Methyleugenol (MEG), a constituent of human food, induces malignant tumors in multiple tissues of rats and mice. Although MEG forms DNA adducts and induces unscheduled DNA synthesis in rat liver, it is negative in many in vitro genetic toxicity assays. In the present study, we evaluated MEG-induced DNA damage in the rat using (1) the alkaline Comet assay, (2) the oxidative Comet assay, and (3) expression profiling of genes associated with DNA damage pathways. Male F344 rats received single oral doses of 400 or 1000 mg/kg body weight (bw) MEG and DNA damage was assessed by the Comet assay in liver, bladder, bone marrow, kidney, and lung 3 h and 24 h later. MEG failed to produce any increase in DNA damage. In addition, rats were given a single oral dose of 2000 mg/kg bw MEG, and Comet assays were performed with liver, bone marrow, and bladder 1, 3, 6, and 8 h later. With one exception (bone marrow at 8 h), no DNA damage was detected. Enzyme-modified Comet assays were conducted in parallel with standard Comet assays in liver. Whereas no MEG-induced DNA damage was detected following formamidopyrimidine DNA glycosylase digestion, digestion with endonuclease III resulted in increases in DNA damage at the 6- and 8-h sampling times. Gene expression analysis on the livers from MEG-exposed rats showed significant reduction in genes associated with DNA repair. The results indicate that MEG induces DNA damage in rat liver and that oxidative DNA damages may be partly responsible for the genotoxicity of MEG in rodents.

Key Words: Comet assay; gene expression; genotoxicity; methyleugenol; methyl methanesulfonate; oxidative DNA damage.

Methyleugenol (MEG) is a colorless to pale yellow, oily liquid with a clove-carnation odor and a bitter taste. It occurs naturally in many essential oils, blackberry essence, bananas, black pepper, and bilberries (NTP, 2000). In addition, MEG is produced synthetically; its uses extend to research laboratories where MEG has been administered as an anesthetic to rodents (Carlini et al., 1983). On its presence in many different herbs and spices, and its widespread use by industry and in food and cosmetic products, it is probable that humans are exposed to MEG on a daily basis. Thus, any health risks associated with exposure to MEG should be carefully evaluated (Smith et al., 2002). The potential carcinogenicity of MEG is of particular concern. For instance, studies conducted by the National Toxicology Program (NTP) in F344/N rats and B6C3F1, mice indicate that chronic oral intake of high doses of MEG results in hepatotoxicity and stomach neoplasms (NTP, 2000). Based on evidence from carcinogenicity studies in multiple species of experimental animals indicating that MEG increases the incidence of malignant tumors, and/or a combination of malignant and benign tumors, at multiple tissue sites, MEG was classified by the NTP as a “reasonably anticipated carcinogen” (NTP, 2005). Studies of metabolic enzyme induction in rodents and humans have demonstrated the conversion of MEG by cytochromes P450 (CYP1A2, CYP2A6, CYP2C9, and perhaps CYP2D6 and CYP2C19) to several metabolites, including the presumed proximal carcinogen, 1′-hydroxymethyleugenol (Chan and Caldwell, 1992; Gardner et al., 1997; Jeurissen et al., 2006). MEG metabolites bind covalently to DNA at the N2-position of guanine and the N4-position of adenine ( Muller et al., 2004), and MEG-DNA adducts have been detected both in vivo and in vitro, with adduct formation being linearly correlated with MEG tumorigenic activity (Gardner et al., 1997; Phillips et al., 1984). It seems possible that the carcinogenicity of MEG may be operating through a genotoxic mode of action; however, the...
existing studies on MEG genotoxicity have yielded inconclusive results. Although MEG induced chromosomal recombination in Saccharomyces cerevisiae (Brennan et al., 1996; Schiestl et al., 1989), it has been negative in most standard in vitro genotoxicity assays, including the Ames test (Brennan et al., 1996; Dorange et al., 1977; Mortelmans et al., 1986; Schiestl et al., 1989; Sekizawa and Shibamoto, 1982). In in vivo studies, mice dosed orally for 14 weeks with MEG had no increase in the frequency of micronucleated normochromatic erythrocytes (NTP, 2000) and repeated dosing with MEG failed to increase liver lacI mutant frequency in female Big Blue mice (Tyrrell et al., 2000). In contrast, high doses of MEG induced unscheduled DNA synthesis (UDS) in hepatocytes derived from male Fischer 344 rats (Chan and Caldwell, 1992) and increased liver lacI mutant frequency in female Big Blue rats dosed similarly to the negative Big Blue mouse assay (Tyrrell et al., 2000). These findings indicate that the contribution of MEG genotoxicity to the carcinogenesis process is not well understood. Understanding the genotoxicity of MEG is important not only for identifying it as a potential genotoxin but also for establishing its cancer mode of action, which will facilitate a better risk characterization for humans.

