 1.15×10^{-3}. In addition, a statistically significant increase in mutation frequency from that of non-treated cells was observed in HepG2/NAT1 cells treated with 0.6 and 1.0 µg/ml (1.8 and 3 µM) 3,6-DNBeP and in the HepG2/pcDNA cells treated with 1.0 µg/ml (3 µM) 3,6-DNBeP. In ANOVA, there was a significant ($P < 0.05$) effect of cell type overall.

SCE test and detection of γH2AX foci

The SCE frequency in HepG2 cells after treatment of 3,6-DNBeP is shown in Table II. SCE frequencies increased ~2- to 3-fold compared with the control level in cells treated with 0.2–1.0 µg/ml (0.6–3 µM) 3,6-DNBeP. An increase in the frequency was also observed in HepG2/NAT1 and HepG2/NAT2 cells, and these frequencies were similar to those in HepG2 cells (data not shown).

We measured phosphorylation levels of histone H2AX as a marker of DNA strand breaks. Table II shows that 3,6-DNBeP (1 µg/ml, 3 µM) induced H2AX phosphorylation 8-fold higher than the background level, which was statistically higher than the background level ($P < 0.05$).

Micronucleus assay

Figure 3 shows that 3,6-DNBeP increased the proportion of micronucleated cells in CHL/IU and HepG2 cells. In these cells, the background frequency of micronucleated cells was 0.9–1.2%, and the frequency increased to 2.3–7.6% after 1 µg/ml (3 µM) 3,6-DNBeP treatment. The frequency of micronucleated cells was statistically higher than that of the control at all concentrations tested ($P < 0.05$). The treatment inhibited cell growth slightly; the growth rates of CHL/IU and HepG2 cells with 1 µg/ml (3 µM) 3,6-DNBeP were 0.93 ± 0.034-fold and 0.86 ± 0.006-fold, respectively, that of the control cells.

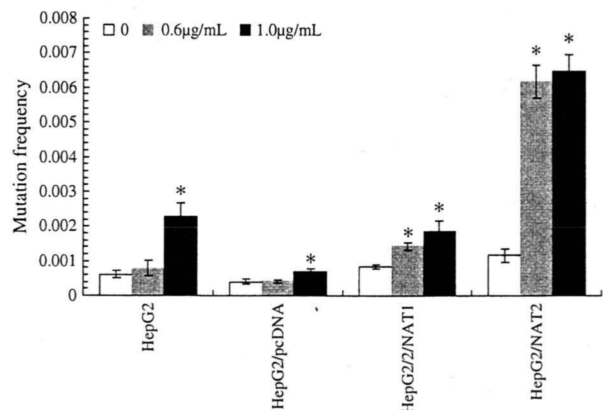

Fig. 2. Mutation frequency at the *hprt* locus in HepG2 cells and their derivatives after treatment with 3,6-DNBeP. Mean ± SD of at least three independent experiments is shown. *$P < 0.05$ (versus control).

DNA-damaging activity of 3,6-DNBeP

The DNA tail moment of the comet assay was determined as the DNA-damaging activity of 3,6-DNBeP in various mouse organs, i.e. liver, kidney, lung and bone marrow. As shown in Figure 4, the DNA tail moment values increased significantly in all tested organs at 40 and 80 mg/kg (0.12 and 0.23 mmol/kg) body weight and in a dose-dependent manner. The highest mean tail moment (1.44; control 0.94) was detected in the lung at 80 mg/kg (0.23 mmol/kg) body weight.

Discussion

Various mutagenic and carcinogenic chemicals have been discharged into ambient air from anthropogenic sources such as industrial power plants (20,21), furnaces for metal smelting (22,23), municipal incinerators (24) and the engines of motor vehicles (25–27). These pollutants are eventually deposited on the ground and accumulate in surface soil; therefore, surface soil is thought to contain pollutants with mutagenic and carcinogenic activities (9). In fact, mutagenicity and DNA-damaging activity were observed in organic extracts of soils from roadsides (28–30), parks (31,32), agricultural land (33–35) and residential sites (36,37). 3,6-DNBeP was identified as one of the major contributors to soil mutagenicity in a *Salmonella* assay and then detected in ambient air as well (7). The current study investigated the genotoxicity of 3,6-DNBeP in mammalian cells *in vitro* and *in vivo* and showed that 3,6-DNBeP significantly increased genotoxic indicators in all assays.

Previous studies indicated that HepG2 cells retain the activities of certain phase I and phase II enzymes and can metabolically activate DNA-damaging compounds more than other cultured cells (17). HepG2 cells have been used for successfully detecting genotoxic carcinogens, including compounds giving false-negative results in other *in vitro* assays (17). Therefore, HepG2 cells were used to examine the genotoxicity of 3,6-DNBeP. 3,6-DNBeP is highly mutagenic in bacteria, being activated through nitroreduction and *N,O*-esterification. In a test using *S. typhimurium* TA98 and its *N,O*-acetyltransferase overproducing derivative YG1024 (38), 3,6-DNBeP induced 285 $\times$ 10^3 and 955 $\times$ 10^3 revertants/nmol, respectively (7). Furthermore, oxidation of the benzo[*e*]pyrene moiety is unlikely to exert its mutagenicity because the mutagenic activities in each strain without S9 mix were higher than those with S9 mix (7). Therefore, *N,O*-acetyltransferase could be required for the mutagenicity of 3,6-DNBeP. Although HepG2 cells have various kinds of metabolic activities, several reports indicate low activity of NATs in HepG2 (17–19). Although HepG2 cells were generated with increased activity of NATs in the present study, enhanced genotoxicity was observed only in the *hprt* mutation assay. Induction of micronuclei, SCE and γH2AX might be mediated by a different mechanism from metabolic activation through *N,O*-acetyl transfer.

