Etomoxir-Induced Oxidative Stress in HepG2 Cells Detected by Differential Gene Expression Is Confirmed Biochemically

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Although they are known to be effective antidiabetic agents, little is published about the toxic effects of carnitine palmitoyltransferase-1 (CPT-1) inhibitors, such as etomoxir (ET). These compounds inhibit mitochondrial fatty acid β-oxidation by irreversibly binding to CPT-1 and preventing entry of long chain fatty acids into the mitochondrial matrix. Treatment of HepG2 cells with 1 mM etomoxir for 6 h caused significant modulations in the expression of several redox-related and cell cycle mRNAs as measured by microarray analysis. Uregulated mRNAs included heme oxygenase 1 (HO1), 8-oxoguanine DNA glycosylase 1 (OGG1), glutathione reductase (GSR), cyclin-dependent kinase inhibitor 1A (CDKN1 [p21\(^{CIP1}\)]) and Mn+ superoxide dismutase precursor (SOD2); while cytochrome P450 1A1 (CYPIA1) and heat shock 70K protein 1 (HSPA1A) were downregulated. Real time quantitative PCR (RT-PCR) confirmed the significant changes in 4 of 4 mRNAs assayed (CYPIA1, HO1, GSR, CDKN1), and identified 3 additional mRNA changes; 2 redox-related genes, γ-glutamylcysteine ligase modifier subunit (GCLM) and thioredoxin reductase (TXNRD1) and 1 DNA replication gene, topoisomerase IIα (TOP2A). Temporal changes in selected mRNA levels were examined by RT-PCR over 11 time points from 15 min to 24 h post-dosing. CYPIA1 exhibited a 38-fold decrease by 4 h, which rebounded to a 39-fold increase by 20 h. GCLM and TXNRD1 exhibited 13- and 9-fold increases, respectively at 24 h. Etomoxir-induced oxidative stress and impaired mitochondrial energy metabolism were confirmed by a significant decrease in reduced glutathione (GSH), reduced/oxidized glutathione ratio (GSH/GSSG), mitochondrial membrane potential (MMP), and ATP levels, and by concurrent increase in oxidized glutathione (GSSG) and superoxide generation. This is the first report of oxidative stress caused by etomoxir.

Key Words: etomoxir; toxicity; oxirane-carboxylates; oxidative stress; gene expression.

Etomoxir is a member of a family of substituted 2-oxirane-carboxylic acids that inhibit mitochondrial long-chain fatty acid β-oxidation (FAO), ketogenesis, and gluconeogenesis (Wolf, 1992). Once converted to its CoA ester, etomoxir irreversibly binds to the CPT-1 catalytic site and prevents long chain fatty acids from entering the mitochondrion. Along with this inhibition of FAO, etomoxir causes a shift in energy substrate utilization from fatty acids to glucose, leading to systemic hypoglycemia, hypoketonemia, and hypotriglyceridemia (Wolf, 1992). These effects make etomoxir potentially useful in the treatment of noninsulin-dependent diabetes mellitus (NIDDM). However, the compound has been shown to induce cardiac and hepatic hypertrophy in animals and therefore has not been fully developed as an antidiabetic agent to date (Rupp and Jacob, 1992; Vetter et al., 1995; Yotsumoto et al., 2000). The mechanism(s) of the respective hypertrophies have not yet been worked out, but are shown to be different from those involved in hyperthyroidism and hypertension (Rupp et al., 1992; Vetter et al., 1995).

Reactive oxygen species (ROS) are implicated in the development of numerous pathological conditions including inflammatory disease, cancer, ischemia-reperfusion injury, and metabolic diseases such as diabetes mellitus. Emerging evidence links the presence of free radicals to the progression of diabetes and its complications. There is much evidence that hyperglycemia and advanced glycation end products (AGE) promote the formation of ROS in the diabetic patient and several markers of oxidative stress, such as 8-hydroxydeoxyguanosine, hydroperoxide and oxidized lipoprotein (oxLDL), are increased in these patients (Rosen et al., 2001).