In the present study, we have exposed male Fischer 344 rats to different doses of MEG and the resulting DNA damage was assessed by the Comet assay. The in vivo Comet assay is capable of detecting the early stages of DNA damage that frequently leads to genotoxicity in various organs (Sasaki et al., 2000). This assay has been reported to be sensitive to genotoxic rodent carcinogens and relatively insensitive to nongenotoxic rodent carcinogens (Sasaki et al., 2000). Partial guidelines for conducting both the in vitro and in vivo Comet assay have been published (Kirkland and Speit, 2008), and a collaborative study has been conducted on 15 compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies (Rothfuss et al., 2010). It is therefore an appropriate assay for investigating the genotoxicity of a compound like MEG to which humans are frequently exposed.

The standard alkaline version of the Comet assay detects DNA strand breaks (SB) and alkali-sensitive sites induced directly by the test agent. An approach that we have used in this present study is to digest the samples with specific DNA repair enzymes prior to analysis for DNA SB. Digestion with lesion-specific endonucleases induces SB that can be detected by the assay; this approach also increases the mechanistic information derived from the assay. The repair enzymes that we have used for this study are formamidopyrimidine DNA glycosylase (Fpg), which primarily detects 8-oxo-7,8-dihydro guanine (8-oxoGua), and endonuclease III (Endo III), which recognizes and breaks DNA at altered pyrimidines (Azqueta et al., 2009; Collins, 2004). In addition to performing the in vivo Comet assay, we used rat DNA damage and repair SuperArrays to assess the expression of DNA damage/repair genes in liver at early times following treatments with MEG. Gene expression analysis may be useful for understanding the pathways involved in the genotoxicity of MEG and for identifying early molecular biomarkers associated with MEG-induced carcinogenicity in rodents.

MATERIALS AND METHODS

Animals. The maintenance, handling, treatment, and sacrifice of animals were approved by the National Center for Toxicological Research (NCTR) Institutional Animal Care and Use Committee. Male F344 rats were obtained from the NCTR breeding colony at 4 weeks of age and housed in rooms maintained at 20°C–24°C, 55–65% humidity, and with a 12-h light-dark cycle. The animals were housed two per cage, and the cages were arranged in close proximity in order to minimize possible effects due to cage placement. All rats were fed NIH-31R Purina Rodent Chow (pellets) and received Millipore-filtered drinking water ad libitum throughout the experiment.

Chemicals/treatments/tissue harvesting. Methyl methanesulfonate (MMS) (CAS 66-27-3) was purchased from Sigma (St Louis, MO), whereas MEG (CAS 95-15-2) was supplied by Elan Chemical Company (Newark, NJ). Methylcellulose (USP/FCC grade) was obtained from Spectrum (Gardena, CA). All other chemicals and reagents were of analytical grade and were obtained from commercial sources. MEG solutions were prepared in 0.5% aqueous methylcellulose, and MMS was dissolved in phosphate-buffered saline, pH 7.4 (PBS).

In the initial experiment, rats received single doses of 400 or 1000 mg/kg bw MEG by gavage, whereas a higher dose of 2000 mg/kg bw MEG was given in the subsequent time-course experiments. The choice of these doses was based on previous studies that evaluated the genotoxicity of estragole, a derivative of MEG (Muller et al., 1994); in addition, Organization for Economic Cooperation and Development guidelines recommend 2000 mg/kg bw as the maximum acute dose for toxicological evaluations conducted in rodents (OECD, 2001) The most likely route of human exposure is oral exposure. Oral gavage was chosen as the most appropriate method of dosing for this study as it offers the advantages of precisely measured doses that can be administered at precise times and because of the unpalatability of the applied doses of MEG in feed. In addition to the test agent treatments, additional rats were treated by gavage with single doses of 100 mg/kg bw MMS or a 0.5% aqueous solution of methylcellulose to serve as positive and vehicle controls, respectively.

