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Notes

DMPO-OH Radical Formation from 5,5-Dimethyl-1-pyrroline *N*-Oxide (DMPO) in Hot Water

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When an aqueous solution of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was heated at 70°C for 30 min, formation of DMPO-OH was observed by ESR. This DMPO-OH radical formation was suppressed under an argon atmosphere. When water was replaced with ultra-pure water for ICP-MS experiments, DMPO-OH radical formation was also diminished. Under an argon atmosphere in ultra-pure water, the intensity of the DMPO-OH signal decreased to about 1/20 of that observed under aerobic conditions with regular purified water. The addition of hydroxyl radical scavengers such as mannitol did not affect the formation of DMPO-OH, but the signal turned faint in the presence of EDTA. We suggest that DMPO reacted with dissolved oxygen to form DMPO-OH.

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Introduction

The spin-trapping method with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has been widely accepted as an assay method to measure hydroxyl radical formation, and to detect the hydroxyl radical scavenging activity of a compound. Although unstability of DMPO is claimed because it turns yellow with time even at −20°C in a sealed tube under vacuum,[1] the basic chemistry of DMPO and DMPO-OH has not been well understood.

In a previous work,[2] we noticed very weak DMPO-OH signals in the baseline of a negative control spectrum which was obtained after standing DMPO solution at 37°C for 24 h. We suspected that in a DMPO aqueous solution at a higher concentration and at a higher temperature, an appropriate amount of DMPO-OH might generate. And we considered that this reaction should provide a practical preparation method to obtain DMPO-OH radical as a simple aqueous solution which contains no other materials except unreacted DMPO. Also this DMPO-OH aqueous solution may be utilized in the investigation of the chemical or physicochemical properties of DMPO-OH radicals.

When DMPO was dissolved in purified water and the solution was heated at 70°C for 30 min, a sufficient amount of DMPO-OH radical formation was observed by ESR (Fig. 1). Under an argon atmosphere, DMPO-OH formation was minimized to 1/4, and this slightly increased under a dioxygen atmosphere. This indicates that dioxygen participates in the reaction. Previously, Makino *et al.* observed DMPO-OH formation in an aqueous mixture of DMPO and 1 mM $FeCl_3$. Based on this observation of the formation of iron chelate with DMPO at 77 K, and DMPO-OCH_3 formation in the presence of CH_3OH, researchers elucidated that nucleophilic attachment of water to DMPO should occur in the presence of Fe^{3+} ion.[3] To study the effect of a small number of metal ions in purified water, we replaced the purified water with ultra-pure water for ICP-MS measurements; the latter should contain iron of no more than 1 ppb. In ultra-pure water, and under an argon atmosphere, DMPO-OH signal was still observed after heating, although its intensity decreased to about 1/20. In this communication, we describe DMPO-OH radical formation in hot water, in which dissolved dioxygen participates in the reaction and metal ions such as Fe^{3+} might catalyze DMPO-OH formation.

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Experimental

Chemicals

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from

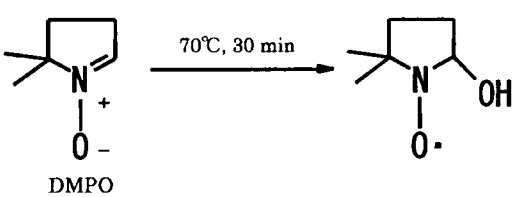

Fig. 1 DMPO-OH radical formed in a heated aqueous solution of DMPO.

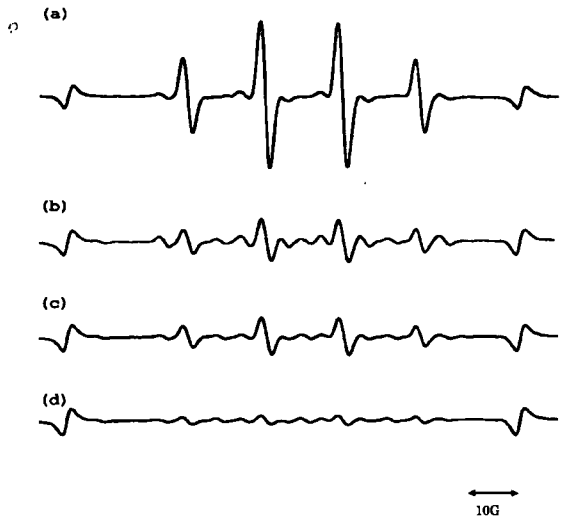

Fig. 2 The ESR spectrum of DMPO-OH radical observed in an aqueous solution of DMPO after heating at 70°C for 30 min (a). Four strong signals in the mid-section are of the DMPO-OH radical (A_H 1.50 mT, A_N 1.50 mT), and two side signals are of Mn used as an external reference. The ESR spectrum of DMPO aqueous solution heated under an argon atmosphere (b); under aerobic conditions in ultra-pure water (c); under an argon atmosphere in ultra-pure water (d).

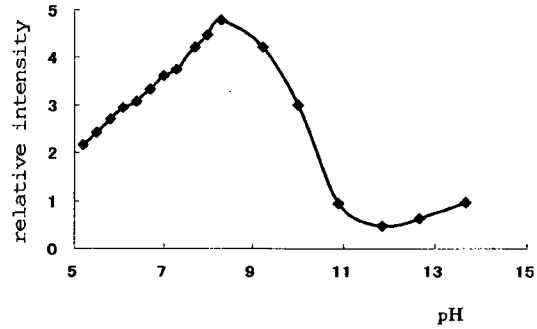

Fig. 3 Effect of pH on the relative intensity of DMPO-OH *vs.* Mn (external reference), which is the highest at around pH 8.5.

Labotec Co. (Tokyo, Japan), and used without further purification.

The water used in this study was purified by ion-exchange resin (18.2 MΩ). Ultra-pure water for ICP-MS was obtained by two successive distillations of purified water with an all-quartz apparatus, in which Fe^{3+} ion was not detected (at most 1 ppb). In experiments under an argon atmosphere, water was deaerated under reduced pressure using an aspirator, and then stored under argon by attaching an argon balloon.

ESR measurement

An electron spin resonance spectrometer, JES-RE1X (JEOL, Tokyo, Japan), and a JEOL flat quartz cell were used. The conditions were: field, 336 ± 5 mT width; power, 4 mW; field modulation, 0.200 mT; time constant, 0.1; and amplitude, 500. A manganese signal was used for the external standard. Pyrex glassware was washed with 1 M nitric acid by sonication, rinsed well with purified water, and air dried.

DMPO-OH radical formation in hot water

Neat DMPO was dissolved in purified water to make a 2.5% aqueous solution. In cases of pH controlled experiments, 2.5% DMPO solution in an acetate buffer or a phosphate buffer was used. A 2-mL volume of sample was heated in a test tube for 30 min in a water bath at 70°C. Under an argon atmosphere or a dioxygen atmosphere, 10 mL of 2.5% DMPO aqueous solution was introduced into a 25 mL round-bottomed flask, and a three-way stopcock with an argon (or dioxygen) balloon was attached. The solution was deaerated under reduced pressure (aspirator), and argon (or dioxygen) was introduced from the balloon. After heating at 70°C for 30 min, an aliquot was removed and was measured by ESR just 1 min after the tube was taken out of the water bath.

Results and Discussion

After heating the aqueous solution of DMPO at 70°C for 30 min, we observed typical ESR signals of DMPO-OH radical with an intensity ratio of 1:2:2:1 (A_H 1.50 mT, A_N 1.50 mT; Ref. 4, A_H 1.53 mT, A_N 1.53 mT) (Figs. 1 and 2).

It was reported that Fe^{3+} ions catalyze the addition of water to DMPO to produce DMPO-OH radical.[3] In Fig. 2, however, its formation decreased under Ar atmosphere (Figs. 2(b) and (d)), also in ultra-pure water under aerobic conditions (Fig. 2(c)). In all spectra in Fig. 2, unknown weak signals can be seen. DMPO-OH radical formation by heating was not suppressed by the addition of a hydroxyl radical scavenger, mannitol (1 - 100 mM) or DMSO (1 - 100 mM). This indicates that DMPO-OH radical should not be formed *via* hydroxyl radical attachment to DMPO. However, DMPO-OH signal intensity was decreased to about 1/6 in the presence of 1 mM of EDTA. Thus, it was suspected that dissolved dioxygen, which is a biradical molecule, should be added directly to DMPO, where catalytic participation of metal ions might exist (Eq. (1)). Biradical might produce a DMPO-OOH radical, and this would develop into a DMPO-OH radical quickly at higher temperatures.

