

Fig. 1. Three possibilities in DMPO-OH signal decay. (1) The reaction is suppressed, (2) hydroxyl radicals are scavenged, or (3) DMPO-OH radical is quenched by the additive. Chelating agents such as EDTA should participate in this process (3), not in (1). Four peaks in the middle are the signals of DMPO-OH radical ($A_H = 1.50$ mT, $A_N = 1.50$ mT), and the two signals of both ends are those of Mn (external reference).

seemed to scavenge hydroxyl radicals, but this resulted from the enhancement of the DMPO-OH radical quenching activity of Fe(II) ions in the presence of phosphate ions.

The first chelating agent reported to suppress the iron-catalyzed reaction was diethylenetriaminepenta acetic acid (DTPA) [2]. Deferoxamine (DFO) also showed reduced reactivity in $\cdot\text{OH}$ radical formation [3], and is widely used as a “probe” for iron-dependent radical reactions, mostly because its complex with Fe(III) is very difficult to reduce [3,4]; however, ambiguous results have been reported on the effect of EDTA or DTPA on iron-dependent hydroxyl radical-generating systems, varying from stimulation to no effect, to inhibition [5]. Similar conflicting results were observed by Jay [6], who measured the amount of hydroxyl radical formed by Fenton reaction by the deoxyribose degradation method and also ESR measurement with PBN. However, they could not clarify why hydroxyl radical formation was underestimated by ESR measurement, and they showed that the instability of PBN-OH \cdot radical was plausible. Similar variability in the effect of iron-chelating agents was observed by voltammetry [7].

We suspected that these ambiguous effects of iron-chelating agents in hydroxyl radical formation reaction may have resulted from the assay method used. Our previous results indicated that Fe(II) quenched DMPO-OH radicals, and this quenching of [DMPO-OH] \cdot by Fe(II) became much stronger in phosphate buffer where phosphate ions could form a chelate with Fe(II) [1], although we could not demonstrate whether hydroxyl radical formation could be suppressed by chelation. On the other hand, we developed an HPLC assay method for hydroxyl radicals using terephthalic acid as a fluorescent probe [8]. Thus, we tried to confirm the effect of iron-chelating agents in Fenton reaction by measuring two different methods, ESR spin-trapping with DMPO and HPLC.

This report demonstrates that chelating agents with iron ions such as EDTA, NTA and DTPA seem “to suppress the Fenton reaction”; however, hydroxyl radical formation was “not suppressed” by the addition of these chelating agents when observed by the HPLC method, but the addition of these chelating agents enhanced [DMPO-OH] \cdot signal decay greatly by ESR measurement. Thus, it has been clarified that iron-chelating agents enhanced the quenching of DMPO-OH \cdot radical with Fe(II), and the trapped signal diminished in a short period by ESR spin-trapping measurement, which may lead to the mistaken conclusion: “Fenton reaction should be suppressed by iron-chelating agents by forming strong chelation.” This work should be an example that evaluation of the results obtained by a routinely used assay method could clarify an underlying problem.

2. Experiment

2.1. Chemicals

Ammonium iron(II) sulfate (99.999%) was obtained from Aldrich–Sigma Japan (Tokyo, Japan). Hydrogen peroxide (without stabilizer), and other chemicals, such as citric acid, were purchased from Wako Pure Chemicals Co. Ltd. (Osaka, Japan). Most of the chelating agents were obtained from Dojin Chemical Co., (Kyusyu, Japan). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Labotec Co. (Tokyo, Japan). Sodium terephthalate was obtained from Tokyo Kasei Kogyo (Tokyo, Japan), and purified by re-precipitation from ethanol and water [8].

The water used in this study was prepared by Milli-Q (Millipore Japan Co. Ltd., Tokyo, Japan), and de-aerated under reduced pressure using an aspirator, then stored under argon by attaching an argon balloon.

2.2. 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 300. A manganese signal was used for the external standard.

2.3. Solution used for Fenton reaction

Solution A: 5 mmol L⁻¹ DMPO in acetate buffer (117 mmol L⁻¹, pH 5.0).

Solution B: Ammonium ferrous sulfate solution (1 mmol L⁻¹).

Solution C: Hydrogen peroxide solution (0.5 mmol L⁻¹, titrated with permanganate).

Solution D: The additive solution (EDTA, nitrilotriacetic acid (NTA), citrate, oxalate or DTPA at ambient concentration).

Solution E: 5 mmol L⁻¹ sodium terephthalate solution in acetate buffer (117 mmol L⁻¹, pH 5.0).

Table 1
50%-inhibition concentration (IC_{50}) of DMPO-OH radical formation in Fenton reaction

Compound	$IC_{50} \times 10^{-2}$ (mmol L ⁻¹)
DFO	6.1
EDTA	4.8
DTPA	2.1
NTA	1.8
citrate	1.8
oxalate	1.2
Fe(II)	3.0 ^a
Fe(III)	1000.0 ^a
Mannitol	300.0 ^a
Phosphate	10.0 ^a

By ESR measurement, DMPO-OH radical formation was suppressed by the addition of Fe(II) or iron-chelating agents. *Abbreviations:* DFO, deferoxamine; EDTA, ethylenediamine tetraacetic acid; DTPA, diethylenetetramine pentaacetic acid; NTA, nitylotriacetic acid. All data are the average of three measurements.

^a These data are obtained from the previous report [1].

2.4. Measurement of the “suppression” of Fenton reaction

The Fenton reaction was started by mixing 100 μ L of solution C to a mixture of 1.7 mL of solution A, 100 μ L of solution B and 100 μ L of D. The spin-trapped [DMPO-OH][•] signal was measured 2 min after the addition of hydrogen peroxide solution (solution C). In the previous report [1], the signal was recorded 1 min after the addition of H₂O₂; however, signal intensity could be observed more reproducibly after 2 min. The concentrations of the additive changed in the range at which a 50% decrease in the DMPO-OH signal could be observed. The concentration at which 50% scavenging was obtained was expressed as IC_{50} (50% inhibition concentration for OH radical scavenging) in Table 1.

2.5. Observation of DMPO-OH radical “quenching”

The Fenton reaction was performed by mixing 1.7 mL of solution A, 100 μ L of B, and 100 μ L of C, which was allowed to stand at room temperature for 10 min. DMPO-OH[•] formation almost ceased after 6 min (96%), and was considered to have completed after 10 min (Fig. 2). Then, 100 μ L of a solution of chelating agent, solution D (concentration is IC_{50} , shown in Table 1) was added, and the solution mixed. The [DMPO-OH][•] signal was measured 1 min after the addition of solution D, that is, 11 min after hydrogen peroxide solution (solution C) was added, and successive signal decay was recorded over the next 12 min. The natural logarithm of the signal ratio of [DMPO-OH][•]/Mn was plotted versus time after adding solution D.

2.6. Observation of hydroxyl radical formation by HPLC-fluorescence detection

For HPLC analysis, a JASCO 980 HPLC system (Japan Spectroscopic Co. Ltd., Tokyo) was used, equipped with a Rheodyne sampling valve (20 μ L) and a reverse phase column (Develosil

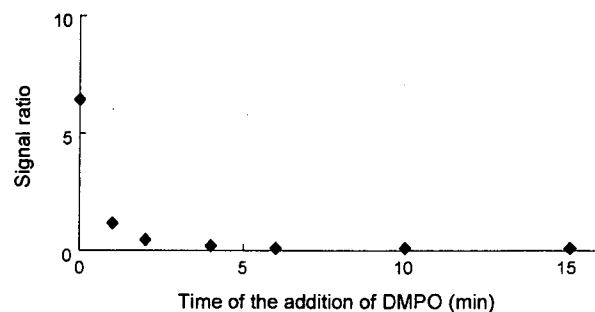


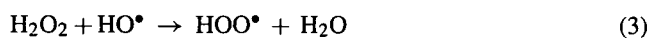
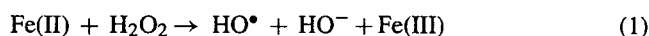
Fig. 2. Fenton reaction almost ceased in ten minutes after the addition of H₂O₂. The datum at 0 min represents the signal ratio of DMPO-OH radical vs. Mn (external standard) 1 min after the Fenton reaction started (by the addition of H₂O₂) in the presence of DMPO. The other data represent the signal ratio of DMPO-OH radical vs. Mn when the reaction had started without DMPO, then DMPO solution was added after the period indicated. Thus, hydroxyl radical formation can be negligible 10 min after the reaction started. Although, the signal of DMPO-OH radical could be detected with an appropriate intensity after 10 min after the reaction started in the presence of DMPO.

