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VIA E-Mail and Federal Express

Dr. C.W. Jameson
National Toxicology Program
Report on Carcinogens
79 Alexander Drive
Building 4401, Room 3118
P.O. Box 12233
Research Triangle Park, NC 27709

Re: National Toxicology Program: Call for Public Comment on 16 Substances, Mixtures and Exposure Circumstances Proposed for Listing in the Report on Carcinogens, Eleventh Edition: 66 Fed. Reg. 38430 (July 24, 2001)

Dear Dr. Jameson:

The Alkanolamines Panel (Panel) of the American Chemistry Council submits the enclosed paper by Dr. Lois Lehman-McKeeman et al in response to the National Toxicology Program's call for comments on the proposal to list diethanolamine (DEA) in the Eleventh Edition of the *Report on Carcinogens* (*RoC*). 66 Fed. Reg. 38430 (July 24, 2001). This paper recently was accepted for publication in *Toxicological Sciences*. It may take two to three months before the paper actually gets into press. This paper supplements the comments submitted by the Panel on September 24, 2001. Please direct any questions that you might have concerning these comments to me at (703) 741-5633.

Sincerely,

Jonathon T. Busch

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Manager, Alkanolamines Panel
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Diethanolamine Induces Hepatic Choline Deficiency in Mice

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Running Title: DEA-Induced Choline Deficiency

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ABSTRACT

Lehman-McKeeman, L. D., Gamsky, E. A., Hicks, S. M., Vassallo, J. D., Mar, M-H, and Zeisel, S. H. (2001). Diethanolamine induces hepatic choline deficiency in mice. Toxicol. Sci. 00, 00-00.

The purpose of the present experiments was to test the hypothesis that diethanolamine (DEA), an alkanolamine shown to be hepatocarcinogenic in mice, induces hepatic choline deficiency and to determine whether altered choline homeostasis was causally-related to the carcinogenic outcome. To examine this hypothesis, the biochemical and histopathological changes in male B6C3F1 mice made choline deficient by dietary deprivation were first determined. Phosphocholine (PCho), the intracellular storage form of choline was severely depleted, decreasing to about 20% of control values with 2 weeks of dietary choline deficiency. Other metabolites, including choline, glycerophosphocholine (GPC) and phosphatidylcholine (PC) also decreased. Hepatic concentrations of S-adenosylmethionine (SAM) decreased, whereas levels of S-adenosylhomocysteine (SAH) increased. Despite these biochemical changes, fatty liver, which is often associated with choline deficiency, was not observed in the mice. The dose-response, reversibility and strain-dependence of the effects of DEA on choline metabolites were studied. B6C3F1 mice were dosed dermally with DEA (0, 10, 20, 40, 80 and 160 mg/kg) for 4 weeks (5 days/week). Control animals received either no treatment or dermal application of 95% ethanol (1.8 ml/kg). PCho was most sensitive to DEA treatment, decreasing at dosages of 20 mg/kg and higher and reaching a maximum 50% depletion at 160 mg/kg/day. GPC, choline and PC also decreased in a dose-dependent manner. At 80 and 160 mg/kg/day, SAM levels decreased while SAH levels increased in liver. A no-observed effect level (NOEL) for DEA-induced changes in choline homeostasis was 10 mg/kg/day. Choline metabolites, SAM and SAH returned to control

levels in mice dosed at 160 mg/kg for 4 weeks and allowed a 2-week recovery period prior to necropsy. In a manner similar to dietary choline deficiency, no fatty change was observed in the liver of DEA-treated mice. In C57BL/6 mice, DEA treatment (160 mg/kg) also decreased PCho concentrations, without effecting hepatic SAM levels, suggesting that strain specific differences in intracellular methyl group regulation may influence carcinogenic outcome with DEA treatment. Finally, in addition to the direct effects of DEA on choline homeostasis, dermal application of 95% ethanol for 4 weeks decreased hepatic betaine levels, suggesting that the use of ethanol as a vehicle for dermal application of DEA may exacerbate or confound the biochemical actions of DEA alone. Collectively, the results demonstrate that DEA treatment causes a spectrum of biochemical changes consistent with choline deficiency in mice and demonstrate a clear dose-concordance between DEA-induced choline deficiency and hepatocarcinogenic outcome.