DMPO-OH signals were also observed in acetate buffer (pH 5.0 - 6.8), phosphate buffer (pH 7.0 - 10.0) or disodium phosphate solution of which the pH was adjusted with sodium hydroxide solution (pH 13.0) (Fig. 3). The half-lives of DMPO-OH[5] or DMPO-OOH[6,7] at basic pH are much shorter than those at neutral or acidic conditions, and the decrease of the intensity of DMPO-OH signal can be considered as the unstability of these radical species. The optimum pH was found to be around pH 8.5, thus, the catalytic action of H^+ may not be plausible.

$$\text{DMPO} + O_2 \longrightarrow {}^{\bullet}[\text{DMPO-OO}]^{\bullet} \quad (1)$$

$${}^{\bullet}[\text{DMPO-O}_2]^{\bullet} + H_2O \longrightarrow [\text{DMPO-OOH}]^{\bullet} + {}^{\bullet}OH \quad (2)$$

$$[\text{DMPO-OOH}]^{\bullet} \longrightarrow [\text{DMPO-OH}]^{\bullet} \quad (3)$$

Even at low concentrations, transition metal ions such as Fe^{3+} may catalyze the first step (Eq. (1)), or catalyze the reaction of [DMPO-O_2] biradical with water to [DMPO-OOH]$^{\bullet}$ (Eq. (2)), although we could not detect [DMPO-OOH] signals nor OH radical formation by the addition of DMSO. DMPO-OOH radical is known to be labile so as to decompose to DMPO-OH with a half-time of 50 s at pH 7[6,7] (Eq. (3)), and the decomposition mechanisms of DMPO-OOH adducts to DMPO-OH are discussed by Villamena *et al.*[8]

The formation of DMPO-OH with time was traced at 70°C (Fig. 4). It was found that the reaction reached almost steady state after 60 min. After dioxygen in the reaction mixture is consumed, the supply of dioxygen to the reaction solution

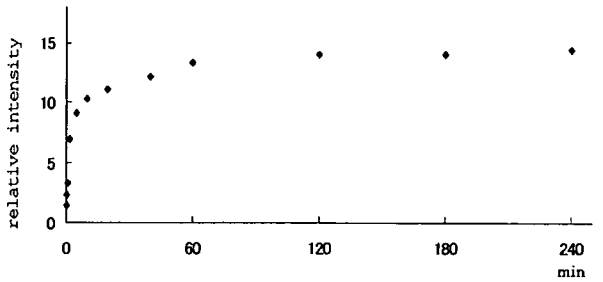

Fig. 4 The relative intensity of DMPO-OH *vs.* Mn (external reference) with time. The ESR signal of DMPO-OH radical in a 2.5% DMPO solution adjusted at pH 7.4 using a phosphate buffer, was recorded just 1 min after taking out the reaction tube which was heated at 70°C and then cooled at 0°C for 15 s in a water-bath.

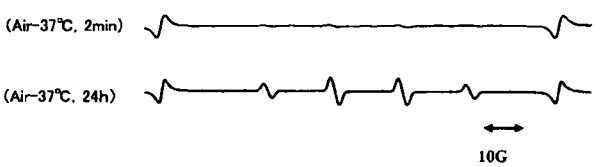

Fig. 5 ESR signal of DMPO-OH radical observed in an aqueous solution of DMPO (dissolved in milliQ water) under aerobic conditions.

should be the rate-determining step.

DMPO-OH formation in an aqueous solution of DMPO was also observed at 37°C after 24 h, indicating that radical trapping experiments in an intact animal using DMPO over 20 h may provide misleading results (Fig. 5). However, in the standard experimental procedure, spin-trapping experiments should be carried out at room temperature or lower, and over a short time, DMPO-OH formation could be neglected.

The mechanism of DMPO-OH radical formation remains uncertain; however, we report here the observed evidence that DMPO should react with dioxygen in water to generate DMPO-OH radical under aerobic conditions at 70°C.

Conclusions

In conclusion, DMPO-OH radical was detected after heating a DMPO aqueous solution at 70°C for 30 min. In the presence of dioxygen, DMPO-OH production was enhanced. The catalytic participation of metal ions was also suspected, because decreased formation of DMPO-OH was observed when regular purified water was replaced with ultra-pure water.

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Regular article

Differential Gene Expression Induced by Two Genotoxic *N*-nitroso Carcinogens, Phenobarbital and Ethanol in Mouse Liver Examined with Oligonucleotide Microarray and Quantitative Real-time PCR[1]

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It is known that genotoxic *N*-nitroso carcinogens induce DNA damage in mouse liver within a few hours and induce mutations within 28 days after their administration. However, related-gene expression changes at these time points in liver were not fully elucidated. Differential gene expression induced by two genotoxic *N*-nitroso carcinogens in mouse liver was examined 4 h and 28 days after their administration with in-house oligonucleotide microarray (268 genes) and quantitative real-time PCR, and compared to that of a non-genotoxic carcinogen and a non-carcinogenic toxin. Diethylnitrosamine (DEN, 80 mg/kg bw), dipropylnitrosamine (DPN, 250 mg/kg bw), phenobarbital sodium (30 mg/kg bw) and ethanol (1000 mg/kg bw) were injected intraperitoneally into groups of male 9-week-old B6C3F1 mice and liver was dissected after 4 h and 28 days. mRNA from pooled livers was reverse-transcribed to cDNA, and Cy3- and Cy5-labeled cDNA was competitively hybridized with in-house made microarray, scanned and analyzed; additionally, quantitative real-time PCR was performed for selected genes. Differential gene expression between two genotoxic *N*-nitroso carcinogens and phenobarbital and ethanol was observed in 11 genes 4 h after administration, including seven tumor suppressor *p53* target genes, viz. *c-Jun*, *Ccng1*, *Mdm2*, *p21*, *Bax*, *Hsp27* and *Snk*; the other genes were *Mbd1*, *Hmox-1*, *Ccnf* and *Rad52*. However, only some degree of differential gene expression of *p21*, *Ccng1* and *Snk* was observed 28 days after administration; no other differentially-expressed genes were evident. The present results suggest that DEN and DPN induce differential gene expression in *p53* target genes in liver within a few hours after administration and that these acute responses remained only partially in liver after 28 days.

Key words: quantitative real-time PCR, oligonucleotide microarray, toxicogenomics, genotoxic carcinogens, non-genotoxic tumor promoter

Introduction

Toxicogenomics is a rapidly developing discipline to aid understanding the molecular and cellular effects of chemicals in biological systems. DNA microarray is a powerful technology for characterizing gene expression on a genome scale (1), although issues of reliability, reproducibility and correlation of data produced across the different DNA microarrays are still being addressed (2). There are several commercially available microar-

[1]This work was partly a JEMS/MMS/Toxicogenomics group collaborative study (C. Furihata, T. Watanabe, M. Nakajima, S. Hamada, C. Namiki, T. Suzuki and A. Hyogo).

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rays (3-5) but which are very expensive and time-consuming. It may therefore be economical to prepare in-house microarray that contains a small number of selected genes for a specific purpose and which is easy to analyze. We tried preparing in-house oligonucleotide microarray useful for characterizing mutagenic and carcinogenic chemicals and for differentiating from non-genotoxic carcinogens and non-carcinogenic toxic compounds, and applied it to the study of early response in mouse liver in order to clarify key word genes and to develop chemical risk assessment microarray. Quantitative real-time PCR (qPCR) is an alternative technology for toxicogenomics (6). qPCR is thought to be a reliable quantitative method but takes time to analyze a large number of genes. DNA microarray and qPCR would be complementary to each other.

It is known that genotoxic *N*-nitroso carcinogens induce DNA damage and repair in mouse liver in a matter of a few hours after their administration; DNA adducts (7), unscheduled DNA synthesis (8), DNA lesions (Comet assay) (9) and other lesions have been reported. It is also known that mutations are induced in transgenic mouse liver 28 days after genotoxic *N*-nitroso carcinogen administration (7,10,11). This coincides with the time at which chronic changes leading to carcinogenesis are known to begin. However, related-gene expression changes at these time points are not yet fully elucidated.

We compared gene expression profiles after mouse liver exposure to typical carcinogenic *N*-nitroso compounds (diethylnitrosamine and dipropylnitrosamine), to a non-genotoxic carcinogen (phenobarbital) and to a non-carcinogenic liver toxin (ethanol). This is the first trial DNA microarray in our JEMS/MMS/Toxicogenomics group collaborative study. We first selected candidate genes for our in-house microarray from gene classes of DNA repair, DNA recombination, DNA methylation, DNA transcription, apoptosis, cell cycle, extracelluar matrix protein, inflammation, dendritic cell, oncogene, tumor development, tumor growth factor, tumor suppressor, xenobiotic metabolism and signal transduction, and then added genes from our preliminary results of Affymetrix GeneChip Mu74AV2. (See online supplemental Table S1 at http://www.chem.aoyama.ac.jp/Chem/ChemHP/Furihatalab/).

Materials and Methods

Animals: Eight-week-old male B6C3F1/Crj mice were purchased from Charles River Japan, Inc. (Yokohama, Japan). They were kept in plastic cages with hard wood chips as bedding in an air-conditioned room at 23±2°C and 55±5% humidity with a 12 h light/dark cycle. Food (Oriental MF, Oriental Yeast Co., Tokyo) and tap water were available *ad libitum*. All animal experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at the Biosafety Research Center, Foods, Drugs, and Pesticides.

Chemicals: Diethylnitrosamine (DEN, CAS No. 55-18-5) and dipropylnitrosamine (DPN, CAS No. 621-64-7), were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Phenobarbital sodium (PB, CAS No. 57-30-7) and ethanol (EtOH, CAS No. 64-17-5) were obtained from Wako Pure Chem. Ind. Ltd. (Osaka, Japan).

Animal experiments: Experiment I. Main lobe of liver was dissected from untreated control 9-week-old mice and immediately frozen and stored at −80°C until use for validation experiments in Fig. 1. Experiment II. Test chemicals in sterile saline were given to groups of 9-week-old mice (5 per group) intraperitoneally. Doses of chemicals were: DEN, 80 mg/kg bw, 0.2 to 0.3 mL per mouse; DPN, 250 mg/kg bw; PB, 30 mg/kg bw; EtOH, 1000 mg/kg bw. Sterile saline was injected ip into control groups of mice. Main lobe of liver was dissected after 4 h and 28 days, was pooled and immediately frozen and stored at −80°C until use for experiments in Figs. 2-4. Doses of DEN and DPN were selected that induced mutation in transgenic mice (MutaMouse or Big Blue mice) at 28 days after their administration in previous studies (10,11). The doses of PB and EtOH were half their respective LD_{50}. Experiment III. DEN and saline were given to the other groups of 9-week-old mice (5 per group) at the same dose as in experiment II. Main lobe of liver was dissected after 4 h and 28 days, was immediately frozen individually and stored at −80°C until use for individual experiment in Fig. 5.

RNA isolation: Experiment I and II. Frozen pooled main lobes of liver from 5 mice were placed directly in TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA), homogenized for 2 min on ice, and total RNA was isolated using manufacturer's protocol (12). RNA was quantified based on 260 nm absorbance reading of the solution. Quality of the RNA was assured by measuring 260 nm: 280 nm absorbance ratio and reviewing integrity on agarose denaturing gels. TRIzol reagent-isolated total RNA was additionally purified with Isogen-LS (Nippon Gene, Co. Ltd., Tokyo, Japan) (13) for real-time PCR experiments. Experiment III. Total RNA was extracted from individual mouse main lobe liver by a similar method and was examined only with qPCR.