Induction of histone H2AX phosphorylation (γH2AX) is triggered by DNA double-strand breaks (DSBs) and DNA replication fork arrest. Many reports have shown that various chemical and physical treatments cause H2AX phosphorylation (39–41). PAHs do not cause DSB directly but produce bulky DNA adducts in cellular DNA and induce H2AX phosphorylation. A previous study showed that treatment of HeLa cells with 3-nitrobenzanthrone, one of the potent mutagenic Nitro-PAHs, and benzo[*a*]pyrene increased phosphorylation levels 24 and 30 times higher than the background level (42). In contrast, 3,6-DNBeP augmented the phosphorylation level eight times higher than the background level in the present study.

The comet assay has been developed as a sensitive method that is capable of detecting various ranges of DNA damage, including double- and single-strand DNA breaks, which include indirect origins from incomplete excision repair and alkali-labile sites (43). The comet assay can be applied to many tissues in an experimental animal (44) and has been suggested to be a promising method for detecting the organ-specific genotoxicity of chemical mutagens and carcinogens such as Nitro-PAHs (44). 3,6-DNBeP induced significantly higher amounts of DNA damage in various mouse organs, i.e. liver, kidney, lung and bone marrow, by i.p. injection, and the highest amount of damage was observed in the lung. These results may indicate that 3,6-DNBeP is absorbed after i.p. injection, and 3,6-DNBeP and/or its active metabolites are distributed to various organs. The absorption, distribution, metabolism and clearance of 3,6-DNBeP *in vivo* remain to be clarified in future studies.

Table II. SCEs and H2AX phosphorylation after 3,6-DNBeP-treatment in HepG2 cells

Treatment (μg/ml)	Mean number of SCEs/chromosome[a]	Relative γH2AX level (green area/blue area)[ab]
0	0.27 ± 0.03	0.00197 ± 0.00100
0.2	0.70 ± 0.17*	NA
1.0	0.84 ± 0.15*	0.0157 ± 0.00195

NA, not assayed.
[a]Mean ± SD of at least three independent experiments.
[b]HepG2/pCDNA cells were used as HepG2 cells.
*$P < 0.01$ (versus control).

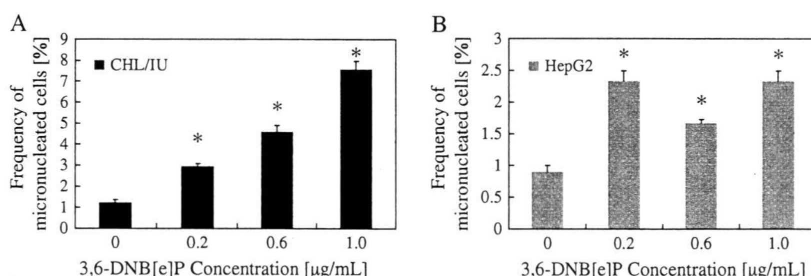

Fig. 3. Micronuclei induced by 3,6-DNBeP in CHL/IU (**A**) and HepG2 (**B**) cells. Mean ± SD of micronucleated frequency based on 1000-cell observation in at least three independent experiments. *$P < 0.05$ (versus control).

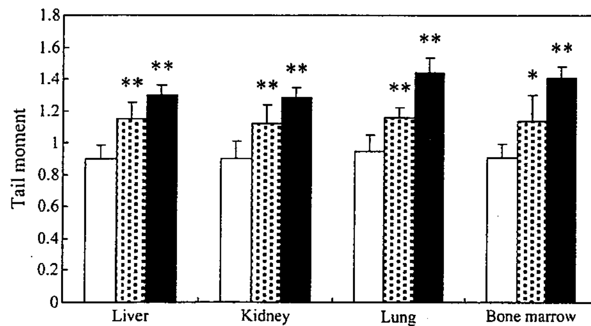

Fig. 4. DNA-damaging activity of 3,6-DNBeP. Mice were treated i.p. with 3,6-DNBeP at doses of 40 mg/kg (dotted column) and 80 mg/kg (closed column) body weight. Control mice (open column) were treated with olive oil. Tail moments of EMS-treated (160 mg/kg) mice were 2.42 ± 0.60 in liver, 2.45 ± 0.41 in kidney, 2.52 ± 0.39 in lung and 2.49 ± 0.42 in bone marrow. Organs were removed 3 h after injection. One hundred cells were observed per mouse. Tail moment = DNA migration × intensity. $^{*}P < 0.05$ (versus control); $^{**}P < 0.01$ (versus control).

Because 3,6-DNBeP is widely detected in ambient air and surface soil (7–9), humans may be exposed to 3,6-DNBeP. The present study demonstrated that 3,6-DNBeP is genotoxic in mammalian cells *in vitro* and *in vivo*. To fully estimate the impact of 3,6-DNBeP on human health, further studies on its biological activities, including carcinogenicity, are necessary. In addition, more detailed quantification of exposure level to 3,6-DNBeP is required to assess the risk on human health.

