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Ethylene Glycol Monomethyl Ether–Induced Toxicity Is Mediated through the Inhibition of Flavoprotein Dehydrogenase Enzyme Family

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Ethylene glycol monomethyl ether (EGME) is a widely used industrial solvent known to cause adverse effects to human and other mammals. Organs with high metabolism and rapid cell division, such as testes, are especially sensitive to its actions. In order to gain mechanistic understanding of EGME-induced toxicity, an untargeted metabolomic analysis was performed in rats. Male rats were administrated with EGME at 30 and 100 mg/kg/day. At days 1, 4, and 14, serum, urine, liver, and testes were collected for analysis. Testicular injury was observed at day 14 of the 100 mg/kg/day group only. Nearly 1900 metabolites across the four matrices were profiled using liquid chromatography-mass spectrometry/mass spectrometry and gas chromatography-mass spectrometry. Statistical analysis indicated that the most significant metabolic perturbations initiated from the early time points by EGME were the inhibition of choline oxidation, branched-chain amino acid catabolism, and fatty acid β-oxidation pathways, leading to the accumulation of sarcosine, dimethylglycine, and various carnitine- and glycine-conjugated metabolites. Pathway mapping of these altered metabolites revealed that all the disrupted steps were catalyzed by enzymes in the primary flavoprotein dehydrogenase family, suggesting that inhibition of flavoprotein dehydrogenase–catalyzed reactions may represent the mode of action for EGME-induced toxicity. Similar urinary and serum metabolite signatures are known to be the hallmarks of multiple acyl-coenzyme A dehydrogenase deficiency in humans, a genetic disorder because of defects in primary flavoprotein dehydrogenase reactions. We postulate that disruption of key biochemical pathways utilizing flavoprotein dehydrogenases in conjugation with downstream metabolic perturbations collectively result in the EGME-induced tissue damage.

Key Words: **metabolomics; ethylene glycol monomethyl ether; mode of action.**

Ethylene glycol monomethyl ether (EGME) is an industrial solvent used widely for the production of cellulose acetate, resins, paints, inks, and stains. It is also used in jet fuels and hydraulic fluids as an antifreeze. EGME is colorless and volatile and can be absorbed readily by inhalation and skin contact. It has been well established that EGME causes toxicity to several mammalian species including human. Tissues and organs with rapidly dividing cells and high metabolism, such as testes and thymus, are known to be particularly vulnerable (Bagchi and Waxman, 2008; Boatman, 2005; Johanson, 2000). In human studies, various reports have linked occupational exposure to EGME with hematological, neurological, and reproductive tissue abnormalities (Cohen, 1984; Cook *et al.*, 1982; El-Zein *et al.*, 2002; Larese *et al.*, 1992; Welch and Cullen, 1988; Welch *et al.*, 1988). In laboratory animals, reproductive tissue damage, including testicular atrophy and impairment of female fertility, has been found to be the most significant outcome of EGME treatment (Berndtson and Foote, 1997; Chapin and Lamb, 1984; Dodo *et al.*, 2009; Doe *et al.*, 1983; Hanley *et al.*, 1984). In addition, EGME can also impact the immune system, causing decreased thymus weight, thymic atrophy, and reduced spleen cell number (Exon *et al.*, 1991).

In spite of the substantial EGME literature and the well-documented histopathological findings, little has been elucidated about the molecular mechanisms of EGME-induced toxicity. Transcriptomic analyses have been conducted to understand gene changes following EGME treatment (Fukushima *et al.*, 2005; Jindo *et al.*, 2001; Syed and Hecht, 1998; Tirado *et al.*, 2003; Wang and Chapin, 2000). Among genes regulated by EGME were various protein kinases and genes associated with oxidative stress, thus leading to the hypothesis that EGME-induced apoptosis is mediated through oxidative stress (Bagchi and Waxman, 2008). However, in these studies, a single high dose of EGME was used, which could result in acute cell injury. With the exception of one study (Jindo *et al.*, 2001), all the transcriptomic analyses of EGME toxicity

examined a single time point after exposure, further limiting the scope of these data sets. In another well-designed study, a microarray analysis of the effect of methoxyacetic acid (MAA), a metabolite of EGME treatment in rats over a time course, suggested that histone hyperacetylation could be an underlying cause of the toxicity (Wade *et al*., 2008).

Biochemical profiling, or metabolomics, is a rapidly evolving technology that has been used increasingly in disease characterization and drug development. Hence, we attempted to gain further understanding of the molecular mechanism of EGME-induced toxicity by an unbiased biochemical profiling approach based on liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry (LC/MS and GC/MS). For this study, male rats were treated with two doses of EGME (30 and 100 mg/kg/day) and vehicle control. Serum, urine, liver, and testes were collected at days 1, 4, and 14 for metabolic profile analyses.

MATERIALS AND METHODS

Animal studies. Male Fischer 344 rats of 9 weeks of age used in this study were from Charles River Japan, Inc. (Atsugi Breeding Center, Tokyo, Japan). Animals were housed in stainless steel cages in a room that was lit for 12 h (0700 h–1900 h) daily, ventilated with an air exchange rate of 10–20 times/h, and maintained at 20°C–26°C with a relative humidity of 30–70%. Rats were housed individually in metabolic cages during the period when urine samples were collected, and urine collection began after transfer to these cages. All animals were allowed free access to water and food (Charles River Formula 1, sterilized by radiation; Oriental Yeast Co., Tokyo, Japan) except for a 4-h fasting period prior to plasma and tissue collection. After a 5-day acclimatization period, rats were randomly assigned to nine groups ($n = 5$ for each group) by body weight. Three groups received EGME at 30 mg/kg/day, three groups received EGME at 100 mg/kg/day, and the other three groups received a vehicle control (0.5% carboxymethyl cellulose sodium aqueous solution) by oral gavage. The initial dosing day was designated as day 0, and various samples were collected at days 1, 4, and 14. Urine was collected over 24 h with the collection vessels surrounded by dry ice during the collection period. After urine collection, rats were maintained under fasting condition for 4 h after which serum samples were collected. Animals were then euthanized for histopathological examination and liver and testes harvested. The samples were stored in a freezer at −80°C. All animal study procedures were performed in accordance with the rules of the Institutional Animal Care and Use Committee at the study facility.

Metabolomic profiling platform. The untargeted metabolic profiling platform employed for this analysis was based on a combination of three independent platforms: ultrahigh-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) optimized for basic species, UHPLC/MS/MS optimized for acidic species, and GC/MS. The details of this platform were described in a previous publication (Evans *et al*., 2009).