C30-UG5, 4.6 mm i.d. \times 250 mm, Nomura Chemical Co., Seto, Aichi, Japan; this column could be used without any organic additive in high reproducibility in retention profile [9]). It was eluted with 200 mmol L⁻¹ potassium hydrogen phosphate containing 2% of KCl (pH 4.37) and monitored with a UV detector 750-UV (290 nm, Japan Spectroscopic Co. Ltd., Tokyo) and a fluorescence detector, FP1520-S (Japan Spectroscopic Co. Ltd., Tokyo) in series. The excitation and emission wavelengths were: Ex, 309 nm and Em, 412 nm, respectively.

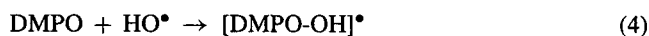
Fenton reaction was performed as described in the Section 2.4, except for the use of a solution of sodium terephthalate, solution E, instead of solution A (DMPO solution).

3. Results and discussion

The initial reaction of the Fenton reaction is described as Eq. (1), accompanied by reactions (2) and (3) [10,11]. Yamazaki and Piette [12] found that the stoichiometry of oxidant formation in the reaction between Fe(II) and H₂O₂ often shows marked deviation from the expected value of 1:1, in the observation with ESR. Sharpe and Cooper also reported that the Fenton reaction became unstable when the ratio of Fe(II) to H₂O₂ exceeded 1.0 [13]; however, under our reaction conditions, we used Fe(II):H₂O₂ = 2:1, because the reaction reached a plateau at this molar ratio by observation of the ESR method.



In the presence of DMPO, hydroxyl radicals were trapped by DMPO (Eq. (4)).



The signal of [DMPO-OH][•] could be observed for 30 min after the addition of hydrogen peroxide under the conditions described above; however, in the presence of EDTA, NTA,

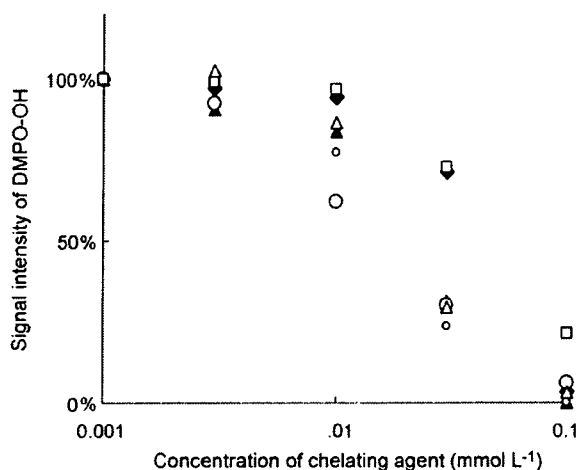


Fig. 3. Iron-chelating agents inhibited DMPO-OH formation in Fenton reaction in the presence of DMPO (citrate (Δ), oxalate (\circ), NTA (\circ), EDTA (\blacklozenge), DFO (\square), and DTPA (\blacktriangle)). The 50%-inhibition concentration (IC_{50}) of each chelating agent was obtained, and was shown in Table 1.

DTPA, citrate or oxalate at the beginning of the Fenton reaction, the signal of DMPO-OH diminished depending on the concentration of these additives (Fig. 3). Thus, 50% suppression concentration (IC_{50}) of each compound was determined (Table 1), where Fe(II) and Fe(III) were also studied for their scavenging effects. It seems that Fe(II) and these chelating agents are efficient hydroxyl radical “scavengers” or “suppressors of the Fenton reaction”.

Iron-chelating agents, such as deferoxamine (DFO), are known to prevent Fenton reaction by complex formation with Fe(II) ion [3]. This was deduced from observation of ESR spin-trapping measurements with DMPO and lipid oxidation measured by red pigment formation with *thiobarbituric acid* (TBA); however, EDTA solely inhibited the TBA formation reaction [14]. Also, Aitken et al. [15] reported that EDTA suppressed malondialdehyde formation in vivo, but hydroxyl radical formation was enhanced by the addition of EDTA. Thus, the “chelating effect” must be checked by a different analytical method.

Under the conditions for the Fenton reaction described here, the reaction almost ceased about 10 min after the addition of hydrogen peroxide solution (Fig. 4). When the Fenton reaction started in the presence of DMPO, the hydroxyl radical formed should be trapped by DMPO, and its formation should almost cease by 10 min, but the DMPO-OH signal could be detected for more than 20 min thereafter. If the DMPO-OH \cdot signal disappeared by the addition of a compound to the reaction mixture, we could suspect that the additive should decompose [DMPO-OH] \cdot . Thus, the signal decay of [DMPO-OH] \cdot was monitored after the addition of a chelating agent 10 min after the Fenton reaction started. It was found that the [DMPO-OH] \cdot signal diminished in a short period after the addition of these chelating agents, whereas the addition of mannitol and dimethyl sulfoxide, which are hydroxyl radical scavengers, or acetate buffer (pH 5.0, as control), did not affect signal intensity (Fig. 5). When these chelating agents in a fresh Fe(II) solution were added, the

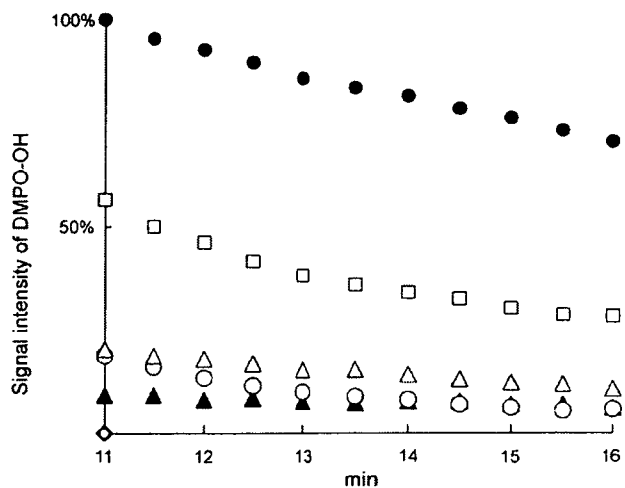


Fig. 4. ESR signal of DMPO-OH radical decreased rapidly by the addition of chelating agents (DFO (\square), citric acid (Δ), oxalic acid (\circ), DTPA (\blacktriangle)) after the Fenton reaction finished (10 min after the reaction started), but changed little by the addition of water (\bullet). With NTA (\circ) and EDTA (\blacklozenge), the DMPO-OH signal disappeared completely (0% at 11 min). These results indicate that these chelating agents should not suppress the Fenton reaction, but may diminish DMPO-OH radical.

DMPO-OH \cdot signal disappeared quite rapidly (Fig. 6). Thus, it was suspected that [DMPO-OH] \cdot is decomposed to a diamagnetic specimen by Fe(II) in the reaction mixture. We speculate that these chelating agents enhance the quenching of [DMPO-OH] \cdot with Fe(II).

