Hsia-lien Lin, Elizabeth S. Roberts and Paul F. Hollenberg

Department of Pharmacology, University of Michigan, 1150 West Medical Center Drive, Ann Arbor, MI 48109–0632, USA

*To whom correspondence should be addressed
Email: phollen@umich.edu

GM0637, a human fibroblast cell line, was transfected with pCMV2E1, an expression vector containing the full length cDNA for rat cytochrome P450 2E1 (P450 2E1), and with pCMVneo, which contained vector alone, and the selected clones were designated GM2E1 and GMneo, respectively. Western blot analysis showed that GM2E1, but not GMneo, expressed a protein that reacted with anti-human P450 2E1 antibody. The 7-ethoxycoumarin O-deethylase, p-nitrophenol hydroxylase, and N-nitrosodimethylamine (NDMA) demethylase activities of the P450 in these cells were measured in monolayer cell cultures without preparing microsomes. Exposure of the GM2E1 cells to NDMA for 4 days caused severe decreases in cell viability, as determined by crystal violet uptake, and showed a sigmoidal dose–response curve with a median lethal dose of 17 μM. In contrast, the viability of GMneo cells was not altered by NDMA even at concentrations up to 10 mM. Time- and concentration-dependent methylation of DNA, RNA, and protein by [14C]NDMA was only observed in cells expressing P450 2E1. Inhibitors of P450 2E1 activity such as ethanol, 4-methylpyrazole, and isoniazid caused a 90% decrease in the methylation of cellular macromolecules and also completely protected the cells against NDMA-mediated toxicity. The cytoxicity due to exposure to NDMA was partially inhibited by antioxidants such as N-acetylcysteine, ascorbic acid, butylated hydroxyanisole and N-v-buty1-α-phenyl nitrotrite but was not potentiated upon glutathione depletion. These results document the ability of rat P450 2E1 to metabolize NDMA to toxic reactive intermediates and demonstrate that this cell line provides a useful model for studying the mechanisms of metabolism-mediated toxicity and carcinogenesis.

Introduction

Cytochrome P450 2E1 (P450 2E1*), one of the P450 isofoms, is considered to be a major catalyst responsible for the metabolism of many low molecular weight carcinogens and potentially toxic chemicals including ethanol, nitrosamines, halogenated alkanes and aromatic compounds (1,2). P450 2E1 can be induced by ethanol resulting in enhanced susceptibility to the hepatic damage caused by these compounds (3–7).

N-Nitrosodimethylamine (NDMA), the simplest and most widely occurring nitrosamine, has been shown to be an acute hepatotoxin and a potent carcinogen in many animal species and humans (8–10). In experiments with human liver microsomes, it has been demonstrated that the major enzyme responsible for the oxidative demethylation of NDMA at very low concentrations is P450 2E1 (11). Experiments using P450 2E1 in a reconstituted system containing purified NADPH-cytochrome P450 reductase and lipid have shown that P450 2E1 is an efficient catalyst of NDMA metabolism at low concentrations of the carcinogen (12,13).

The hepatotoxic and genotoxic effects of NDMA are dependent on metabolic activation by P450 2E1 (14). The key step is believed to be the hydroxylation of the α-carbon atom to an unstable intermediate, which decomposes to formaldehyde and an electrophilic methylating agent, the diazonium ion. The methylating agent has been implicated as the primary toxic metabolite in the liver and the methylated macromolecules were found in both animal and human target tissues (7,15–18). Possible mechanisms of NDMA hepatotoxicity such as covalent binding, oxidative stress, glutathione (GSH) depletion, lipid peroxidation, and the disruption of calcium homeostasis have been reviewed by Archer et al. (19). In short, despite several decades of research examining the metabolism of NDMA and its relation to hepatotoxicity and carcinogenicity, relatively little is known about the molecular mechanisms involved in its toxicity.

Recently, human P450 2E1 has been stably expressed in several mammalian cells for the characterization of catalytic activity and some cytotoxicity studies (20–26). In this study, we describe the stable expression of rat P450 2E1 in GM0637, a human fibroblast cell line. We have characterized the in situ metabolism of 7-ethoxycoumarin (7EC), p-nitrophenol (PnP) and NDMA, and studied NDMA-mediated cytotoxicity in these P450 2E1 expressing cells. The possible mechanisms of cytotoxicity of NDMA were evaluated by the addition of P450 2E1 inhibitors, antioxidants or GSH depleting agents and by measuring the methylation of cellular macromolecules.