Microarray preparation: Mounted genes (268 genes, Table S1, http://www.chem.aoyama.ac.jp/Chem/ChemHP/Furihatalab/) such as suggested genes of DNA repair, DNA recombination, DNA methylation, DNA transcription, cell cycle, apoptosis, inflammation, dendritic cell, extracelluar matrix protein,

oncogene, signal transduction, tumor development, tumor growth factor, tumor suppressor and xenobiotic metabolism were selected mainly for determining genotoxic carcinogens. 40–47mer unmodified oligonucleotide sequences for probes were determined in accordance with the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Accession No. and name of genes are summarized in Table S1. Three negative control plant genes were included: [1] *A. thaliana gene* (*LHCP AB 140*) for chlorophyll a/b binding protein, [2] *Gossypium hirsutum* cellulose synthase (*celA2*) mRNA, and [3] *A. thaliana Lhb1B2* gene for photosystem II chlorophyll a binding protein. Probes were selected in 3'-end region and BLAST search (http://www.ncbi.nlm.nih.gov/) was performed respectively. Nucleotide sequences were applied to Japan Patent No. 2005-99843. 40–47mer unmodified oligonucleotides were obtained from Invitrogen (Invitrogen Japan K.K, Tokyo, Japan). Probes (50 μM) in 1×Micro Spotting Solution (ArrayIt Division, TelChem International, Inc., Sunnyvale CA, USA) and 0.2% Tween 20 were spotted on polycarbodiimide-coated slides (CarboStation-U, Nisshinbo Ind. Inc., Tokyo, Japan) with RIKEN arrayer. Spotted microarrays were rehydrated in a humid chamber with 1×SSC, UV-crosslinked (600 mJ) and washed with MilliQ water. Microarrays were stored under vacuum at 4°C.

Labeling and hybridization: Experiment I (microarray validity study): Untreated control total RNA (20 μg) was reverse-transcribed to cDNA and labeled with Alexa fluor dyes using SuperScript plus indirect cDNA labeling system with Alexa fluor dyes (Invitrogen, Corp., Carlsbad, CA, USA) in experiments in Fig. 1. Experiment II. mRNA was purified from total RNA with Oligotex-dT30 (Takara Bio Inc., Otsu, Shiga, Japan). mRNA (1 μg) of DEN, DPN, PB, EtOH and control samples (4 h and 28 days) was reverse-transcribed to cDNA and labeled with CyDye (Cy3 and Cy5) using CyScribe cDNA post labeling kit (Amersham Biosciences, Piscataway, NJ, USA) as described in manufacturer's protocol. CyDye-labeled cDNA (150 μL) was hybridized with microarray in Gene Tac Hybridization Station (Genomic Solutions, Ann Arbor, MI, USA) at 55°C overnight, washed in 2×SSC-0.1% SDS at 45°C, 1×SSC-0.1%SDS at 40°C and 0.2×SSC at 40°C, dried by centrifugation and scanned by DNAscope (Gene Focus, Waterloo, Ontario, Canada).

Data analysis of microarray results: Scanning data were analyzed using MACROview (Biomedical Photometrics Inc., Waterloo, ON, Canada) by global normalization method and further analyzed using Cluster and TreeView (http://rana.lbl.gov/EisenSoftware.htm). Gene network was analyzed using Ingenuity Pathways Analysis (Ingenuity Systems, Inc. Redwood City, CA, USA).

Table 1. qPCR primer sequences

Gene	Primer sequence
Bax 5'	gattgctgacgtggacacggac
Bax 3'	cagggccttgagcaccagtttg
Ccnf 5'	ggagctctcaagtgaagacagcac
Ccnf 3'	ggcctcacacaccattaggctac
Ccng1 5'	tggccgagatttgaccttctgg
Ccng1 3'	gtgcttcagttgccgtgcagtg
c-Jun 5'	aggcagagaggaagcgcatg
c-Jun 3'	tgtgccacctgttccctgag
Gapdh 5'	tctggaaagctgtggcgtgatg
Gapdh 3'	tcccgttcagctctgggatgac
Hmox-1 5'	gtggcctgaactttgaaaccagc
Hmox-1 3'	cgtggtcagtcaacatggatgc
Hprt 5'	cttgctcgagatgtcatgaaggag
Hprt 3'	taatccagcaggtcagcaaagaac
Hsp27 5'	ctcacagtgaagaccaaggaag
Hsp27 3'	ggatagggaagaggacactagg
Mbd1 5'	tacagccctacacgaaccagc
Mbd1 3'	gaatttgggcttgtcgcagcag
Mdm2 5'	ttgatccgagcctgggtctgtg
Mdm2 3'	aagatcctgatgcgagggcgtc
p21 5'	tccgcacaggagcaaagtgtg
p21 3'	acgcgctcccagacgaagttg
p53 5'	ttggaccctggcacctacaatg
p53 3'	gcagacaggctttgcagaatgg
Rad52 5'	gagaaccagcccaaacttctgc
Rad52 3'	gaacatgctggttggtgttgc
Snk 5'	ctgttgagagcgtcttcagttg
Snk 3'	ccatagttcacagttaagcagc

Quantitative real-time PCR (qPCR): For qPCR, 2 μL of the cDNA product solution were used to measure gene expression using DNA Engine Opticon 2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as the housekeeping normalized gene for each sample. Nucleotide sequence of qPCR primers was determined in accordance with Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), ncbi DNA database (http://www.ncbi.nlm.nih.gov/) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Primers resulted in a single product which could be visualized on a 1.2% agarose gel. Single peak of melting curve for each primer set was confirmed using DNA Engine Opticon 2. Primer sequences are summarized in Table 1. RNA (2.5 μg) was reverse-transcribed with SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA, USA) and 250 μL cDNA product solution (12) was prepared as described in the manufacturer's protocol.

Data analysis of qPCR results: Some of the data were statistically analyzed by Student's t-test.

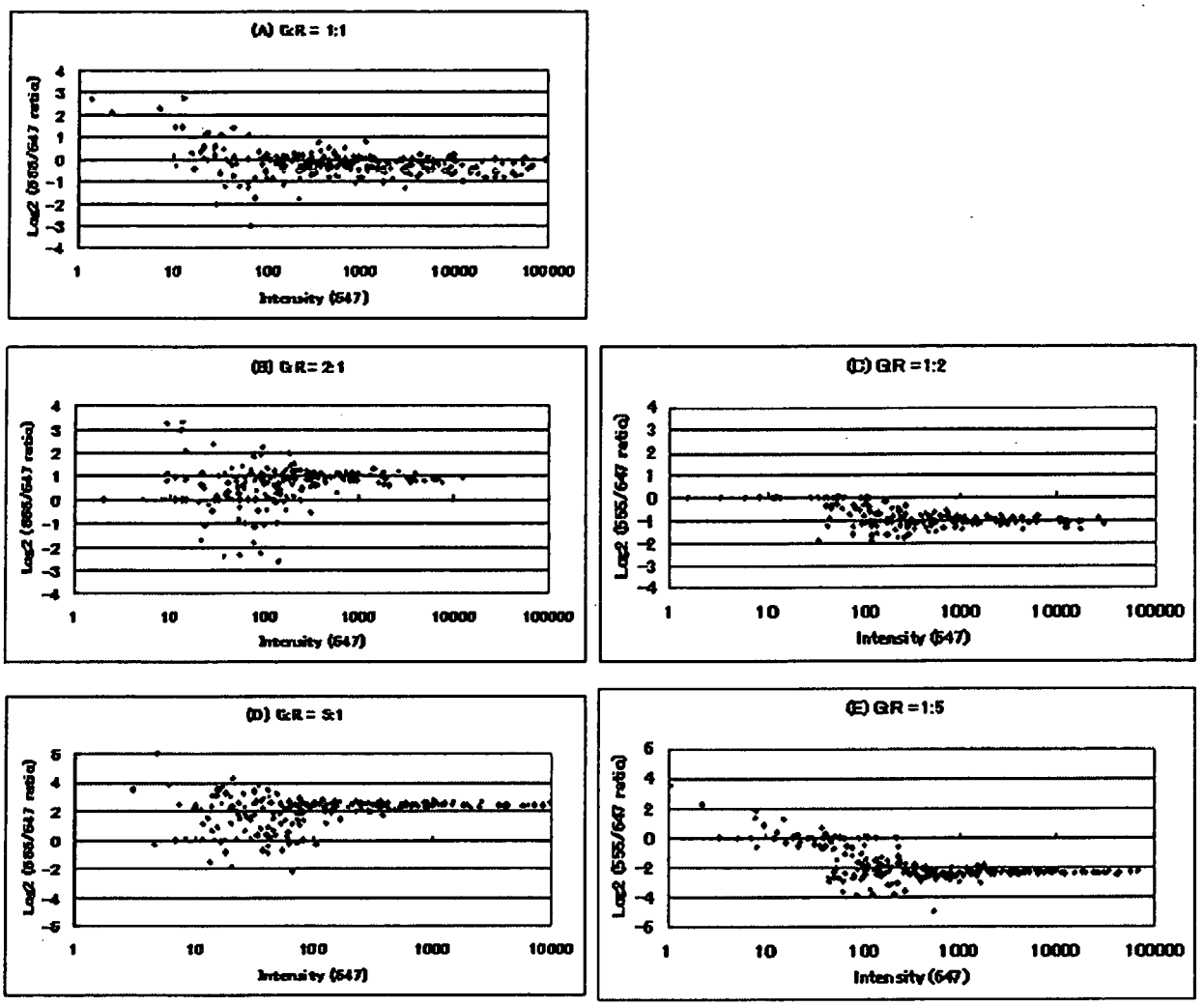


Fig. 1. The validity of the in-house made microarray expressed as Scatter pattern (Experiment I). Results of hybridization with the same control samples labeled with Alexa555 (G) and Alexa647 (R) with the ratio of Alexa555/Alexa647 of A):5/1, B):2/1, C):1/1, D):1/2 and E):1/5. Results show the expected ratio of fluorescence for each sample, demonstrating the quantitative capability of our in-house microarrays.