In the compounds listed in Table 1, EDTA, NTA and DTPA were subjected to the HPLC assay of hydroxyl radical formation. As shown in Fig. 4, hydroxyl radical formation detected by the hydroxylation reaction of terephthalic acid [8] showed that these chelating agents had little effect on hydroxyl radical formation and, with DTPA, enhancement of hydroxyl radical formation was observed at a higher concentration. It has been reported that

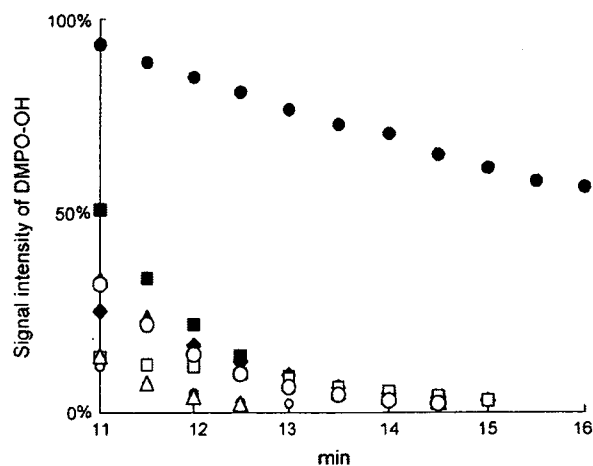


Fig. 5. The signal of DMPO-OH radical quenched by the addition of Fe(II) (\blacksquare). The DMPO-OH radical quenching of Fe(II) was enhanced by the simultaneous addition of Fe(II) and chelating agents (citrate (Δ), oxalate (\circ), NTA (\circ), EDTA (\blacklozenge), DFO (\square), and DTPA (\blacktriangle)), although it was not affected by the addition of water (\bullet).

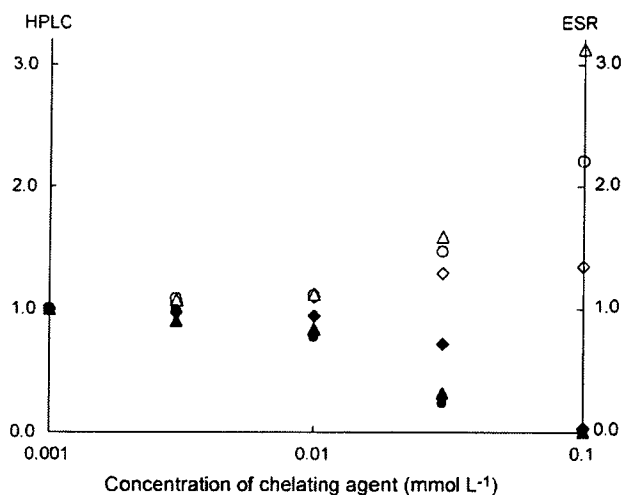


Fig. 6. Measurements of hydroxyl radical formation in Fenton reaction by two different methods. Formation ratios of hydroxyl radical indicators are measured relative to that in the presence of iron-chelating agent of $0.001 \text{ mmol L}^{-1}$; formation of DMPO-OH by ESR spin-trapping method with DMPO (DTPA (Δ), EDTA (\diamond), NTA (\bullet)), and formation of hydroxyl terephthalate detected by HPLC with terephthalic acid (DTPA (Δ), EDTA (\diamond), NTA (\circ)). Fenton reaction was not suppressed by these iron-chelating agents measured by HPLC.

DMPO forms [DMPO-OH] \cdot in Fe(III) solution [16], or Fe(III) in the presence of chelating agents such as DTPA or DFO [17]. Accordingly, the increase of hydroxylated terephthalate may be considered as the contribution of Fe(III)-aided hydroxyl radical formation in the presence of these strong iron-chelating agents.

It must be pointed out that hydroxyl radical scavenging measurements by the ESR-spin trapping method with DMPO may lead to the mistaken conclusion that “the chelating agents with iron ions should suppress Fenton reaction by chelate formation,” when the Fenton reaction is used as the hydroxyl radical source with an iron-chelating agent.

4. Conclusions

In conclusion, it was demonstrated that hydroxyl radical formation in the Fenton reaction increased in the presence of chelating agents with iron ions such as EDTA, whereas the

ESR signal of DMPO-OH radicals disappeared rapidly with Fe(II) ions, and this quenching effect was enhanced by chelating agents. When we measure changes in the amount of hydroxyl radical formed in the Fenton reaction, it must be measured by several different assay methods, such as ESR, HPLC, TBA reaction and so on, and we must be careful in our assessment of the results obtained.

Acknowledgements

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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 instability 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 FeCl₃. Based on this observation

of the formation of iron chelate with DMPO at 77 K, and DMPO-OCH₃ formation in the presence of CH₃OH, researchers elucidated that nucleophilic attachment of water to DMPO should occur in the presence of Fe³⁺ 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 Fe³⁺ might catalyze DMPO-OH formation.

Experimental

Chemicals

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

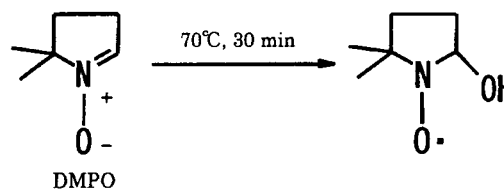


Fig. 1 DMPO-OH radical formed in a heated aqueous solution of DMPO.

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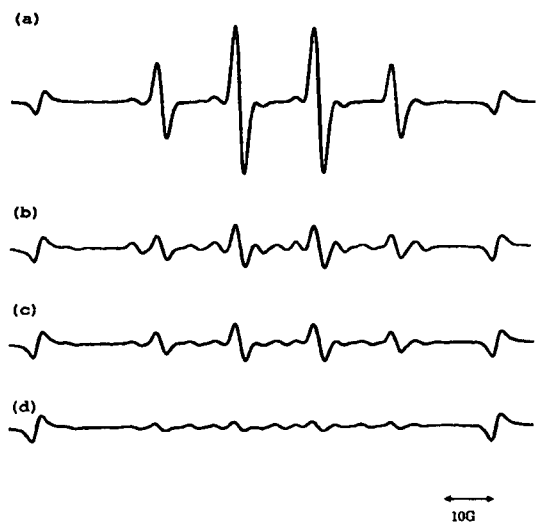


Fig. 2 The ESR spectrum of DMPO-OH radical observed in an aqueous solution of DMPO after heating at 70°C for 30 min (a). Four strong signals in the mid-section are of the DMPO-OH radical (A_H 1.50 mT, A_N 1.50 mT), and two side signals are of Mn used as an external reference. The ESR spectrum of DMPO 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).

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

The water used in this study was purified by ion-exchange resin (18.2 M Ω). Ultra-pure water for ICP-MS was obtained by two successive distillations of purified water with an all-quartz apparatus, in which Fe³⁺ 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 \pm 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

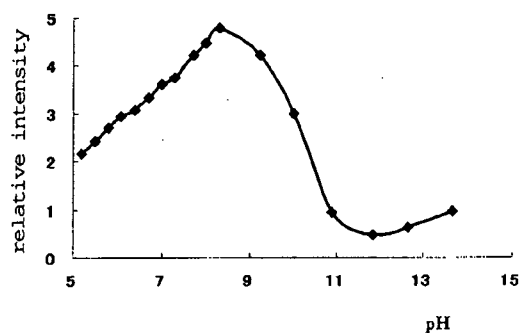
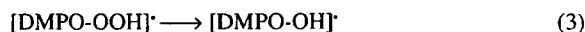


Fig. 3 Effect of pH on the relative intensity of DMPO-OH vs. Mn (external reference), which is the highest at around pH 8.5.

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 Fe³⁺ 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-OH⁵ 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 instability 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.