Materials and methods

Chemicals

Restriction enzymes, DNA ligase, media for tissue culture, fetal calf serum and G418 were from Life Technologies, Inc. (Gaithersburg, MD). NDMA (99% pure), acetaminophen, N-acetylcycteine, (±)-α-tocopherol (vitamin E), PnP, and 4-nitrocatechol were purchased from Sigma Chemical Co. (St Louis, MO). 7EC and 7-hydroxy coumarin were purchased from Aldrich Chemical Co. (Milwaukee, WI). [14C]NDMA (55 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO). All other chemicals and solvents used were of the highest purity available from commercial sources.

Construction of the rat P450 2E1 expression vector

The 1.6 kb full length rat P450 2E1 cDNA cloned into pBluescript II KS(+) vector, designated pBS2E1, was obtained from Dr Xiao Dong Lu (University of Michigan). For expression studies, we used a pCMVneo plasmid containing the cytomegalovirus promoter and the gene for neomycin phosphotransferase.
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(kindly provided by Dr Michael Uhler, University of Michigan). To generate pCMV2E1 constructs, the Not I-Xho I fragment from pBS2E1 was inserted into a unique Bgl II restriction site immediately 3’ to the CMV promoter. The proper orientation of the P450 2E1 cDNA with respect to the CMV promoter was confirmed by restriction enzyme analysis.

Cell culture and transfection

SV40 transformed human skin fibroblasts (GM0637) were obtained from the NIGMS Human Genetic Mutant Cell Repository and maintained in α-MEM containing 10% fetal calf serum and 10 mM HEPES, pH 7.2, in a humidified incubator at 37°C with a 5% CO2 atmosphere. The plasmids, pCMVneo and pCMV2E1, were transfected separately into the GM0637 cells (0.5 × 10⁶ cells/100-mm dish) with calcium phosphate–DNA co-precipitation technique (27,28). Two days later, the cells from each 100-mm dish were subcultured into ten 100-mm dishes and incubated with fresh medium containing 400 µg G418/ml. Two weeks later, G418-resistant colonies were picked and transferred to single wells in a 24-well plate for propagation. The cells from a single well were expanded in a six-well plate and grown to ~80% confluence. P450 2E1 expression was screened by monitoring 7EC O-deethylase activity (21,29). The colonies with the highest 7EC activity were grown to large scale cultures for further investigation. The selected clones, which were successfully transfected with pCMVneo and pCMV2E1, were designated GMneo and GM2E1, respectively.

Assay for 7EC O-deethylase activity

To assay for metabolism of 7EC in situ, both GMneo and GM2E1 cells were seeded in 60-mm dishes (1–2 × 10⁶ cells/100-mm dish) 1 day prior to the assay (21). The culture medium was then replaced with 1 ml of Opti-MEM I (Life Technologies, Gaithersburg, MD) without phenol red and without calf serum (assay medium). The assay was initiated by adding 7EC to the intact cell cultures. After incubation for the times indicated, the medium was harvested and centrifuged at 3000 g for 5 min. The supernatants were mixed with 1/5 volume of 2 M glycine–NaOH buffer, pH 10.3, and the 7EC O-deethylase activity was evaluated by measuring the fluorescence (390 nm excitation, 440 nm emission) of the 7-hydroxycoumarin on a SLM Amicon spectrofluorometer (29). A standard curve was generated by adding known amounts of 7-hydroxycoumarin to the assay medium and assaying as described above.

Assay for PNP hydroxylase activity

The assay was adapted for in situ cell culture by modification of previously described methods (23,30). Cells were prepared as described for the determination of 7EC O-deethylase activity except that the reactions were initiated by adding PNP to the cell cultures. After incubation for the times indicated, media were harvested and centrifuged at 3000 g for 5 min. The supernatants were mixed with 1/10 volume of 10 N NaOH and centrifuged again to sediment undissolved particles. The absorbances of the clarified supernatants were measured at 510 nm, and the amount of product formed was quantitated using the calibration curve generated with 7-hydroxycoumarin.

Assay for glutathione (GSH) content

Cells were plated at a density of 3 × 10⁶ cells/25-cm² flask 1 day prior to the assay. The reactions were initiated by adding GSH and 10 mM semicarbazide–HCl to the assay medium (31,32). After incubation for the times indicated in the presence or absence of NMDA, the media were harvested and centrifuged at 3000 g for 5 min. The supernatants, which contained the semicarbazone derivative, were mixed with 1/10 volume of 50% ZnSO₄, neutralized with 1/10 volume of saturated Ba(OH)₂ in capped microcentrifuge tubes, and then centrifuged to pellet the precipitated protein. The resulting supernatants were mixed with Nash reagent in a 4:1 ratio (33). The mixtures were incubated at 50°C for 30 min, and the absorbance at 412 nm was measured (34). Standard curves were prepared by adding known amounts of formaldehyde to the assay medium and assaying as described above.