In the initial experiment, the rats were euthanized 3 and 24 h after the treatment, whereas in the time-course experiments, the animals were euthanized at 1, 3, 6, and 8 h following the acute treatments. The 3- and 24-h time points were based on general recommendations for conducting the in vivo Comet assay (Tice et al., 2000), whereas the 1, 3, 6, and 8 h time points were chosen to maximize the chances of detecting an early genotoxic response to the MEG treatment. Pharmacokinetic studies indicate that MEG, like the other structurally related allylbenzenes, is rapidly absorbed, metabolized, and excreted. After oral administration of MEG in humans via consumption of gingersnap cookies, the half-life is about 90 min, whereas in rodents the time is shorter (Johnson et al., 2000; Schecter et al., 2004; Smith et al., 2002).

Upon sacrifice, the organs/tissues (liver, kidney, bladder, lung, and bone marrow) were aseptically removed and stored in cold tissue dissociation buffer (Hanks Balanced Salt Solution, 20mM EDTA, and 10% dimethyl sulfoxide) for isolation of single cells as described below. It should be noted that stomach, fore stomach, mammary gland, and sc skin are among the target organs for MEG carcinogenicity (Johnson et al., 2000); however, they were excluded from the analysis because in preliminary studies, cells obtained from these tissues often resulted in hedgehog or ghost cells.

Tissue dissociation. For liver and lungs, cells were dissociated by mincing using sharp scissors and then filtered through a 40-μm cell strainer (Fisher Scientific, Pittsburgh, PA). Kidney and bladder were dissociated by incubation with 0.5 mg/ml Collagenase I (Worthington Biochemical, Lakewood, NJ) at 37°C.
37°C for 1 h, with gentle shaking. Bone marrow cells were isolated by aspiration. For all the samples, cell viability was measured by Trypan Blue dye exclusion and the % cell viability for tissues ranged from 75 to 95% irrespective of the treatment and tissue dissociation method used.

In vivo alkaline Comet assay. The standard alkaline Comet assay was performed using established methods (Singh et al., 1988; Tice et al., 2000), with appropriate modifications. Briefly, 100 µl of the single-cell suspensions derived from different organs were mixed with 900 µl 1% low-melting-point agarose in PBS at 37°C, and 200 µl of this suspension were applied to microscope slides (Fisher Scientific, St Louis, MO) previously coated with 1% agarose. Coverslips were placed on the slides, and the slides were stored at 4°C for 30 min to solidify the agarose. With the coverslips gently removed, the slides were placed in freshly prepared lysis buffer (2.5M NaCl, 0.1M EDTA, 10mM Tris, with 10% dimethyl sulfoxide and 1% Triton X-100 added just before use) and stored at 4°C overnight. The slides then were transferred into a chilled alkaline solution (300mM NaOH, 1mM EDTA, pH 13) and allowed to remain in the solution for 40 min in the dark to unwind DNA. After unwinding, electrophoresis was performed in the same solution at 4°C in the dark for 30 min at 25 V and ~300 mA. The slides then were washed with neutralizing buffer (0.4M Tris, adjusted to pH 7.5 with HCl) 3× for 5 min each to neutralize the remaining alkali and remove detergent; the slides next were fixed with ice-cold ethanol (100%) and dried overnight. Prior to scoring, the slides were stained with SYBR Gold (Invitrogen, Carlsbad, CA; 1:10,000 dilutions in tris-cold ethanol (100%) and dried overnight. Prior to scoring, the slides were stained with SYBR Gold (Invitrogen, Carlsbad, CA; 1:10,000 dilutions in tris-cold EDTA buffer). Two slides were scored from each tissue/treatment/sampling time; 100 cells were selected randomly from each slide and scored using a system consisting of a Nikon 501 fluorescent microscope and Comet IV digital imaging software (Perceptive Instruments, Wiltshire, UK). Percent DNA (% DNA) in tail, defined as the fraction of DNA in the tail divided by the total amount of DNA associated with a cell multiplied by 100, was used as the parameter for DNA damage analysis.