Results

The validity of the in-house made DNA microarray (Experiment I): Fig. 1 shows the results of hybridization with the same control cDNA samples labeled with Alexa555 (green: G) and Alexa647 (red: R) with the ratio of Alexa555/Alexa647 of 5/1, 2/1, 1/1, 1/2 and 1/5. Figs. 1A to 1E show scatter plots which reveal an equivalent ratio of fluorescence for each sample, thereby confirming the quantitative capability of our in-house DNA microarrays. Data of fluorescence intensity above 200 show good convergence which suggests high reliability in this region.

Gene expression analysis determined with DNA microarray expressed as hierarchical clustering (Experiment II): Fig. 2A shows gene expression profile in liver 4 h after administration determined with in-house DNA microarray. Only 10 differentially expressed genes and additional 3 genes are presented. Differential gene expression was observed between two genotoxic *N*-nitroso carcinogens (DEN and DPN) and PB and EtOH in 10 genes including six *p53* target genes, namely *c-Jun*, *Ccng1*, *Mdm2*, *Bax*, *Snk* and *Hsp27*; the other differentially expressed genes were *Mbd1*, *Hmox-1*, *Ccnf*, and *Rad52*. Gene expression changes were very similar for DEN and DPN. However, differential gene expression of *p53* itself was not observed 4 h after treatment in the present experimental conditions. *Gapdh* and *Hprt*, house-keeping genes, did not change their expressions 4 h after administration. Specific expression-changed genes for PB or EtOH were not found in the present 268 gene-list.

Fig. 2B shows gene expression profile for the same 13

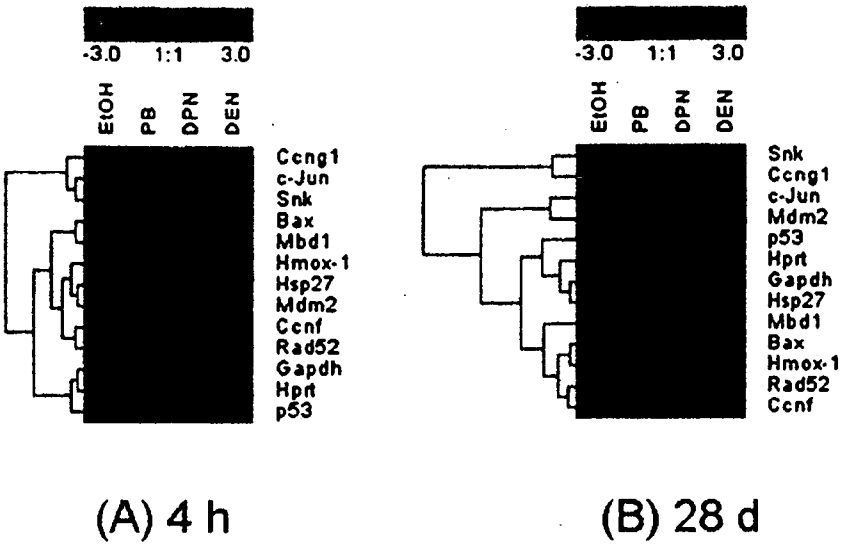


Fig. 2. Gene expression analysis determined with DNA microarray expressed as hierarchical clustering (Experiment II). Eleven differentially expressed genes and 3 expression-unchanged genes are presented. A): at 4 h, B): at 28 days. Livers were from B6C3F1 mice treated with DEN, DPN, PB or EtOH for 4 h and 28 days. Genes are listed in rows, and chemicals are listed in columns. The ratio of gene expression levels (experiment group/control group) is color coded. Green indicates down-regulation, black means no change, and red indicates up-regulation of expression. Color shows gradation of −3 or less (decrease, green) to 1 (black) to 3 or more (increase, red), as log2 expression. *Gapdh* and *Hprt* are house-keeping genes.

genes in liver 28 days after administration determined with in-house DNA microarray. There was a small degree of differential gene expression of *Ccng1* and *Snk* observed at 28 days. Expression of other genes returned to control level at 28 days after administration of DEN and DPN, and there were no newly-appeared differentially expressed genes by DEN and DPN at this time. PB and EtOH did not show significant increase or decrease of gene expression 28 days after administration in these 13 genes examined with DNA microarray. *Gapdh* and *Hprt*, house-keeping genes, did not change their expressions 28 days after administration. Specific expression-changed genes for PB or EtOH at that time point were not found in the present 268 gene-list.

Verification of DNA microarray results with qPCR (Experiment II): Figs. 3A to 3O show qPCR verification of DNA microarray results (Fig. 2A) of 10 expression-changed genes and three expression-unchanged genes 4 h after chemical administration. Differential gene expression was observed with qPCR between two genotoxic *N*-nitroso carcinogens (DEN and DPN) and PB and EtOH in 10 genes including six *p53* target genes. Generally, qPCR results coincided with DNA microarray results for these genes, suggesting considerable reliability of our DNA microarray results. An important *p53* target gene, *p21*, was additionally examined with qPCR, and a very large increase in *p21* expression (120-fold) for DEN was observed with qPCR. Relative expression (experiment group/control group) of 11 expression-changed genes was generally in some degree greater with DEN than with DPN.

Figs. 4A to 4O show qPCR verification of DNA microarray results (Fig. 2B) of the same 13 genes 28 days after chemical administration. Generally, qPCR results coincided with DNA microarray results for these genes, suggesting considerable reliability of our DNA microarray results. While there was a small degree of differential gene expression of *Ccng1* and *Snk* observed at 28 days, distinctive differences between *N*-nitroso carcinogenic compounds and PB and EtOH were not observed in the other 8 genes that were differentially expressed at 4 h after administration. *p21* was additionally examined with qPCR, and differential gene expression of *p21* was observed between *N*-nitroso carcinogenic compounds and PB and EtOH, but the increase was about 1/10 of that at 4 h (Fig. 4O vs. Fig. 3O). *Gapdh* and *Hprt*, house-keeping genes, did not change their expressions 28 days after administration. Specific expression-changed genes for PB or EtOH were not found in the present 268 gene-list.

Variation among individuals determined with qPCR (Experiment III): Fig. 5 (individual results) and Table 2 (mean ± SD) show individual relative expression of 5 genes presented in Fig. 3 and Fig. 4. Figs. 5A and 5B show relative expression of *Gapdh* in samples of 5 control-4 h, DEN-4 h, control-28 d and DEN-28 d individual livers determined with qPCR. This reveals that *Gapdh* expression was similar in each liver, i.e. variation among individuals was small, and that cDNA preparation was appropriate. In subsequent experi-

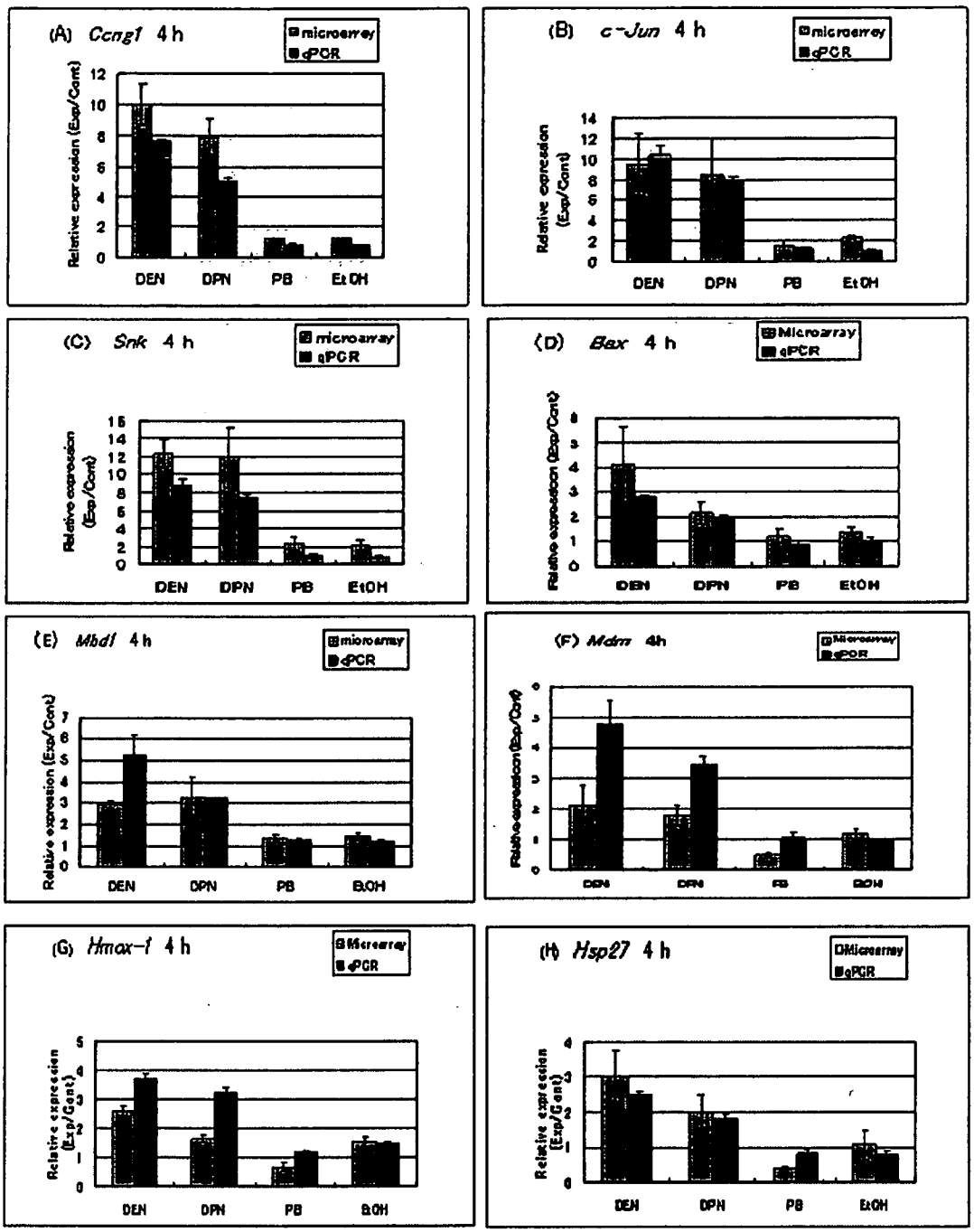


Fig. 3.