Western blot analysis and immuno-quantitation of P450 2E1

Cell pellets were disrupted by three cycles of freezing and thawing and then homogenized in 100 mM potassium phosphate buffer, pH 7.4, containing 150 mM KC1, 1 mM EDTA and 250 µM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 10 000 g for 30 min, and the resulting supernatant fraction was centrifuged at 100 000 g for 30 min. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol and 20% glycerol. Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL), with bovine serum albumin as the standard. Proteins were separated on a 10% SDS-polyacrylamide gel prepared according to Laemmli (35) and electrophoretically transferred to a nitrocellulose membrane. Non-specific binding sites on the membrane were blocked with 1% BSA and then the membranes were incubated with the appropriate primary antibody. The membranes were washed with a modified buffer containing 0.1% Tween 20 (Oxford Biomedical Research, Oxford, MI) at a 1:5000 dilution for 18 h. After washing, the blots were incubated with rabbit anti-goat horseradish peroxidase conjugate (Bio-Rad, Hercules, CA) at a 1:3000 dilution and the immunoreactive bands were developed using the enhanced chemiluminescence (ECL) detection method (Amersham, Arlington Heights, IL). Microsomal P450 2E1 content was determined using purified bovine P450 2E1 kindly provided by Dr Minor J.Coon (University of Michigan) as a standard. The ECL films were scanned using a Scan Jet IIC (Hewlett-Packard, Palo Alto, CA) desktop scanner and analyzed using the IMAGE QUANT program (Molecular Dynamics, Sunnyvale, CA).

Cytotoxicity assays

To test for cytotoxicity, 2 × 10⁴ cells were seeded per well in 12-well plates and cultured in G418-free medium. On the following day, NMDA (0.001–10 mM) and other agents being tested for cytotoxicity were added to the cells and incubated for 1–4 days. The media were removed at the end of incubation and the cells that attached to the plates were rinsed with PBS, fixed with 4% formaldehyde for 1 h, and stained with 0.1% crystal violet for 20 min. The stained cells were washed with water and air-dried. Crystal violet was extracted from the stained cells with 5% SDS, and the intensities were quantitated spectrophotometrically at 550 nm (36). Four replicate wells were monitored for each concentration of NMDA, and the relative survival (% of control) was determined using an untreated set of cells as control (100% survival). For investigation of the possible cytotoxic effects of NMDA on GM2E1 cells, 1000 cells were plated in 6-well plates with a density of 1 × 10⁵ cells in medium alone or in the presence of 1 mM NMDA for 4 days. Cells attached on plates were photographed using a Leitz-Orthoplan phase contrast microscope and Leitz-Orthomatt camera (Leitz, Rockleigh, NJ).

Methylation of cellular macromolecules by [14C]NMDA

Cells were subcultured in 60-mm dishes at a density of 2 × 10⁶ cells per dish. The next day, the medium was replaced with 1 ml of assay medium containing [14C]NMDA. After the indicated times of incubation, the medium was removed, and the cells were lysed by adding 1 ml of TRIZol Reagent (Life Technologies, Gaithersburg, MD) to isolate and separate total DNA, RNA and protein (37). The radioactivity associated with each of these cellular macromolecules was determined by liquid scintillation counting. The methylation levels were calculated from the radioactivity incorporated into the macromolecules, specific activity of [14C]NMDA, and the concentrations of DNA, RNA and protein. The purity of the DNA and RNA samples was verified by monitoring the A260/A280 ratio.

Assay for glutathione (GSH) content

Cell pellets (3 × 10⁵ cells) were resuspended in 0.5 ml phosphate-buffered saline and 50 µl of a 1.5 mM γ-Glu-Glu solution in 0.3% perchloric acid was added as an internal standard. Intracellular GSH was measured using the technique described by Faris and Reed (38). The dinitrophenyl derivatives of amino acids were separated on an Econosphere NH₂ column (5 × 4.6 mm) using reverse-phase HPLC and were detected by monitoring the absorbance at 365 nm. The solvent system consisted of buffer A, 80% methanol and buffer B, 0.5 M sodium acetate in 64% methanol, at a flow rate of 1.5 ml/min. The gradient conditions were 10% B for 5 min followed by a linear gradient to 99% B over 10 min. The mobile phase was held at 99% B and maintained at this final concentration. The gradient was then decreased to 10% B over 4 min and held at 10% B for 5 min.