Enzyme-modified Comet assay. For detecting oxidative DNA damage, cells were embedded in agarose on microscope slides and stored in the lysis buffer as described above for the standard assay. After removing the slides from the lysis buffer, they were washed 3× for 5 min each with an enzyme buffer (40mM HEPES, 0.1M KCl, 0.5mM EDTA, 0.2 mg/ml bovine serum albumin, with the solution adjusted to pH 8.0 with KOH). After the last wash, excess liquid was removed, and 200 µl of the following solutions were applied to slides prepared from each treatment group: 200 µl of enzyme buffer alone (reference slides, two slides), 200 µl of enzyme buffer containing Fpg (1:1000, two slides) or 200 µl of enzyme buffer containing Endo III (1:1000, two slides). The slides were kept in a moist box and incubated at 37°C for 45 min for Endo III and reference slides, 30 min for Fpg. After the enzyme treatment, the slides were placed in a horizontal electrophoresis chamber to perform DNA unwinding and electrophoresis as described above for the standard assay. The slides were neutralized, dried, and stained as described for the standard Comet assay.

Gene expression analysis. Gene expression analysis was performed on liver samples from the MEG time-course experiment using the rat DNA damage and repair array (SABiosciences, Frederick, MD). Liver samples were used because liver is a major target organ for tumor induction by MEG in rats and because studies with [3H]-labeled MEG indicate that the liver is highly exposed immediately following oral dosing of rats with MEG (NTP, 2000) and was thus the most likely place to search for a detectable signal.

RNA isolation and reverse transcription. Approximately 30 mg of liver tissue were harvested from rats treated with 2000 mg/kg bw MEG or vehicle control, which were harvested 1, 3, 6, and 8 h after treatment. Tissues were stored in RNA Later at -80°C before processing (Ambion, Austin, TX). The tissues were minced and transferred to lysing matrix D tubes (MP Biomedical, Solon, OH) containing 600 µl of lysis buffer (RLT buffer [Qiagen, Valencia, CA] and 1% β-mercaptoethanol), minced, and homogenized for 40 s using a FastPrep FP120 homogenizer (Thermo Savant, Sugar Land, TX). The liver samples were kept on ice before and after the homogenization step. The homogenates were transferred to clean 1.5 ml tubes and centrifuged at 10,000 × g for 3 min; the supernatants were removed and placed in a new clean tube. An equal volume of 50% ethanol was added to the supernatants and mixed by pipetting. Total RNA was isolated using the AllPrep kit (Qiagen) according to the manufacturer’s instructions. Total RNA was treated with DNase I (Qiagen) before reverse transcription. From each sample, approximately 1.0 µg of RNA was reverse transcribed with random hexamer primers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA); the cDNAs produced were used as a template for quantitative real-time polymerase chain reaction without further purification.

Gene expression profiles were constructed using the RT² profiler PCR array for rat DNA damage and repair signaling pathways (SABiosciences). The expression arrays consisted of 84 genes associated with apoptosis, the cell cycle, and DNA repair pathways, as well as 5 housekeeping genes, a genomic DNA control, 3 reverse transcription controls, and 3 positive PCR controls genes. The reactions were processed using a BioRad CFX C1000 (BioRad, Hercules, CA). The results of the assays were analyzed with the software provided with the instrument. The 2 -ΔΔCt method (Livak and Schmittgen, 2001) was used to calculate the fold-change in the normalized Ct values: 2 − (GOIcntr/GOIexp) − (GOIcntr/GOIcntr), where GOI represents the Ct value for the gene of interest, RG represents the reference gene (housekeeping genes) Ct value, exp represents the experimental MEG-treated animals, and cntr represents vehicle control animals.

Statistical analysis. Oxidative DNA damage calculation was based on the method described in the protocol by Dusinska (2000). Briefly, the control gels (no enzyme treatment) provide an estimate of the background of DNA SB. The enzyme-treated gels reveal SB and oxidized bases (SB + OX). Assuming a linear dose-response while using % DNA in tail or other arbitrary units, subtraction of SB from SB + OX gives a measure of oxidized pyrimidines/ altered purines.

DNA damage measured as % DNA in tail was analyzed as a function of dose or time by one-way ANOVA followed by the Holm-Sidak test to evaluate the differences in DNA damage among groups. Because the standard deviations of the DNA damage tended to increase with the magnitude of the response, a logarithmic transformation was performed before conducting the analyses. A P-value of less than 0.05 was considered statistically significant.