Even at low concentrations, transition metal ions such as Fe³⁺ may catalyze the first step (Eq. (1)), or catalyze the reaction of [DMPO-O₂] biradical with water to [DMPO-OOH] (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

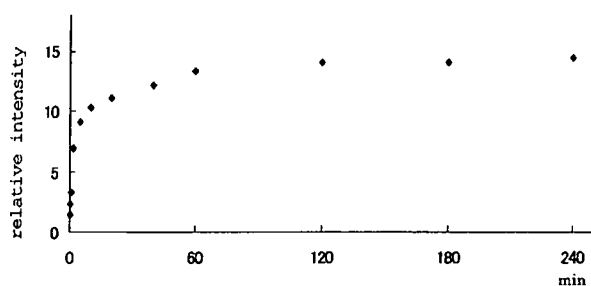


Fig. 4 The relative intensity of DMPO-OH vs. Mn (external reference) with time. The ESR signal of DMPO-OH radical in a 2.5% DMPO solution adjusted at pH 7.4 using a phosphate buffer, was recorded just 1 min after taking out the reaction tube which was heated at 70°C and then cooled at 0°C for 15 s in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

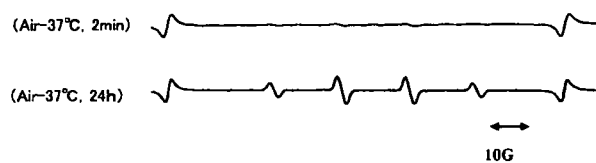


Fig. 5 ESR signal of DMPO-OH radical observed in an aqueous solution of DMPO (dissolved in milliQ water) under aerobic conditions.

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 PCR¹

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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-

¹This 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, extracellular 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 $23 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ 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, extracellular 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 μ M) in 1 \times Micro Spotting Solution (ArrayIt Division, TelChem International, Inc., Sunnyvale CA, USA) and 0.2% Tween 20 were spotted on polycarbodiimide-coated slides (CarboStation-U, Nissinbo Ind. Inc., Tokyo, Japan) with RIKEN arrayer. Spotted microarrays were rehydrated in a humid chamber with 1 \times 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 μ g) 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 μ g) 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 μ L) was hybridized with microarray in Gene Tac Hybridization Station (Genomic Solutions, Ann Arbor, MI, USA) at 55°C overnight, washed in 2 \times SSC-0.1% SDS at 45°C, 1 \times SSC-0.1% SDS at 40°C and 0.2 \times SSC at 40°C, dried by centrifugation and scanned by DNA-scope (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/Eisen-Software.htm>). Gene network was analyzed using Ingenuity Pathways Analysis (Ingenuity Systems, Inc.

Table 1. qPCR primer sequences

Gene	Primer sequence
<i>Bax</i> 5'	gattgctgacgtggacacggac
<i>Bax</i> 3'	cagggccttgagcaccagittg
<i>Ccnf</i> 5'	ggagctctcaagtgaagacagcac
<i>Ccnf</i> 3'	ggcctcacacacccatlaggctac
<i>Ccng1</i> 5'	tggccgagattgacctctctgg
<i>Ccng1</i> 3'	gtgcttcagttgccgtgcagtg
<i>c-Jun</i> 5'	aggcagagaggaagcgcgatg
<i>c-Jun</i> 3'	tgtgccacctgttccctgag
<i>Gapdh</i> 5'	tctggaaagctgtggcctgatg
<i>Gapdh</i> 3'	tcccgttcagctctgggatgac
<i>Hmox-1</i> 5'	gtggcctgaacttgaaccagc
<i>Hmox-1</i> 3'	cgtggtcagtcacatggatgc
<i>Hprt</i> 5'	cttgctcagatgatgaaggag
<i>Hprt</i> 3'	taatccagcaggtcagcaagaac
<i>Hsp27</i> 5'	ctcacagtgaagaccaaggaag
<i>Hsp27</i> 3'	ggataggaagaggacactagg
<i>Mbd1</i> 5'	tacagccctacacgaaccagc
<i>Mbd1</i> 3'	gaatttggcttgcgcagcag
<i>Mdm2</i> 5'	ttgatccgagcctggctctgtg
<i>Mdm2</i> 3'	aagatcctgatcgaggggcgtc
<i>p21</i> 5'	tcccacagagcaaatgttg
<i>p21</i> 3'	acgcgtcccagacgaagtgtg
<i>p53</i> 5'	tggaccctggcaccctacaatg
<i>p53</i> 3'	gcagacagccttgcagaatgg
<i>Rad52</i> 5'	gagaaccagcccaactctgc
<i>Rad52</i> 3'	gaacatgctggtgggtgtgc
<i>Snk</i> 5'	ctgttgagagcctctcagttg
<i>Snk</i> 3'	ccatagttcagatlaagcagc

Redwood City, CA, USA).

Quantitative real-time PCR (qPCR): For qPCR, 2 μ L 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 μ g) was reverse-transcribed with SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA, USA) and 250 μ L 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.

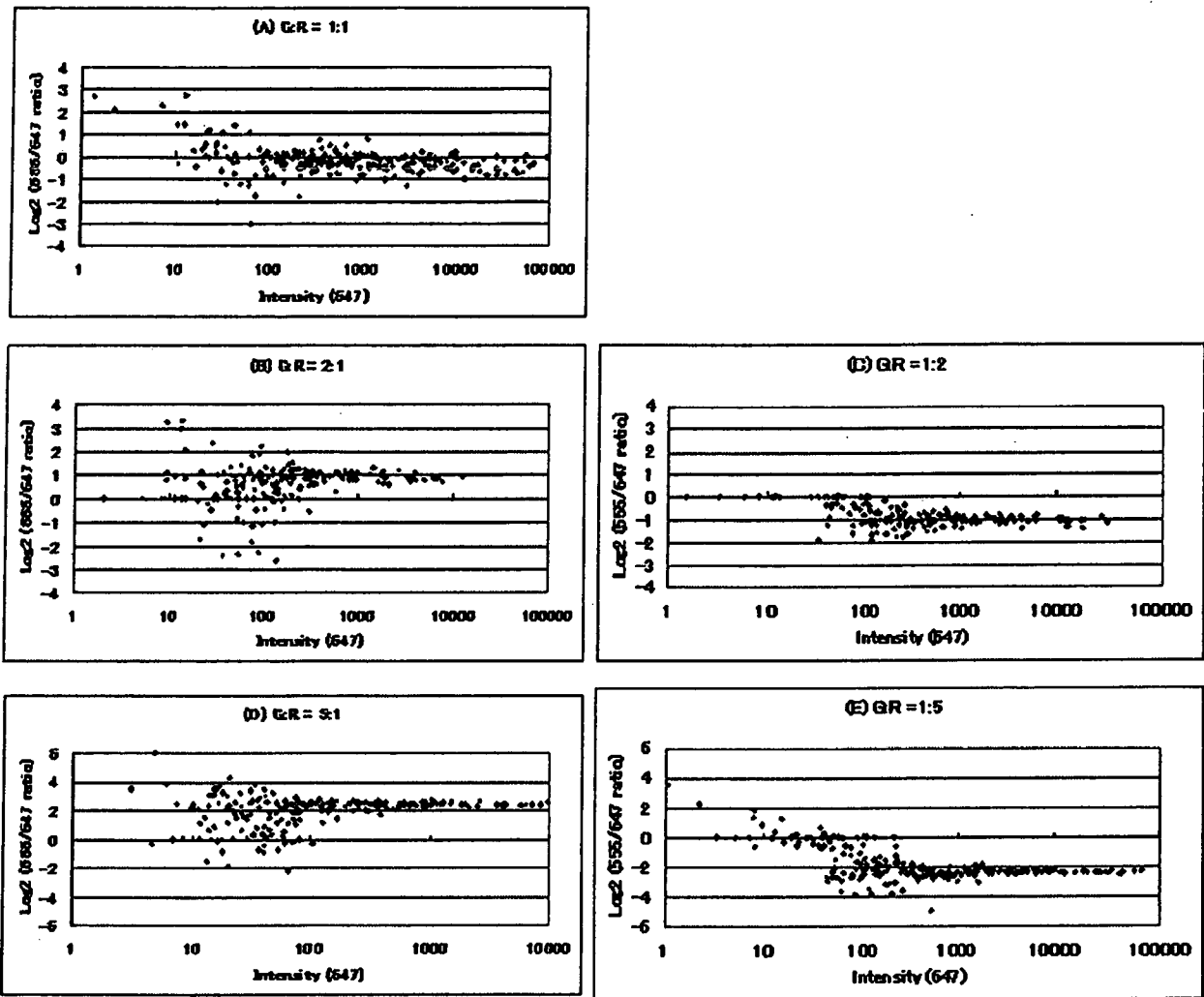


Fig. 1. The validity of the in-house made microarray expressed as Scatter pattern (Experiment I). Results of hybridization with the same control samples labeled with Alexa555 (G) and Alexa647 (R) with the ratio of Alexa555/Alexa647 of A):5/1, B):2/1, C):1/1, D):1/2 and E):1/5. Results show the expected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