Data analysis

The dose–response curves for NMDA-mediated cytotoxicity were determined by non-linear regression using the computer program GRAPH PAD. Results are the mean ± SD and statistical evaluations were based on the unpaired, two-tailed Student’s t-test.

Results

Selection of clones expressing P450 2E1 and in situ catalytic activity

Thirty-two independently isolated G418-resistant colonies were screened for P450 2E1 expression by measuring the in situ 7EC O-deethylase activity. This simple and sensitive assay only requires 1 × 10⁶ cells cultivated in six-well plates to detect the 7EC O-deethylase activity directly after adding 100 µM 7EC to the assay medium. Eleven of the 32 clones were found to de-ethylate 7EC. One particular clone, subsequently referred to as GM2E1, was the most active and was chosen for use in future studies. To confirm P450 2E1 expression levels, 7EC O-deethylase activity was monitored routinely and was found to be high in this clone.
to be maintained at about the same level for >6 months of continuous culture. Therefore, GM2E1 cells are suitable for long-term metabolism-mediated cytotoxicity studies. GMneo cells, transfected with the expression vector alone, were used as controls.

In addition to 7EC O-deethylase activity, both PNP hydroxylase and NDMA demethylase activities were characterized in these cells. Studies with the GM2E1 cells demonstrated that 7EC de-ethylation was linear with time up to 5 h, while the PNP hydroxylation and NDMA demethylation were linear for up to 20 h (Figure 1). In contrast, the GMneo cells did not exhibit catalytic activity for the metabolism of any of these substrates (Figure 1). The addition of hemin or 4MP to the growth medium a day prior to the activity assays has been shown to greatly increase the expression levels for some isozymes of P450 (23,39,40). However, P450 2E1-dependent activity determined by 7EC de-ethylation or by PNP hydroxylation was only enhanced by a maximum of 16 ± 2% in GM2E1 cells (data not shown). Therefore, hemin and 4MP were not included in the culture medium in the present study. In addition, since the phenol red and fetal calf serum in the culture medium interfered with the fluorescent and spectrophotometric detection of products, Opti-MEN I, a medium devoid of these interferences, was used for the activity assays. The results of studies on the substrate dependence and rates of metabolite formation for 7EC de-ethylation, PNP hydroxylation and NDMA demethylation in the GM2E1 cells are illustrated in Figure 2. The insets in Figure 2 are plots of velocity against substrate concentration. Both 7EC de-ethylation and PNP hydroxylation exhibited saturation at a concentration of 0.2 mM, whereas NDMA demethylation reached saturation level at a concentration of 1 mM. The kinetic parameters $K_m$ and $V_{max}$ were determined from Eadie–Hofstee plots (V versus V/S). The products and kinetic constants for 7EC de-ethylation, PNP hydroxylation and NDMA demethylation are summarized in Table I.

**Table I.** Kinetic parameters for the P450 catalytic activities determined in cells expressing P450 2E1$^a$

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Product</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (pmol/min per 10$^6$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7EC de-ethylation</td>
<td>7-Hydroxycoumarin</td>
<td>0.052</td>
<td>9.55</td>
</tr>
<tr>
<td>PNP hydroxylation</td>
<td>4-Nitrocatechol</td>
<td>0.074</td>
<td>24.30</td>
</tr>
<tr>
<td>NDMA demethylation</td>
<td>Formaldehyde</td>
<td>0.737</td>
<td>5.90</td>
</tr>
</tbody>
</table>

$^a$Kinetic constants were determined by linear regression analyses of the data from the Eadie–Hofstee plots (Figure 2).

**Western blot analysis and quantitation of P450 2E1**

The cellular expression of P450 2E1 was further assessed by SDS–polyacrylamide gel electrophoresis followed by immunoblot analysis of microsomes prepared from the GM2E1 and GMneo cells (Figure 3). Microsomes from the GM2E1 cells contained a polypeptide band at ~52 kDa, which co-migrated with the P450 2E1 in microsomes prepared from pyridine-induced rat livers and purified rabbit P450 2E1. In contrast, there was no detectable P450 2E1 in cells transfected with the vector alone. Based on densitometric scans of immunoblots using purified rabbit P450 2E1 as a standard (data not shown), the P450 2E1 content in GM2E1 cells was estimated to be 3 pmol/mg microsomal protein.