ments, *Gapdh* was used as the house-keeping normalized gene for each sample. Figs. 5C to 5J show relative gene expression of *c-Jun, Ccngl, Hsp27* and *Rad52* normalized to *Gapdh*. Variation among individual gene expression of *c-Jun, Ccngl, Hsp27* and *Rad52* (Fig. 5C to 5J) was small. Increase in gene expression by DEN at 4 h was 12-fold for *c-Jun*, 6-fold *Ccngl*, and around 2-fold for *Hsp27* and *Rad52*. In the main, these increases returned to about control level 28 days after DEN administration; *Ccngl* was slightly elevated over control at this time point. These results show that there was minor variation in the gene expression in individual livers for the various treatment groups.

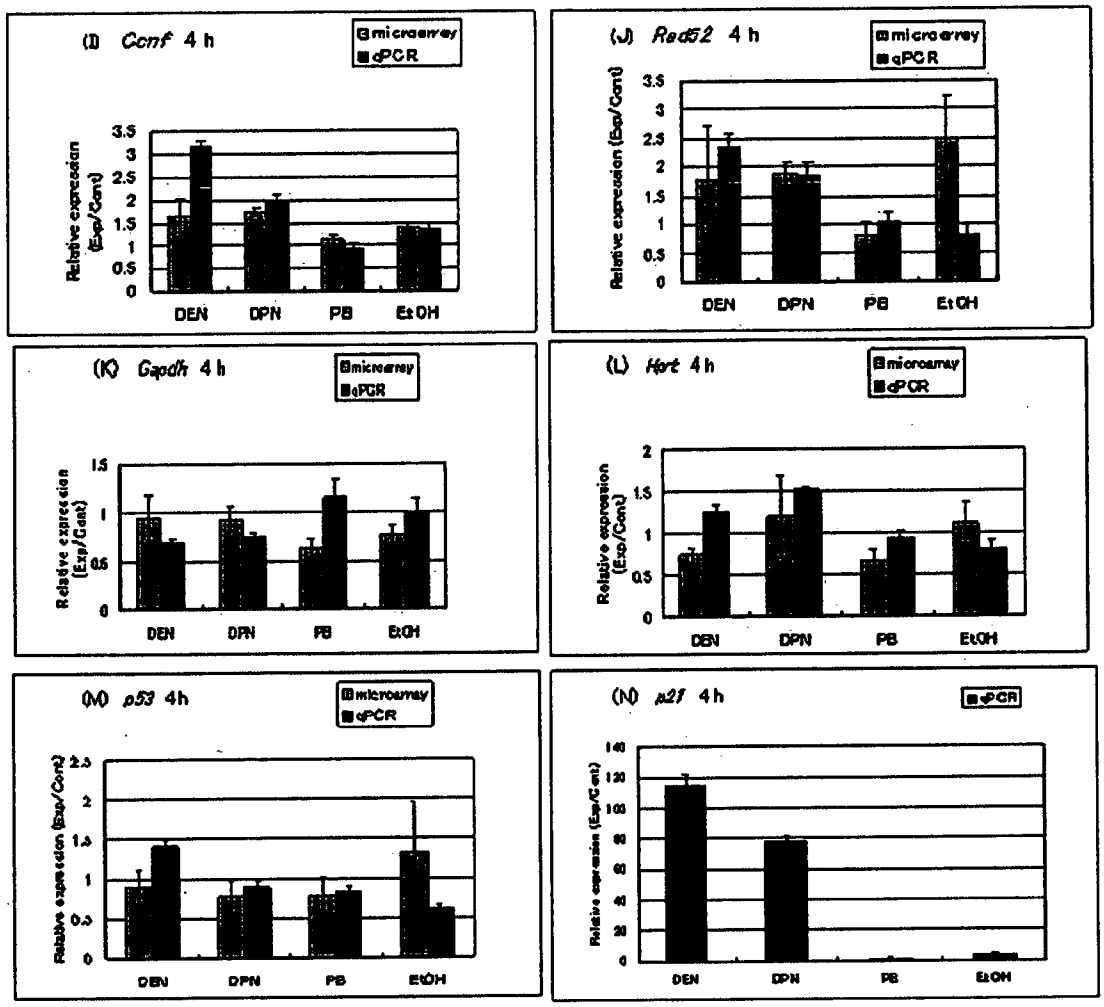


Fig. 3. Verification of DNA microarray results with qPCR 4 h after chemical administration (Experiment II). Results are shown as average and SD (error bar), and expressed as relative expression (experiment group/vehicle control group). Microarray: data are from 4 spots from two microarrays for DEN and DPN, and from 4 spots from one microarray for PB and EtOH. qPCR: results from triplicate assays. mRNA or total RNA from pooled livers from 5 mice was used as material. Only qPCR result is shown for *p21*.

Discussion

In the present study we examined differential gene expression in mouse liver 4 h and 28 days after administration of genotoxic *N*-nitroso carcinogens, DEN and DPN, compared to non-genotoxic carcinogen PB and non-carcinogenic toxin EtOH using in-house DNA microarray and qPCR. Four hours is a time at which genotoxic *N*-nitroso carcinogens induce DNA damage (7-9). We found differential gene expression between the two *N*-nitroso carcinogens and PB and EtOH in 11 genes 4 h after administration. The most characteristic genes were seven *p53* (14) target genes, viz. *c-Jun* (*c-jun* proto oncogene) (15), *Ccng1* (cyclin G1) (16), *Mdm2* (transformed 3T3 cell double minute 2, *p53* binding protein) (17), *p21* (cyclin-dependent kinase inhibitor 1A) (18), *Bax* (*Bcl2*-associated X protein) (19), *Hsp27* (heat shock protein HSP27) (20) and *Snk* (serum-inducible protein kinase) (21). A suggested gene network is shown in Fig. 6. There are about 40 DEN- and DPN-related direct or indirect *p53* target genes in the 268 gene-list analyzed using Ingenuity Pathways Analysis. Results of differentially expressed genes are presented in this manuscript.

These *p53* target genes may lead to either DNA repair or apoptosis according to the seriousness of DNA damage by DEN and DPN. However, changes in gene expression of *p53* itself were not observed in the present experimental conditions. It might be possible that this "chief protector" *p53* gene is always expressed adequately in mouse liver to function against DNA damaging hazards. Another possibility might be that *p53* expression changed earlier than 4 h and returned to