Data imputation and statistical analysis. After the data were corrected for minor variation resulting from instrument interday tuning differences (Evans *et al*., 2009), the missing values for a given metabolite were imputed with the observed minimum detection value on the assumption that they were below the limits of detection. For the convenience of data visualization, the raw area counts for each metabolite were rescaled by dividing each samples value by the median value for the specific metabolite.

Statistical analysis of the data was performed using JMP (SAS, http://www.jmp.com), a commercial software package, and "R" (http://cran.r-project.org/), which is a freely available open source software package. A log transform was applied to the observed relative concentrations for each metabolite because, in general, the variance increased as a function of a metabolite's average response. Welch's *t*-tests were performed to compare data between experimental groups. Multiple comparisons were accounted for with the false discovery rate (FDR) method, and each FDR was estimated using *q* values.

TABLE 1
Histopathological Findings in Rats Treated with 100 mg/kg/day of EGME at Day 14. There Were No Pathological Changes in Other Time Points and with 30 mg/kg/day Doses

Tissue	Number of rats affected	Histopathological changes
Testis	3/5	Single-cell necrosis of dividing spermatocytes, cell debris in tubules, decreased number of spermatocytes, round spermatids, and elongate spermatids
Epididymis	2/5	Decreased number of spermatozoa in duct and cell debris in duct
Liver	0/5	Within normal limits
Kidney	0/5	Within normal limits
Thymus	0/5	Within normal limits

RESULTS

Dose Regimen and Histopathological Observations

In this study, we treated male rats with two doses (30 and 100 mg/kg/day) and selected three time points (days 1, 4, and 14) for analysis. As summarized in Tables 1 and 2, body, liver, kidney, thymus, epididymis, and testes weights as well as organ histopathology were examined. At the 30 mg/kg/day dose, there were no significant differences between the treatment and the control groups at all three time points. At the 100 mg/kg/day dose, a significant decrease in testis weight was observed at day 14. Consistent with this finding, single-cell necrosis of dividing spermatocytes (stage XIV), decreased number of spermatocytes, rounded and elongated spermatids (stages I–VI), and ductal cell debris were observed (Fig. 1). Another significant difference was the decreased thymus weight at both day 4 and day 14. Also, at the 100 mg/kg/day dose, decreased number of spermatozoa and cell debris in epididymal ducts were observed. In addition, slight and transient decreases in liver and kidney weight were observed at day 4 only. However, no abnormal histopathological changes were noted in either organ.

Metabolomic Profiles and Statistical Analysis

A combination of two LC/MS/MS platforms and a GC/MS platform were used to profile the metabolites present in the serum, urine, liver, and testes. The detailed method is described in a previous publication (Evans *et al*., 2009). In brief, the samples were extracted, analyzed over the three MS platforms, ion peaks matched to standards in a reference library for structural identification, and relative levels quantitated. Across

TABLE 2

Tissue Weights of Rats Treated with EGME. Significant Difference from Control Group in Each Dosing Period: *$p < 0.05$, **$p < 0.01$ by Dunnett's Test. Parenthesis Shows Relative Weight of Tissue to Body Weight. The Bold Font Type Signifies Statistically Significant Results ($p < 0.05$)

Treatment time	Day 1			Day 4			Day 14		
Dose (mg/kg)	0	30	100	0	30	100	0	30	100
Number of animals	4	5	5	5	5	5	5	5	5
Body (g)	230.8 ± 11.32	231.4 ± 5.46	233.2 ± 4.55	243.0 ± 7.42	238.6 ± 4.93	235.0 ± 8.25	267.0 ± 6.00	266.4 ± 13.58	264.2 ± 9.15
Testis (right) (g)	1.378 ± 0.0532	1.388 ± 0.0370	1.392 ± 0.0683	1.451 ± 0.0631	1.429 ± 0.0283	1.401 ± 0.0746	1.472 ± 0.0397	1.458 ± 0.0383	**1.287 ± 0.1997***
Epididymis (right) (g)	0.275 ± 0.0129	0.262 ± 0.0286	0.254 ± 0.0270	0.265 ± 0.0194	0.289 ± 0.0315	0.246 ± 0.0224	0.357 ± 0.0235	0.370 ± 0.0190	0.356 ± 0.0236
Liver (g)	8.243 ± 0.6615	8.216 ± 0.3798	8.192 ± 0.3398	8.616 ± 0.3144	8.146 ± 0.2364	**7.584 ± 0.5871****	8.782 ± 0.4870	8.748 ± 0.9380	8.727 ± 0.3212
Kidney (right) (g)	0.833 ± 0.0826	0.818 ± 0.0319	0.854 ± 0.0503	0.860 ± 0.0163	0.847 ± 0.0362	**0.799 ± 0.0322***	0.887 ± 0.0247	0.875 ± 0.0494	0.840 ± 0.0271
Thymus (g)	0.265 ± 0.0289	0.290 ± 0.0245	0.262 ± 0.0319	0.263 ± 0.0202	0.259 ± 0.0112	**0.202 ± 0.0123****	0.232 ± 0.0291	0.232 ± 0.0162	**0.149 ± 0.0298****

FIG. 1. Histopathological observations of the testis in day 14 group. Representative light micrographs are shown. Seminiferous tubules at stages XIV (A and B) and I–VI (C and D) from the 100 mg/kg–treated group (B and D) and the control (A and C) are shown. The dividing spermatocytes are indicated by arrows. Single-cell necrosis and cell debris in the tubules were observed at stage XIV (B). Spermatocytes and round and elongate spermatids were decreased or lost in the 100 mg/kg–treated group (C) compared with the control group (D).

the four matrices, nearly 1900 metabolites were profiled by this method (391, 575, 422, and 418 metabolites from the serum, urine, liver, and testes, respectively).

Five rats per experimental group were used in this study. The changes of the metabolites between EGME-treated groups and their control at each time point were calculated by the ratio of their group means. The statistical significance of the changes was analyzed by Welch's *t*-test, with $p < 0.05$ deemed to be significant. In addition, multiple comparisons for this data set were accounted for with the FDR method, and each FDR was estimated using q values. The full statistical table is included in the Supplementary data. After mapping the metabolites into general biochemical pathways as illustrated in the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) and analyzing the statistical significance (see the statistical table under Supplementary data), it became clear that the pathways perturbed by the EGME in the early time points and persisted throughout the course of the experiment were those of choline oxidation, branched-chain amino acid (BCAA) catabolism, and fatty acid β-oxidation.