**Characterization of NDMA-mediated cytotoxicity**

Time-course and concentration-dependence experiments were conducted to characterize the cytotoxic effect of NDMA in
GM2E1 and GMneo cells. Since a 1 day exposure of GM2E1 cells to 1 mM NDMA had little effect on cell viability, as determined by crystal violet staining, the time-course experiment was determined starting with a 2-day treatment with NDMA. A similar delayed onset of NDMA-induced toxic effect has been observed in rat hepatocytes (10,41). As shown in Figure 4, the relative survivals of GM2E1 cells exposed to 1 mM NDMA for 2, 3 or 4 days, was 67, 47 or 28%, respectively, compared with the survival of GM2E1 cells not exposed to NDMA. In contrast, GMneo cells exhibited no significant toxicity even at concentrations of 1 mM NDMA. Decreases in the viability in GM2E1 cells were observed at concentrations as low as 0.02 mM NDMA.

The cytotoxic effect of NDMA at concentrations ranging from 1 mM to 10 mM was investigated after incubation for 4 days (Figure 5). In the GM2E1 cells, the dose–response curve for cytotoxicity was sigmoidal with a median lethal dose of 17 µM, and maximum cytotoxicity was observed at 1 mM NDMA. When the NDMA concentration was increased from 1 to 10 mM, the toxicity for the GM2E1 cells remained at the same level and at least 25% of the cells were refractory to nitrosamine toxicity. Our observation that ~25% of GM2E1 cells survive after 10 mM NDMA exposure is in line with results from two other studies (25,26). In cells transfected with vector alone, the viability was not decreased by NDMA even at concentrations exceeding 10 mM. The requirement for relatively long incubation times in order to achieve large reductions in surviving cells and the limit of toxicity observed at 1–10 mM NDMA is most likely a result of less than optimal rates of metabolism of NDMA in the GM2E1 cells.

The cytotoxicity of NDMA for GM2E1 cells was also investigated by phase contrast microscopy. The untreated cultures in Figure 6A showed a typical pattern of fibroblast monolayers in which contact with the adjacent cells was retained. In contrast, as shown in Figure 6B, cell damage and the loss of cell-to-cell contact was observed after exposure to 1 mM NDMA for 4 days.

Methylation of cellular macromolecules by [14C]NDMA
To further investigate the cellular metabolism of NDMA by P450 2E1 and the covalent interactions of the reactive metabolites with cellular macromolecules, experiments were performed in which radiolabeled NDMA was added to the intact cell cultures and the time and concentration dependence of labeling were determined. As shown in Figure 7A, there was a variable lag period for the incorporation of [14C]NDMA into DNA, RNA and protein. The length of the lag period was dependent on the identity of the labeled macromolecule. Following the lag period, there was an increase in incorporation of the radiolabel for at least 22 h. In contrast, there was no significant incorporation of [14C]NDMA into cellular macromolecules in GMneo cells even up to 22 h. The methylation of DNA, RNA and protein in cells expressing P450 2E1 was linear up to 0.08 mM NDMA and then began to level off at concentrations above 0.16 mM NDMA (Figure 7B).

Role of P450 2E1 in NDMA cytotoxicity
The results in Figure 4 and Figure 5 demonstrated that NDMA cytotoxicity was only observed in GM2E1 cells but not in GMneo cells. The only difference between GM2E1 and GMneo cells is that the former cells express P450 2E1 as determined by catalytic activity, immunoblot analysis and macromolecular methylation. P450 2E1 inhibitors or ligands (3,10,24) such as ethanol, 4-methylpyrazole (4MP), and isoniazid (ISON) were added to the GM2E1 cells in culture in the presence of NDMA to see if they would protect against NDMA-mediated cytotoxicity. As shown in Figure 8A, the co-incubation of NDMA with ethanol, 4MP or ISON resulted in the complete prevention of the NDMA toxic effect on the cells. The mechanism of protection appeared to be via the loss of P450 2E1 catalytic activity due to the presence of these inhibitors. This was demonstrated in that the NDMA demethylation activity in the cells challenged with inhibitors was <10% of that of control groups (data not shown). In addition, the
P450 2E1 expression and toxicity

Fig. 6. Morphological changes in untreated and NDMA-treated GM2E1 cells. Cells were plated at the same density and the adherent cells were observed under a phase contrast microscope (×225) after 4 days of culture in the absence (A) or in the presence (B) of 1 mM NDMA.