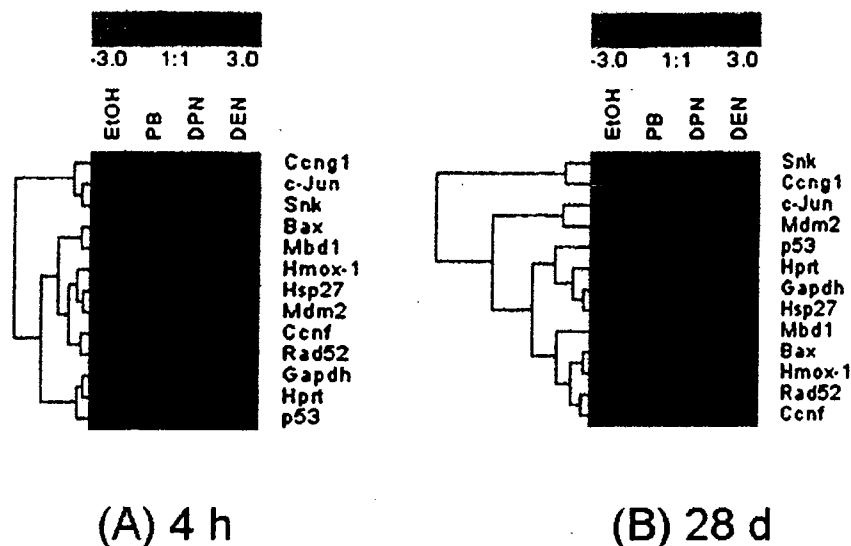


Fig. 2. Gene expression analysis determined with DNA microarray expressed as hierarchical clustering (Experiment II). Eleven differentially expressed genes and 3 expression-unchanged genes are presented. A): at 4 h, B): at 28 days. Livers were from B6C3F1 mice treated with DEN, DPN, PB or EtOH for 4 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 \log_2 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 \pm 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-

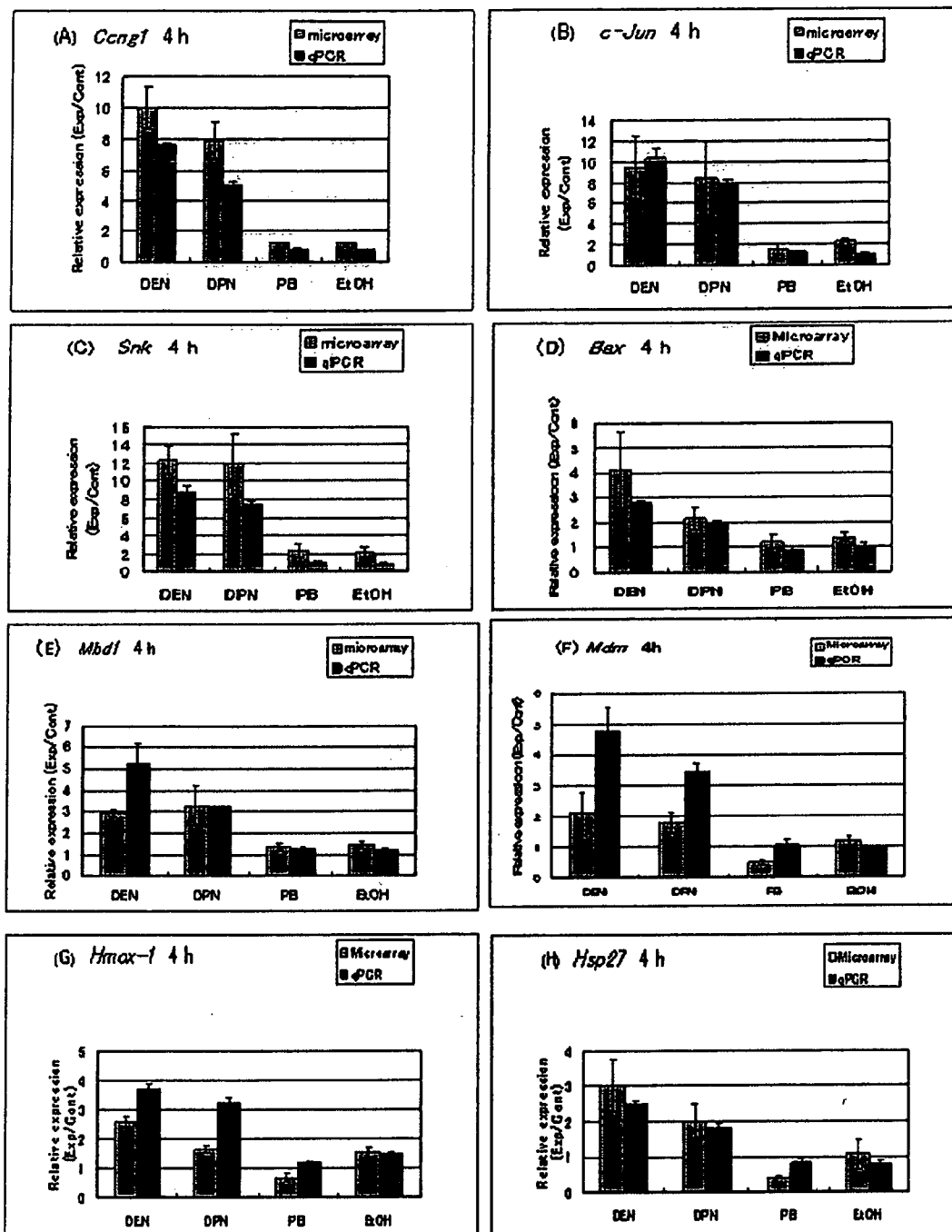


Fig. 3.

ments, *Gapdh* was used as the house-keeping normalized gene for each sample. Figs. 5C to 5J show relative gene expression of *c-Jun*, *Ccng1*, *Hsp27* and *Rad52* normalized to *Gapdh*. Variation among individual gene expression of *c-Jun*, *Ccng1*, *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 *Ccng1*, and around 2-

fold for *Hsp27* and *Rad52*. In the main, these increases returned to about control level 28 days after DEN administration; *Ccng1* 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.