INTRODUCTION

Diethanolamine (DEA) is an alkanolamine used in the synthesis of fatty acid condensates that are present in many consumer products. With lifetime dermal exposure, DEA increased the incidence and multiplicity of liver tumors in mice, but no carcinogenic activity was observed in rats (NTP, 1999). DEA has shown no evidence of DNA reactivity (Knaak et al., 1997; NTP, 1999), suggesting that secondary, non-genotoxic mechanisms are likely contributing to the hepatocarcinogenic response.

DEA is structurally-similar to ethanolamine and choline, important endogenous precursors in the synthesis of phospholipids that are essential for normal membrane structure and function (Pelech and Vance, 1984; Zeisel and Blusztajn, 1994). Other choline metabolites include acetylcholine, a vital neurotransmitter, platelet-activating factor, a phospholipid that mediates many processes of inflammation and allergy, and sphingomyelin, an important precursor involved in cell signaling and differentiation (Zeisel and Blusztajn, 1994). Choline is also oxidized to betaine, an essential methyl group donor (Finkelstein et al., 1982).

The pathways of choline utilization and 1-carbon metabolism are shown in Figure 1. Importantly, choline utilization intermingles with the metabolic pathways that serve to protect the capacity to donate methyl groups. The interaction between these pathways is demonstrated by the fact that dietary choline deficiency in rats not only depletes hepatic choline and choline metabolites, but decreases S-adenosylmethionine (SAM) concentrations as well (Shivapurkar and Poirier, 1983; Zeisel et al., 1989; Pomfret et al., 1990). The liver is distinguished from most other tissues in that it has an active alternate pathway to synthesize PC by sequential methylation of phosphatidylethanolamine (PE) by the action of PE N-methyltransferase (Vance et al., 1997). This alternate biosynthetic process requires 3 moles of SAM, representing another example of

the inter-dependence between the choline and the methylation pathways. Furthermore, the methylation of PE to PC is thought to be the only pathway for *de novo* choline biosynthesis in mammals (Zeisel and Blusztajn, 1994).

Previous studies have shown that DEA is incorporated into rat liver phospholipids (Barbee and Hartung, 1979; Mathews et al., 1995) and can alter the biosynthesis of hepatic PE and PC (Barbee and Hartung, 1979). In cultured cells, DEA inhibited cellular uptake of choline, decreased PC synthesis and became incorporated into phospholipids fractions. These in vitro effects were prevented by culturing cells in the presence of excess choline (Lehman-McKeeman and Gamsky, 1999, 2000). Collectively, these data indicate that DEA treatment alters phospholipid biosynthesis and may disrupt choline homeostasis.

Dietary choline deprivation is hepatocarcinogenic in rodents (Newberne et al., 1982; Mikol et al., 1983; Ghoshal and Farber, 1984; Newberne and Rogers, 1986; Zeisel, 1996) and promotes liver tumor formation following initiation by a chemical carcinogen (Sells et al., 1979; Lombardi and Shinozuka, 1979; Yokoyama et al., 1985). In contrast, dietary supplementation of choline with or without methionine reduces liver tumor incidence in carcinogen-treated mice (Newberne et al., 1990; Fullerton et al., 1990). The mechanisms by which choline deficiency is thought to be carcinogenic include enhanced cell proliferation, altered methylation status, and altered signal transduction (Zeisel, 1996; Zeisel and Blustjazn, 1994; Rogers, 1995). DEA caused morphological transformation in the Syrian hamster embryo (SHE) cell transformation assay, supporting its carcinogenic activity. However, this response was prevented when SHE cells were cultured in a medium containing excess choline (Lehman-McKeeman and Gamsky, 2000), supporting a role for altered choline homeostasis as a mode of action for the carcinogenic effects of DEA.

Although the precise mechanisms underlying the hepatocarcinogenic action of dietary choline deficiency are not fully established, it is clear that choline deficiency is a mode of action by which liver tumors develop in rodents. Given that DEA disrupts choline homeostasis in cultured cells, the purpose of the present work was to test the hypothesis that DEA treatment could produce biochemical changes consistent with choline deficiency in mice. To this end, the biochemical changes observed in mice made choline deficient by dietary choline deprivation were compared to the effects observed in DEA-treated mice. Experiments were also conducted to define the dose-response relationship, mouse strain dependence and possible influence of an ethanol vehicle on these biochemical effects.

MATERIALS AND METHODS

Animals. Male B6C3F1 mice, approximately 5 weeks of age at receipt, were obtained from Charles River Laboratories (Portage, MI). Mice were housed in a temperature- and humidity-controlled environment and acclimated for approximately 1 week prior to study initiation. Unless otherwise noted, mice were allowed rodent chow (Purina 5001; Ralston-Purina, St. Louis, MO) and water *ad libitum* throughout the study. Dietary concentrations of choline and methionine in the Purina diet were 0.2 and 0.4%, respectively, whereas the fat composition was approximately 5%.

Chemicals. DEA (99% purity) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Absolute ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY) was used to prepare 95% ethanol used as the vehicle in these studies. Betaine (HCl salt) was obtained from Aldrich, SAM and SAH were obtained from Sigma Chemical Co (St. Louis, MO), and all standards for choline analyses were as described by Pomfret et al. (1989).

Dietary Choline Deficiency. Male B6C3F1 mice (approximately 6 weeks old at study initiation) were allowed *ad libitum* exposure to a control or choline-devoid diet (Dyets, Bethlehem, PA) for a period of two weeks (n = 8/group). The control diet contained 0.25% choline (as choline bitartrate) and the methionine content in the control and choline-devoid diets was 0.6%. At the end of the 2-week exposure period, livers were rapidly removed under anesthesia, immediately flash-frozen in liquid N_2 and stored at -80°C pending analysis of choline metabolites. In a separate group of mice (n = 4/group), blood was collected for clinical chemistry analyses, and livers were weighed and fixed in 10% neutral buffered formalin for histopathological assessment.

DEA Dose-Response Study. Male B6C3F1 mice (approximately 6 weeks old at study initiation) were housed individually and dosed dermally with DEA dissolved in 95% ethanol at 0, 10, 20, 40, 80 or 160 mg/kg (n = 6/group) for a period of 4 weeks (5 days/week). These dosages were selected to include those shown to be carcinogenic (40, 80 and 160 mg/kg/day; NTP, 1999). There were two control groups for this study including untreated and ethanol-treated mice. A separate group of mice was dosed with the ethanol vehicle or 160 mg DEA/kg/day for 4 weeks (5 days/week) and then allowed a two-week recovery prior to necropsy. For dosing, the backs of all mice were shaved, and the dosing solutions (1.8 ml/kg) were applied to a region approximately $2cm^2$ (from the mid-back to the interscapular region) using a pipette. All mice, including the untreated controls, were shaved on an as-needed basis during the study period. At the end of the dosing or recovery period, livers were harvested as described above and analyzed for choline metabolites.

To evaluate potential strain differences in the response to DEA, male C57BL/6 mice, also about 6 weeks old at study initiation, were dosed with 0 or 160 mg DEA/kg for a similar 4-week period. The untreated and ethanol-treated control groups were also used in this study.

Clinical and Histopathological Evaluations. Formalin-fixed livers were processed using standard conditions and stained with hematoxylin and eosin. Histopathological evaluations were made without knowledge of treatment. Clinical analyses were conducted on freshly isolated serum with a Hitachi 717 chemistry analyzer (Boehringer-Mannheim Corp., Indianapolis, IN).

Analysis of Hepatic Choline Metabolites. Phospholipids and aqueous choline metabolites were analyzed according to the method outlined by Pomfret et al., (1989). Briefly, frozen liver

samples were pulverized under liquid N_2 after which lipids were extracted according to the method of Bligh and Dyer (1959). The organic phase (for PC) was separated by thin layer chromatography (TLC), whereas the aqueous phase was injected onto a silica HPLC column, and the appropriate internal standards labeled with stable isotopes were added to calculate and correct for recovery. Following the TLC analyses, the segments representing PC were visualized, scraped and hydrolyzed in 6 M methanolic-HCl to liberate free choline. From the HPLC analyses, the peaks representing PCho, GPC and choline were collected, dried and hydrolyzed to liberate choline. In all cases, choline was converted to its propionyl ester and demethylated with sodium benzenethiolate. This volatile derivative was isolated by gas chromatography and quantified with a mass selective detector.

SAM and SAH concentrations were determined by a high-performance liquid chromatographic (HPLC) method based on a modification of Wagner et al (1984). Briefly, frozen liver samples were homogenized in 2.5% perchloric acid containing 10 mM Na metabisulfite and 1 mM EDTA. After centrifugation, protein-free supernatants were injected onto a Waters Symmetry column (2.1 x 100 cm) and SAM and SAH were separated with an acetonitrile gradient in 100 mM phosphate buffer (pH 3.0), acetonitrile and 8 mM octanesulfonic acid. Buffers A and B contained 5 and 50% acetonitrile, respectively, and a linear gradient system of 0 to 70% buffer B over a 20 minute interval was used. SAH and SAM, detected at 254 nm, eluted at approximately 10.4 and 12.7 minutes, respectively. The detection limit for both analytes was 0.1 nmol/g liver.

For betaine analysis, frozen liver samples were homogenized in 4 volumes of 15% trichloracetic acid, after which the protein-free supernatants were subjected to exhaustive extraction and periodide complexation as described by Barak and Tuma (1979). Betaine

periodide was precipitated, resuspended in dichloroethane and analyzed spectrophotometrically at 365 nm. The detection limit for this assay was 0.3 μmol betaine/g liver.

Statistical Analysis. Data were analyzed for statistical significance by Students' t test or analysis of variance (ANOVA) followed by a multiple comparison test (Fischer's PLSD) using StatView 4.1 (Abacus Concepts, Berkeley, CA). For statistical comparisons, a 5% significance level ($p<0.05$) was used.

RESULTS

Previous studies in rats have shown that 2 weeks of dietary choline deprivation is sufficient to deplete hepatic choline metabolites (Pomfret et al., 1990). In a similar manner, when male B6C3F1 mice were fed a choline-devoid diet for two weeks, choline-containing metabolites in liver were decreased (Table 1). PCho, the intracellular storage form of choline decreased to the greatest extent, representing a 75% reduction relative to control. PC levels also decreased, although not to the magnitude observed for aqueous choline metabolites. Hepatic SAM concentrations were reduced by approximately 20% during the 2-week period of choline deficiency, whereas SAH levels increased. Despite these biochemical changes, body weight gain and liver weights were unaffected.

Choline deficiency is typically associated with histological evidence of fatty liver. However, although the choline-devoid diet caused marked changes in choline metabolites, no fatty change was evident when evaluated histologically (Figure 2). Several clinical parameters, including total bile acids and serum triglycerides were affected by choline deficiency. Most notably, serum triglycerides decreased to about 50% of control values (97 ± 12 versus 41 ± 12 mg/dL in control and choline deficiency, respectively.)

The biological half-life of DEA is about 1 week, with a steady state for bioaccumulation reached at approximately 4 weeks of dosing (Mathews et al., 1997). Dose-related changes in hepatic choline levels following 4 weeks of DEA treatment are shown in Table 2. There were no differences in choline or SAM levels between the untreated versus ethanol-treated control mice (results not shown). PCho, was most sensitive to DEA, showing a 20% reduction at the 20 mg/kg dosage. Higher dosages decreased PCho levels further, reaching a maximum 50% decrease at 160 mg DEA/kg. GPC and choline concentrations were also decreased in a dose-

dependent manner, whereas PC concentrations decreased only at the highest dosage of DEA. DEA treatment decreased hepatic SAM levels at 80 and 160 mg/kg with a concurrent increase in SAH concentrations. Despite the biochemical changes, there was no evidence of fatty liver in DEA-treated mice and serum triglyceride levels were not altered (results not shown).

A separate group of mice was dosed at 160 mg/kg/day for 4 weeks and allowed a 2-week recovery period prior to necropsy. Choline metabolite, SAM and SAH concentrations in these mice, also shown in Table 2, returned to control values during the recovery period.

One way in which B6C3F1 mice are distinguished from the maternal C57BL/6 strain is the ability to maintain nascent methylation status, a characteristic which is dependent on hepatic SAM levels (Counts et al., 1996). Figure 3 illustrates that when dosed with DEA at 160 mg/kg/day, C57BL/6 mice were sensitive to changes in PCho levels, but unlike the B6C3F1 mice, no change in hepatic SAM levels were noted in C57BL/6 mice.

DEA was applied dermally in these studies using an ethanol vehicle (95% ethanol). Although PC precursors such as PCho or choline were not altered by ethanol treatment, hepatic betaine levels were decreased by ethanol treatment. After 4 weeks of treatment, betaine concentrations were reduced by approximately 20% (Figure 4). DEA treatment did not cause any further decrease in hepatic betaine levels.

DISCUSSION

The results presented herein establish the biochemical manifestations of choline deficiency in mice. With 2 weeks of dietary choline deficiency in B6C3F1 mice, a major reduction in PCho levels was observed, whereas other choline metabolites were less affected. SAM levels decreased, whereas SAH levels increased. Overall, the pattern of change in choline metabolites, SAM and SAH levels in mice is similar to that previously reported for choline-deficient rats (Pomfret et al., 1990). As illustrated in Figure 1, there is considerable interaction between the pathways of choline metabolism and 1-carbon metabolism, and it is generally agreed that this interplay contributes to the hepatocarcinogenic action of dietary choline deficiency. Specifically, when choline availability is limited, there is a compensatory activation of the PE N-methyltransferase pathway to form PC to limit the magnitude of depletion of PC and other phospholipids (Schneider and Vance, 1978; Cui and Vance, 1996). This activation increases the cellular demand and utilization of SAM by the methyltransferase, thereby depleting this important methyl group donor. Altered methylation reactions, particularly DNA methylation, are thought to play an important role in hepatocarcinogenesis resulting from choline deficiency (Wainfan and Poirer, 1992; Christman, 1995; Rogers, 1995).

The pattern of changes observed in choline metabolites after DEA treatment was very similar to those observed in choline deficient mice. Specifically, hepatic PCho levels, decreased reaching a maximum 50% reduction within 4 weeks of dermal dosing. Choline metabolites and SAM levels also decreased. Therefore, these data indicate that DEA treatment produces biochemical changes in liver that are consistent with choline deficiency. Furthermore, the effects of DEA were dosage-dependent. All carcinogenic dosages of DEA (40, 80 and 160 mg/kg/day) altered choline homeostasis, supporting the hypothesis that choline deficiency is the mode of

action by which DEA is hepatocarcinogenic in mice. Importantly, these changes occurred in mice consuming a choline-replete diet. Previous studies have demonstrated that DEA selectively inhibits the uptake of choline into cells and competes for the normal utilization of choline in the CDP-choline pathway (Lehman-McKeeman and Gamsky, 1999, 2000). Thus, it is likely that the actions of DEA limit intracellular choline availability in a manner that leads to a choline-deficient condition even when choline is available in the diet. The effects of DEA on choline uptake and utilization have also been shown to be competitive and reversible, as choline supplementation can inhibit the effects of DEA in cultured cells (Lehman-McKeeman and Gamsky, 2000). Similarly, the ability of DEA to alter choline homeostasis *in vivo* reversed when treatment was stopped for 2 weeks prior to necropsy, demonstrating that DEA must be continuously present in order to exert these effects.

The histopathological hallmark of choline deficiency in rodents is fatty liver. This feature, which results from the inability of the liver to synthesize adequate PC for the formation of very low density lipoproteins required for triglyceride secretion (Yao and Vance 1988, 1990), has been documented repeatedly for rats made choline deficient by dietary deprivation. The possibility that DEA could produce choline deficiency has been discounted by some groups due to the lack of fatty liver (NTP, 1999). However, the present results demonstrate that no fatty change was observed in mice after 2 weeks of dietary choline deficiency. It is generally recognized that the mouse model of chronic choline deficiency differs from rats because most mouse strains do not develop liver cirrhosis and hemorrhagic necrosis that is typical of chronic choline deficiency in rats (DeCarmago et al., 1985). Moreover, two other experimental conditions can contribute to the development and severity of fatty liver in response to choline deficiency. First, sensitivity to and severity of choline deficiency is age-dependent. Weanling

animals are highly susceptible to choline deficiency, and most studies conducted to date have initiated choline deficiency in animals ranging from 3-4 weeks of age (Chandar and Lombardi, 1988; Fullerton et al., 1990; daCosta et al., 1995). Susceptibility to choline deficiency declines rapidly, and animals that are 10-12 weeks of age are unlikely to develop features of fully-expressed choline deficiency (Rogers et al., 1987). Secondly, dietary fat composition also contributes to the development of fatty liver in choline deficiency. Specifically, the fat composition of choline deficient diets is often augmented to at least 20% of the diet, whereas standard laboratory chows contain about 5% fat (Ghoshal and Farber, 1984; Rogers et al., 1987). Additionally, variations in fatty liver development are observed when the fat is derived from animal or plant origins (Rogers et al., 1987; DeCamargo et al., 1985).

In the present studies, mice were approximately 6 weeks of age at the initiation of choline deficiency or DEA treatment. This age was chosen intentionally to mimic the conditions of the chronic bioassays conducted with DEA. Furthermore, the synthetic diet contained less than 10% fat and the laboratory chow used in the DEA experiments contained only 5% fat which is derived from soybean oil. Consequently, experimental conditions, namely species used, animal age and dietary composition may have decreased the likelihood of fatty liver development, but do not detract from the biochemical evidence that DEA causes hepatic choline deficiency.

Chronic ethanol ingestion has been shown to increase hepatic choline requirements (Barak et al., 1973), and the major manifestation of this action is a reduction in hepatic betaine levels (Barak et al., 1984; Chern et al., 2000). As illustrated in Figure 1, betaine, the oxidation product of choline, is an important 1-carbon donor utilized in the maintenance of cellular methylation status. In the conduct of the chronic bioassays for DEA, ethanol was used as the vehicle for dermal application of DEA, with the daily dermal dosage reaching nearly 1.5 g

ethanol/kg/day (NTP, 1999). The results presented herein indicate that the internal dosage of ethanol from dermal dosing was sufficient to reduce hepatic betaine levels. DEA treatment had no further effect on hepatic betaine levels. Given the substantial interplay between choline and methyl metabolism, it is highly likely that the hepatic effects of DEA, including its carcinogenic action, may be exacerbated by co-administration of ethanol.

In addition to providing a mode of action by which DEA is carcinogenic, the present data have important implications for human cancer risk assessment. First, from a qualitative perspective the sensitivity of the B6C3F1 mouse strain to choline deficiency and hepato-carcinogenesis must be considered. In the present study, choline deficiency, as evidenced by changes in PCho concentrations, was produced in both B6C3F1 and C57BL/6 mice. However, unlike the B6C3F1 mouse, DEA did not alter SAM concentrations in the C57BL/6 strain, which is considered resistant to liver tumor development (Goodman et al., 1991; Counts et al., 1996). Although we have not conducted chronic studies to evaluate the carcinogenic potential of DEA in C57BL/6 mice, the results denote important strain differences in potential susceptibility that should be considered when evaluating human risk.

From a qualitative and quantitative perspective, there are marked species differences in susceptibility to choline deficiency, with rats and mice being far more susceptible than other species including humans (Zeisel and Blusztajn, 1994). In fact, rats and mice have a higher dietary choline requirement than humans in large part because rodents oxidize choline more rapidly than humans (Sidransky and Farber, 1960). In the carcinogenicity studies, DEA was only carcinogenic in mice, not in rats. The lack of carcinogenicity in rats, a species that is highly susceptible to choline deficiency, should be an important consideration in the overall evaluation of human cancer risk. Disposition data indicate that DEA is less readily absorbed across rat skin

than mouse skin, and the resulting blood and tissue concentrations of DEA are at least 3-times lower in rats than in mice at similar dosages (Mathews et al., 1997). The present work has determined the NOEL for DEA-induced choline deficiency in mice (based on PCho concentrations) to be 10 mg/kg/day. Thus, there is a critical concentration of DEA that must be reached in order to affect choline homeostasis. In rats, the lack of a carcinogenic response suggests that it is unlikely that exposure to DEA reached this critical or that rats are not as susceptible as mice to the effects of DEA on hepatic choline metabolism.

In summary, the results of the present study provide evidence that 4 weeks of DEA treatment leads to a biochemical condition of hepatic choline deficiency in mice. The changes, which provide strong evidence for a non-genotoxic mode of action, were dose-dependent, strain-dependent and reversible. Moreover, there was a consistent dose-concordance between the biochemical and hepatocarcinogenic effects of DEA. Mice, especially the B6C3F1 strain are particularly susceptible to altered choline and methyl group homeostasis after DEA treatment, but a clear a no-observed effect level for DEA-induced changes in hepatic choline concentrations was established. The results also demonstrate that dermally-applied ethanol decreased hepatic betaine concentrations and may confound or exacerbate the hepatic effects of DEA. Overall, the results suggest that the hepatocarcinogenic effects of DEA in mice are not predictive of similar susceptibility in other laboratory animals or humans.

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LEGENDS

Figure 1. Schematic representation of the inter-relationship between the intracellular pathways for the utilization of choline and methionine. Choline is utilized in PC biosynthesis or oxidized to betaine, which serves as the methyl donor in the conversion of homocysteine to methionine. In this manner, the generation of methionine from homocysteine intersects choline and 1-carbon metabolic pathways. Methionine, as SAM is also an important precursor for the conversion of PE to PC, a pathway that is most active in liver. The contribution of tetrahydrofolate in the regeneration of methionine in cells is not shown in this metabolic scheme.

Figure 2. Histopathological evaluation of hepatic changes associated with dietary choline deficiency in mice. Mice were fed a diet devoid of choline for 2 weeks prior to evaluation. The upper panel shows a typical liver from a control mouse, and the histological appearance of the liver of the choline-deficient mice (lower panel) was indistinguishable from the controls (Magnification = 200x).

Figure 3. PCho and SAM levels in DEA-treated C57BL/6 mice. Mice were treated for 4 weeks with DEA (160 mg/kg/day; 5 days/week) prior to analysis. PCho levels were decreased by DEA treatment, whereas SAM levels were unaltered. Results represent the mean ± SE of 6 mice/group, and (*) denotes statistical significance ($p < 0.05$).