Fig. 7. Methylation of cellular macromolecules in cell cultures treated with [14C]NDMA. Cells were plated in 60-mm dishes at 90% confluency at 1 day prior to the start of the experiment. For the time-course studies (A), 0.02 mM [14C]NDMA was added to the GM2E1 and GMneo cells and the incorporation of the radiolabel into cellular macromolecules was determined at the time points indicated. The data are the mean ± SD from three separate experiments. For the concentration dependent labeling (B), concentrations of [14C]NDMA indicated were added to the cell cultures and incubated for 16 h. The DNA, RNA and protein were separated using the TRIzol Reagent as described in Materials and methods.

methylation of cellular macromolecules in the presence of the P450 2E1 inhibitors was also decreased to <10% of that of control groups (Figure 8B).

Fig. 8. The effect of inhibitors of P450 2E1 on NDMA-mediated cytotoxicity and methylation of cellular macromolecules in GM2E1 cells. For the cytotoxicity studies (A), 100 mM ethanol, 2 mM 4MP or 2 mM ISON was added to cell cultures in the absence (control) or presence of 0.02, 0.1 mM or 1 mM NDMA and incubated for 4 days. The data are the mean ± SD from three separate experiments. For the alkylation studies (B), 0.02 mM [14C]NDMA was added to the cell cultures in the absence (control) or in the presence of 100 mM ethanol, 2 mM 4MP or 2 mM ISON and incubated for 16 h. The concentrations of ethanol, 4MP or ISON used in these studies did not affect the cell viability.

Table II. The modulation of NDMA cytotoxicity in GM2E1 cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative survival (% of control)</th>
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<tbody>
<tr>
<td>0.02 mM NDMA</td>
<td>57 ± 8 (14)</td>
</tr>
<tr>
<td>0.1 mM NDMA</td>
<td>38 ± 7 (14)</td>
</tr>
<tr>
<td>Butylated hydroxyanisole (0.2 mM</td>
<td>80 ± 3 (3) *</td>
</tr>
<tr>
<td>N-t-Butyl-α-phenylnitrone (2 mM)</td>
<td>84 ± 6 (3) *</td>
</tr>
<tr>
<td>N-Acetylcysteine (4 mM)</td>
<td>81 ± 3 (4) *</td>
</tr>
<tr>
<td>Ascorbic acid (0.25 mM)</td>
<td>77 ± 2 (3) **</td>
</tr>
<tr>
<td>Vitamin E (0.25 mM)</td>
<td>86 ± 9 (9)*</td>
</tr>
</tbody>
</table>

*At the concentration used in the present studies, none of the compounds tested significantly inhibited P450 2E1 activity as determined by assays for 7EC de-ethylation and NDMA demethylation activities, and the compounds were not toxic to the cells by themselves as determined by cell viability.

Values presented are the mean ± SD and the number of independent experiments are shown in parentheses. *P < 0.001; **P < 0.01, compared with cells treated with NDMA only. The control represents the GM2E1 cells treated with the various additions in the absence of NDMA.

A butylated hydroxyanisole stock solution was prepared in methanol, and the vitamin E stock was prepared in acetone. The final concentrations of methanol and acetone were 0.05 and 0.025%, respectively.

Modulation of NDMA-mediated cytotoxicity in GM2E1 cells

Metabolism by the cytochrome P450 mixed function oxidase system is believed to be required for both the hepatotoxic and carcinogenic effects of NDMA (14). To determine if electrophilic intermediates are responsible for the toxic effects, several antioxidants and nucleophiles were added to the cells exposed to NDMA and their ability to protect against NDMA-induced cytotoxicity was evaluated. As shown in Table II, antioxidants such as butylated hydroxyanisole, N-acetylcysteine, ascorbic acid and vitamin E had a protective effect against NDMA toxicity. N-t-Butyl-α-phenylnitrone, a spin-trapping agent, also inhibited the cytotoxic effects of NDMA. The protection of these agents against the toxicity of 0.02 mM and 0.1 mM NDMA, although significant, was not as effective as that seen with P450 2E1 inhibitors or ligands such as ethanol, 4MP or ISON (Figure 8A).