In one study, hyperglycemia was shown to induce mitochondrial superoxide overproduction, which in turn activated the hexosamine pathway, thought to be a major factor in the pathogenesis of diabetic complications (Du et al., 2000). Lipid peroxidation and antioxidant depletion, which strongly contribute to the development of atherosclerosis, are both present in diabetes mellitus (Cominacini et al., 1994; Dimitriadis et al., 1995; Leonhardt et al., 1996; Schoen and Cotran, 1999; Srinivasan et al., 1997). In addition to vascular dysfunction, oxidative stress is involved in the pathogenesis of diabetic polyny-
ropathy, retinopathy and the expansion of extracellular matrix components, type IV and VI collagen, fibronectin, and laminin (Rosen et al., 2001). Diabetes appears to be associated with increased levels of oxidative stress. It would be best, therefore, if a drug designed to treat this metabolic disease did not contribute to such stress. Etomoxir has been shown to reduce hyperglycemia in diabetic humans and animals (Wolf, 1990), and hyperglycemia has been shown to increase oxidative stress (Rosen et al., 2001); therefore, etomoxir might theoretically decrease oxidative stress. Morgan et al., however, determined that another CPT-1 inhibitor, tolbutamide, caused upregulation of the oxidative stress gene transcripts GSR and SOD2 in HepG2 cells (Morgan et al., in press).

The purpose of this study was to evaluate ET-induced mechanisms of toxicity in HepG2 cells, with special emphasis on oxidative stress. Gene expression strongly suggestive of oxidative stress was observed and supported by decreased levels of reduced glutathione, reduced/oxidized glutathione ratio (GSH/GSSG), concurrent increase in oxidized glutathione (GSSG), and superoxide generation. Impairment of mitochondrial energy metabolism was implicated by a significant decrease in mitochondrial membrane potential and ATP levels. Other gene expression findings suggested activation of p53, DNA repair, and cell cycle arrest. This is the first report of oxidative stress caused by etomoxir.

MATERIALS AND METHODS

Chemicals. Etomoxir was obtained from Research Pharmaceuticals, Alensbach, Germany, and other chemicals were obtained from Clontech Laboratories (Palo Alto, CA), ResGen (Huntsville, AL), and Ambion (Austin, TX).

Cell culture. Human hepatocellular carcinoma (HepG2) cells (ATCC 1998, CRL-10741, Homo sapiens, HepG2/C3A, Rockville, MD) were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with Glutamax and 10% fetal bovine serum (FBS), under standard cell culture conditions (37°C, humidified, 5% CO₂) without antibiotics. For this experiment, time zero (T₀) represents the time at which treatment was first begun. At time –48 h, HepG2 cells were seeded at a density of 1 × 10⁶ in 3 control and 3 treated collagen coated (Vitrogen-100, Cohan, Palo Alto, CA) 150 mm diameter cell culture dishes (non-treated, nonprogeny polystyrene, Corning, Inc., Corning, NY) with 26 ml medium. A sterile, numbered, collagen-coated glass coverslip was placed in the bottom of each dish for use in the light microscopic determination of cell morphology. Cells were fed at –24 h and re-fed at 0 h with either untreated media (control) or 1 mM etomoxir in media (treated). Treatment duration in the first experiment was 6 h. In the second experiment, multiple time points were used (0.25, 0.5, 1, 2, 4, 6, 8, 12, 16, 20, and 24 h) in order to track mRNA expression changes chronologically (time course study).

Selection of exposure concentration. As previously described (Morgan et al., in press), ET exposure concentration was selected to be that which caused 50% reduction in MTS assay (Cell Titer 96™ AQueous Non-radioactive Cell Proliferation Assay, Promega) in HepG2 cells at 24 h. MTS is bioreduced by dehydrogenase enzymes in cells into a formazan that is soluble in cell culture medium. The quantity of formazan product as measured by 490 nm absorbance is directly proportional to the number of living cells in culture (Cory et al., 1991; Marshall et al., 1995). HepG2 cells were cultured in 96 well plates with 20,000 cells and 50 μl of DMEM + 10% FBS per well. ET was added in fresh medium at serial 10-fold dilutions (6 replicates per dilution). Following exposure for 24 h, 10 μl of MTS reagent were added to each well, the plate was incubated for 1 h at 37°C and the spectrophotometric absorbance read at 490 nm. A 1 mM exposure concentration was selected and confirmed in six 150-mm plates seeded with 1 × 10⁶ cells (per RNA collection protocol) and MTS assay was run on media collected from plates. Light microscopy was used to confirm the toxic responses in the cells as measured by the MTS assay.