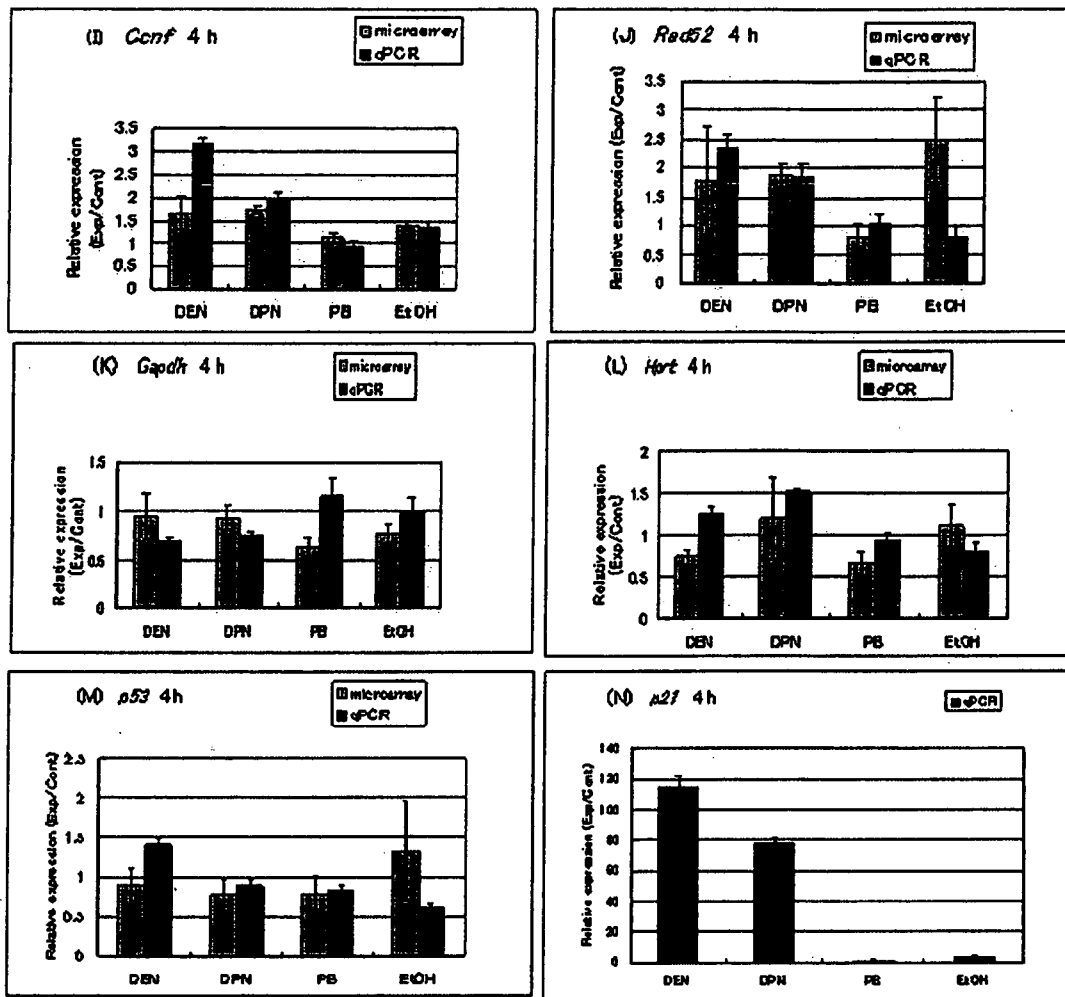


Fig. 3. Verification of DNA microarray results with qPCR 4 h after chemical administration (Experiment II). Results are shown as average and SD (error bar), and expressed as relative expression (experiment group/vehicle control group). Microarray: data are from 4 spots from two microarrays for DEN and 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

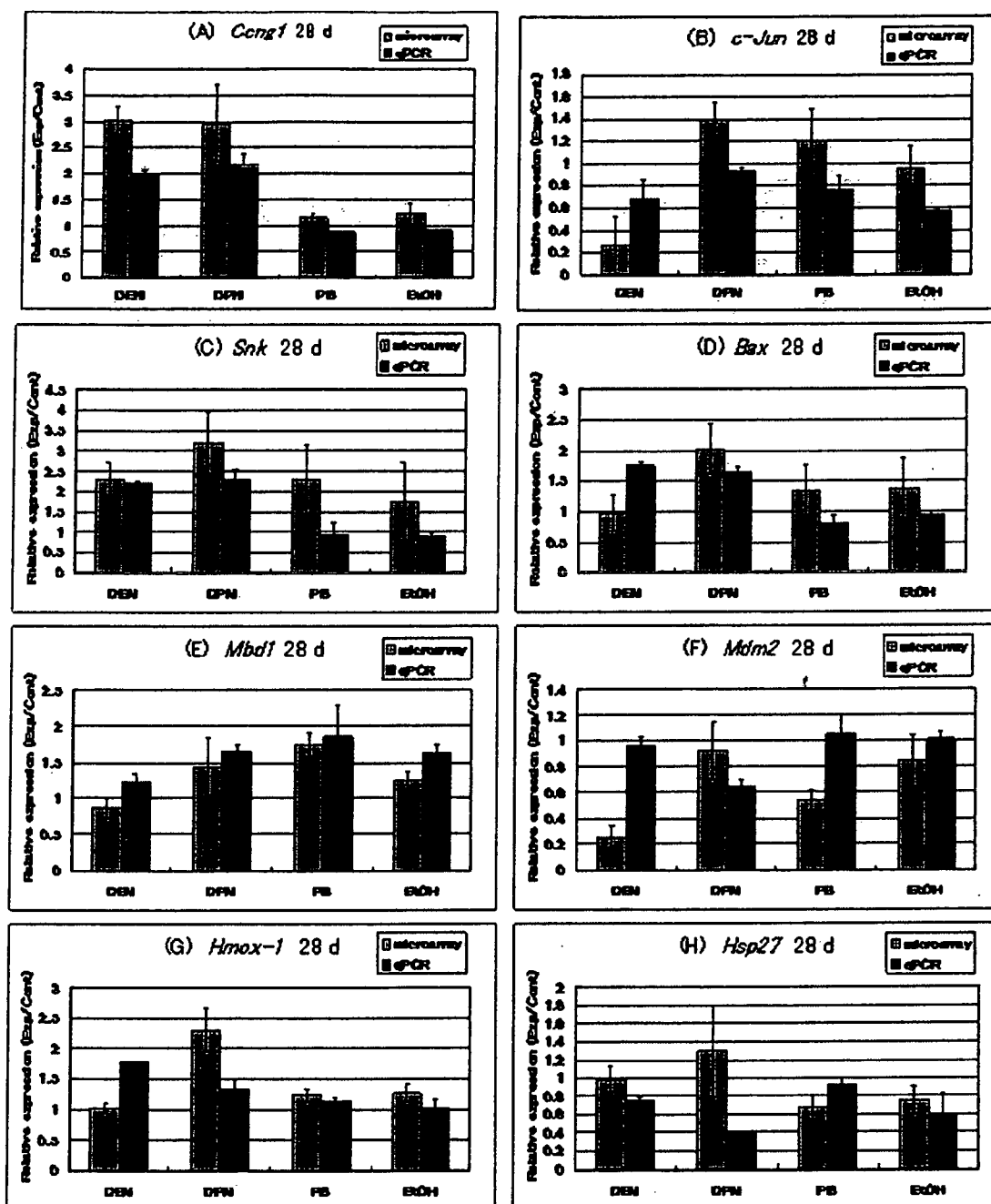


Fig. 4.