To evaluate the role of cellular glutathione in protecting against NDMA toxicity, cell cultures were exposed to GSH-depleting compounds such as DL-buthionine-[S,R]-sulfoximine...
Exposure of cells to NDMA (0.1 mM) had no effect on the GSH content in GM2E1 cells, the cellular GSH content was measured in the presence and absence of BSO. In order to investigate directly whether or not BSO had an effect on GSH levels (3.59 ± 0.01 fmol/cell) for up to 2 days. Although a slight decrease was observed after 3 days, this decrease was not significant (P > 0.05, Student’s t-test). Furthermore, the co-incubation of 20 μM BSO with NDMA for 2 and 3 days completely depleted the GSH content. Similar results were obtained with cells treated with acetaminophen (0.5 nM) in the presence or absence of BSO for 3 days. The cellular GSH levels were 3.75 ± 0.10 fmol/cell in the cells treated with acetaminophen in the absence of BSO, while the GSH levels were not detectable after co-incubation of acetaminophen with BSO in GM2E1 cells. These results indicate that the reactive metabolites of NDMA did not conjugate with GSH and that the toxic effects were not potentiated even when the GSH was completely depleted by the addition of BSO. Although the reactive metabolite generated from acetaminophen at concentration of 0.5 mM did not affect the GSH content, the cytotoxicity was potentiated significantly upon GSH depletion (Figure 9).

Discussion

We report here the stable expression of rat P450 2E1 in a human fibroblast cell line, GM0637. Previously, this cell line has been stably transformed with a controllable P450 1A1 expression construct and proven to be a reliable tool in testing the cytotoxicity and mutagenicity of polycyclic aromatic hydrocarbons requiring bioactivation by P450 1A1 (43).

The expression of P450 2E1 in GM2E1 cells was confirmed by in situ enzymatic activities including measurement of 7EC de-ethylation, PNP hydroxylation and NDMA demethylation. In addition, the level of P450 2E1 apoprotein was also quantitated by Western blot analysis using purified rabbit 2E1 as a standard. Although P450 2E1 has been expressed in several mammalian cells (20–26), this is the first time the kinetic parameters for the P450 2E1 catalytic activities have been characterized in an intact cell culture. There are some difficulties in comparing kinetic constants for substrates metabolized by P450 2E1 using different experimental paradigms such as intact cell cultures, liver microsomal preparations or purified proteins. These can include availability of substrate in the vicinity of active site, level of P450, P450-reductase or cytochrome b5 and stability of metabolites. In addition, the expression system described here is heterologous, in that the rat P450 2E1 is expressed in a human fibroblast cell. Since the rat P450 2E1 is 75% homologous with human P450 2E1 (44), the interaction among rat P450 2E1 and human NADPH-cytochrome P450 reductase and cytochrome b5 may not be optimal. Using intact cell cultures, we obtained an apparent K_m of 0.074 mM for PNP hydroxylation as compared with a value of 0.028 mM (45) or 0.055 mM (46) using rat liver microsomes. For the determination of the kinetic constants for NDMA demethylation, semicarbazide, a P450 2E1 inhibitor (47), was added to the assay medium to trap formaldehyde (31,32). Therefore, the apparent K_m value of 0.737 mM determined for NDMA demethylase activity in GM2E1 cells may be too large. A number of laboratories have implicated the existence of two major forms of rat microsomal NDMA demethylase, a high affinity enzyme with an apparent K_m value in the 0.02–1.5 mM substrate range (10,13,34–50) and a low affinity enzyme with an apparent K_m in the 30–50 mM substrate range (10, 13, 14, 48–50). It may be suggested that the P450 2E1 expressed in the GM2E1 cells is the low K_m isoform from the following: (i) the apparent K_m is 0.737 mM; (ii) the median lethal dose is 17 μM NDMA, and maximal cytotoxicity is reached at 1 mM NDMA; and (iii) the methylation of macromolecules is saturated at 0.16 mM NDMA. Thus, these findings support the previous studies which suggest....
that only the high affinity low $K_m$ form of P450 2E1 plays a significant role in NDMA metabolism and is also responsible for the carcinogenic, cytotoxic and genotoxic effects (11–14).

In the past several years, human P450 2E1 has been successfully expressed in mammalian cells and used for studying the metabolic activation and cytotoxic effects of NDMA (21,25,26). Nouso et al. have shown the formation of a covalent adduct with cellular DNA in the cells treated with NDMA (21). Schmalix et al. (25) and Nakagawa et al. (26) have demonstrated the dose-dependent cytotoxic effects of NDMA. In the present study, the correlation between the cytotoxicity and the level of macromolecular methylation of NDMA in a time- and concentration-dependent manner was shown in GM2E1 cells. The linkage between NDMA-mediated metabolism and cytotoxicity was further demonstrated with the use of the P450 2E1 inhibitors, which abolished the methylation of cellular macromolecules and also prevented the cytotoxicity of NDMA in the GM2E1 cells. Therefore, these findings provide additional evidence that P450 2E1 metabolizes NDMA to a methylating agent that results in the formation of covalent adducts, which may play an important role in exerting the toxic effects of this nitrosamine (14–18).