Cell morphology and viability. Coverslips were removed from plates at the 6-h time point or at time course time points (listed above) and fixed in ethanol. Coverslips were hematoxylin and eosin stained and evaluated by light microscopy. Using standard cell counting procedures, cells were counted and fixed in 5 reproducible sites on each 6- and 24-h time course coverslip (control and 1mM ET; n = 3). Utilizing an Olympus 25 grid reticle (total area 25 mm²), cell nuclei within each of the 5 sites were counted for total cell counts. Mitotic figures and apoptotic/necrotic cells within the same sites comprised the mitotic index or apoptotic/necrotic index (number of affected cells/1000 cells counted). Apoptotic cells were characterized by cell shrinkage, chromatin condensation, formation of cytoplasmic blebs, and phagocytosis by adjacent healthy cells. Necrotic cells exhibited loss of nuclear integrity, increased eosinophilia, +/− cell swelling. It was frequently difficult to distinguish these 2 forms of cell death, therefore apoptotic and necrotic cells were lumped together during cell counts. Release of lactate dehydrogenase (LDH) enzyme activity was used as a measure of cell viability. LDH activity was determined by monitoring the enzymatic formation of NADH from NAD+ in the presence of L-lactic acid. At the indicated time points, media samples were withdrawn and centrifuged to remove viable cells. Postcentrifugation supernatants were diluted 5 times with phosphate buffered saline (PBS), pH 7.4. An aliquot of 100 μl diluted sample was mixed with 100 μl reagent to give a final concentration of 3.75 mM NAD+ and 25 mM L-lactic acid in 125 mM Tris-HCl buffer, pH 8.9 in a 96 well plate. The increase in absorbance at 340 nm was immediately monitored at room temperature using a SPECTRAmax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The percent LDH leakage was calculated by comparing values with total LDH activity. Total LDH activity was measured from untreated HepG2 cells lysed with a final concentration of 0.2% Triton X-100 in PBS.

Gene expression arrays. Cultured cells, after removal of overlying media, were lysed with Trizol Reagent™ and lysate was frozen at –80°C until use. Total RNA was isolated by a chloroform/isopropanol/ethanol extraction and RNA quality and quantity were assessed using aarose gel electrophoresis and spectrophotometric 260/280 nm absorbance. 3P-labelled cDNA probes were prepared using a modification of the Clontech protocol and hybridized to Clontech Atlas™ Human Stress Toxicology cDNA Arrays (234 genes). Denaturation (4 μl) was carried out at 70°C for 10 min using 6 μg total RNA and 1 μl CDS Atlas specific primers (0.2 μM each). The annealing and extension reactions (22 μl, 35 min at 49°C) contained 0.5 mM each dATP, dGTP, dTTP, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 4.5 mM, 100μCi 3P-dCTP (3000 Ci/mmol, 1 μCi/μl, NEN) and 200 units Super Script II™ reverse transcriptase (Gibco-BRL, MD). Extension was terminated by heating to 94°C for 5 min. Unincorporated 3P-dCTP was removed using G-50 MicroSpin columns (Amersham Biosciences, Inc., Piscataway, NJ). Hybridization was carried out at 64°C for 16 h, in 6.5 ml MicroHyb™, 3.25 μl poly-dA (1 μg/μl, ResGen) and 6.5 μl Human Cot-1 DNA (1 μg/μl, Clontech) and heat denatured 3P-dCTP. Arrays were washed at 64°C following manufacturer’s instructions. Phosphor imaging screens were exposed to the arrays for 24–48 h and the optical density was acquired using OptiQuant and a Cyclone scanner (Packard BioScience Co., Meriden, CT). Image files generated from phosphorimager scans were analyzed using Clontech AtlasImage Software™.

After background subtraction, nonnormalized data were analyzed statistically using Normalization and Local Regression (NLR) software (Tom Kepler, Santa Fe Institute), which is downloadable from the internet at ftp://ftp.santeafe.edu/pub/kepler/). NLR was used to compare control with treated groups (n = 3/group), generate p values, mean log intensity (MLI) to provide an indication of signal strength, and ratio of differences between groups.