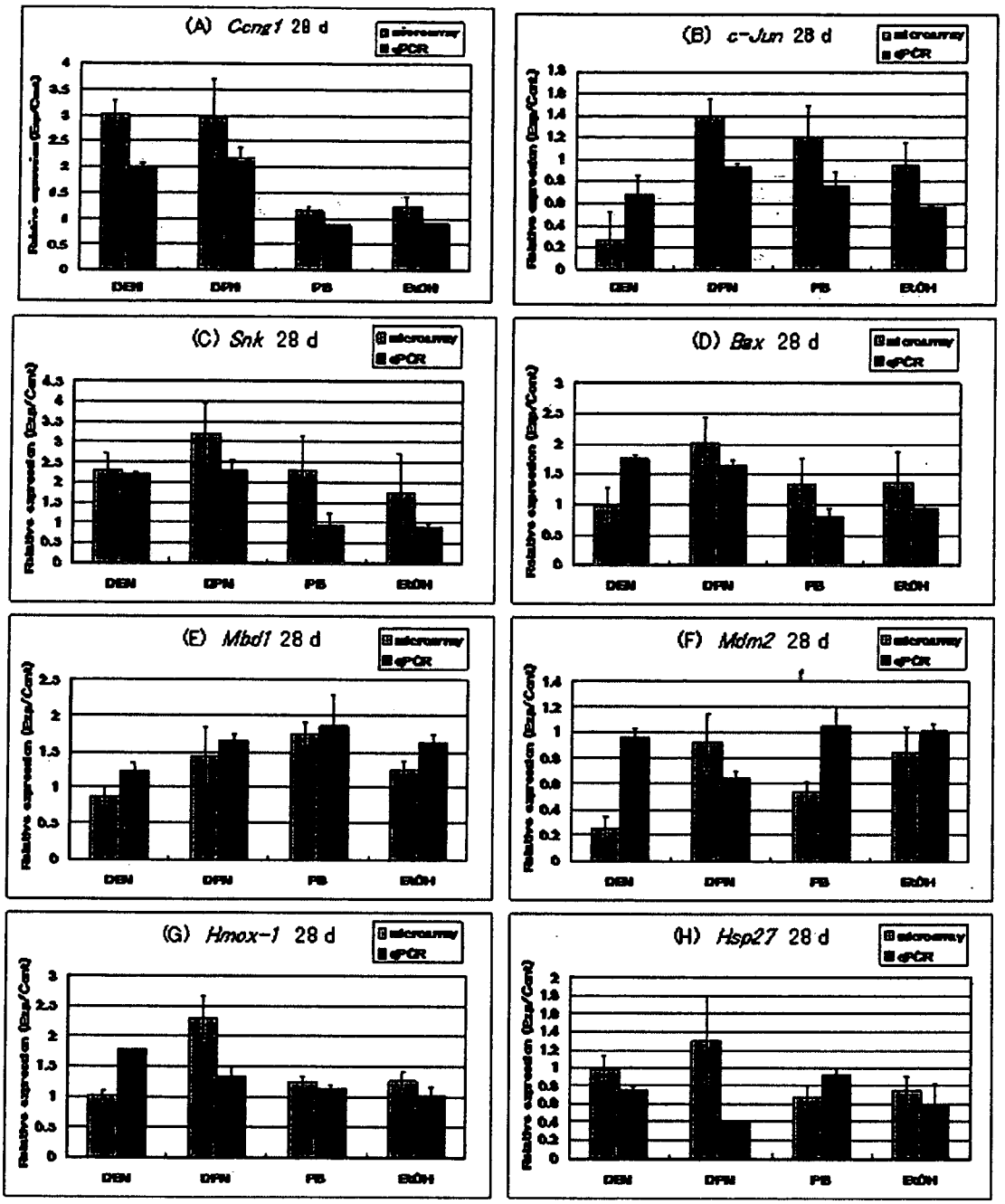
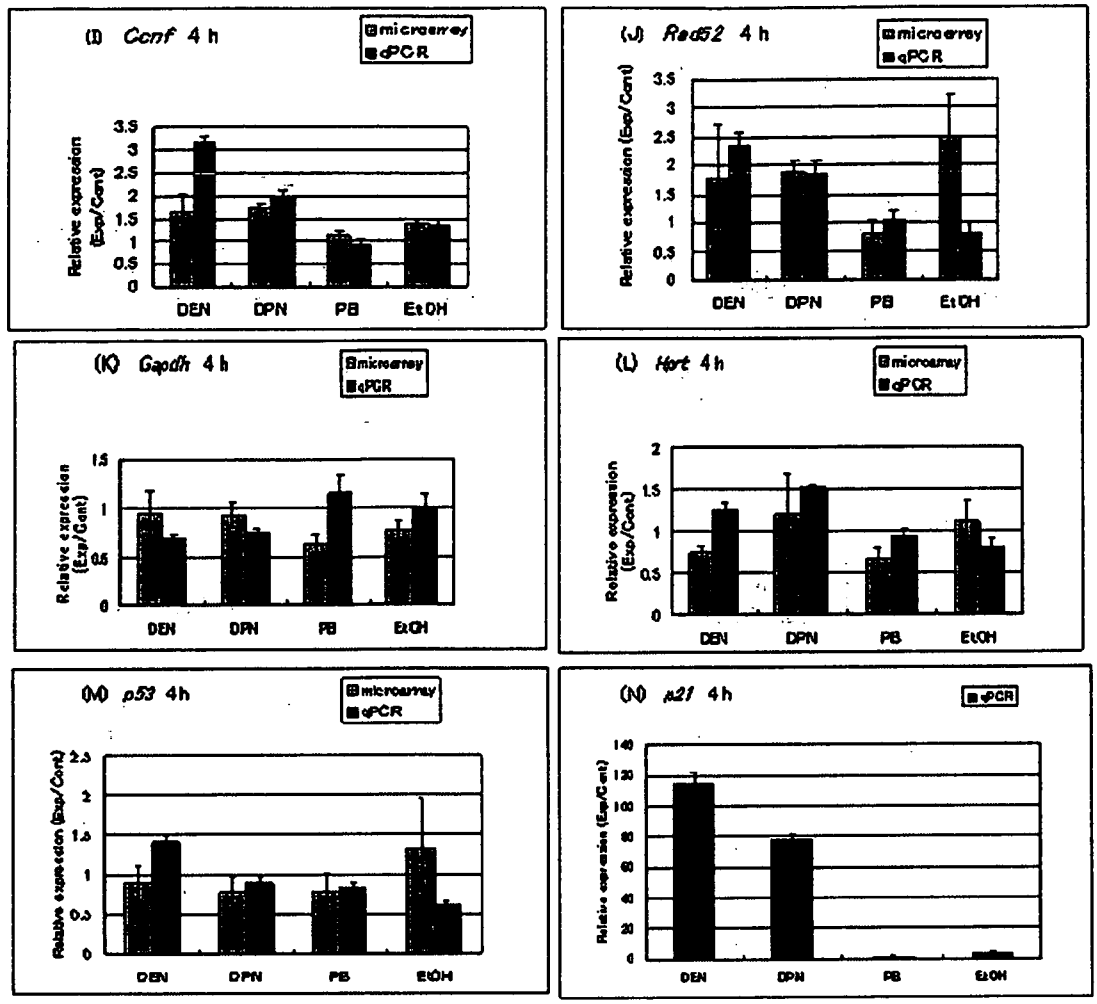


Fig. 4.

control level by 4 h. Differential gene expression of DNA repair enzymes was not detected, against expectations. DNA damage in liver by PB or EtOH at 4 h after their administration has not been reported.

Regarding differential gene expression in mouse liver 28 days after chemical exposure, it was previously reported that few DNA adducts were observed at this time after DEN administration but that mutations were observed (7). Our results showed that *p21* retained high gene expression but was reduced to one-tenth compared to 4 h, and that *Ccng1* and *Snk* showed minor increase in gene expression 28 days after administration of DEN and DPN. Expression of other genes which had elevated expression at 4 h returned to control level 28 days after administration of DEN and DPN. Differential expression of all other genes in the 268 gene-list examined did



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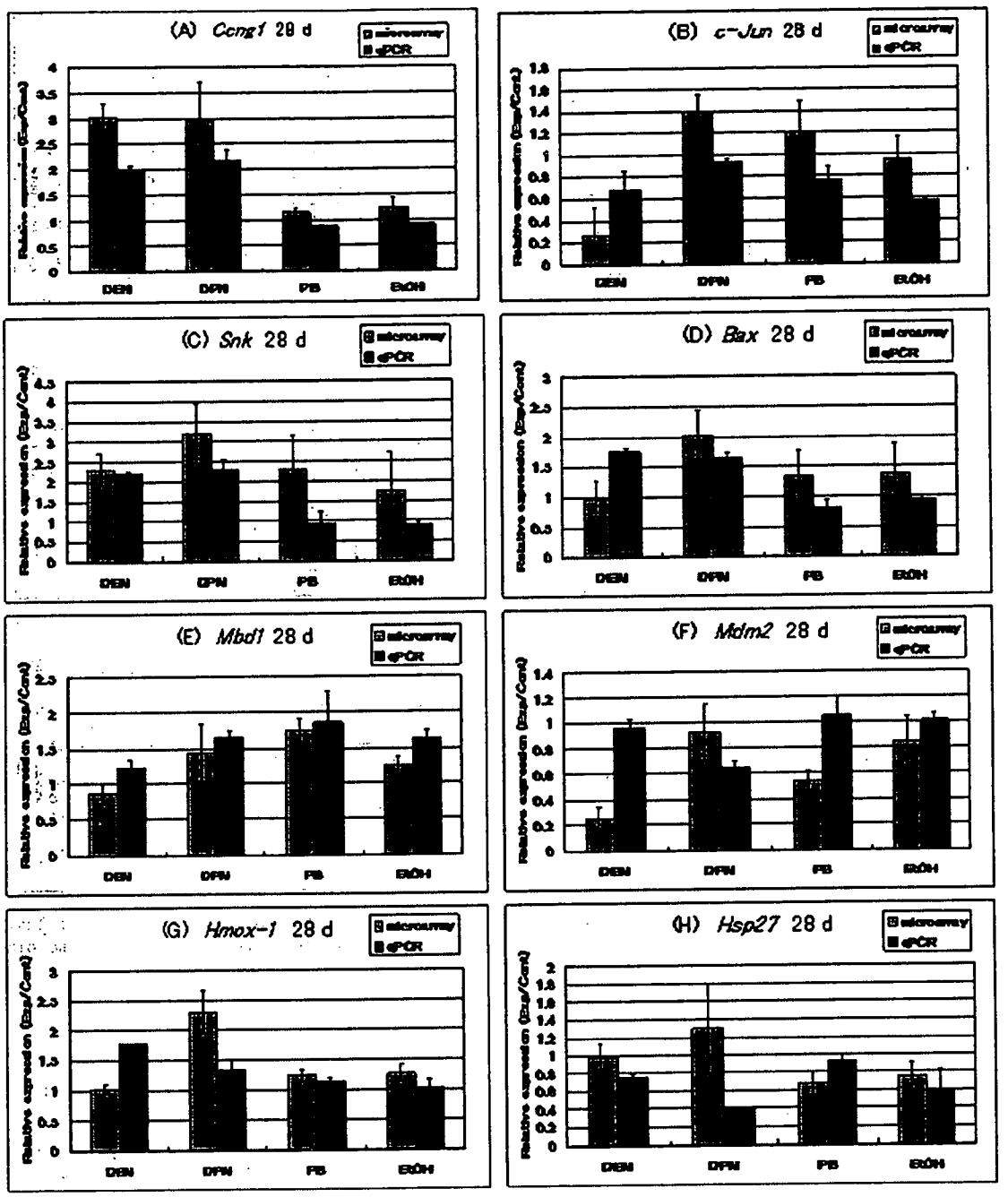

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Regarding differential gene expression in mouse liver 28 days after chemical exposure, it was previously reported that few DNA adducts were observed at this time after DEN administration but that mutations were observed (7). Our results showed that *p21* retained high gene expression but was reduced to one-tenth compared to 4 h, and that *Ccng1* and *Snk* showed minor increase in gene expression 28 days after administration of DEN and DPN. Expression of other genes which had elevated expression at 4 h returned to control level 28 days after administration of DEN and DPN. Differential expression of all other genes in the 268 gene-list examined did