Choline Oxidation

The catabolism of choline occurs through a series of demethylation steps to produce betaine, dimethylglycine, sarcosine, and glycine (Fig. 2). In this study, increases of urinary

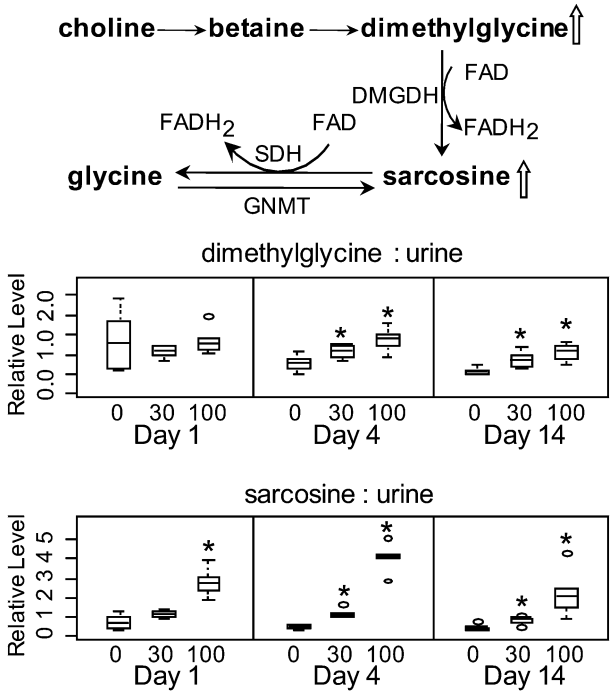

FIG. 2. Choline oxidation pathway and the box plots of dimethylglycine and sarcosine in the urine. Asterisk represents statistically significant to the control group. The box represents the middle 50% of the distribution, and upper and lower ''whiskers'' represent the entire spread of the data. The hyphen refers to the mean and the circle the outlier. The name of each metabolite is indicated on the top of the plot. The y-axis references the median scaled value. The *p* values for all comparisons are referenced in Supplementary data and, if less than 0.05, are marked with an asterisk. GNMT, glycine N-methyltransferase; SDH, sarcosine dehydrogenase.

dimethylglycine and sarcosine were among the most dramatic changes induced by EGME treatment (Fig. 2). Sarcosine was elevated significantly at day 1 of the 100 mg/kg dose and then at days 4 and 14 of both doses. Dimethylglycine was elevated significantly at days 4 and 14 of both doses.

Amino Acid Catabolism and Fatty Acid β-Oxidation

Significant changes in the isoleucine pathway by EGME were observed. As shown in a condensed scheme (Fig. 3), the catabolism of isoleucine produces succinyl-coenzyme A (CoA), which is further incorporated into the tricarboxylic acid (TCA) cycle for energy production (The S-pathway). Alternatively, isoleucine can be degraded by the R-pathway to produce metabolites. A portion of the R-pathway overlaps with fatty acid β-oxidation because butyrylglycine, butyrylcarnitine, and ethylmalonate can be derived from both pathways. In this study, the levels of 2-methylbutyrylglycine, 3-hydroxy-2-ethylpropionate, ethylmalonate, and butyrylglycine were increased in the urine following the EGME treatment. In addition, the levels of butyrylcarnitine, 2-methylbutyrylcarnitine, and 3-hydroxy-2-ethylpropionate were elevated in the serum, testes, and liver (Fig. 3). Many of these changes were significant as early as day 4 at both the 30 and the 100 mg/kg doses.

Similar to isoleucine metabolism, leucine catabolism was altered by EGME treatment. Isovalerylglycine was significantly elevated in the urine at day 4 by the 100 mg/kg doses and at day 14 by both the 30 and the 100 mg/kg doses. The increase of hydroxyisovaleroylcarnitine was observed in the testes at day 14 of the 100 mg/kg dose (Fig. 4).

Another significant change of EGME treatment was observed in lysine catabolism. In liver, a significant increase of glutarylcarnitine was found at day 14 of the 100 mg/kg group (Fig. 5). Glutarylcarnitine also showed a trend of increases in a dose-dependent manner at day 4, although the *p* values were slightly beyond the significance cutoff of 0.05.

Notable Metabolic Changes at Day 14

Creatine was increased significantly in the urine, serum, and liver. Arginine, guanidinoacetate, and ornithine were also elevated in various matrices, suggesting that the biosynthesis of creatine was altered following EGME treatment (Fig. 6). These changes were primarily significant at day 14 of the 100 mg/kg/day group.

Additional metabolites likely associated with cell and tissue damage, including dihydrouracil, pyroglutamine, hydroxyproline, and dimethylarginine were found to be elevated by EGME treatment in various matrices (Fig. 7). Ophthalmate, on the contrary, was decreased by EGME treatment (Fig. 7). Dihydrouracil is a catabolite of RNA degradation. Pyroglutamine is a posttranslational protein modification of N-termini. Its presence is likely derived from protein degradation. Hydroxyproline is a marker for collagen degradation. Ophthalmate and dimethylarginine are markers for oxidative stress (Beltowski and Kedra, 2006; Soga *et al*., 2006). These changes were mostly observed at day 14 of the 100 mg/kg/day dose.

DISCUSSION

We performed a multidose and multi–time point analysis of serum, urine, liver, and testicular tissues collected from EGME-treated rats. The doses and time points were chosen so that only the last time point of the high-dose group would display tissue injury by histopathological analysis. By this experimental design, we hoped to identify EGME's mechanism of action and the metabolic effects leading to tissue injury. Statistical analysis of the data and pathway mapping of the metabolites clearly found that the primary perturbation by EGME was centered on the inhibition of choline oxidation, BCAA catabolism, and fatty acid β-oxidation pathways.

In the choline oxidation pathway, sarcosine was elevated at day 1 by the 100 mg/kg EGME treatment. At days 4 and14, dimethylglycine and sarcosine were both increased significantly by the 30 and 100 mg/kg EGME. These changes strongly suggested that the inhibition of the enzymes converting the two metabolites, dimethylglycine dehydrogenase (DMGDH) and