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

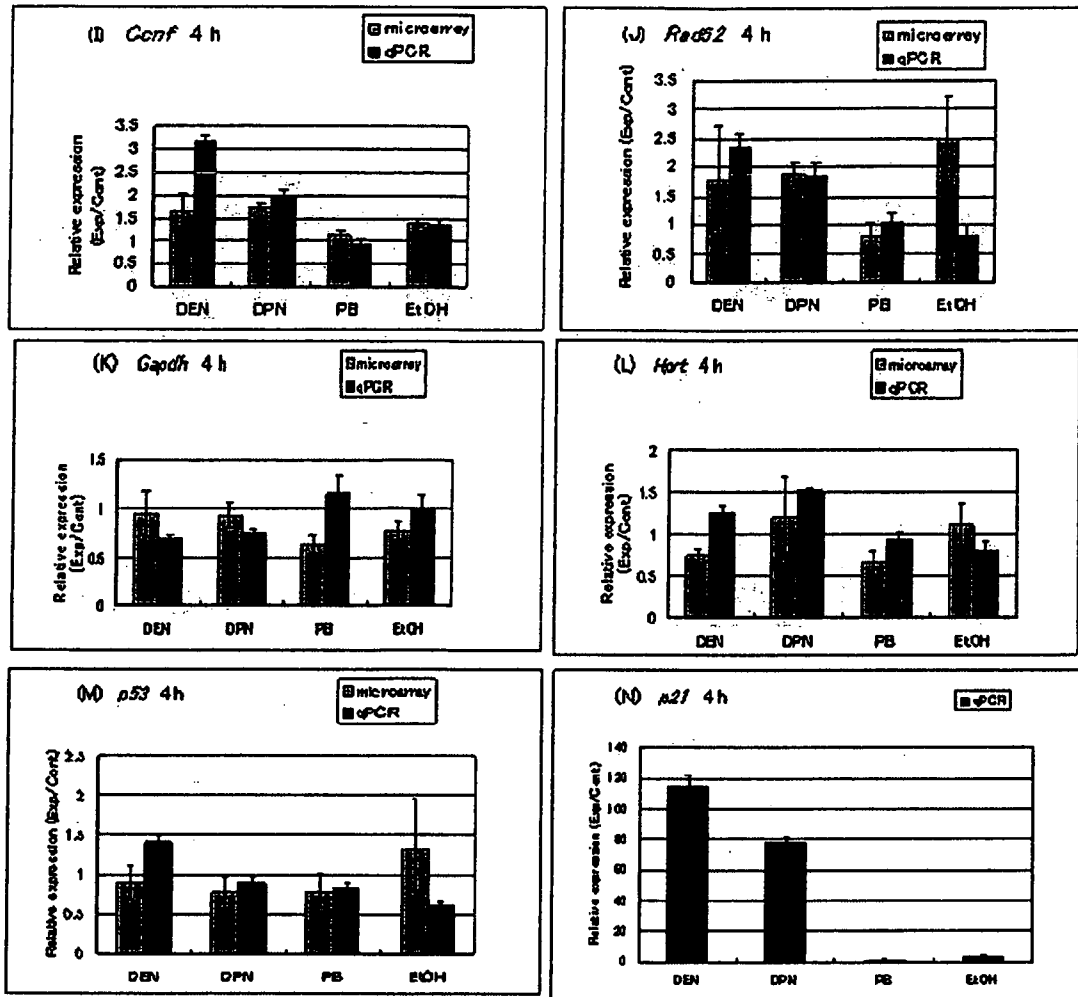


Fig. 3. Verification of DNA microarray results with qPCR 4 h after chemical administration (Experiment II). Results are shown as average and SD (error bar), and expressed as relative expression (experiment group/vehicle control group). Microarray: data are from 4 spots from two microarrays for DEN and 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

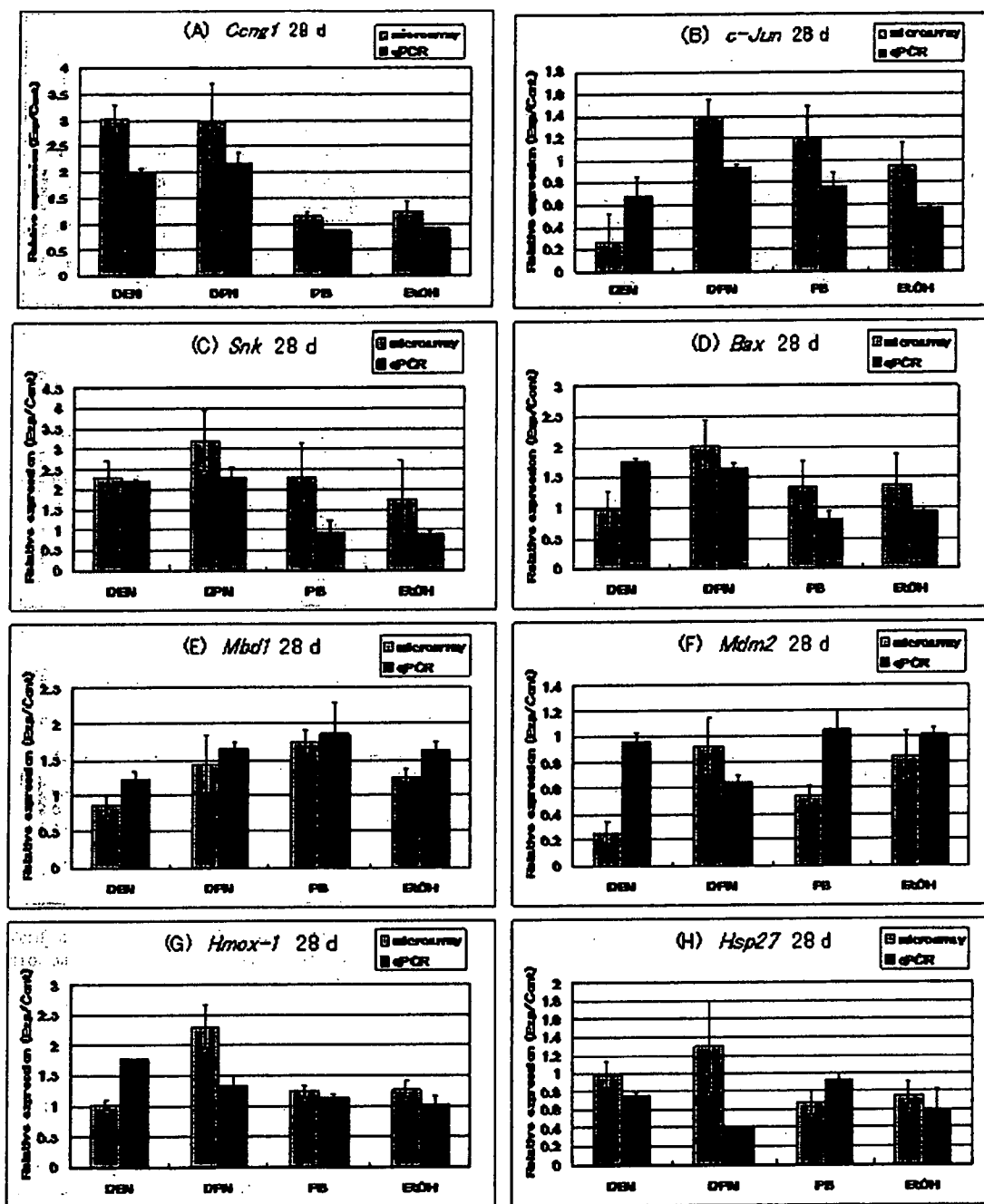


Fig. 4.