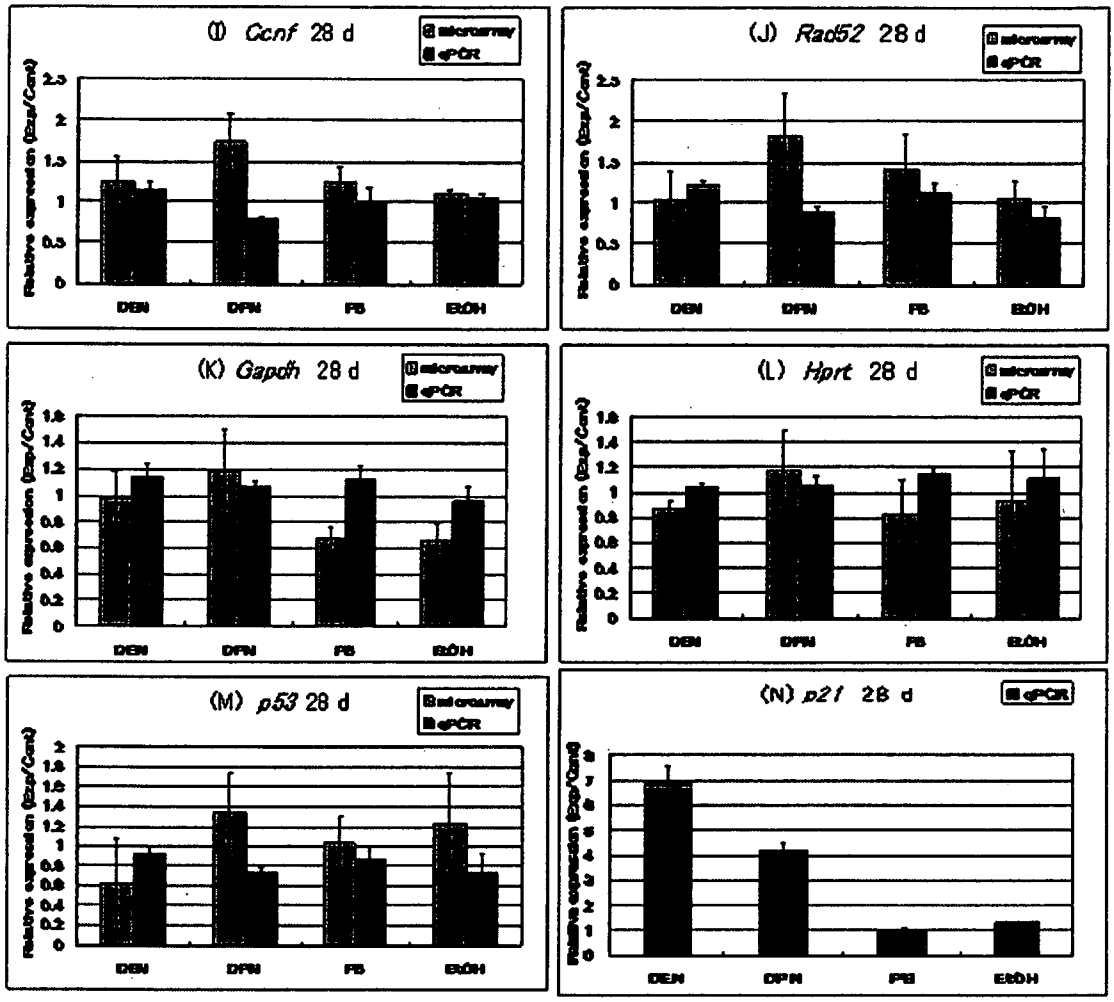

Fig. 4. Verification of DNA microarray results with qPCR 28 days after chemical administration (Experiment II). Results are shown as average and SD (error bar), and expressed as relative expression (experimental group/vehicle control group). Microarray: data are from 4 spots from two microarrays for DEN and DPN, and from 4 spots from one microarray for PB and EtOH. qPCR: results from triplicate assays. mRNA or total RNA from pooled livers from 5 mice was used as material. Only qPCR result is shown for *p21*.

not newly appear at 28 days after administration. PB and EtOH did not show significant increase or decrease of gene expression 28 days after administration. In conclusion, acute responses to the genotoxic carcinogens remained only partially in liver 28 days after administration, while PB and EtOH did not induce significant acute or longer term gene expression changes in the 268 gene-list.

Figs. 3 and 4 show that standard deviation in data sets was generally smaller in qPCR (3 tubes per experiment) than DNA microarray (4 spots per experiment). Fluorescence intensity was very similar in another experiment of the same gene (*Gapdh, Ccngl, c-Jun, Rad52, Hsp27* and others) in qPCR, indicating good reproducibility. However, fluorescence intensity in a repeat DNA microarray experiment was less reproducible, likely due to variability in fluorescent labeling of cDNA and washing of DNA microarray even if a similar amount of DNA is used for hybridization with the same lot of DNA microarray. The reliability and reproducibility of experiments are higher with qPCR than with DNA microarray in the present experimental conditions.

We demonstrated that inter-individual variation was small for all 5 genes assayed with qPCR and examined 4 h and 28 days after administration in 5 control and 5 DEN-treated mice (Fig. 5). These individual results matched well the qPCR results in Fig. 3 and Fig. 4, where liver samples of 5 different mice were pooled and assayed. Based on this, we mainly conducted experiments using pooled liver samples of 5 mice (Experiment II), although experiments using individual animal

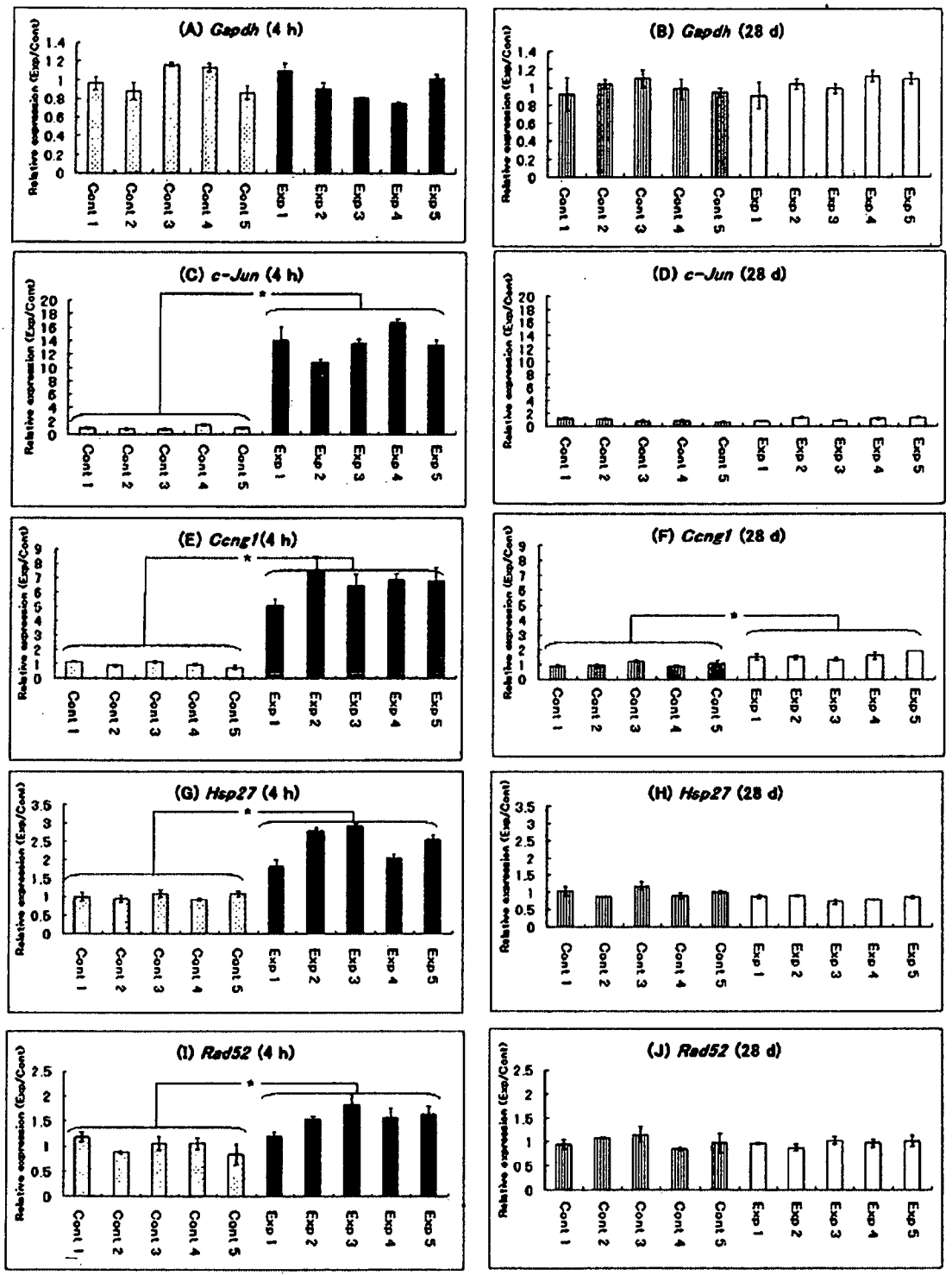

Fig. 5. Variation among individual mice (relative liver gene expression) (Experiment III). DEN (80 mg/kg bw) and saline were given to 9-week-old mice (5 per group), main lobe of liver was dissected individually after 4 h and 28 days and individual gene expression was determined with qPCR. cDNA from individual liver from 5 mice for each group was used as material. Gene expression of *Ccngl*, *c-Jun*, *Rad52* and *Hsp27* was normalized with gene expression of *Gapdh*. Values in *c-Jun* (4 h), *Ccngl* (4 h and 28 d), *Hsp27* (4 h) and *Rad53* (4 h) in DEN group were significantly different ($p < 0.01$) from control group.