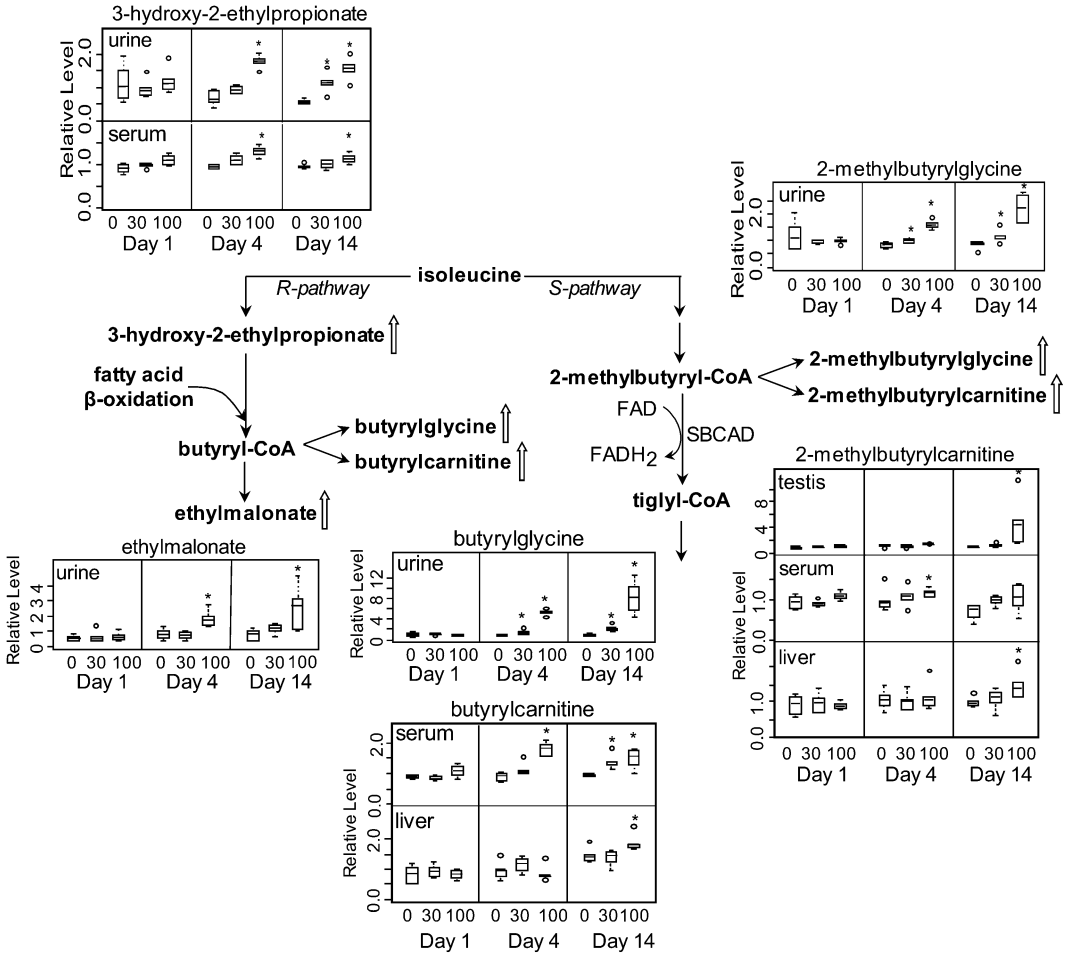

FIG. 3. Isoleucine metabolism and the box plots of isoleucine catabolites altered by EGME treatment. Asterisk represents statistically significant to the control group. SBCAD, short/branched-chain acyl-CoA dehydrogenase.

sarcosine dehydrogenase (SDH) (Fig. 2), was an early action of the EGME treatment.

Similarly, many intermediates in the isoleucine and leucine catabolism were accumulated at the early time points and the low-dose EGME treatment. As illustrated in Figures 3 and 4, the catabolism of isoleucine and leucine generates various CoA intermediates. The CoA intermediates can be rapidly converted into their corresponding carnitine esters (which can often be measured in serum and tissues) and/or glycine esters (which can often be measured in urine). Under normal conditions, the levels of carnitine and glycine CoA esters are low because the CoA intermediates are efficiently converted to produce acetyl-CoA and succinyl-CoA, which are incorporated into the TCA cycle for energy generation. Hence, the serum and urinary profiles of carnitine and glycine metabolites can reflect impaired BCAA metabolism and have been used as biomarkers for genetic disorders associated with BCAA metabolic pathways (Bennett *et al.*, 1994; Korman, 2006; Tanaka *et al.*, 1966). The accumulation of 2-methylbutyrylcarnitine and 2-methylbutyrylglycine in the isoleucine catabolic pathway suggests that short/branched-chain acyl-CoA dehydrogenase, the enzyme converting 2-methylbutyryl-CoA, was inhibited by EGME. Consistent with this conclusion, 3-hydroxy-2-ethylpropionate, ethylmalonate, butyrylglycine, and butyrylcarnitine were increased because of the inhibition of the S-pathway, thus shunting isoleucine into the R-pathway. Similarly, the increases of isovalerylglycine and hydroxyisovaleroylcarnitine in the leucine catabolic pathway suggested that isovaleryl-CoA dehydrogenase was inhibited by EGME.

Although choline oxidation and isoleucine/leucine catabolism were not directly associated in a metabolic cascade, the fact that the pathways were simultaneously affected by EGME suggested that they shared a common linkage. Upon examining the EGME-inhibited enzymes in the pathways, it became apparent that they all belonged to a small enzyme family of primary flavoprotein dehydrogenases with flavin adenine dinucleotide (FAD) as the prosthetic factor. This family of enzymes is involved in fatty acid β-oxidation (short-, medium-, long-, and very long–chain acyl-CoA dehydrogenases [LCAD]), amino acid metabolism (isovaleryl-CoA dehydrogenase for

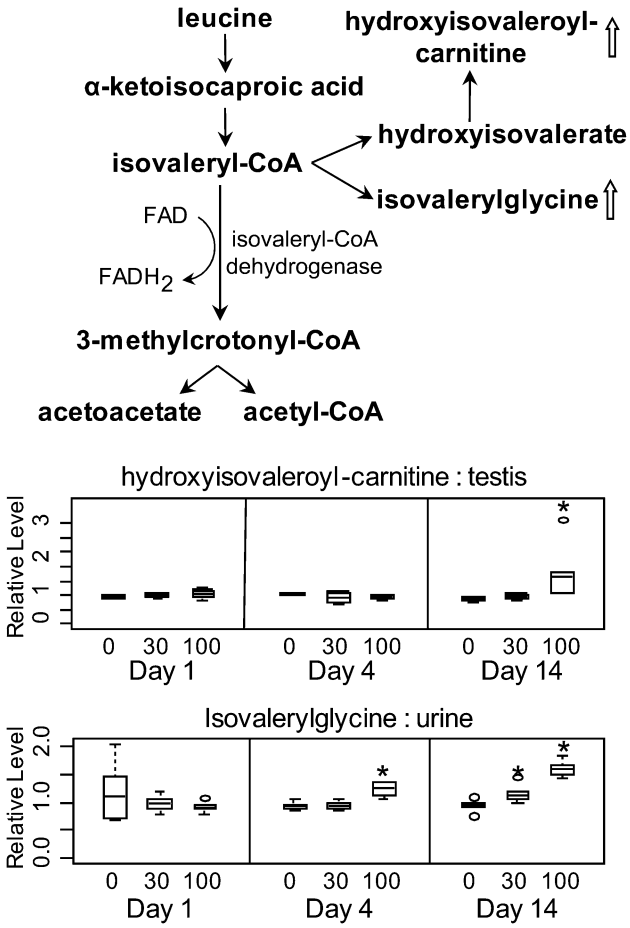

FIG. 4. Leucine metabolism and the box plots of leucine catabolites altered by EGME treatment. Asterisk represents statistically significant to the control group.