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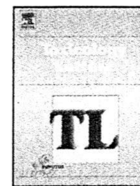

Genotoxic activation of the environmental pollutant 3,6-dinitrobenzo[*e*]pyrene in *Salmonella typhimurium umu* strains expressing human cytochrome P450 and *N*-acetyltransferase

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ABSTRACT

3,6-Dinitrobenzo[*e*]pyrene (DNBeP) is a potent mutagen identified in surface soil in two metropolitan areas of Japan. We investigated whether DNBeP can cause genotoxicity through any metabolic activation pathway in bacteria using the parental strain *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) TA1535/pSK1002, nitroreductase (NR)-deficient strain NM1000, the *O*-acetyltransferase (*O*-AT)-deficient strain NM2000, bacterial *O*-AT-overexpressing strain NM2009, and bacterial NR- and *O*-AT-overexpressing strain NM3009 established in our laboratory. To further clarify the role of human cytochrome P450 (P450 or CYP) and *N*-acetyltransferase (NAT) enzymes in the bioactivation of DNBeP to genotoxic metabolites, we determined the genotoxicity of DNBeP using a variety of *umu* tester strains expressing human P450 and NAT enzymes. The dose-dependent induction of *umuC* by DNBeP was observed at concentrations between 0.01 and 1 nM in the *O*-AT-expression strain, but not in the *O*-AT-deficient strain. In the CYP3A4-, CYP1A2-, CYP1A1-, and CYP1B1-expressing strains, DNBeP was found to be activated to reactive metabolites that cause the induction of *umuC* gene expression compared with the parent strain. The induction of DNBeP in the NAT2-expressing strain had a 10-fold lower concentration than that in the NAT1-expressing strain. Collectively, these results suggest that nitroreduction by human CYP1A2, CYP3A4, and CYP1A1 and *O*-acetylation by human NAT2 contributed to the genotoxic activation of DNBeP to its metabolites.
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1. Introduction

Nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are a class of environmental contaminants that have potent mutagenicity in bacterial and mammalian cells and carcinogenicity in laboratory animals (Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986). Recently, 3,6-dinitrobenzo[*e*]pyrene (DNBeP) has been identified in large amounts of surface soil samples collected in Takatsuki, Osaka Prefecture and four other cities by the bioassay directed fractionation method (Watanabe et al., 2005; Hasei et al., 2006). Extracts from these surface soils showed potent mutagenicity in the Ames assay. DNBeP is highly mutagenic in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) TA98 without the mammalian metabolic system (rat S9 mix), inducing 285,000 revertants/nmol in TA98, and its potency is comparable to those of 1,6-dinitropyrene and 1,8-dinitropyrene, which are the most potent mutagens reported to date (Watanabe et al., 2005).

DNBeP has direct-acting mutagenicity in *S. typhimurium* strains (Watanabe et al., 2005, 2008). This chemical is converted by bacterial nitroreductase (NR) to *N*-hydroxylated and then by *O*-acetyltransferase (*O*-AT) to esterified metabolites in *S. typhimurium* strains YG1021 overexpressing NR and YG1024 overexpressing *O*-AT (Watanabe et al., 2005). In contrast, although the genotoxic activation of nitro-compounds, such as 1,8-dinitropyrene and 3-nitrobenzanthrone, in mammals, including humans, is mainly via nitroreduction catalyzed by cytosolic xanthine oxidase and microsomal NADPH-cytochrome P450 reductase (NPR), and the participation of cytochrome P450 (CYP) enzymes in the oxidative metabolism of DNBeP has been suggested (Borlak et al., 2000; Arlet et al., 2003; Yamazaki et al., 2000; Bieler et al., 2003), there is no direct evidence of the involvement of CYP enzymes in the nitroreduction of DNBeP. The role of specific CYP enzymes in the metabolic pathways of DNBeP remains unclear.

In the present study, we newly constructed the OY1022 strain by selecting resistant clones of TA1535NR capable of growth in the presence of 1,8-dinitropyrene to reduce direct sensitivity to DNBeP. Using this strain as a host, we established different strains expressing four recombinant human CYPs by introducing two plasmids into *S. typhimurium* OY1022, one carrying both CYP and NPR

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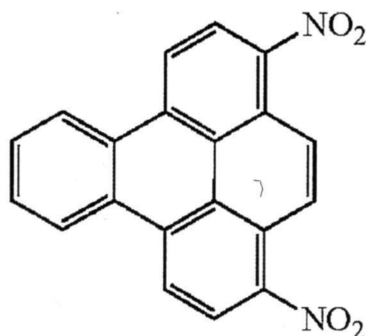

Fig. 1. Chemical structure of 3,6-dinitrobenzo[*e*]pyrene.

cDNA in a biocistronic construct under control of an isopropyl β-D-thiogalactoside (IPTG)-inducible double *tac* promoter and the other, pOA102, carrying *O*-AT and *umuC'-'lacZ* fusion genes, and investigated whether any human CYP enzymes and human *N*-acetyltransferases (NATs) are involved in the genotoxic activation of DNBeP to its metabolites. Genotoxicity of DNBeP was investigated by using *S. typhimurium* strains expressing bacterial *O*-AT, and eight human CYP enzymes, NPR and *O*-AT, and human NAT1 and NAT2.

2. Materials and methods

2.1. Chemicals

3,6-Dinitrobenzo[*e*]pyrene was synthesized as previously described (Watanabe et al., 2005) (Fig. 1). 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) was purchased from Wako Pure Chemical Industry (Osaka). Aflatoxin B_1 (AFB_1) was obtained from Makor Chemicals Ltd. (Israel). All other chemicals and reagents were of the highest purity commercially available (Wako Pure Chemical Industries, Osaka).