For gene expression, ANOVA with Tukey’s post hoc test was applied for gene-specific as well as time-point comparisons. To identify differentially expressed pathways and to determine functionally related sets of genes that are expressed differently in different experimental groups, MANOVA method (Tsi and Chen, 2009) was used in this study. The level of significance was set at a P-value of ≤ 0.05.

RESULTS

Dose- and Time-Dependent Studies

DNA damage was analyzed in tissues at 3 and 24 h following treatment of rats with 400 or 1000 mg/kg bw MEG. Tissues from rats treated with 100 mg/kg bw MMS were used as positive controls. After dissociating the tissues into single cells, cell viability for all samples, as determined by the Trypan Blue dye exclusion test, was > 75% (data not shown). As illustrated in Figure 1, MMS treatment produced significant increases (p ≤ 0.05) in DNA damage compared with the vehicle control in all tissues examined, namely liver, lung, kidney, bladder, and bone marrow, with the greatest levels of DNA damage noted in bladder and kidney. In general, the responses were higher at 3 h after treatment than at 24 h, suggesting that there was a time-dependent reduction in DNA damage, which may also suggest a time-dependent DNA damage
repair (Fig. 1A). For rats exposed to 400 or 1000 mg/kg bw MEG, however, no statistically significant differences in DNA damage were observed compared with responses in the vehicle control group. The DNA damage caused by MEG exposure was either equal to or slightly but not significantly higher than control values, and the responses were not affected appreciably by the sampling times (Figs. 1B and 1C).

A second set of experiments was conducted that measured DNA damage using 2000 mg/kg bw, the maximum test agent dose recommended for acute treatments in toxicological studies, and using different sampling times after the treatment to maximize the possibility of detecting an early, transient response for DNA damage induction. In these studies, rats were treated with either a single dose of the vehicle, 2000 mg/kg
bw MEG, or 100 mg/kg bw MMS (positive control) and sacrificed at 1, 3, 6, and 8 h following the treatment; liver, bone marrow, and bladder were collected for analysis. As in the first study, MMS treatment resulted in a significant increase in DNA damage compared with vehicle-treated control rats for all tissues examined. A time-related decrease in DNA damage over the 8 h of sampling was evident only in the liver (Fig. 2A). In rats exposed to MEG, DNA damage was similar to the control at all time points and for all tissues. The only exception was in the bone marrow, where a slight but statistically significant ($p < 0.05$) increase in DNA damage was observed at the 8-h sampling time (Figs. 2A–C). Due to the high turn over rate of bone marrow cells, we would prefer to interpret this increase as a coincidence instead of a real increase in DNA damage.4

**Oxidative DNA Damage**

Fpg- and Endo III–modified Comet assays were performed in parallel with the standard Comet assay with liver samples. No difference in the frequency of Fpg-sensitive sites was detected between samples from MEG-treated and vehicle control rats (Fig. 3A). In contrast, Endo III digestion, which cleaves DNA at oxidized pyrimidines, resulted in higher levels of % DNA in tail for liver samples from MEG-treated rats compared with liver from control rats ($p < 0.05$) at both the 6- and 8-h sampling times (Fig. 3B). Due to the fact that Endo III recognizes and breaks DNA at altered pyrimidines (Azqueta et al., 2009; Collins, 2004), we suspected MEG or its metabolite induced some changes at pyrimidines which were recognized and cut into DNA strand break by Endo III.

**Gene Expression Analysis**

Liver samples from the time-course study also were analyzed to determine gene expression changes following short-term exposure to MEG. Out of the 84 genes investigated, 9 were differentially expressed at the different sampling times post-MEG treatment (Brcal, Rad1, and Rad3 genes were repeatedly altered), with a majority of the genes (Brcal, Rad1, Rad9, Rad52, Mpg, Oggl1, Pms1, Pms2, and Pold3) being involved in DNA base excision repair pathways in rats. The relative fold-change in the treated samples compared with controls was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Fig 4). A decrease in expression of most genes was observed 3 h after MEG treatment. This decrease or suppression was unexpected and may suggest that inhibition of DNA damage repair may be one of the potential mechanisms for MEG-induced genotoxicity and carcinogenicity.

**DISCUSSION**

The present study used the Comet assay and pathway-focused gene expression profiling to investigate the genotoxicity of MEG in the rat. Although MEG is considered a potent rodent carcinogen that induces tumors at multiple sites (NTP, 2000), previous studies on its genotoxicity have yielded inconclusive results. Thus, its cancer mode of action has not been clearly defined. With one exception (bone marrow, 8 h following a dose of 2000 mg/kg bw MEG), exposure of male F344 rats to single oral doses of up to 2000 mg/kg bw MEG failed to produce DNA damage that could be detected by the standard alkaline Comet assay. These negative responses contrasted with the strong positive responses produced by MMS; responses that were consistent with earlier results using this compound (Oshida et al., 2008; Sasaki et al., 2000). These generally negative responses with MEG also contrast with positive responses for DNA adduct formation, UDS, and gene mutation in MEG-treated rats (Burkey et al., 2000; Chan and Caldwell, 1992; Gardner et al., 1997; Howes et al., 1990; NTP, 2000; Phillips et al., 1984) and the determination that it is carcinogenic in multiple tissues in rats and mice (NTP, 2000).

DNA adducts formation and UDS induction reflect early DNA damage which is mechanistically important in mutation and chemical carcinogenesis. Although DNA damage and mutation induction are fundamentally distinct, DNA damage often causes errors during repair or replication, and these errors constitute major sources of mutation. Thus, detection of early endpoints such as SB, DNA adducts, and UDS is important because they could signal the beginning of the carcinogenesis process. The existence of a linear relationship between MEG-induced DNA adducts/UDS and tumor response in rodents (Howes et al., 1990; Waddell et al., 2004) is consistent with this possibility. In the case of our negative findings with the standard alkaline Comet assay, we speculate that the repair synthesis measured by the UDS assays either did not occur in vivo or occurred in a way that did not generate SB detectable by our assay. An important difference between the two assays is that UDS measures all repair integrated over the hours or days to which the cells are exposed to the radiolabeled marker whereas the Comet assay measures unrepaired damage a discrete point in time. Although the alkaline Comet assay is likely to pick up DNA damages associated with single or double-strand breaks, abasic sites, the failure to detect MEG-induced DNA damage suggests that the damages if initiated by MEG took place outside of the time frame of our observations or occurred at too slow a pace. The observation from our pathway-focused gene expression profiling that DNA damage and repair pathways were perturbed by MEG exposure (see below) is consistent with the hypothesis that DNA damage occurred, as observed in in vitro UDS experiments, but that the damage repair was suppressed such that without the repair the damage was minor and not detectable by the in vivo Comet assay.

The types of DNA damages (such as oxidative DNA damage) being detected by alkaline Comet assay can be expanded by incubating the lysed cells with lesion-specific endonucleases that recognize specific damaged bases and create breaks (Collins, 2000; Tice et al., 2000). In the present
Fpg- and Endo III–modified Comet assays were performed in parallel with the alkaline Comet assay. These two endonucleases are used in the Comet assay to detect oxidative DNA damage: Endo III detects damaged pyrimidine bases, whereas Fpg recognizes oxidized purines, including 8-oxoGua. In our study, digestion with Endo III resulted in significantly increased levels of DNA damage at 6 and 8 h after exposure to 2000 mg/kg bw MEG; digestion with Fpg only resulted in a slight (nonsignificant) increase in DNA damage and only at the 1-h sampling time (Figs. 3A and 3B). However,

![Graphs showing DNA damage in different tissues](https://example.com/graphs.png)

**FIG. 2.** Time-dependent study of MEG-induced DNA damage in male F344 rats. Rats were treated with 100 mg/kg bw MMS or 2000 mg/kg bw MEG by gavage. Rats were sacrificed 1, 3, 6 and 8 h after dosing. Rats exposed to the MEG vehicle were used as the negative control. Single-cell suspensions were prepared from liver (A), bone marrow (B), and bladder (C) immediately after animal sacrificing. For each tissue, 100 cells were analyzed using the Perceptive Assay IV Image Analysis System to determine the extent of DNA damage expressed as % DNA in tail. Each group included four rats. Error bars represent the SD; **p** < 0.05 compared with corresponding control.
the fact that this weak response found for Fpg decreased to control levels at the later sampling times could be indicative of this oxidative DNA damage that was repaired (Fig. 3A). These findings suggest that these two lesion-specific endonucleases have different sensitivity toward MEG-induced DNA alkylation and that MEG predominantly induces DNA lesions sensitive to Endo III digestion in the rat liver. Although preliminary, this study is the first to suggest that MEG can induce oxidative DNA damage and that this may be one of its carcinogenic modes of action.