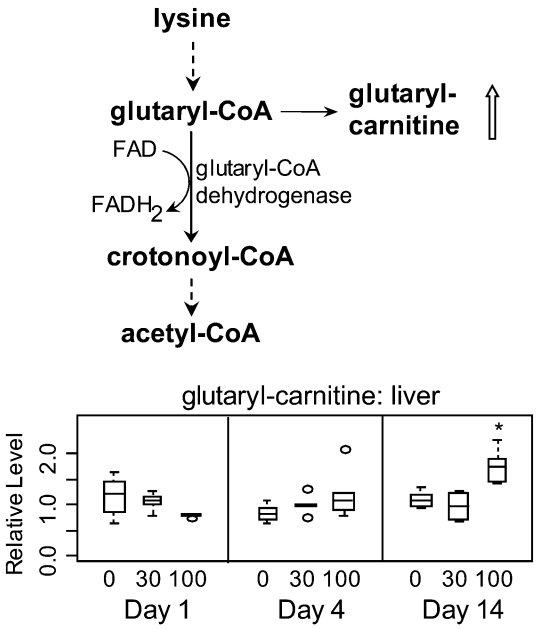

FIG. 5. Lysine degradation pathway and the box plot of glutarylcarnitine in the liver. Asterisk represents statistically significant to the control group.

leucine, branched-chain acyl-CoA dehydrogenase for isoleucine, isobutyryl-CoA dehydrogenase for valine, and glutaryl-CoA dehydrogenase for lysine), and choline oxidation (DMGDH and SDH) (Swigonova *et al.*, 2009). In order to assess the degree to which these enzymes were affected by EGME, we performed a detailed examination of the metabolomic data with the focus on the rest of enzymes in this family: glutaryl-CoA dehydrogenase, isobutyryl-CoA dehydrogenase, and enzymes of the fatty acid β-oxidation pathway. As a consequence, there was clear evidence that this family of enzymes was indeed broadly inhibited by EGME.

In the liver, both glutarylcarnitine (Fig. 5) and isobutyrylglycine (Supplementary data) were increased significantly at day 14 of the 100 mg/kg EGME group. These changes were consistent with the inhibition of glutaryl-CoA dehydrogenase and isobutyryl-CoA dehydrogenase, respectively, leading to the conversion of the CoA intermediates to their carnitine or glycine esters. In the fatty acid β-oxidation pathway, several species of fatty acid–derived carnitines were not altered significantly (Supplementary data). However, it is worth noting that the most significant biochemical sources for butyrylglycine, butyrylcarnitine, and ethylmalonate are derived from fatty acid β-oxidation rather than isoleucine degradation. Increases of butyrylglycine, butyrylcarnitine, and ethylmalonate have been regarded as hallmarks of inhibition of fatty acid β-oxidation because of short-chain acyl-CoA dehydrogenase deficiency (van Maldegem *et al.*, 2006).

The conclusion that EGME inhibits the primary flavoprotein dehydrogenase reactions is supported by published studies on genetic disorders of acyl-CoA dehydrogenases in human. In patients with multiple acyl-CoA dehydrogenase deficiency (MADD), primary flavoprotein dehydrogenase reactions are impaired. Although the clinical features of MADD can vary among patients, they always manifest themselves with muscle, liver, heart, and congenital anomalies (Ghisla and Thorpe, 2004; Kim and Miura, 2004; Olsen *et al.*, 2007). The urinary and serum profiles of MADD patients are found to share remarkable similarities with the ones observed in this study. It has been well established that elevated dimethylglycine and sarcosine (Burns *et al.*, 1998; Goodman *et al.*, 1980), ethylmalonate, butyrylcarnitine, butyrylglycine (Amendt *et al.*, 1987; Bhala *et al.*, 1995; Birkebaek *et al.*, 2002; Jethva *et al.*, 2008; Koeberl *et al.*, 2003; Przyrembel *et al.*, 1976; van Maldegem *et al.*, 2006), 3-hydroxy-2-ethylpropionate, isobutyrylcarnitine, 2-methylbutyrylcarnitine, 2-methylbutyrylglycine, and glutarate (Andresen *et al.*, 2000; Bennett *et al.*, 1994; Bonafe *et al.*, 2000; Korman, 2006; Korman *et al.*, 2005; Tanaka *et al.*, 1966; Vockley and Ensenauer, 2006) are hallmarks in patients with varying degrees of MADD.

For the primary flavoprotein dehydrogenases, the reducing equivalents are transferred sequentially to the electron transfer flavoprotein (ETF), ETF-ubiquinone oxidoreductase (ETF:QO),

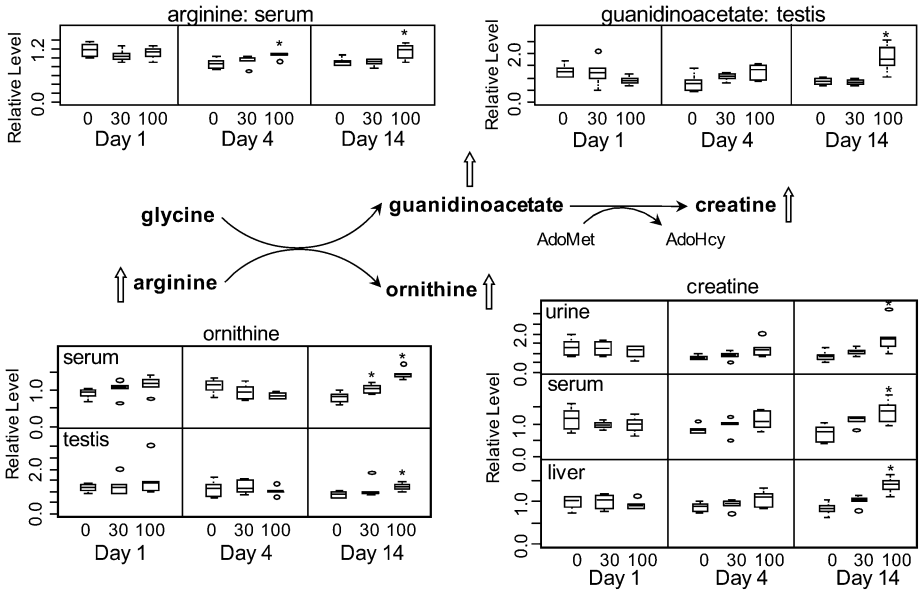

FIG. 6. Creatine biosynthesis and the box plots of its metabolites altered by EGME treatment. Asterisk represents statistically significant to the control group. AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine.