2.2. Isolation of a dinitropyrene-resistant clone from the TA1535NR strain

A dinitropyrene-resistant clone was isolated as described by McCoy et al. (1981). Briefly, overnight cultures of *S. typhimurium* TA1535NR (Watanabe et al., 1989) were plated onto Columbia base containing 5 μg of 1,8-dinitropyrene/mL. After incubation in the dark for two days at 37 °C, colonies were picked, recloned, and tested for a deep-rough character (sensitivity to crystal violet) (Ames et al., 1973) and sensitivity to 1,8-DNP on L-agar. In addition, we examined the induction of *umuC* gene expression by 1,8-DNP using a 1,8-DNP-resistant clone harboring pSK1002 and NM2000. Both strains showed similar sensitivity to 1,8-DNP; therefore, this 1,8-DNP-resistant clone appeared to have an *O*-acetyltransferase-deficient character. The 1,8-DNP-resistant clone (TA1535NR/1,8-DNP) was designated *S. typhimurium* OY1022.

2.3. Bacterial strains and their construction

The *umu* tester strains used in the present study are *S. typhimurium* TA1535/pSK1002 (parental strain), NM1000 (nitroreductase-deficient strain), NM2000 (*O*-acetyltransferase-deficient strain), NM2009 (*O*-acetyltransferase-overproducing strain), and NM3009 (nitroreductase- and *O*-acetyltransferase-overproducing strain) (Oda et al., 1993) and NM6000 (*O*-acetyltransferase-deficient parental strain), NM6001 (human acetyltransferase type 1-expressing strain), and NM6002 (human acetyltransferase type 2-expressing strain) (Oda et al., 1999). To establish *S. typhimurium* tester strains expressing human cytochrome P450 (CYP) and NADPH-cytochrome P450 reductase (NPR) genes, we employed four plasmids: pCW'/1A1/hNPR, pCW'/1A2/hNPR, pCW'/1B1/hNPR, and pCW'/3A4/hNPR (provided by Dr. F.P. Guengerich, Vanderbilt University School of Medicine, USA) (Sandhu et al., 1994; Parikh et al., 1997). These plasmids were then introduced into *S. typhimurium* TA1535NR/1,8-DNP (harboring pOA102) and the resulting strains were referred to as *S. typhimurium* OY1022/1A1, OY1022/1A2, OY1022/1B1, and OY1022/3A4, respectively. In a similar way, the parental *S. typhimurium* OY1022/pCW strain was established by introducing the null vector pCW and pOA102 (Table 1).

2.4. Determination of expressions of CYP and NPR in S. typhimurium OY1022 cells

The expression of different CYP enzymes and NADPH-cytochrome P450 reductase in *S. typhimurium* strains was determined according to the method previously described (Oda et al., 2001). The contents of CYP P450 in *S. typhimurium* cells were determined by Fe^{2+} CO versus Fe^{2+} difference spectra, according to established procedure (Omura and Sato, 1964). The difference spectra were recorded using a UV–visible spectrophotometer model MPS-2000 (Shimazu, Kyoto, Japan). To determine NPR expression levels, the activity of NPR in sonicated bacterial cells was measured with cytochrome *c* as an electron acceptor by measuring absorbance change at 550 nm at 20 °C using spectrophotometer according to the method of Phillips and Langdon (1962).

2.5. Umu assay using strains expressing bacterial nitroreductase and/or O-AT enzymes

Umu assay was carried out according to the procedure described previously (Oda et al., 1985). The overnight culture was diluted 100-fold with TGA medium (1% bactotryptone (w/v), 0.5% NaCl (w/v), 0.2% glucose (w/v), and 20 μg/mL ampicillin). The culture was incubated for 1 h at 37 °C and 1-mL aliquots of TGA culture (OD_{600}: 0.25–0.3) and 10 μL tester compound dissolved in DMSO were mixed and further incubated for 2 h. Induction of *umuC* gene expression as a response to DNA damage was determined by measuring cellular β-galactosidase activity, as reported previously. The cytotoxic effect of the chemicals on bacterial cells was determined in the reaction mixture by measuring optical density change at 600 nm using spectrophotometer. The results are presented as the means of two or three independent experiments in Figs. 2 and 3.

Table 1
Umu tester strains and plasmids used in the present study.