The partial protection by the antioxidants and the spin-trapping agent against the NDMA-mediated toxicity for the GM2E1 cells suggests that free radicals or reactive oxygen species such as $\cdot$OH, $\cdot$O$_2^-$, $\cdot$NO, and H$_2$O$_2$ produced during NDMA demethylation and denitrosation (51–53) may contribute to its toxic actions. The reactive oxygen species may alter the target molecules via non-covalent interactions or covalent binding to DNA, RNA, proteins or lipids and thus result in toxic consequences (54–56). The 8-hydroxyguanine adduct is widely used as a biomarker of oxidative DNA damage induced by reactive oxygen species (57). Recently, the involvement of N-nitrosodimethylamine-induced 8-hydroxyguanine formation in rat liver carcinogenesis was reported (58). It is possible that one or more reactive oxygen species generated during NDMA bioactivation may lead to DNA damage and cause cell death in GM2E1 cells. Furthermore, Garland et al. have reported that N-acetylcysteine inhibits in vitro covalent binding to microsomal protein and mutagenicity and in vivo hepatotoxicity caused by NDMA (59).

The demonstration in this cell model that NDMA-mediated toxicity was not affected by completely depleting the GSH protective system in cells expressing P450 2E1 agrees with in vivo studies in mouse liver (9) and in vitro studies in isolated rat hepatocytes (10,60). The chemical nature of the reactive metabolite is an important factor in determining the influence of GSH status on toxicity (55,60,61). The alkylating intermediate of NDMA is believed to be CH$_3$N$_2^+$, a relatively hard electrophile, which results in the attack on hard nucleophilic sites (particularly oxygen and certain nitrogens of nucleic acids), but not the soft nucleophilic site (the thiol sulfur of GSH) (55,60). Therefore, in the GM2E1 cells, the GSH contents did not decrease significantly and the toxicity was not potentiated regardless of the depletion of GSH following treatment with NDMA. In contrast, acetalaminophen is metabolized to a soft electrophile, which will preferentially covalently bind to soft nucleophilic sites in the cells including the thiol groups of cysteinyl residues in proteins or GSH (55,60). Therefore, GSH depletion potentiated the toxic action of acetalaminophen in the GM2E1 cells. These results indicate that the GSH-dependent detoxification mechanism plays a major role in removing the reactive metabolite of acetalaminophen but not the reactive metabolites of NDMA in GM2E1 cells.

Studies on the alkylation of cellular macromolecules in primary hepatocyte cultures have previously been performed by using TRIZol Reagent in our laboratory. Therefore, we compared the time courses for NDMA methylation in the two cell culture systems. In primary cultures from pyridine-induced rats, which have elevated levels of P450 2E1, methylation was maximal at 3 h and the order of the methylation level was DNA > RNA > protein (37). Whereas in GM2E1 cells, the methylation increased with increasing time for ~22 h and the order of methylation level was RNA > DNA > protein. In both studies, the methylation of nucleic acids was much greater than protein. This supports the hypothesis that a hard electrophile such as the methycarbonium ion produced from NDMA reacts predominantly with hard nucleophiles such as the oxygen of purines and pyrimidines in DNA and RNA (55,60).

Corcoran and co-workers have recently reported the involvement of a Ca$^{2+}$-endonuclease in NDMA-induced DNA damage in vivo (62) and in cultured mouse hepatocytes (63). We have also demonstrated a role for a Ca$^{2+}$-endonuclease in NDMA-induced toxicity in GM2E1 cells (manuscript in preparation). Studies are currently in progress to characterize the DNA adducts and to detect levels of reactive oxygen species and lipid peroxidation in order to elucidate the mechanism of NDMA-mediated toxicity.

In summary, these studies demonstrate that GM2E1 cells can metabolize NDMA to toxic reactive metabolites that interact with the target molecules either covalently or non-covalently to cause cell death. These results also demonstrate a strong correlation between the methylation of cellular macromolecules and cytotoxicity following NDMA treatment and that a reactive oxygen species, as evidenced by the protection afforded by the antioxidant defense system, may be responsible for the NDMA-mediated toxic effect. Whereas, the GSH dependent cytoprotective mechanism does not appear to play a role in protecting against NDMA toxicity.

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References

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