Quantitative real time PCR. Total RNA prepared above from 3 control and 3 ET-treated samples was DNase-treated (Ambion DNAase I) according
etomoxir’s protocol. RNA was quantified using the Molecular Probes Ribogreen™ assay and a Cytofluor 2350 fluorometer. Samples were diluted to 10 ng/μl prior to Taqman™ analysis (Perkin Elmer ABI Prism 7700 Sequence Detection system). A 7-gene plate, described by Morgan et al. was used to quantify the mRNA expression of the following genes: HO1, CYP1A1, TOP2A, CDKN1, GSR, GCLM, and TXNRD1 (Morgan et al., in press). The plate was arranged with 1 row allotted for each gene to be assayed and 1 row of water blanks, and duplicate columns assigned to each treated or control sample. Primers were designed with Perkin Elmer Primer Express™ software. Forward and reverse primers and probes were diluted to the appropriate concentrations to make the probe/primer master mix. The master mix was prepared according to the manufacturer’s protocol (without probes and primers) and 30 μl of master mix, 5 μl of RNA, and 15 μl of probe/primer mix were aliquotted per well into the 7-gene plate. The plate was sealed with Optical Adhesive Covers (PE Biosystems™), centrifuged at 3000 × g for 10 s and the reaction was incubated at 48°C for 30 min (reverse transcriptase [RT] step), denatured at 95°C for 10 min (Amplitaq activation and RT denaturation), and then subjected to 40 PCR cycles of 94°C for 15 s and 60°C for 1 min. Values of fold change in expression were graphed for comparison purposes. Statistical significance was determined for control versus treated groups using a t-test (two-tailed, pooled, assuming normal distribution and variances equal) and significance selected at p < 0.05.

**Oxidative Stress Assays**

**GSH and GSSG levels.** GSH and GSSG levels were assayed by HPLC according to the method of Martin and White (1991).

**Superoxide radical.** Hydroethidine (HE), a sodium borohydride-reduced form of ethidium bromide (EB) was used to evaluate generation of superoxide ($O_2^•−$) upon exposure to etomoxir in HepG2 cells. HE, a specific and sensitive indicator of $O_2^•−$ (Rothe and Valet, 1990) is cell permeable and can be directly oxidized to EB by $O_2^•−$ produced by the cell (Carter et al., 1994). Intracellular EB tightly binds DNA, and is fluorescent (610 nm) on excitation with the 488 nm line of a FACSCalibur (Becton-Dickinson, San Jose, CA). Ten μM HE was added to the cell suspension for dye-loading. Fifteen min after incubation at 37°C with HE, HepG2 cells were incubated for an additional 6 h in the presence or absence of ET (500 and 1000 μM) after which sample collection was started immediately on the FACSCalibur bench-top flow cytometer (Becton-Dickinson).

**Mitochondrial Energy Metabolism Assays**

**ATP.** Cellular ATP levels were measured with an ATP Bioluminescent Somatic Cell Assay Kit obtained from Sigma (St. Louis, MO) according to the manufacturer’s instructions with slight modifications. HepG2 cells plated on a 96-well plate were incubated with 500 μM etomoxir in media with serum. After 24 h, the media was removed and cells were lysed by adding 100 μl of ATP releasing reagent and 100 μl of water. Aliquots of 100 μl were transferred to white 96 well assay plates. Luminescence was monitored on a Gemini XS SPECTRAmax dual scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) in the luminescence mode following the addition of 100 μl luciferin and luciferase.

**Mitochondrial membrane potential (MMP).** Disruptions in MMP can be measured using cationic lipophilic fluorochromes such as Mitotracker Red (Chloromethyl-X-Rosamine, MR). These probes diffuse passively across the plasma membrane and accumulate in the negatively charged mitochondrial matrix. The extent of dye uptake depends on the potential difference ($ΔΨ$); dissipation of MMP results in a decrease in cell-associated fluorescence that can be detected by flow cytometry. To determine changes in MMP, 1 × 10⁶ cells were incubated with 100 nM Mitotracker Red for 15 min at 37°C in the dark and then treated with ET (250, 500, and 1000 μM) for 1 h at 37°C. Samples were analyzed immediately by flow cytometry on a FACSCalibur.

**RESULTS**

**Morphology and Cell Viability**

Examination of HepG2 cells treated with 1 mM ET for 6 and 24 h revealed clear morphologic changes (Fig. 1) including loss of large cytoplasmic vacuoles, mitotic figures, and few apoptotic bodies. (B) 1 mM etomoxir-treated HepG2 cells have a loss of cytoplasmic vacuoles, cell shrinkage and rounding-up, marked decrease in mitotic figures and increased numbers of apoptotic bodies (arrow). H & E, original magnification ×400.
cells were increased ~1200%. LDH enzyme activity was used as a measure of cell viability at 6 and 24 h with significance determined by Dunnett’s Test (n = 3). Of the 3 concentrations evaluated (0.25, 0.5 and 1 mM), only the 24 h, 1 mM combination caused significant cell death (21%).

**Clontech Human Stress Arrays**

Statistically significant changes (p < 0.05; Table 2) in mRNA levels relative to controls were induced by treatment of HepG2 cells with 1 mM ET for 6 h.

**Redox-related genes.** HO1, GSR, OGG1, and SOD2 were upregulated and CYP1A1 was downregulated.

**Cell cycle/apoptosis genes.** Growth arrest- and DNA damage-inducible gene 153 (GADD153), growth arrest- and DNA damage-inducible gene 45 (GADD45), CDKN1, mouse double minute 2 homolog (MDM2), ubiquitin and ubiquitin protein ligase were upregulated, while proliferating cell nuclear antigen (PCNA), extracellular signal-regulated kinase 1 (ERK1) and mitogen-activated protein kinase P38 (MAP kinase p38) were downregulated.

**Heat shock proteins.** Heat shock protein 40 homolog (HSP40 homolog) and heat shock 70kD protein 1 (HSP70A1) were upregulated.

**DNA synthesis and repair.** Excision repair cross-complementing rodent repair deficiency, complementation group 2 (ERCC2, aka xeroderma pigmentosum group D complementing protein) and UV excision repair protein (RAD23A) were upregulated.

**Peroxisome proliferator activated receptor (PPAR) genes.** PPAR-alpha mRNA was decreased.

**Quantitative Real Time-PCR**

RT-PCR was used to confirm selected mRNA expression changes identified by microarray analysis. The following RT-PCR (fold expression) changes in the 6 h, 1 mM ET experiment indicate oxidative stress and cell cycle modulation: CYP1A1 (-9.6), HO1 (+1.5), TOP2A (-1.6), GSR (+1.3), CDKN1 (+2.6), GCLM (+3.4), and TXNRD1 (+2.7; Fig. 2, Table 2). Direction of change and statistical significance (p < 0.05) of the expression of 4 mRNA transcripts (HO1, CDKN1, and GSR upregulated, and CYP1A1 downregulated) present on the Clontech Human Stress Array were confirmed by RT-PCR. The mRNA expression of TOP2A was downregulated on both platforms (RT-PCR and microarray), but only exhibited statistical significance on RT-PCR. The 2 genes not present on the arrays (GCLM and TXNRD1) were both significantly (p < 0.05) upregulated at 6 h.

The ET time course study followed expression of these 7 genes over 11 time points from 0.25 to 24 h (Fig. 3). Note the severe swing of CYP1A1 from a decrease of 38-fold at 4 h to an increase of 39-fold at 20 h. GCLM and TXNRD1 proved to be good indicators of oxidative stress as they were significantly increased above controls at 6 h and continued to rise through the 24-h time point to 13- and 9-fold increases, respectively.

**Oxidative Stress Assays**

**GSH and GSH:GSSG ratio.** Intracellular reduced GSH levels decreased relative to time-matched controls upon treatment with ET (Fig. 4). At 6 h, only the 1 mM dose group was significantly decreased; control 53.7 ± 6.4 nmol/mg protein

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**Table 2**

<table>
<thead>
<tr>
<th>HepG2 Cell Counts, Mitotic Index, and Apoptotic/Necrotic Index after 24 h of 1 mM ET Treatment</th>
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<tbody>
<tr>
<td><strong>24 h HepG2</strong></td>
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<tr>
<td>Cell count</td>
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<tr>
<td>Mitotic index</td>
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<td>Apoptotic/necrotic index</td>
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*Note. Values are mean ± SD. Mitotic index, mitotic figures/1000 cells. Apoptotic/necrotic index, apoptotic and necrotic cells/1000 cells.*

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**Table 2**

<table>
<thead>
<tr>
<th>Etomoxir-Induced mRNA Expression Compared with Control</th>
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<tr>
<td><strong>Microarray</strong></td>
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<tr>
<td><strong>Fold change</strong></td>
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<tr>
<td>Redox and related genes</td>
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<tr>
<td>HO1</td>
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<td>GSR</td>
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<td>SOD2</td>
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<td>OGG1</td>
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<td>CYP1A1</td>
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<td>TOP2A</td>
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<td>GCLM</td>
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<td>TXNRD1</td>
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<td><strong>Cell cycle/apoptosis</strong></td>
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<td>GADD153</td>
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<td>GADD45</td>
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<tr>
<td>CDKN1</td>
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<tr>
<td>MDM2</td>
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<tr>
<td>Ubiquitin</td>
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<td>Ubiquitin protein ligase</td>
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<tr>
<td>PCNA</td>
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<tr>
<td>ERK1</td>
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<td>MAP kinase p38</td>
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<td><strong>Heat shock</strong></td>
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<tr>
<td>HSP40 homolog</td>
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<tr>
<td><strong>DNA synthesis and repair</strong></td>
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<td>XP gp D complementing protein</td>
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<tr>
<td>RAD23A</td>
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<tr>
<td><strong>PPAR</strong></td>
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<td>PPARα</td>
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*Note. Etomoxir treatment was 6 h, 1 mM. Fold change values are ratio of treated/control.
and 1 mM ET group 40.1 ± 11.1 nmol/mg protein (−25%). The GSH depletion was more pronounced at the 24-h time point with significant reduction in the 0.5 and 1 mM groups; control 55.0 ± 13.5 nmol/mg protein, 0.5 mM ET 24.6 ± 2.8 nmol/mg protein (−55%) and 1 mM ET 24.3 ± 3.9 nmol/mg protein (−56%). There was a significant increase in GSH at 24 h with 0.25 mM ET. The ratio of GSH:GSSG, an accepted measure of oxidative stress (Armstrong, 1998), mirrored GSH changes with significant declines at 6 h 1 mM (−56%), and at 24 h 0.5 mM ET (−93%) and 1 mM ET (−84%).

Superoxide radical. At 6 h, superoxide radical generation was increased in 0.5 mM ET-treated cells by ~350% versus untreated controls (Fig. 5).

Mitochondrial Energy Metabolism Assays

ATP. Treatment of HepG2 cells for 24 h with 0.5 mM ET was associated with an 84% decrease in ATP levels (Fig. 6). This dose was chosen because it was the highest nontoxic dose (0.5 mM) of ET as measured by lactate dehydrogenase activity.

Mitochondrial membrane potential. At the 0.5 mM and 1 mM ET concentrations, MMP is reduced by 16 and 28%, respectively (Fig. 7). The 0.25 mM ET concentration does not change MMP relative to the untreated control.

**DISCUSSION**

Etomoxir, an effective hypoglycemic, hypotriglyceridemic, and hypocholesterolemic compound, has not been fully developed as an antidiabetic drug due to its toxic effects on the liver and heart of some animal species (Anderson, 1998). Hypertrophy of both the heart and liver have been reported with supra-clinical doses of etomoxir, but the pathophysiological mechanisms are unclear (Rupp and Jacob, 1992; Yotsumoto et al., 2000). In this study, gene expression patterns were profiled to identify potential toxic pathways associated with the treatment of HepG2 cells with etomoxir. Patterns consistent with oxidative stress, cell cycle arrest, apoptosis, and DNA and protein damage were identified by microarray and RT-PCR assays, and confirmed morphologically and biochemically. In addition, the decreases in ATP concentration and mitochondrial membrane potential are indicative of dysregulation of mitochondrial energy metabolism.

Several genes associated with redox imbalance were significantly altered, including HO1, SOD2, GSR, OGG1, and...
CYP1A1. HO1 is the rate-limiting enzyme in heme degradation, catalyzing the cleavage of the heme ring to form ferrous iron, carbon monoxide, and biliverdin. HO1, biliverdin, and carbon monoxide have been shown to have protective effects during oxidative stress (Duckers et al., 2001). SOD2 is an intramitochondrial free radical scavenging enzyme that serves as the first line of defense against superoxide produced during oxidative phosphorylation. This enzyme catalyzes the dismutation of 2 superoxide anions to hydrogen peroxide. During redox imbalance, excess H2O2 produced in HepG2 cells is most likely converted to H2O by glutathione peroxidase, as there is little measurable catalase in the mitochondria (Bai and Cederbaum, 2000). In the presence of glutathione peroxidase and H2O2, 2 molecules of reduced glutathione react to form a molecule of glutathione disulfide (GSSG) causing the concurrent reduction of H2O2 to H2O. The glutathione disulfide molecule is converted back to 2 molecules of reduced glutathione by glutathione reductase and NADPH + H+. In our study, glutathione reductase mRNA was increased 1.6-fold (p < 0.012) while glutathione peroxidase and catalase were not significantly altered with ET-treatment. A major mutagenic base lesion in DNA caused by exposure to reactive oxygen species is 8-oxoguanine. This damaged base is excised by OGG1 (Lu et al., 1997). Although we have no direct evidence of DNA damage in this study, upregulation of OGG1 and direct evidence of oxidative stress gives us strong hypothetical evidence that there is oxidative damage to DNA secondary to ET treatment. CYP1A1, a P450 enzyme known to generate reactive oxygen species (Morel et al., 2000, 1999), was markedly downregulated in the first 6 h. This P450 monoxygenase has recently been shown to exhibit negative autoregulation, when the reactive oxygen species (ROS) generated by action of the enzyme repress the CYP1A1 gene promoter (Morel et al., 1999). Taken together, the altered gene expression pattern of the above-mentioned 5 genes is highly suggestive of oxidative stress.

Because microarray technology remains somewhat untested, selected significantly altered stress gene transcripts were quantitated by RT-PCR using the 7-gene plate described by Morgan et al. and compared to the results of the microarray gene expression analysis (Morgan et al., in press). Dysregulation of 3 redox-sensitive genes described above (HO1, GSH, and CYP1A1) and a cell cycle gene (CDKN1) were confirmed by RT-PCR analysis of the 6-h single time point experiment. TOP2A, downregulated, though not significantly according to microarray analysis, was significantly decreased as measured by RT-PCR. The expression levels of GCLM and TXNRD1, redox-sensitive genes not present on the Clontech Atlas Human Stress Array, were also upregulated via RT-PCR analysis.

The time course experiment was performed to track the expression of the 7 genes mentioned above over a 24-h period by RT-PCR. By following the gene expression changes over time, we not only provide a more complete gene expression profile but we also prevent inaccurate conclusions based on a single “snapshot”-type experiment. The time course RT-PCR data confirmed the gene expression changes noted in the 6-h microarray and RT-PCR assays; however, the chronology of these transcript changes did not always correlate well between experiments. For example, GSR transcripts did not reach values significantly increased above controls until 8 h in the time course experiment but obtained significance at 6 h in the single time point experiment. Given the dynamic nature of gene transcript expression, multiple time points are recommended.

Changes in transcription cannot be equated to changes in functional pathways because transcripts have to be translated, and proteins may require posttranslational modification in order to become functional. Because the above-mentioned changes in mRNA do not necessarily represent actual changes in the protein profile of the cell, the premise of ET-induced oxidative stress identified by gene expression profiling, needed the support of functional biochemical assays before it could be accepted. At 6 h and 1 mM (ET), depletion of intracellular GSH, accumulation of GSSG, and decreased GSH/GSSG ratio
confirmed the uncompensated redox imbalance first suggested by analysis of the oxidative stress gene expression. This allows the potential detection of toxic endpoints by evaluation of gene expression prior to morphologic changes in the cells. The 0.5 mM (ET), which failed to cause significant cell death at 24 h, caused oxidative stress detectable by decreased GSH, increased GSSG, and decreased GSH:GSSG ratio. In contrast to the higher concentrations, 0.25 mM (ET) at 24 h gave a significant increase in GSH. This result might be explained by ability of ET to induce GCLM, the rate-limiting step in GSH synthesis, causing an increase in intracellular GSH with (ET) insufficient to deplete GSH.

Decrease in ATP levels may be due to oxidative stress-associated protein cross-linking within the electron transport chain complexes and subsequent decrease in oxidative phosphorylation due to a decrease in mitochondrial membrane potential. Alternatively, the decrease in ATP levels and mitochondrial membrane potential might be due to an ET-induced intracellular increase in acyl-CoA esters which have been shown to be potent inhibitors of the mitochondrial adenine nucleotide translocase (ANT; Shrago et al., 1995). ANT ex-

FIG. 5. 0.5 mM ET for 6 h causes a marked induction in superoxide radical generation as measured by the oxidation of hydroethidine to ethidium bromide with subsequent fluorescence (610 nm) on excitation (confocal microscopic image). (A) Untreated HepG2 cells; (B) 035 mM ET.

FIG. 6. HepG2 cells administered a nontoxic dose of 0.5 mM ET exhibit an 84% reduction in ATP at 24 h. Data are the means of 5 separate experiments.

FIG. 7. ET-treated HepG2 cells exhibit a dose dependent reduction in mitochondrial membrane potential. 1 × 10⁶ cells were incubated with 100 nM Mitotracker Red for 15 min at 37°C in the dark and then treated with ET (250, 500, and 1000 μM) for 1 h at 37°C. Samples were analyzed immediately by flow cytometry; n = 2 replicates per concentration.
changes mitochondrial ATP for cytosolic ADP, considered to be the overall rate limiting step in energy metabolism (Lemasters and Sowers, 1979). The inability to bring ADP into the mitochondrion would halt the activity of Complex-V, allowing the proton gradient across the inner mitochondrial membrane to increase to dangerous levels. Uncoupling protein-3, known to be upregulated by ET-treatment (Cabrero et al., 1999, 2001), would dissipate the proton gradient and decrease the mitochondrial membrane potential. We know that oxidative stress is present in the ET-treated HepG2 cells, but we do not know if the ATP depletion or decrease in mitochondrial membrane potential is directly related to oxidative stress or if these changes are ET-specific.

Modulation of additional stress gene transcripts was also observed secondary to ET-treatment. GADD45, GADD153, GADD45, CDKN1, and MDM2 were upregulated, suggesting that there was DNA damage and p53-activation (Friedberg et al., 1995; Lewin, 2000; Walsh et al., 1995). Ubiquitin and ubiquitin protein ligase were also upregulated suggesting increased turn-over of proteins, which may be secondary to increased cytosolic protein volume or to misfolded or damaged proteins. Downregulated genes included PCNA, ERK1, and MAP kinase p38. Both ERK1 and p38 MAP kinase are responsive to oxidative stress (Ogura and Kitamura, 1998) and MAP kinase p38 is negatively regulated by thioredoxin (Hashimoto et al., 1999). ET-treated (1 mM) HepG2 cells exhibited a significant increase in cell death at 24 h with concurrent reduction in mitotic figures and increase in apoptotic/necrotic cells. All of these morphologic changes in the cells correlate well with downregulation of PCNA, ERK1 and MAP kinase p38. All of these changes in gene expression might be attributable to oxidative stress leading to DNA damage and protein cross-linking with subsequent cell cycle arrest and apoptosis and necrosis, however more work should be done to confirm these speculative conclusions.

Etomoxir and other CPT-1 inhibitors are known activators of peroxisome proliferator-activated receptor α (PPARα; Brandt et al., 1998; Djouadi et al., 1999; Forman et al., 1997; Portilla et al., 2000). The cytoplasmic accumulation of long-chain fatty acids secondary to inhibition of CPT-1 serves as a trigger for PPARα activation (Brandt et al., 1998; Forman et al., 1997). In addition, several CPT-1 inhibitors are structural analogs of long chain fatty acids, and therefore can bind directly to and activate PPARα (Forman et al., 1997). Skorin et al. (1992) showed that 2-tetradeccylglycidic acid (TDGA) and etomoxir enhance production of H$_2$O$_2$ in peroxisome- proliferated rat primary hepatocytes when fatty acids of 12 carbons or more were utilized as substrate. The increase in PPARα activity and subsequent H$_2$O$_2$ generation might be expected to induce oxidative stress in cells treated with CPT-1 inhibitors like etomoxir. Since ET mRNA levels are decreased in our study, it is unclear what role, if any, PPARα plays in generating oxidative stress secondary to ET-treatment of HepG2 cells, but the possible contribution of PPARα cannot be ignored.

In summary, the results of the current study demonstrate etomoxir-induced oxidative stress in a human liver cell line, first identified by analysis of stress gene transcript expression and then confirmed by multiple biochemical oxidative stress/ mitochondrial function assays. Alterations in the expression of genes related to cell cycle arrest and apoptosis are also observed and supported by morphological evidence. This is the first report of oxidative stress secondary to etomoxir administration, while the contribution of this oxidative stress to known toxic endpoints remains to be evaluated. This study illustrates the potential for gene expression profiling to serve as a tool to identify toxic mechanisms and pathways.

REFERENCES