S. typhimurium	Relevant genotype	Source or reference
Bacteria or plasmid		
TA1535	*hisG46, gal, del (chl, uvrB, bio), rfa*	Ames et al. (1973)
TA1535NR	As TA1535 but is deficient in classical nitroreductase	Watanabe et al. (1989)
TA1535/pSK1002	As TA1535 but pSK1002 is contained	Oda et al. (1985)
NM1000	As TA1535/pSK1002 but deficient in classical nitroreductase	Oda et al. (1993)
NM2000	As TA1535/1,8-DNP/pSK1002 but deficient in *O*-acetyltransferase (*O*-AT)	Oda et al. (1993)
NM2009	As TA1535/pSK1002/pNM12: a *O*-AT-overexpressing strain	Oda et al. (1993)
NM3009	As TA1535/pSK1002/pNM13: a nitroreductase- and *O*-AT-overexpressing strain	Oda et al. (1993)
OY1022	As TA1535NR, but is resistant to 1,8-DNP probably due to *O*-AT deficiency	Present study
OY1022/pCW	As TA1535 NR/1,8-DNP but harbors pCW and pOA102	Present study
OY1022/1A1	As TA1535 NR/1,8-DNP but harbors pCW'/1A1/hNPR and pOA102	Present study
OY1022/1A2	As TA1535 NR/1,8-DNP but harbors pCW'/1A2/hNPR and pOA102	Present study
OY1022/1B1	As TA1535 NR/1,8-DNP but harbors pCW'/1B1/hNPR and pOA102	Present study
OY1022/3A4	As TA1535 NR/1,8-DNP but harbors pCW'/3A4/hNPR and pOA102	Present study
NM6000	As TA1538/1,8-DNP/pSK1002 but is deficient in *O*-AT	Oda et al. (1999)
NM6001	As TA1538/1,8-DNP/pSK1002/pNM63: a human *N*-acetyltransferase 1-expression strain	Oda et al. (1999)
NM6002	As TA1538/1,8-DNP/pSK1002/pNM64: a human *N*-acetyltransferase 2-expression strain	Oda et al. (1999)
Plasmids		
pOA102	As pACYC184, but has *umu* "*lacZ* gene and *O*-AT gene, Tet	Aryal et al. (1999)
pCW	Ampr	Shet et al. (1993)
pCW'/1A1/hNPR	As pCW, but has human CYP 1A1 and NADPH-P450 reductase genes, Ampr	Parikh et al. (1997)
pCW'/1A2/hNPR	As pCW, but has human CYP 1A2 and NADPH-P450 reductase genes, Ampr	Parikh et al. (1997)
pCW'/1B1/hNPR	As pCW, but has human CYP 1B1 and NADPH-P450 reductase genes, Ampr	Shimada et al. (1998)
pCW'/3A4/hNPR	As pCW, but has human CYP 3A4 and NADPH-P450 reductase genes, Ampr	Parikh et al. (1997)

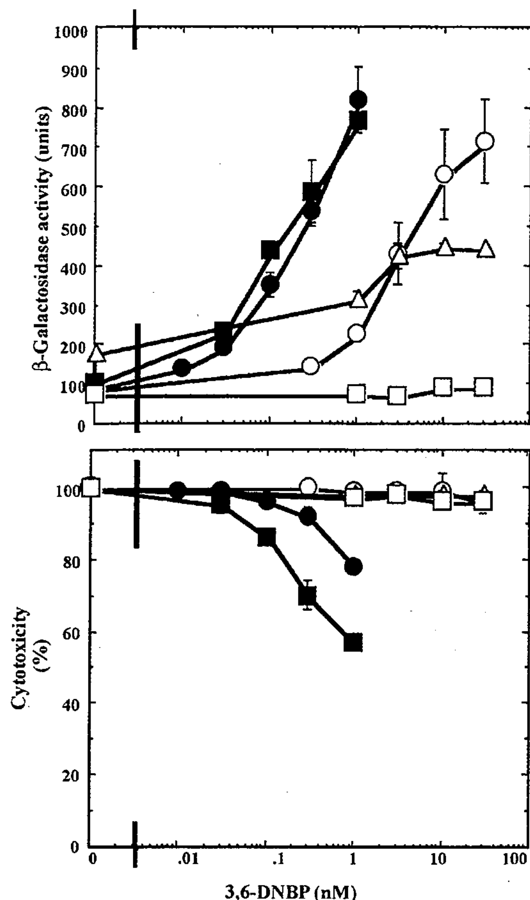

Fig. 2. Induction of *umuC* gene expression and cytotoxicity response by 3,6-dinitrobenzo[*e*]pyrene in *S. typhimurium* TA1535/pSK1002 (open circles), NM2009 (filled circles), NM3009 (filled squares), NM1000 (open triangles) and NM2000 (open squares). β-Galactosidase activity (units) was determined as described in Materials and methods. Cytotoxicity activities are expressed as optical density change (%) at 600 nm. Each point represents the means ± SD of duplicate or triplicate determinations.

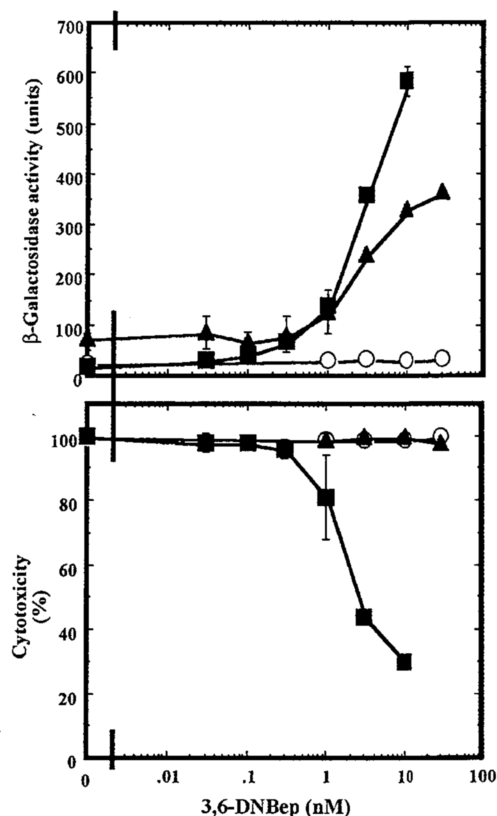

Fig. 3. Induction of *umuC* gene expression by 3,6-dinitrobenzo[*e*]pyrene in *S. typhimurium* NM6000 (open circles), NM6001 (filled triangles), and NM6002 (filled squares). Other details are as in the legend to Fig. 2.

2.6. Umu assay using strains expressing human CYP and O-AT enzymes

We used the method as previously described (Aryal et al., 1999) with a slight modification. The overnight culture of tester strains was diluted 100-fold in TGlyT medium (1% bactotryptone (w/v), 0.5% NaCl (w/v), 0.2% glycerol (v/v), and 1 μg tetracycline/mL) supplemented with 1.0 mM isopropyl β-D-thiogalactoside, 0.5 mM δ-aminolevulinic acid, and 250 μL of trace elements/L (Sandhu et al., 1994). The culture was incubated for 2 h (37 °C, 165 rpm) and then 1-mL aliquots of TGlyT culture (OD_{600}: 0.25–0.3) and 10 μL of tester compound dissolved in DMSO were mixed and further incubated for 3 h at 37 °C.

2.7. Umu assay using strains expressing human NAT

Umu assay was carried out as described previously (Oda et al., 1999). The bacterial cells were grown overnight at 37 °C in LB broth containing tetracycline HCl (1 μg/mL) and/or ampicillin (25 μg/mL). The overnight culture was diluted 50-fold with TGA medium (1% bactotryptone (w/v), 0.5% NaCl (w/v), 0.2% glucose (w/v), and 20 μg/mL ampicillin). The culture was incubated for 1 h at 37 °C and 1-mL aliquots of TGA culture (OD_{600}: 0.25–0.3) and 10 μL tester compound dissolved in DMSO were mixed and further incubated for 2 h.

3. Results

3.1. Expression of CYPs together with OR in S. typhimurium strains

The expression level of CYPs and the level of OR in bacterial cells are summarized in Table 2. The expression levels of CYP1A1, 1B1, 1A2 and 3A4 enzymes were 110, 330, 110, and 110 nmol/L culture, respectively. NADPH-cytochrome *c* reduction activities in OY1022/1A1, OY1022/1B1, OY1022/1A2, and OY1022/3A4 strains were found to be 120, 30, 26, and 23 units/L culture, respectively. Neither CYP protein nor NADPH-cytochrome *c* reduction activity was detected in the *S. typhimurium* OY1022/pCW strain.

3.2. Effects of NR and O-AT on cytotoxicity and induction of umuC gene expression by DNBeP in Salmonella tester strains

The sensitivity of DNBeP induced cytotoxicity and genotoxicity was examined in *S. typhimurium* TA1535/pSK1002 harboring the *umuC'-lacZ'* fusion gene, NM1000, NM2000, NM2009 and NM3009 strains. DNBeP was found to be highly cytotoxic and genotoxic in a concentration-dependent fashion in the *O*-AT-overexpressing strain NM2009, and NR- and *O*-AT-overexpressing strain NM3009. Cytotoxicity and genotoxicity responses in NM2009

Table 2
Expression of P450 and NADPH-P450 reductase in bacterial culture.

Strains	Bacterial culture	
	P450 (nmol/L culture)	NPR (nmol/L culture)
OY1022/pCW	–	–
OY1022/1A1	110	120
OY1022/1B1	330	30
OY1022/1A2	110	26
OY1022/3A4	110	23

P450 concentrations were determined spectrally. NPR contents were determined on the basis of NADPH-cytochrome *c* reduction activities, assuming 3.0 mmol cytochrome *c*-reduced/min/nmol NPR.

Table 3
Genotoxicity of 3,6-dinitrobenzo[*e*]pyrene and other compounds in five *S. typhimurium* tester strains.

Chemical	Concentration (nM)	β-Galactosidase activity (units)				
		OY1022/pCW	OY1022/1A1	OY1022/1A2	OY1022/1B1	OY1022/3A4
DNBeP	0	196 ± 28 (1.0)	99 ± 12 (1.0)	88 ± 6 (1.0)	138 ± 11 (1.0)	84 ± 9 (1.0)
	0.03	211 ± 35 (1.1)	159 ± 4 (1.6)	118 ± 6 (1.3)	149 ± 1 (1.1)	127 ± 7 (1.5)
	0.1	287 ± 16 (1.5)	233 ± 48 (2.4)	182 ± 43 (2.1)	203 ± 19 (1.5)	193 ± 44 (2.3)
	0.3	356 ± 118 (1.8)	401 ± 13 (4.1)	333 ± 28 (3.8)	240 ± 21 (1.7)	358 ± 40 (4.3)
	1.0	494 ± 68 (2.5)	598 ± 39 (6.0)	558 ± 34 (6.3)	382 ± 87 (2.8)	586 ± 70 (7.0)
	3.0	633 ± 6 (3.2)	903 ± 57 (9.1)	948 ± 25 (10.8)	682 ± 132 (4.9)	811 ± 8 (9.7)
Control	0	320 ± 28 (1.0)	105 ± 3 (1.0)	124 ± 2 (1.0)	143 ± 7 (1.0)	158 ± 8 (1.0)
IQ	5000	344 ± 1 (1.1)	113 ± 5 (1.1)	450 ± 11 (3.6)	146 ± 3 (1.0)	161 ± 1 (1.0)
AFB_1	30000	460 ± 6 (1.4)	243 ± 3 (2.3)	264 ± 17 (2.1)	308 ± 16 (2.2)	346 ± 0 (2.2)

Numbers in parentheses represent the ratio which was calculated by dividing β-galactosidase activity for chemicals by activity in the control. Data are the means of two or three determinations (±S.D.).

and NM3009 were higher than those in TA1535/pSK1002 (Fig. 2). In contrast, NM2000 (*O*-AT-deficient strain) did not show any reactivity towards DNBeP, and NM1000 (nitroreductase-deficient strain) showed lower sensitivity to the chemical than NM2009, NM3009, and TA1535/pSK1002.

3.3. Roles of P450 enzymes in the metabolic activation of DNBeP

We compared the sensitivity of five tester strains (OY1022/1A1, OY1022/1A2, OY1022/1B1, OY1022/3A4, and OY1022/pCW) for the induction of *umuC* gene expression by DNBeP (Table 3). Background levels of β-galactosidase activities in strains expressing hNPR-CYP were lower than those in the parental strain; therefore, the genotoxicity of DNBeP in these strains was expressed by the ratio to the control (0 nM of DNBeP). DNBeP at a maximum concentration of 3 nM induced 9.7-, 10.8-, 9.1-, 4.9- and 3.2-fold increases in β-galactosidase acitivity in OY1022/3A4, OY1022/1A2, OY1022/1A1, OY1022/1B1 and OY1022/pCW, respectively (Table 3). These results indicate that DNBeP was activated at a much higher degree in strains OY1022/3A4, OY1022/1A2 and OY1022/1A1, and almost at similar low level in OY1022/1B1 and the parental strain. In addition, β-galactosidase acitivities induced by IQ and AFB_1 in OY1022/3A4, OY1022/1A2, OY1022/1A1, OY1022/1B1 and OY1022/pCW, respectively, were included for comparison.

3.4. Roles of NAT enzymes in the metabolic activation of DNBeP

We compared the sensitivities of two tester strains expressing different acetyltransferases and the parent strain towards the effect of DNBeP on the induction of *umuC* gene expression. As shown in Fig. 3, it was found that NAT2-expressing strain NM6002 exhibited stronger induction of *umuC* gene expression by DNBeP than NAT1-expressing strain NM6001.

4. Discussion

We have previously reported a simple SOS/*umu* assay, which uses the measurement of β-galactosidase activity to detect *umuC* gene expression (Oda et al., 1985). This system was developed to detect a wide range of carcinogenic mutagens (Oda et al., 1985; Nakamura et al., 1987) as well as the genotoxic activity of complex mixture samples such as environmental pollution (Ong et al., 1987), river and industrial wastewater (Reifferscheid et al., 1991; Dizer et al., 2002; Yamaguchi, 1989). Furthermore, we have shown that the *umu* test system, for which we constructed the nitroreductase- and *O*-acetyltransferase-overproducing NM3009 strain and *O*-acetyltransferase-overproducing NM2009 strain, is hypersensitive to the genotoxicity of nitroarenes (Oda et al., 1993) and aromatic amines (Oda et al., 1995).

3,6-Dinitrobenzo[*e*]pyrene is one of the potent bacterial mutagens identified in surface soil samples in two metropolitan areas of Japan (Watanabe et al., 2005) and its percent contribution to the total mutagenicity of soil extracts was 22–29%. In this study, we determined the genotoxicity of DNBeP by using *umu* tester strains constructed in our laboratory. We found that the NM2009 and NM3009 strains exhibited high sensitivity to the cytotoxic and genotoxic effects of DNBeP (Fig. 2). The results suggest that DNBeP is bioactivated to the ultimate reactive metabolite(s) via nitroreduction followed by *O*-acetylation. The result is consistent with the results of an early study (Watanabe et al., 2005), which reported that DNBeP is a potent mutagen in the *S. typhimurium* YG1024 strain overexpressing *O*-acetyltransferase. The order of sensitivity in the cytotoxicity of bacterial strains is almost parallel to that in genotoxicity. Molecular species that exhibit cytotoxicity might be the same as those exhibiting genotoxicity.

In the previous study, we showed that 1-nitropyrene (1-NP), a nitropolycyclic aromatic hydrocarbon, can be activated by human CYP1B1 to a genotoxic compound via nitroreduction/*O*-acetylation at low substrate concentrations by using a *umu* test system that added CYP enzymes from the outside (Yamazaki et al., 2000). Although human cells are nitroreductase deficient, 1-NP is activated by nitroreductase and *O*-AT existed in bacteria. Therefore, we developed a strain OY1022 that is deficient in nitroreductase and *O*-AT. To clarify the roles of CYPs and NPR in the metabolic activation of DNBeP, we used the OY1022 strain, which is deficient in NR and probably also in *O*-AT. We introduced two plasmids into the OY1022 strain. One plasmid carried both CYP and NPR cDNA in a biocistronic construct under control of an IPTG-inducible double *tac* promoter and pOA102, and another carrying bacterial *O*-AT and the *umuC′-′lacZ* fusion gene, and established four new *umu* tester strains, namely *S. typhimurium* OY1022/1A1, OY1022/1A2, OY1022/1B1 and OY1022/3A4.

In this study, the genotoxicity of DNBeP was investigated in the *umu* assay using several strains. Among the strains used, OY1022/1A2 (expressing CYP1A2), OY1022/3A4 (expressing CYP3A4), and OY1022/1A1 (expressing CYP1A1) exhibited high genotoxic activity with DNBeP, suggesting that human CYP1A2, CYP3A4, and CYP1A1 are capable of efficiently bioactivating DNBeP with co-expression of NPR (Table 3). In contrast, human CYP1B1 strain and the parental strain OY1022/pCW exhibited almost the same genotoxic activity as DNBeP. Taken together, these results supported the view that DNBeP is mainly bioactivated by CYP1A2, CYP3A4, and CYP1A1 in humans.