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

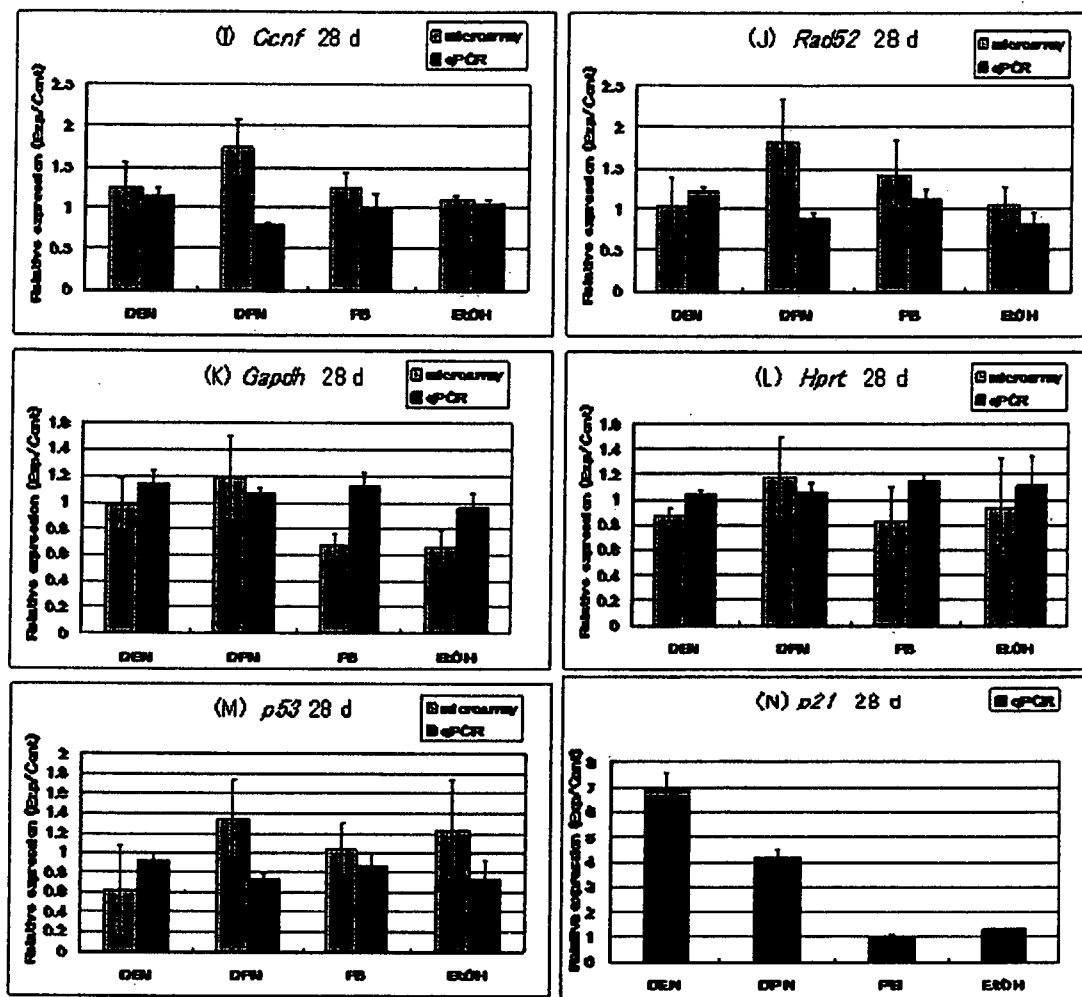


Fig. 4. Verification of DNA microarray results with qPCR 28 days after chemical administration (Experiment II). Results are shown as average and SD (error bar), and expressed as relative expression (experimental group/vehicle control group). Microarray: data are from 4 spots from two microarrays for 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*, *Ccnf*, *c-Jun*, *Rad52*, *Hsp27* and others) in qPCR, indicating good reproducibility. However, fluorescence intensity in a repeat DNA microarray experiment was less reproduc-

ible, 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

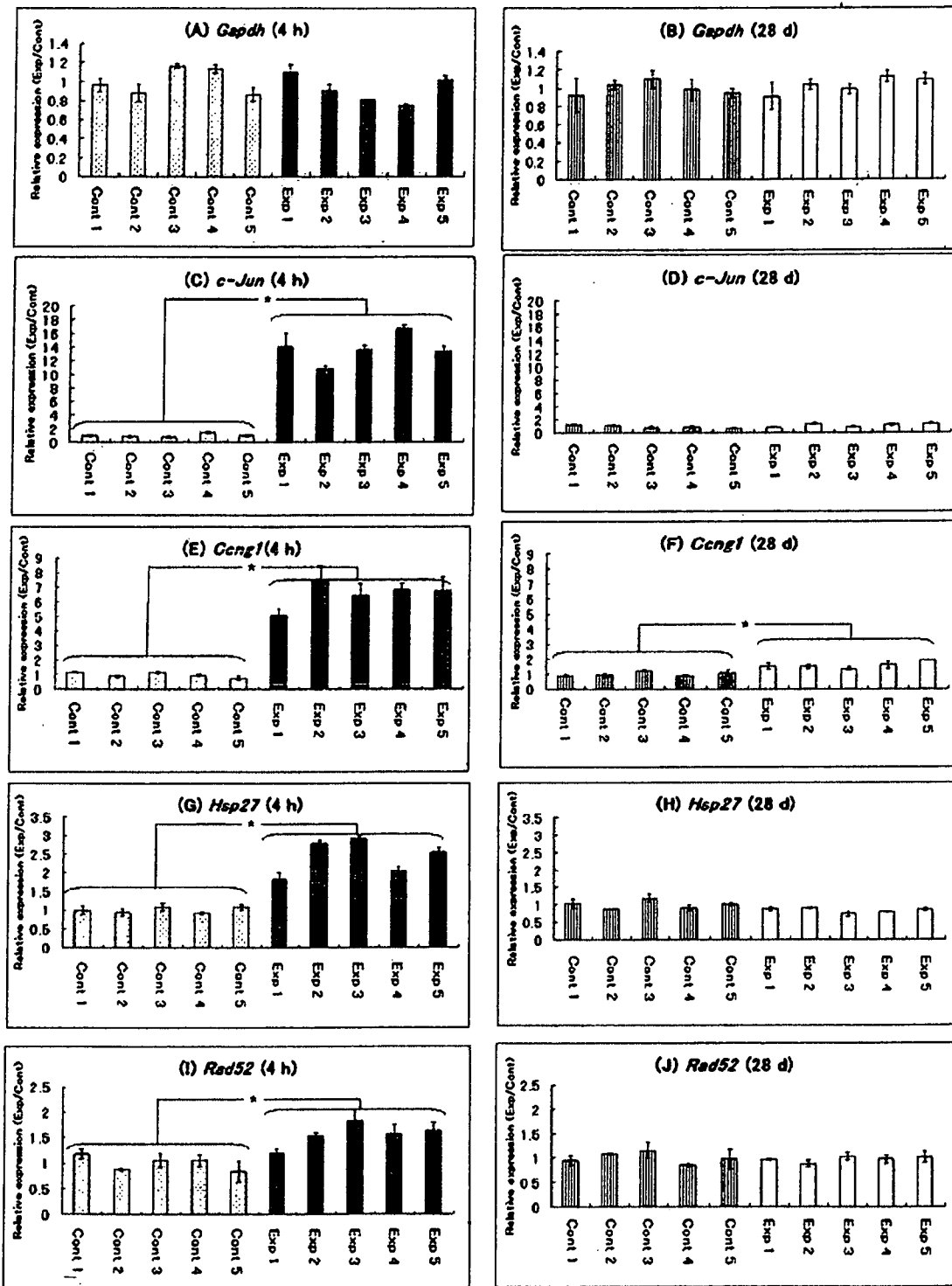


Fig. 5. Variation among individual mice (relative liver gene expression) (Experiment III). DEN (80 mg/kg bw) and saline were given to 9-week-old mice (5 per group), main lobe of liver was dissected individually after 4 h and 28 days and individual gene expression was determined with qPCR. cDNA from individual liver from 5 mice for each group was used as material. Gene expression of *Ccng1*, *c-Jun*, *Rad52* and *Hsp27* was normalized with gene expression of *Gapdh*. Values in *c-Jun* (4 h), *Ccng1* (4 h and 28 d), *Hsp27* (4 h) and *Rad52* (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 \pm SD)				
	<i>Gapdh</i>	<i>c-Jun</i>	<i>Ccng1</i>	<i>Hsp27</i>	<i>Rad52</i>
Saline 4 h	1.00 \pm 0.14	1.00 \pm 0.26	1.00 \pm 0.18	1.00 \pm 0.07	1.00 \pm 0.14
DEN 4 h	0.92 \pm 0.14	13.71 \pm 2.11	6.50 \pm 0.86	2.47 \pm 0.47	1.56 \pm 0.23
Saline 28 d	1.00 \pm 0.07	1.00 \pm 0.24	1.00 \pm 0.16	1.00 \pm 0.12	1.00 \pm 0.12
DEN 28 d	1.03 \pm 0.09	1.15 \pm 0.26	1.57 \pm 0.21	0.83 \pm 0.07	0.97 \pm 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.

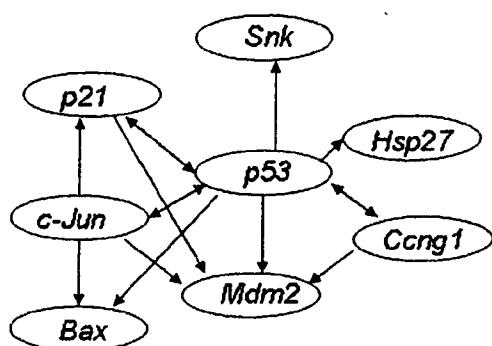


Fig. 6. Suggested gene network of seven *p53* target genes analyzed using Ingenuity Pathways Analysis.

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 EtQH. 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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