ubiquinone pool, and finally to bc1 complex (complex III) (Lenaz, 2001). The molecular mechanism of MADD has been demonstrated to be the consequence of mutations in either the gene encoding ETF or the ETF:QO (Goodman *et al.*, 2002; Loehr *et al.*, 1990). Thus, it is plausible that the inhibition of flavoprotein dehydrogenase reactions by EGME could be mediated by disruptions of FAD-mediated electron transfer. It is not yet clear that, however, how EGME might act in this cascade of events that involve FAD reduction and the subsequent oxidation. It is possible that EGME reduces the interaction of ETF with the acyl-CoA dehydrogenase's FAD or between ETF and the EFT:QO. Future experiments to assess EGME's effect on the electron transfer steps using *in vitro* assays may shed light on EGME's precise site of action. Alternatively, the possibility also exists that EGME's action upon flavoprotein dehydrogenase reactions could be indirect. It has been reported that MAA, the active metabolite of EGME (Johanson, 2000), can inhibit histone deacetylase activity (Wade *et al.*, 2008). Interestingly, the activity of LCAD, a primary flavoprotein dehydrogenase, can be increased by sirtuin-3, an nicotinamide adenine dinucleotide dependent deacetylase (Hirschey *et al.*, 2010). However, it remains unclear the degree to which MAA can impact protein acetylation beyond the histones.

The biochemical pathways utilizing flavoprotein dehydrogenases play critical biological functions in fatty acid β-oxidation, BCAA catabolism, and choline oxidation. Disruption of these functions could conceivably lead to deleterious outcomes. The choline oxidation pathway is a major source of methionine, the precursor of *S*-adenosyl methionine, which is a key methyl donor for many methylation reactions (Zeisel, 2006). An interesting consequence of disrupted choline oxidation was the accumulation of sarcosine (Fig. 2). The biological role of sarcosine had been long ignored, except as a product of the overflow methylation reaction. However, sarcosine was recently found to mediate prostate cancer progression and regulate various processes associated with cell proliferation and invasiveness (Sreekumar *et al.*, 2009). It is plausible that the elevation of the deleterious metabolite sarcosine may further mediate and escalate EGME-induced toxicological consequences.

Collectively, the data strongly suggest that the inhibition of flavoprotein dehydrogenase enzyme family to be the mode of action of EGME-induced toxicity. However, we do not want to completely eliminate the possibility that alternative mechanisms may exist for the biochemical perturbations observed in this study. The EGME-induced accumulation of various metabolites could be because of the upregulations of their biosynthetic enzymes. For example, the increases of dimethylglycine and sarcosine can be explained by the enhanced activities from betaine methyltransferase and glycine N-methyltransferase, respectively. It would be worthwhile to study the impact of EGME on the activities and expression levels of the enzymes in the choline oxidation and BCAA metabolic pathways.

In this study, we also identified several metabolites indicating cell and tissue damage by the EGME treatment at day 14 of the 100 mg/kg dose. Creatine biosynthesis was significantly elevated (Fig. 6). Creatine mediates high-energy phosphate transport between sites of ATP production and ATP consumption. Alterations of creatine biosynthesis, either overproduction or deficiency, are known to produce pathological consequences (Wyss and Kaddurah-Daouk, 2000). Dihydrouracil, pyroglutamine, and hydroxyproline were likely associated with DNA

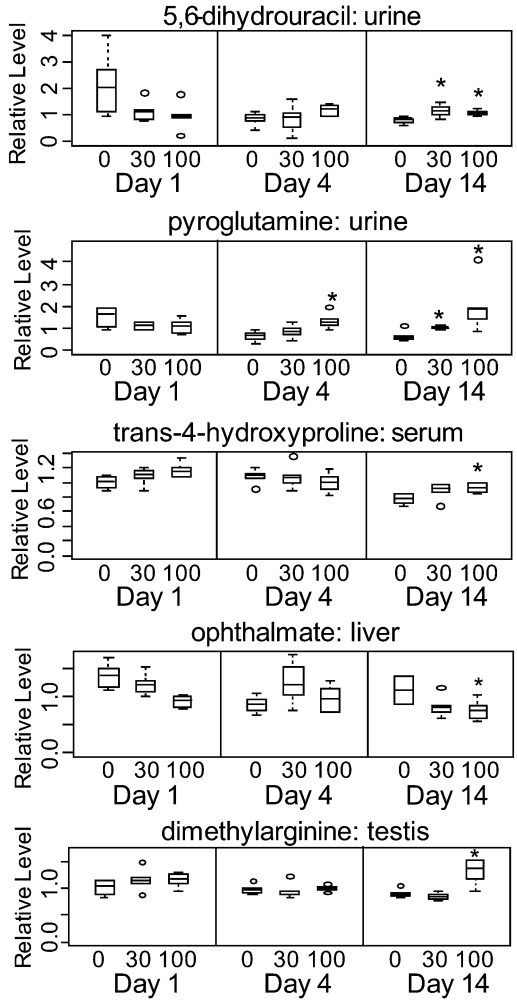

FIG. 7. The box plots for dihydrouracil, pyroglutamine, hydroxyproline, ophthalmate, and dimethylarginine.

breakdown, protein degradation, and tissue damage, respectively. Ophthalmate is a marker for glutathione biosynthesis (Soga *et al*., 2006). The decreased ophthalmate levels suggested that glutathione production was inhibited by the EGME treatment. In addition, N,N-dimethylarginine (ADMA), a marker for oxidative stress (Beltowski and Kedra, 2006), was elevated by EGME treatment (Fig. 7). These results are consistent with published findings that genes related with oxidative stress were upregulated by EGME (Fukushima *et al*., 2005; Syed and Hecht, 1998; Wang and Chapin, 2000).

In summary, we performed an untargeted metabolomic analysis of serum, urine, liver, and testes collected from rats treated with EGME. Based on the results observed, we propose that the mode of action of EGME-induced toxicity is mediated through the inhibition of primary flavoprotein dehydrogenases. The disruption of key biochemical pathways utilizing the flavoprotein dehydrogenases in BCAA metabolism, fatty acid β-oxidation, and choline oxidation, alone or in conjunction with downsteam events such as sarcosine accumulation and alteration of creatine biosynthesis, may precipitate the tissue and cell damages observed as a consequence of EGME exposure.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

Amendt, B. A., Greene, C., Sweetman, L., Cloherty, J., Shih, V., Moon, A., Teel, L., and Rhead, W. J. (1987). Short-chain acyl-coenzyme A dehydrogenase deficiency. Clinical and biochemical studies in two patients. *J. Clin. Invest.* **79,** 1303–1309.

Andresen, B. S., Christensen, E., Corydon, T. J., Bross, P., Pilgaard, B., Wanders, R. J., Ruiter, J. P., Simonsen, H., Winter, V., Knudsen, I., *et al.* (2000). Isolated 2-methylbutyrylglycinuria caused by short/branched-chain acyl-CoA dehydrogenase deficiency: identification of a new enzyme defect, resolution of its molecular basis, and evidence for distinct acyl-CoA dehydrogenases in isoleucine and valine metabolism. *Am. J. Hum. Genet.* **67,** 1095–1103.

Bagchi, G., and Waxman, D. J. (2008). Toxicity of ethylene glycol monomethyl ether: impact on testicular gene expression. *Int. J. Androl.* **31,** 269–274.

Beltowski, J., and Kedra, A. (2006). Asymmetric dimethylarginine (ADMA) as a target for pharmacotherapy. *Pharmacol. Rep.* **58,** 159–178.

Bennett, M. J., Powell, S., Swartling, D. J., and Gibson, K. M. (1994). Tiglylglycine excreted in urine in disorders of isoleucine metabolism and the respiratory chain measured by stable isotope dilution GC-MS. *Clin. Chem.* **40,** 1879–1883.

Berndtson, W. E., and Foote, R. H. (1997). Disruption of spermatogenesis in rabbits consuming ethylene glycol monomethyl ether. *Reprod. Toxicol.* **11,** 29–36.

Bhala, A., Willi, S. M., Rinaldo, P., Bennett, M. J., Schmidt-Sommerfeld, E., and Hale, D. E. (1995). Clinical and biochemical characterization of short-chain acyl-coenzyme A dehydrogenase deficiency. *J. Pediatr.* **126,** 910–915.

Birkebaek, N. H., Simonsen, H., and Gregersen, N. (2002). Hypoglycaemia and elevated urine ethylmalonic acid in a child homozygous for the short-chain acyl-CoA dehydrogenase 625G > A gene variation. *Acta Paediatr.* **91,** 480–482.

Boatman, R. J. (2005). International industry initiatives to improve the glycol ether health effects knowledge base. *Toxicol. Lett.* **156,** 39–50.

Bonafe, L., Troxler, H., Kuster, T., Heizmann, C. W., Chamoles, N. A., Burlina, A. B., and Blau, N. (2000). Evaluation of urinary acylglycines by electrospray tandem mass spectrometry in mitochondrial energy metabolism defects and organic acidurias. *Mol. Genet. Metab.* **69,** 302–311.

Burns, S. P., Holmes, H. C., Chalmers, R. A., Johnson, A., and Iles, R. A. (1998). Proton NMR spectroscopic analysis of multiple acyl-CoA dehydrogenase deficiency—capacity of the choline oxidation pathway for methylation in vivo. *Biochim. Biophys. Acta* **1406,** 274–282.

Chapin, R. E., and Lamb, J. C., IV. (1984). Effects of ethylene glycol monomethyl ether on various parameters of testicular function in the F344 rat. *Environ. Health Perspect.* **57,** 219–224.

Cohen, R. (1984). Reversible subacute ethylene glycol monomethyl ether toxicity associated with microfilm production: a case report. *Am. J. Ind. Med.* **6,** 441–446.

Cook, R. R., Bodner, K. M., Kolesar, R. C., Uhlmann, C. S., VanPeenen, P. F., Dickson, G. S., and Flanagan, K. (1982). A cross-sectional study of ethylene glycol monomethyl ether process employees. *Arch. Environ. Health* **37,** 346–351.

Dodo, T., Taketa, Y., Sugiyama, M., Inomata, A., Sonoda, J., Okuda, Y., Mineshima, H., Hosokawa, S., and Aoki, T. (2009). Collaborative work on evaluation of ovarian toxicity. 11) Two- or four-week repeated-dose studies and fertility study of ethylene glycol monomethyl ether in female rats. *J. Toxicol. Sci.* **34**(Suppl. 1), SP121–SP128.

Doe, J. E., Samuels, D. M., Tinston, D. J., and de Silva Wickramaratne, G. A. (1983). Comparative aspects of the reproductive toxicology by inhalation in rats of ethylene glycol monomethyl ether and propylene glycol monomethyl ether. *Toxicol. Appl. Pharmacol.* **69,** 43–47.

El-Zein, R. A., Abdel-Rahman, S. Z., Morris, D. L., and Legator, M. S. (2002). Exposure to ethylene glycol monomethyl ether: clinical and cytogenetic findings. *Arch. Environ. Health* **57,** 371–376.

Evans, A. M., Dehaven, C. D., Barrett, T., Mitchell, M., and Milgram, E. (2009). Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. *Anal. Chem.* **81,** 6656–6667.

Exon, J. H., Mather, G. G., Bussiere, J. L., Olson, D. P., and Talcott, P. A. (1991). Effects of subchronic exposure of rats to 2-methoxyethanol or 2-butoxyethanol: thymic atrophy and immunotoxicity. *Fundam. Appl. Toxicol.* **16,** 830–840.

Fukushima, T., Yamamoto, T., Kikkawa, R., Hamada, Y., Komiyama, M., Mori, C., and Horii, I. (2005). Effects of male reproductive toxicants on gene expression in rat testes. *J. Toxicol. Sci.* **30,** 195–206.

Ghisla, S., and Thorpe, C. (2004). Acyl-CoA dehydrogenases. A mechanistic overview. *Eur. J. Biochem.* **271,** 494–508.

Goodman, S. I., Binard, R. J., Woontner, M. R., and Frerman, F. E. (2002). Glutaric acidemia type II: gene structure and mutations of the electron transfer flavoprotein:ubiquinone oxidoreductase (ETF: QO) gene. *Mol. Genet. Metab.* **77,** 86–90.

Goodman, S. I., McCabe, E. R., Fennessey, P. V., and Mace, J. W. (1980). Multiple acyl-CoA dehydrogenase deficiency (glutaric aciduria type II) with transient hypersarcosinemia and sarcosinuria; possible inherited deficiency of an electron transfer flavoprotein. *Pediatr. Res.* **14,** 12–17.

Hanley, T. R., Jr., Young, J. T., John, J. A., and Rao, K. S. (1984). Ethylene glycol monomethyl ether (EGME) and propylene glycol monomethyl ether (PGME): inhalation fertility and teratogenicity studies in rats, mice and rabbits. *Environ. Health Perspect.* **57,** 7–12.