Table 2. Variation among individuals determined with qPCR*

Group	Relative expression (Exp/Cont, Mean ± SD)				
	Gapdh	*c-Jun*	*Ccng1*	*Hsp27*	*Rad52*
Saline 4 h	1.00±0.14	1.00±0.26	1.00±0.18	1.00±0.07	1.00±0.14
DEN 4 h	0.92±0.14	13.71±2.11	6.50±0.86	2.47±0.47	1.56±0.23
Saline 28 d	1.00±0.07	1.00±0.24	1.00±0.16	1.00±0.12	1.00±0.12
DEN 28 d	1.03±0.09	1.15±0.26	1.57±0.21	0.83±0.07	0.97±0.06

*Experiment III, livers from 5 mice each of experimental groups (4 h and 28 days) and control group were used. Mean of control group is presented as 1.00.

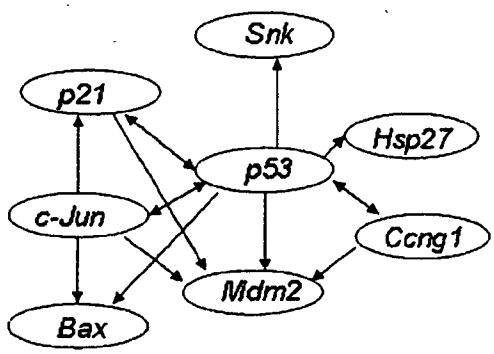

Fig. 6. Suggested gene network of seven *p53* target genes analyzed using Ingenuity Pathways Analysis.

material were desirable.

Recent reports have described changes in gene expression in DEN-induced mouse liver tumors (22-24) and DEN-treated rat liver (25) with different DNA microarrays containing different genes, but no studies have focused on the DNA damaging time of 4 h nor the mutation fixing time of 28 days; thus, different gene expressions were detected. As well, there are no reports which compare changes in gene expression in mouse liver for DEN and DPN exposures. The present study showed that DEN and DPN induced very similar changes in gene expression in mouse liver at 4 h and 28 days after their administration, suggesting a similar response mechanism. Gene expression changes with PB (25-27) and EtOH (28) were previously studied in mouse or rat liver at different time points with different DNA microarrays containing different genes, resulting in different gene expressions being detected.

In the present DNA microarray we mainly selected genes that have been linked with the action of genotoxic carcinogens. As a result we could find characteristic gene expression increases for DEN and DPN, but we could not find genes that were specifically responsive to the non-genotoxic carcinogen PB or to the non-carcinogenic toxin EtOH. Decreased-expression genes were observed among the 268 genes in the present results (not shown). However, characteristically decreased genes were not found for DEN, DPN, PB or EtOH in the present 268 gene-list.

In the first step of characterizing our microarray system, we examined 20mer, 30mer and 40-47mer oligonucleotides as probes (data not shown) and concluded that 40-47mer oligonucleotides showed sufficient strength of fluorescence. In an early trial, we prepared cDNA microarray with PCR products of 400 bp length. However these cDNA microarrays were less sensitive than the present oligonucleotide microarray (data not shown).

In conclusion, we have used DNA microarray and qPCR to show (1) that in comparison to PB and EtOH, the genotoxic carcinogens DEN and DPN induced differential gene expression in *p53* target genes in mouse liver 4 h after chemical administration (a time when DNA damage is induced by *N*-nitroso carcinogens), and (2) that these acute responses remained only partially in liver 28 days after administration, a time when little DNA damage remains but mutations are observed. We will continue further studies to add other useful genes to our DNA microarray, including high throughput DNA microarray studies in mouse liver and preparation of in-house oligonucleotide microarray for characterizing mutagenic and carcinogenic compounds; these data will be applied to the study of chemical risk assessment.

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Potassium bromate treatment predominantly causes large deletions, but not GC > TA transversion in human cells

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Abstract

Potassium bromate ($KBrO_3$) is strongly carcinogenic in rodents and mutagenic in bacteria and mammalian cells in vitro. The proposed genotoxic mechanism for $KBrO_3$ is oxidative DNA damage. $KBrO_3$ can generate high yields of 8-hydroxydeoxyguanosine (8OHdG) DNA adducts, which cause GC > TA transversions in cell-free systems. In this study, we investigated the in vitro genotoxicity of $KBrO_3$ in human lymphoblastoid TK6 cells using the comet (COM) assay, the micronucleus (MN) test, and the thymidine kinase (*TK*) gene mutation assay. After a 4 h treatment, the alkaline and neutral COM assay demonstrated that $KBrO_3$ directly yielded DNA damages including DNA double strand breaks (DSBs). $KBrO_3$ also induced MN and *TK* mutations concentration-dependently. At the highest concentration (5 mM), $KBrO_3$ induced MN and *TK* mutation frequencies that were over 30 times the background level. Molecular analysis revealed that 90% of the induced mutations were large deletions that involved loss of heterozygosity (LOH) at the *TK* locus. Ionizing-irradiation exhibited similar mutational spectrum in our system. These results indicate that the major genotoxicity of $KBrO_3$ may be due to DSBs that lead to large deletions rather than to 8OHdG adducts that lead to GC > TA transversions, as is commonly believed. To better understand the genotoxic mechanism of $KBrO_3$, we analyzed gene expression profiles of TK6 cells using Affymetrix Genechip. Some genes involved in stress, apoptosis, and DNA repair were up-regulated by the treatment of $KBrO_3$. However, we could not observe the similarity of gene expression profile in the treatment of $KBrO_3$ to ionizing-irradiation as well as oxidative damage inducers.

Keywords: Potassium bromate ($KBrO_3$); *TK*-mutation; Loss of heterozygosity (LOH); 8-Hydroxydeoxyguanosine (8OHdG); Gene expression profile

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1. Introduction

Potassium bromate ($KBrO_3$) is used as in bread making a flour improver and in the production of fish-pastes. The EU countries now prohibit its use as a food additive because of its carcinogenicity. Japan and the USA, however, permit its use in bread making on the condition that it never remains in the final product. $KBrO_3$ causes tumors, especially in kidney, in rats, and mice after long-term oral administration in drinking water [1–3]. $KBrO_3$ is also genotoxic. It is positive in in vitro genotoxicity tests – including the bacterial reverse mutation assay [1], the chromosomal aberration test conducted in Chinese hamster cells [4], and the mouse lymphoma assay [5] – and in vivo in the micronucleus test (MN) [6,7].

It has been proposed that $KBrO_3$ induces tumors through the production of oxidative damage to DNA. Oxidative DNA damage can cause mutations that contribute to the activation of oncogenes and/or the inactivation of tumor suppressor genes, thereby leading to tumorigenesis [8,9]. 8-Hydroxydeoxyguanosine (8OHdG) is the main form of oxidative DNA damage induced by $KBrO_3$ [10]. It primarily causes GC > TA transversions (as a result of the pairing of 8OHdG with A) and is believed to be responsible for mutagenesis, carcinogenesis, and aging [11,12]. $KBrO_3$ increases 8OHdG DNA adducts in vivo and in vitro [13–15]. However, $KBrO_3$ induces mutations weakly in microbial mutation assays and the *Hprt* mutation assay in mammalian cells, while it induces chromosome aberrations strongly both in vivo and in vitro [1,16,17]. These findings raise the question of whether 8OHdG is required for the mutagenic process involved in $KBrO_3$-induced carcinogenesis.

In the present study, we examined the genotoxic properties of $KBrO_3$ using the comet assay (COM), the MN test, and thymidine kinase (*TK*) gene mutation assays in human lymphoblastoid TK6 cells [18]. Unlike the X-linked hemizygous *HPRT* gene mutation assay, the *TK* mutation assay can detect not only point mutations, but also large scale chromosomal deletions, recombinations, and aneuploidy [19–21]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumed relevant to carcinogenesis. We analyzed the *TK* mutants induced by $KBrO_3$ at the molecular level and investigated what kind of mutation predominated. We also profiled global gene expression in TK6 cell exposed to $KBrO_3$ using Affymetrix GeneChip® Expression analysis to understand the genotoxic mechanism of $KBrO_3$.

2. Materials and methods

2.1. Cell culture, chemicals, and treatment

The TK6 human lymphoblastoid cell line has been described previously [22]. Cells were maintained in RPMI 1640 medium (Gibco-BRL, Life Technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JR Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were incubated at 37 °C in a 5% CO_2 atmosphere with 100% humidity. $KBrO_3$ (CAS No.7758-01-2) was purchased from Wako Pure Chemical Co. (Tokyo) and dissolved in RPMI medium just before use.

We prepared 20 ml aliquots of cell suspension at a concentration of 5.0×10^5 cells/ml in 50 ml polystyrene tubes. Different concentrations of $KBrO_3$ were added to the tubes, which were then placed on a platform shaker and incubated at 37 °C for 4 h with gentle shaking. At the end of the treatment period, the cell cultures were centrifuged, washed once, and re-suspended in fresh medium. We cultured them in new flasks for the MN assay and *TK* gene mutation assay, or diluted them for plating for survival estimates.

2.2. Genotoxicity assays

After treating cells with $KBrO_3$, we prepared slides for conducting the alkaline and neutral COM assay. The alkaline COM assay was performed as previously reported [23]. For the neutral COM assay, the slide was electrophoresed with chilled neutral solution (pH 8) containing of 90 mM Tris, 2 mM Na_2EDTA, and 90 mM boric acid according to the method by Wada et al. [24]. The COM slides were stained with SYBER green (Molecular Probes, Eugene, OR) and observed by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and tail length of the comet was measured. The relationship between $KBrO_3$ treatment and migration was statistically analyzed by the Dunnett test [25].

We prepared the MN test samples 48 h after treatment, as previously reported [23]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold methanol containing 25% acetic acid, then re-suspended in methanol containing 1% acetic acid. A drop of the suspension was placed on a clean glass slide and air-dried. The cells were stained with 40 μg/ml acridine orange solution and immediately observed with the aid of an Olympus model BX50 fluorescence microscope equipped with a U-MWBV band pass filter. At least 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test [26].

We prepared the *TK* gene mutation assay samples 3 days after treatment. We seeded cells from each culture into 96-well plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluo-