Transcriptional and physiological responses of HepG2 cells exposed to diethyl maleate: time course analysis

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Casey, Warren, Steve Anderson, Tony Fox, Karen Dold, Heidi Colton, and Kevin Morgan. Transcriptional and physiological responses of HepG2 cells exposed to diethyl maleate: time course analysis. Physiol Genomics 8: 115–122, 2002. First published December 18, 2001; 10.1152/physiolgenomics.00064.2001.—Expression levels of 767 genes were measured in HepG2 cells at eight time points (0, 0.5, 1, 6, 12, 16, 20, and 24 h) following exposure to the oxidizing agent, diethyl maleate (DEM). DEM treatment caused an immediate and sustained loss of intracellular GSH, with a concomitant increase in GSSG. From 6–12 h after exposure, there was a substantial increase in the percentage of cells undergoing S phase arrest and apoptosis. Expression profiles of ~90% of the genes fell into one of five clusters generated using hierarchical-clustering software, indicating the well-ordered nature of the stress response. The directional movement and timing of induction for many genes matched closely the known physiological role of the proteins they encode. Inhibitors of the cell cycle (CDKN1, CDKN4D, ATM) were induced, whereas cyclins (proliferating cell nuclear antigen [PCNA], cyclin A, cyclin D1, cyclin K) were downregulated during the period from 6–20 h. Likewise, pro-apoptotic genes such as the caspases (CASP9, CASP3, CASP2) and apoptotic protease activating factor (APAF) were induced during the same period. Results of this study indicate that there is a good correlation between time-dependant physiological, biochemical, and gene expression data.

HepG2 cell cycle; apoptosis

HIGH-DENSITY DNA ARRAYS offer a means for exploring the human genome and expanding our understanding of human disease. The application of these transcrip-tome-based methods to such disciplines as pathological diagnosis and pharmacological safety assessment is already being explored (6, 31, 33, 14, 20). The advent of these information-rich platforms has cast light on our lack of knowledge surrounding the global regulation of gene expression as it relates to the physiology of the systems we are studying. Time course experiments conducted with high-density arrays provide a much-needed dimension to the current paradigms of gene expression. From the relatively few reports published to date, it is apparent that the transcriptome is regulated in a manner that is remarkably well ordered, although the mechanisms used by cells to control large-scale gene expression remain to be elucidated.

The pleiotropic effects of oxidative stress have been well characterized. Redox imbalance has been linked to numerous disease states such as diabetes, cancer, acquired immunodeficiency syndrome (AIDS), Parkinson’s, while oxidative damage on a cellular level has been shown to cause cell cycle arrest and apoptosis (28). The goal of our present work is to determine the time-dependant relationship between biochemical, physiological, and differential gene expression associated with oxidative stress. Although we have performed an initial analysis of the data to demonstrate the general robustness of our approach, our primary intention is to provide the scientific community with a data set that can be analyzed subsequently by many diverse groups using varied techniques.

MATERIALS AND METHODS

Cell culture. Human hepatocellular carcinoma cells were obtained from the American Type Culture Collection (HepG2, ATCC 1998) and maintained on collagen-coated T175 vented flasks (Collagen I Cellware; Becton-Dickinson Labware) in DMEM with Glutamax and 10% fetal calf serum under standard cell culture conditions (37°C, 5% CO2) without antibiotics. For RNA and biochemical analysis, all cultures were grown in collagen (Vitrogen)-coated 150-mm-diameter cell culture dishes (Corning, NY) with 26 ml medium. Plates contained numbered, collagen-coated coverslips for subsequent enumeration and microscopic analyses of cellular morphology. Cultures were seeded at a concentration of 1 × 10⁷ cells per plate. Old medium was removed and 26 ml of fresh medium was added 24 h after seeding. On the day of dosing (48 h after seeding), old medium was removed from
the culture dish and was replaced with either fresh medium or fresh medium containing 1.25 mM of the oxidizing agent, diethyl maleate [DEM; 24 h]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) ED₅₀ concentration, data not shown]. The point at which the cultures were dosed is referred to as time 0, with additional times relative to this point.

Preparation of slides and enumeration of cells. Coverslips were removed from the culture dishes and stained with hematoxylin and eosin. Cells were enumerated using a scored reticule at ×10 magnification. For each slide, 5 squares of the reticule were counted from each of 21 separate areas (4 corners and middle). The average count from three slides was used to calculate the total number of cells per plate for each treatment group at each time point.

Cell cycle, DNA synthesis, and apoptosis analysis. HepG2 cells were grown and treated with 1.25 mM DEM as described above. Cells were collected at the indicated times after a 30-min pulse with 10 μM bromodeoxyuridine (BrdU). Following fixation with ice-cold 4% methanol, cells were incubated as 2 N HCl/0.5% Triton X-100 for 30 min to denature the DNA. The acid was neutralized with 0.1 M sodium borate. The cells were then incubated with anti-BrdU-fluorescein isothiocyanate for 30 min at room temperature, followed by incubation with 5 μg/ml propidium iodide (PI). The samples were analyzed on a FACSort (Becton-Dickinson) instrument. Cell cycle phases from PI-stained cells were quantitated using Modfit software. The percentage of the cell population containing BrdU was quantitated using Cellquest software, as was the sub-2n (apoptotic) population.

RNA preparation. For RNA analysis, three control (fresh medium only) and three treated (1.25 mM DEM in fresh medium) cultures were grown for each time point; 0, 0.5, 1, 6, 12, 16, 20, and 24 h. Total RNA was isolated using Trizol Reagent per the manufacturer’s instructions (GIBCO-BRL). RNA quality was assessed using agarose gel electrophoresis and quantitated spectrophotometrically at 260 and 280 nm.

Membrane hybridization and analysis. For membrane hybridization, the three RNA samples from each time point for control and DEM-treated cells were pooled, and 32P-labeled probes were prepared using 6 μg of total RNA, using the manufacturer’s recommended protocol (Clontech, Palo Alto, CA). Hybridization to Atlas 1.2 Human Arrays (Clontech) was done at 64°C for 16 h in 8 ml of MicroHybe, 4 μg poly-dA (Research Genetics), 8 mg human Cot-1 DNA (Clontech), and heat denatured [32P]cDNA. Arrays were washed at 64°C following the manufacturer’s instructions. The arrays were exposed to phosphor-imaging screens for 16–24 h, and the optical density was acquired using Optiquant and a Cyclone scanner (Packard BioSciences). Image Files from the phosphorimagery were initially analyzed using Clontech Atlas Image Software, which enables global background subtraction, global normalization (sum and median method), gene-specific normalization, and array comparisons. Subsequent analysis was performed using Gene Cluster/Tree View (Stanford University), Atlas Navigator (Clontech Laboratories) and Spotfire (Spotfire).

GSH and GSSG quantification. Control and DEM-treated cultures were grown in triplicate as described above and placed on ice immediately prior to GSH/GSSG extraction. Once cool, the culture medium was rapidly removed, and the cultures were washed once with ice-cold PBS. The PBS was removed, and 1 ml of ice-cold 10% metaphosphoric acid was added to each dish. The cells were then scraped into 16-ml plastic tubes, sonicated, and centrifuged at 12,000 g, and the supernatant was removed and stored at −80°C. GSH and GSSG were quantitated by HPLC on a Zorbax Amino column using the method of Reed et al. (27), with the exception that N-methylmaleimide was used place of iodoacetic acid to prevent oxidation of GSH. The average (n = 3) quantities of GSH and GSSG in each sample were normalized against cell number and expressed as nanomoles per 1 × 10⁶ cells.

RESULTS AND DISCUSSION

Membrane normalization. Adjusted hybridization intensities (background subtracted) were obtained for all 1,197 spots on the array using Atlas Image software. Genes with an adjusted intensity value of less than 50 (twice average background) were not considered for further analysis, leaving 829 genes in the data set. An additional 62 genes were removed from consideration due to their proximity to high-intensity signals and the inherent bleed-over problems associated with these spots (10). The remaining data, representing 767 genes, were normalized using either global (sum) or reference-gene methods.

Conventional “housekeeping genes” such as tubulin (TUBA1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ubiquitin were ruled out as choices for reference genes due to the substantial fluctuation in expression patterns of these genes seen over the course of the experiment. These variations were significantly greater (i.e., >200-fold difference between control and DEM-treated) than those normally encountered due to experiment-associated error (unpublished observation). Changes in expression of these genes would be expected in light of the observed physiological stress and the known roles for these genes in cellular processes such as apoptosis (TUBA1) and bioenergetics (GAPDH). Instead, we identified a “least changed” gene to use as a reference by performing cluster analysis (K-means, Spotfire) on the log₂ ratios (DEM-treated/control) generated using non-normalized adjusted intensity values (data not shown).

A “least changed” cluster composed of 173 genes was identified which showed minimal variation over the time course, and the average profile for this cluster was obtained using Spotfire software. We then searched the data to find a gene whose average intensity across all time points was close to the average intensity of all 767 genes and whose expression profile closely matched the “least changed” average profile. Roundabout homolog 1 (ROBO1), an axon guidance receptor, was the gene we chose as the standard for our reference-gene normalization process.

To access the impact of different normalization techniques, we compared data generated using global (sum) and reference-gene (ROBO1) methods. The expression patterns that resulted from these methods are shown for glutathione synthase (GSH-S) and gastrointestinal glutathione peroxidase (GSHPX-GI), genes representative of the two most divergent clusters (Fig. 1). The expression profiles from both normalization techniques were very similar across the entire time course for the two genes. We analyzed all data using both methods, and the overall conclusions were essentially identical, regardless of the normalization method employed. The global normalization process is based on
the assumption that all genes on the control and treated membranes show equal expression levels. Given the nature of the genes represented on the membrane (e.g., signaling, cell cycle, apoptosis, differentiation, etc.) and the extreme changes in cellular physiology induced by oxidative stress, we feel that this assumption is not accurate. We have therefore chosen to show all data presented herein as normalized to ROBO1, as we feel that the principal behind ROBO1 normalization is more sound than that behind global normalization in under the given circumstances.

Patterns. The ROBO1-normalized data were grouped into five broad clusters (clusters A–E) using software developed by Eisen et al. (12) (Fig. 2). The ~160 genes in cluster A demonstrated a prolonged period of downregulation from 6–16 h, whereas the 54 cluster B genes underwent a more immediate period downregulation from 1–4 h. Cluster C is composed of 170 genes which were immediately upregulated (average 2.6-fold increase at 15 min) and continued to be strongly (>8-fold) upregulated from 6–20 h, whereas the 134 genes in cluster D showed two distinct periods of moderate (~4-fold) upregulation at 6 and 20 h. Cluster E is composed of 150 genes which underwent a slight downregulation at 1 h and an approximate twofold increase in the log2 ratio from 12–16 h, followed by another period of slight downregulation at 20 h. The log2 ratio for almost all genes returned to near zero at the 24-h time point, a fact which demonstrates clearly the importance of time point selection when measuring gene

Fig. 1. Comparison of single gene vs. global normalization methods. Expression profiles of glutathione synthase (GSH-S, A) and gastrointestinal glutathione peroxidase (GSHPX-GI, B) over the 24 h experiment normalized. Log2 ratios (treated/control) were generated using either global or single gene (roundabout homolog 1, ROBO1) methods as described in the text.

Fig. 2. Hierarchical clustering of time course data for all genes. Cluster analysis was performed on the 767 well-measured genes as described in the text. Log2 ratios are shown as a function of time for the five clusters (clusters A–E) identified using clustering software developed by Eisen et al. (12).
expression. It is of interest to note that of the 767 well-measured genes on the membrane, almost all of them underwent a significant change (>2-fold at more than one time point) over the course of the experiment. This is most likely due to the several factors; the nature of the genes on the array (signal transduction, cell cycle, apoptosis, adhesion), the high degree of oxidative stress induced by DEM (MTS 50), and the fact that the dosing coincided with mitogenic stimulation. The rationale behind our experimental design was that by placing a disruptive event close to the source of a signal, the downstream effects of the disruption might be more obvious than if the disruption had been placed further from the source. In this case the “signal” was the mitogenic stimulation brought about by the addition of new media, and the disruption was the chemical insult caused by DEM. Given the infinite number of expression patterns possible with this data set, the five basic patterns we have observed support the emerging realization that the transcriptome is coordinately regulated at a high level (17, 18).

One trait we observed frequently was the “anti-similar” (mirror image) expression patterns of genes which have related physiological functions, two examples of which are given in Fig. 3. The mRNA levels for calcium binding proteins S100-A1 and S100-A9 were substantially changed in response to oxidative stress, with the degree of change being approximately equal for each gene, but in opposite directions across the time course. Another pattern of anti-similar expression is demonstrated with apoptotic protease activating factor-1 (APAF1) and BAD two bcl-binding proteins involved in the pro-apoptotic response. GSH-S and GSH-PX-GI, both involved in GSH utilization are also regulated in an anti-similar manner (see Fig. 1). Although this is not extensively pursued in this report, we propose that anti-similar patterns of expression may provide valuable insight in the coordinated regulation of gene response, raising the possibility that both genes are being affected by a common mediator that has opposite effects on gene expression.

Redox systems. Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) produced in the liver serves as the major redox buffer for mammals and plays a central role in cellular detoxification (13, 15, 23, 24). Treatment with 1.25 mM DEM caused a rapid and sustained depletion of GSH levels in HepG2 cells (Fig. 4A). One hour after treatment, GSH levels in treated cells were reduced to 39% (P = 0.026) of that measured in the control cultures, and these remained below control levels over the course of the 24-h experiment. Oxidized glutathione (GSSG) remained at or below the limit of detection in control cultures until the 16-h time point, whereas DEM-treated cultures showed measurable accumulation of GSSG beginning at 12 h, with a steady increase.

Fig. 3. Anti-similar patterns of expression. Anti-similar (mirror image) patterns of expression were seen for many genes that have related physiological functions. Time-dependent expression patterns are given for genes encoding calcium binding proteins S100-A1 and S100-A9 (A) and pro-apoptotic proteins APAF1 and BAD (B).

Fig. 4. Effects of diethyl maleate (DEM) on intracellular GSH and GSSG level. GSH (A) and GSSG (B) levels were calculated on a per cell basis, as described in the text. Cells treated with 1.25 mM DEM showed a rapid and sustained decrease in the level of intracellular GSH relative to control cultures. GSSG levels in treated cells increased to a detectable level at 12 h and remained elevated throughout the 24-h experiment.
in GSSG concentration seen through 24 h (Fig. 4B). The simultaneous decrease in cellular GSH levels and increase in GSSG levels resulted in DEM-treated cultures having a 4.5-fold lower GSH:GSSG ratio than control cells at the 24-h time point.

GSH is synthesized from its component amino acids in two sequential reactions catalyzed by \(\gamma\)-glutamylcysteine synthase (\(\gamma\)-GCS) and glutathione synthase (GS), respectively. As may be expected in light of the above data, GS, the terminal enzyme in glutathione biosynthesis was upregulated fourfold immediately following DEM treatment and remained upregulated through the 20-h time point (Fig. 5A) (Note: \(\gamma\)-GCS was not represented on the array.) GSH is dimerized during the formation of GSSG, a reaction that can occur spontaneously under oxidizing conditions or which can be catalyzed by one of several glutathione peroxidases (GSHPXs) with the concomitant reduction of hydrogen peroxide to water. GSSG has been shown to stimulate the uptake of \(\text{Ca}^{2+}\), which in turn has numerous physiological consequences such as cell cycle arrest and apoptosis (19, 34, 35). GSHPX1 and GSHPX-G1 were downregulated in treated cells, presumably as a feedback response to the increasing levels of GSSG brought about by the DEM-induced autoxidation of GSH. GSH can be regenerated from GSSG via glutathione reductase (GR) in a reaction requiring NADPH. Interestingly, mRNA levels for GR, a protein which could

Fig. 5. Hierarchical clustering of physiologically related genes. Cluster analysis was performed on physiologically related genes that demonstrated significant change in response to DEM treatment. Groups are divided based on known biological function: redox balance (A), cell cycle control (B), and apoptosis and calcium homeostasis (C).
potentially serve to decrease GSSG and restore GSH levels, were slightly downregulated (cluster B) relative to the control. This could be an indication that the cells are attempting to conserve NADPH to drive other, more physiologically necessary, reactions. Glutathione S-transferases (GSTs) play an important role in the detoxification process by conjugating GSH with hydrophobic and electrophilic compounds. GSTM1, GSTII, GSTA1, GSTT1, and GST12 were all downregulated, a uniform response that is difficult to explain in light of the current knowledge regarding the detoxification function of these proteins.

Thioredoxin, although less abundant than GSH, also plays numerous important biological roles such as growth factor, antioxidant, enzyme cofactor, inhibitor of apoptosis, and transcription factor regulator (reviewed in Refs. 2 and 26). Thioredoxin also plays a crucial role in DNA synthesis by providing reducing equivalents for the synthesis of deoxyribonucleotides via ribonucleotide reductase (16). To exert its beneficial effects, thioredoxin must be in a reduced state, an NADPH-requiring reaction catalyzed by thioredoxin reductase (TRX). Message levels for TRX were substantially upregulated through the 20-h time point (Fig. 5A), most likely a response to the increased requirement for reduced thioredoxin. Selenium binding protein (SBP) was also markedly upregulated, possibly reflecting the important role selenocysteine plays in the activity of GPxs and TRX (reviewed in Refs 3, 4, 32). Heme oxygenase 1 (HO1), a widely accepted marker for oxidative stress important for the formation of the antioxidant, bilirubin, was upregulated, as was B18 (ubiquinone reductase), an enzyme essential for maintaining the antioxidant properties of ubiquinone (25).

Cell cycle and apoptosis. Cell cycle status of control and treated cultures was measured using flow cytometry. Incorporation of BrdU was used as an indicator of active DNA synthesis, while intercalation of PI reflected total DNA content. The percentage of control cells incorporating BrdU paralleled the percentage in S phase as judged by PI staining, indicating normal progression through S phase (Fig. 6A). Cells responded to DEM treatment with an S phase arrest: while the PI data showed a constant percentage of cells in S phase throughout the time course, the BrdU data showed a simultaneous decrease in the percentage of cells incorporating BrdU (Fig. 6B).

The number of cells in the control culture doubled during the 24-h experiment, whereas cell numbers for DEM-treated cultures were reduced by ~50% (Fig. 7A). Microscopic morphological examination of cells demonstrated a substantial increase in the apoptotic index between 6 and 12 h (data not shown), consistent with the observed increase in the sub-2n population as measured by fluorescence-activated cell sorting (FACS) using the PI incorporation (Fig. 7B). These findings are in agreement with previously published reports of oxidative-stress-mediated cell cycle arrest and apoptosis (reviewed in Refs. 7, 11, 21, 22, 28, and 30).

The genes for many proteins involved in cell cycle control were regulated in response to DEM treatment. Cyclin-dependent kinase inhibitor 3 [CDKN1 (waf1)], CDKN2D (ink4d), and ataxia telangiectasia (ATM) were all upregulated significantly (cluster C), whereas proliferating cell nuclear antigen (PCNA), cyclin K, cyclin A, cyclin D1, and cell division cycle protein 25C (CDC25C) were all downregulated (cluster A) (Fig. 5B). The directional movement of the above mentioned genes is both intuitive and consistent with the known function of the proteins. For example, pCDKN1 is known to cause cell cycle arrest, so an increase in the mRNA levels for this protein would be consistent with the observed physiological response to DEM treatment. Conversely, cyclins are known to facilitate progression through the cell cycle, so a decrease in mRNA for these proteins would also be consistent with cell cycle arrest. The change in mRNA levels for several other genes represented a response that could be deemed counterintuitive. CDKNN4, a cell cycle inhibitor, was downregulated (cluster A), whereas cyclin A1 and CDC25A were upregulated (cluster C). These counterintuitive responses may be an attempt by all cells to overcome the physiological damage caused by DEM or
and Bcl-2 family proteins characterized events. The balance between Bax family processes (e.g., cellular dehydration, increased calcium uptake, nuclear disintegration) are clear. We have focused our discussion on genes involved in these well-characterized events. The balance between Bax family and Bcl-2 family proteins figures directly into the activation of apoptotic processes such as the caspase cascade (1, 8, 29). Transcript level for genes in both these families are upregulated to some degree, with the most substantial increases seen in mRNA levels for BAG-1 (Bax family) and MCL-1 (Bcl-2 family) (Fig. 5C). These conflicting signals indicate that the ultimate influence of these proteins is determined at the posttranscriptional level. Message levels for APAF, a protein activated by an increased Bax:Bcl-2 ratio and required for the processing of pro-caspase 9 (CASP9), are also strongly upregulated. Most of the pro-caspases (CASP9, CASP3, CASP2) fall into cluster C, being substantially upregulated over the course of the experiment. WCH4, an aquaporin that could facilitate cellular dehydration, was upregulated in the same cluster at the caspases (cluster C). Both CASP10, a Fas-associated caspase, and the Fas-L receptor gene were downregulated (cluster A). A cellular response to DNA damage was indicated by the upregulation of GADD153 (DNA repair), whereas nuclear disintegration was suggested by the upregulation of DFF40 (DNA fragmentation) and DNase II (Fig. 5C).

Most genes on the array encoding calcium-related proteins were significantly regulated in response to oxidative stress. Calcium binding proteins S100-A9 and S100-A7 were upregulated over the course of the experiment (Fig. 5C), as were apoptosis-associated genes, CANP3 (calcium-activated neutral proteinase), and ALG-2 (calcium binding protein), (cluster C). Calcium binding protein S100-A1 was the only calcium-associated protein on the membrane that showed a marked downregulation.

Summary. HepG2 cells treated with DEM undergo a rapid loss of intracellular glutathione with a concomitant increase in levels of GSSG. These biochemical changes cause a redox imbalance that leads to oxidative stress as determined by a decrease in the GSH:GSSG ratio. The cellular response to this oxidative stress is a massive and highly coordinated change in gene expression, which results ultimately in S phase arrest and apoptosis. By examining the grouping of gene sets and timing of gene expression, it may be possible to elucidate functional relationships that have not previously been realized. For example, the induction and the coregulation of APAF, CASP9, CASP3, CASP2, and DFF40 indicate a clear role for these genes in the observed apoptotic process, whereas the downregulation of CASP10 indicates a distinctly different role from the other caspases. By observing differential activation of metabolic pathways at the transcriptional level, we may also gain insight into the strategic aspects of cellular physiology. In the present study, we hypothesize that cells are attempting to restore GSH levels primarily by de novo synthesis (GS upregulated) as opposed to the reduction of GSSG to GSH (GPX downregulated).

Our data highlight the need for accompanying physiological/biochemical studies when interpreting gene expression data. The change in expression of some genes in this study (e.g., WAF1, CASP9, APAF, cyclin D1) parallels the observed physiological changes, consistent with the function of the respective proteins. Other genes (TRX, CDKN4, CDC25A) have significant changes in expression that do not reflect the observed changes in cellular physiology, but which may be indicative of what the cell is attempting to accomplish. Obviously, further work in needed to understand the meaning behind the directional movement and timing of induction of most gene expression data.

Although we have focused our discussion on a few key cellular processes (redox balance, cell cycle, and...
apoptosis), numerous other processes such as adhesion, intra- and intercellular signaling, membrane channels, and trafficking are represented in the supplemental gene expression data, available as Supplemen
tal Material,1 published online at the Physiological Genomics web site. Indeed, the most valuable aspect of the data presented herein is the availability to other researchers who have expertise in many di
verse aspects of cellular physiology.

REFERENCES


2. Arner ESJ and Holmgren A. Physiological functions of thiore
odoxin and thioredoxin reductase. Eur J Biochem 267: 6102–


12. Eisen MB, Spellman PT, Brown PO, and Botstein D. Clus

13. Fahey RC and Sundquist AR. Evolution of glutathione me


1 Supplementary Material to this article (Microsoft Excel files of the raw data) is available online at http://physiolgenomics.physiology.org/cgi/content/full/8/2/115/DC1.


17. Holter NS, Maritan A, Cieplak M, Fredoroff NV, and Ban


25. Pobezhimo VP and Voinikov VV. Biochemical and physiolo


28. Sen CK, Sies H, and Baererle PA (Editors). Glutathione mod-


34. Xia R, Stangler T, and Abramson JJ. Skeletal muscle ryan
didine receptor is a redox sensor with a well defined redox potential that is sensitive to channel modulators. J Biol Chem 275: 36553–36651, 2000.

35. Zable AC, Favero TG, and Abramson JJ. Glutathione modulo