Hirschey, M. D., Shimazu, T., Goetzman, E., Jing, E., Schwer, B., Lombard, D. B., Grueter, C. A., Harris, C., Biddinger, S., Ilkayeva, O. R., *et al.* (2010). SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. *Nature* **464,** 121–125.

Jethva, R., Bennett, M. J., and Vockley, J. (2008). Short-chain acyl-coenzyme A dehydrogenase deficiency. *Mol. Genet. Metab.* **95,** 195–200.

Jindo, T., Wine, R. N., Li, L. H., and Chapin, R. E. (2001). Protein kinase activity is central to rat germ cell apoptosis induced by methoxyacetic acid. *Toxicol. Pathol.* **29,** 607–616.

Johanson, G. (2000). Toxicity review of ethylene glycol monomethyl ether and its acetate ester. *Crit. Rev. Toxicol.* **30,** 307–345.

Kim, J. J., and Miura, R. (2004). Acyl-CoA dehydrogenases and acyl-CoA oxidases. Structural basis for mechanistic similarities and differences. *Eur. J. Biochem.* **271,** 483–493.

Koeberl, D. D., Young, S. P., Gregersen, N. S., Vockley, J., Smith, W. E., Benjamin, D. K., Jr., An, Y., Weavil, S. D., Chaing, S. H., Bali, D., *et al.* (2003). Rare disorders of metabolism with elevated butyryl- and isobutyryl-carnitine detected by tandem mass spectrometry newborn screening. *Pediatr. Res.* **54,** 219–223.

Korman, S. H. (2006). Inborn errors of isoleucine degradation: a review. *Mol. Genet. Metab.* **89,** 289–299.

Korman, S. H., Andresen, B. S., Zeharia, A., Gutman, A., Boneh, A., and Pitt, J. J. (2005). 2-Ethylhydracrylic aciduria in short/branched-chain acyl-CoA dehydrogenase deficiency: application to diagnosis and implications for the R-pathway of isoleucine oxidation. *Clin. Chem.* **51,** 610–617.

Larese, F., Fiorito, A., and De Zotti, R. (1992). The possible haematological effects of glycol monomethyl ether in a frame factory. *Br. J. Ind. Med.* **49,** 131–133.

Lenaz, G. (2001). A critical appraisal of the mitochondrial coenzyme Q pool. *FEBS Lett.* **509,** 151–155.

Loehr, J. P., Goodman, S. I., and Frerman, F. E. (1990). Glutaric acidemia type II: heterogeneity of clinical and biochemical phenotypes. *Pediatr. Res.* **27,** 311–315.

Olsen, R. K., Olpin, S. E., Andresen, B. S., Miedzybrodzka, Z. H., Pourfarzam, M., Merinero, B., Frerman, F. E., Beresford, M. W., Dean, J. C., Cornelius, N., *et al.* (2007). ETFDH mutations as a major cause of riboflavin-responsive multiple acyl-CoA dehydrogenation deficiency. *Brain* **130,** 2045–2054.

Przyrembel, H., Wendel, U., Becker, K., Bremer, H. J., Bruinvis, L., Ketting, D., and Wadman, S. K. (1976). Glutaric aciduria type II: report on a previously undescribed metabolic disorder. *Clin. Chim. Acta* **66,** 227–239.

Soga, T., Baran, R., Suematsu, M., Ueno, Y., Ikeda, S., Sakurakawa, T., Kakazu, Y., Ishikawa, T., Robert, M., Nishioka, T., *et al.* (2006). Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. *J. Biol. Chem.* **281,** 16768–16776.

Sreekumar, A., Poisson, L. M., Rajendiran, T. M., Khan, A. P., Cao, Q., Yu, J., Laxman, B., Mehra, R., Lonigro, R. J., Li, Y., *et al.* (2009). Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* **457,** 910–914.

Swigonova, Z., Mohsen, A. W., and Vockley, J. (2009). Acyl-CoA dehydrogenases: dynamic history of protein family evolution. *J. Mol. Evol.* **69,** 176–193.

Syed, V., and Hecht, N. B. (1998). Rat pachytene spermatocytes down-regulate a polo-like kinase and up-regulate a thiol-specific antioxidant protein, whereas Sertoli cells down-regulate a phosphodiesterase and up-regulate an oxidative stress protein after exposure to methoxyethanol and methoxyacetic acid. *Endocrinology* **139,** 3503–3511.

Tanaka, K., Budd, M. A., Efron, M. L., and Isselbacher, K. J. (1966). Isovaleric acidemia: a new genetic defect of leucine metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **56,** 236–242.

Tirado, O. M., Martinez, E. D., Rodriguez, O. C., Danielsen, M., Selva, D. M., Reventos, J., Munell, F., and Suarez-Quian, C. A. (2003). Methoxyacetic acid disregulation of androgen receptor and androgen-binding protein expression in adult rat testis. *Biol. Reprod.* **68,** 1437–1446.

van Maldegem, B. T., Duran, M., Wanders, R. J., Niezen-Koning, K. E., Hogeveen, M., Ijlst, L., Waterham, H. R., and Wijburg, F. A. (2006). Clinical, biochemical, and genetic heterogeneity in short-chain acyl-coenzyme A dehydrogenase deficiency. *JAMA* **296,** 943–952.

Vockley, J., and Ensenauer, R. (2006). Isovaleric acidemia: new aspects of genetic and phenotypic heterogeneity. *Am. J. Med. Genet. C Semin. Med. Genet.* **142C,** 95–103.

Wade, M. G., Kawata, A., Williams, A., and Yauk, C. (2008). Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. *Biol. Reprod.* **78,** 822–831.

Wang, W., and Chapin, R. E. (2000). Differential gene expression detected by suppression subtractive hybridization in the ethylene glycol monomethyl ether-induced testicular lesion. *Toxicol. Sci.* **56,** 165–174.

Welch, L. S., and Cullen, M. R. (1988). Effect of exposure to ethylene glycol ethers on shipyard painters: III. Hematologic effects. *Am. J. Ind. Med.* **14,** 527–536.

Welch, L. S., Schrader, S. M., Turner, T. W., and Cullen, M. R. (1988). Effects of exposure to ethylene glycol ethers on shipyard painters: II. Male reproduction. *Am. J. Ind. Med.* **14,** 509–526.

Wyss, M., and Kaddurah-Daouk, R. (2000). Creatine and creatinine metabolism. *Physiol. Rev.* **80,** 1107–1213.

Zeisel, S. H. (2006). Choline: critical role during fetal development and dietary requirements in adults. *Annu. Rev. Nutr.* **26,** 229–250.