Human exposure to DNBeP is thought to occur primarily through the respiratory tract. Although CYP contents in the human lung tissue are lower than in the liver, CYP enzymes in the lung may play a crucial role in extrahepatic activation of DNBeP. Human CYP1A1 is expressed predominantly in extrahepatic organs, such as the lungs

and mammary glands (Huang et al., 1996). Although human CYP3A4 is the most abundant CYP in human liver and the small intestine, it is expressed in only about 20% of individuals (Mace et al., 1998). Thus, bioactivation of DNBeP by human lung cells may be responsible for potential human risk. Furthermore, with tester strains expressing human sulfotransferase enzymes, it is necessary to investigate whether a DNBeP -reduced metabolite is further activated by sulfation.

In this work, DNBeP was considered to be metabolized essentially via two pathways. In bacteria, the compound is mainly activated by nitroreduction and *O*-acetylation, whereas in humans, it is probably activated by nitroreduction due to CYP3A4, CYP1A2, and CYP1A1 and NPR, followed by *O*-acetylation.

Human NAT1 and NAT2 have been reported to play a crucial role as phase II enzymes in the biotransformation of carcinogenic nitroarenes (Oda et al., 1999; Mace et al., 1998). We determined the roles of NAT1 and NAT2 in the genotoxicity of DNBeP with *S. typhimurium* tester strains NM6001 and NM6002, and NM6000 (*O*-AT-deficient parent strain). We found that human NAT2, rather than NAT1, catalyzes the activation of DNBeP to DNA-damaging products in our test system. This result is in good agreement with reports that the dinitro compound 1,8-dinitropyrene exhibited a genotoxic response in a *S. typhimurium* strain expressing NAT2, rather than NAT1 (Oda et al., 1999). These results suggest that NAT2 may play important roles in the genotoxic activation of DNBeP to carcinogenic metabolites.

In summary, we established new *umu* tester strains of *S. typhimurium* OY1022/1A1, OY1022/1A2, OY1022/1B1, and OY1022/3A4, which express four respective human CYP enzymes, NPR and bacterial *O*-AT, to clarify the role of human CYP enzyme in the bioactivation of DNBeP to genotoxic metabolites. Using these tester strains, we demonstrated that genotoxic activation of DNBeP can be catalyzed by human CYP1A2, CYP3A4, and CYP1A1 and NPR to a genotoxin(s) in the presence of bacterial *O*-AT, probably via nitroreduction.

Conflict of interest

None.

Acknowledgments

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Original Article

Modifying Effect of Age on the Association between Ambient Ozone and Nighttime Primary Care Visits Due to Asthma Attack

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ABSTRACT

Background: We examined the association between short-term exposure to outdoor air pollution and nighttime primary care visits due to asthma attack. We also investigated the modifying effects of age on this association.
Methods: A case–crossover study was conducted at a primary care clinic in metropolitan Tokyo. The subjects were 308 children aged 0–14 years and 95 adolescents and adults aged 15–64 years. All subjects made visits to the clinic for an asthma attack at between 7 PM and 12 AM. Data on hourly concentrations of particulate matter with a 50% cut-off aerodynamic diameter ≤2.5 µm ($PM_{2.5}$), ozone, and nitrogen dioxide (NO_2) were obtained. A conditional logistic regression model was used to estimate odds ratios (ORs) of primary care visits per unit increment of each air pollutant.
Results: Among children, the ORs in warmer months per 10 ppb increment of the 24-hour mean concentration of ozone were 1.16 (95% confidential interval [CI], 1.00–1.33) adjusted for temperature, and 1.29 (95% CI, 1.08–1.55) adjusted for $PM_{2.5}$, NO_2, and temperature. With respect to modification of the association by age, the ORs for 24-hour mean concentration of ozone—after adjustment for $PM_{2.5}$, NO_2 and temperature in warmer months—in children aged 0–1 years, 2–5 years, and 6–14 years were 1.06 (95% CI, 0.63–1.78), 1.37 (95% CI, 1.05–1.71), and 1.25 (95% CI, 0.87–1.82), respectively. There was no association between ozone and primary care visits among adults.
Conclusions: An association was found between ozone and nighttime primary care visits for asthma attack in warmer months; the association was greater among preschool children.

Key words: air pollution; asthma; ozone; particulate matter; preschool children

INTRODUCTION

Children's exposure to air pollution is a special concern because their immune system and lungs are not fully developed. Children spend more time outside, where levels of pollution from traffic, power plants, and other sources are generally higher. Exposure to ambient air pollution, including particulate matter (PM), ozone (O_3), and nitrogen dioxide (NO_2), is associated with many adverse health outcomes ranging from increased symptoms of allergic airway disease to increased mortality.[1–3] Children are considered to be more sensitive to air pollution than adults,[4] and asthmatic children are particularly vulnerable to the adverse health effects of air pollution. Studies of asthmatic children conclude that exposure to high concentrations of O_3 or PM significantly increases the risk of respiratory symptoms and asthma medication use, and diminishes lung functions such as peak expiratory flow rate and forced expiratory flow rate.[5–9] However, among children, age-related sensitivity to air pollutants and adverse respiratory effects has been investigated in only a small number of studies. Babin et al[10] observed an association between pediatric emergency room visits for asthma exacerbations and outdoor O_3, especially in children aged 5 to 12 years; however, they noted no such association among children aged 1 to 4 years.

To investigate the associations between short-term exposure to air pollution and adverse pulmonary outcomes—as well as the modifying effects of age—we collected and analyzed hourly air-pollution data and the records of nighttime primary care visits due to asthma attack.

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