The metabolic activation of MEG and its congeners, safrole and estragole, to ultimate carcinogens is known to proceed in several steps (Boberg et al., 1983). Among these steps is cytochrome P450-catalyzed oxidation of the reactive allyl side chain to the 1′-hydroxy derivative, esterification of the 1′-hydroxyl group, mainly by the action of sulfotransferases, formation of electrophilic carbocations, and reaction of the carbocations with exocyclic N-atoms of DNA purines (NTP, 1998). Early work on safrole and related allylbenzenes, including MEG, suggested that DNA adducts were formed not directly by the 1′-hydroxy derivatives but after sulfotransferase-mediated formation of sulfate esters (Wislocki et al., 1976). Conjugation with sulfate is generally considered detoxification because it targets the metabolite for rapid elimination into urine. Thus, it is not without irony that the sulfation might result in a leaving group that accelerates formation of mutagenic DNA adducts via electrophilic attack. The lack of genetic toxicity in short-term in vitro assays may thus be explained by the absence of cofactors that enable sulfotransferase activity. In addition, attempts to circumvent this deficiency also face significant technical obstacles such as an apparent inability of the highly unstable sulfate metabolites to penetrate cell membranes in these in vitro systems (Glatt et al., 1998). There is an indication that eugenol, a derivative of MEG, can induce oxidative DNA damage, possibly through an θ-demethylation reaction (Sakano et al., 2004). The existence of

FIG. 3. Modified Comet assay to determine oxidative DNA damage. Male F344 rats were treated with 2000 mg/kg bw MEG by gavage. Rats were sacrificed 1, 3, 6, and 8 h after dosing. Vehicle-treated rats were used as the negative control. The Fpg-modified (A) and Endo III–modified (B) Comet assay was performed for the liver samples; 100 cells were analyzed using the Perceptive Assay IV Image Analysis System to determine the extent of DNA damage expressed as % DNA in tail. Each group included four rats. Error bars represent the SD; ‘*’ represents \( p < 0.05 \) compared with corresponding control.

FIG. 4. Pathway-focused expression array analysis of DNA damage and repair genes in liver of MEG-treated rats. Fold-change in gene expression was measured in a blocked manner at 1, 3, 6, and 8 h after treatment with 2000 mg/kg bw MEG. Expression of all nine genes (DNA damage–binding genes—Brca1 and Rad1; base excision repair genes—Mpg and Ogg1; double-strand break repair gene—Rad52; mismatch repair genes—Pms1, Pms2, and Polδ3; and cell cycle check point gene—Rad9) was significantly (\( p < 0.05 \)) downregulated at 3 h after MEG treatment. Each group included four rats. Error bars represent the SD.
The absence of a detectable response in liver and other tissues that are targets for MEG-induced malignancy was unexpected. The presumptive mechanism of MEG genetic toxicity is via formation of the DNA adducts known to be formed in Fischer rats at similar doses. Comet does not detect mutations or adducts directly but instead can detect the SB that occur during repair of DNA adducts. That fact that such breaks were not detected could mean that no adducts were formed, that adducts were formed but not repaired, or were formed and repaired but strand breakage occurred at a rate below that detected by the assay. The first of these is less likely than the other two. Further work will be needed to distinguish among these possibilities. Such work will be important not only to investigate the risk of genetic damage from a substance to which there is widespread human exposure but also to investigate the sensitivity of comet as a screen for genotoxic compounds. The outcome of this analysis is relevant to international interest in using comet in regulatory evaluations for genetic toxicity.

In summary, these results indicate that exposure of MEG to male F344 rats at doses that produce tumors in rodents failed to induce DNA damage beyond control levels as measured by the standard alkaline Comet assay. On the other hand, Endo III–modified in vivo Comet assay designed for the detection of oxidative DNA damage at pyrimidines showed a significant increase in DNA damage 6 and 8 h following MEG exposure. Additionally, analysis of gene expression indicated that genes associated with DNA damage and repair in the liver were suppressed following exposure to MEG. Taken together, the results indicate that MEG exposure is associated with suppression of DNA repair gene expression and the induction of oxidative DNA damage which may be at least partially responsible for MEG-induced genotoxicity in rodents. Further studies specifically analyzing a free radical pathway are necessary to confirm the genotoxic mode of action of MEG in rodent carcinogenesis.

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