Figure 4. Effect of dermal application of 95% ethanol on hepatic betaine levels in mice. Betaine concentrations decreased in ethanol-treated mice relative to untreated control mice. DEA

treatment had no further effect on this choline metabolite. Results represent the mean ± SE of 6 mice/group, and (*) denotes statistical significance ($p < 0.05$).

Table 1. Effect of Dietary Choline Deficiency on Hepatic Choline Metabolites[a]

Parameter	Control	Choline-Devoid
Body Weight (g)	24.8 ± 0.4	24.9 ± 0.6
Liver/BW (%)	5.1 ± 0.1	4.8 ± 0.1
PCho[b] (nmol/g liver)	1146 ± 100	297 ± 53*
GPC (nmol/g liver)	615 ± 83	460 ± 27*
Cho (nmol/g liver)	139 ± 4	95 ± 18*
PC (μmol/g liver)	16.6 ± 1.6	15.2 ± 0.6*
SAM (nmol/g liver)	69.2 ± 4.2	56.8 ± 3.3*
SAH (nmol/g liver)	31.8 ± 2.3	41.1 ± 2.4*

[a]Mice were fed choline replete or deficient diets (0.25% or 0%, respectively) for a period of two weeks. Results represent the mean ± 8 mice/group.

[b]Abbreviations used as are follows: PCho, phosphocholine; GPC, glycerophosphocholine; Cho, choline; PC, phosphatidylcholine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

*denotes statistical significance from the control group ($p < 0.05$).

Table 2. Effect of DEA Treatment on Hepatic Choline Metabolites

	DEA (mg/kg/day)[a]						
Parameter	0	10	20	40	80	160	160-R[b]
Body Weight (g)	26.6 ± 0.8	25.9 ± 0.4	26.6 ± 0.1	25.8 ± 0.4	26.1 ± 0.4	27.1 ± 0.6	25.8 ± 0.8
Liver/BW (%)	5.6 ± 0.1	5.6 ± 0.1	5.7 ± 0.1	5.5 ± 0.1	5.5 ± 0.1	6.2 ± 0.1*	5.5 ± 0.1
PCho[c] (nmol/g liver)	1220 ± 44	1192 ± 77	994 ± 78*	959 ± 70*	831 ± 52*	615 ± 37*	1224 ± 114
GPC (nmol/g liver)	372 ± 34	463 ± 34	303 ± 32	275 ± 14*	281 ± 23*	193 ± 20*	318 ± 38
Cho (nmol/g liver)	155 ± 14	137 ± 12	152 ± 11	126 ± 7	94 ± 14*	106 ± 13*	180 ± 21
PC (μmol/g liver)	19.6 ± 1.0	17.7 ± 1.0	17.0 ± 0.4	18.0 ± 1.0	17.4 ± 0.8	16.8 ± 0.4*	17.7 ± 0.7
SAM (nmol/g liver)	83.9 ± 1.1	84.1 ± 4.1	84.7 ± 3.9	82.1 ± 7.2	65.1 ± 6.4*	53.3 ± 4.3*	84.9 ± 1.0
SAH (nmol/g liver)	48.1 ± 1.9	52.2 ± 6.2	49.1 ± 1.8	52.6 ± 1.4	61.8 ± 4.4*	58.5 ± 2.7*	46.1 ± 4.2

[a]Mice were dosed dermally with DEA for 4 weeks at dosages ranging from 10-160 mg/kg/day. Control mice were dosed with 95% ethanol. Results represent the mean ± of 6 mice/group.

[b]Mice were dosed with 160 mg/kg/day for 4 weeks and allowed a 2 week recovery period prior to necropsy.

[c]Abbreviations used as are follows: PCho, phosphocholine; GPC, glycerophosphocholine; Cho, choline; PC, phosphatidylcholine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

(*) denotes statistical significance from the control group ($p < 0.05$).

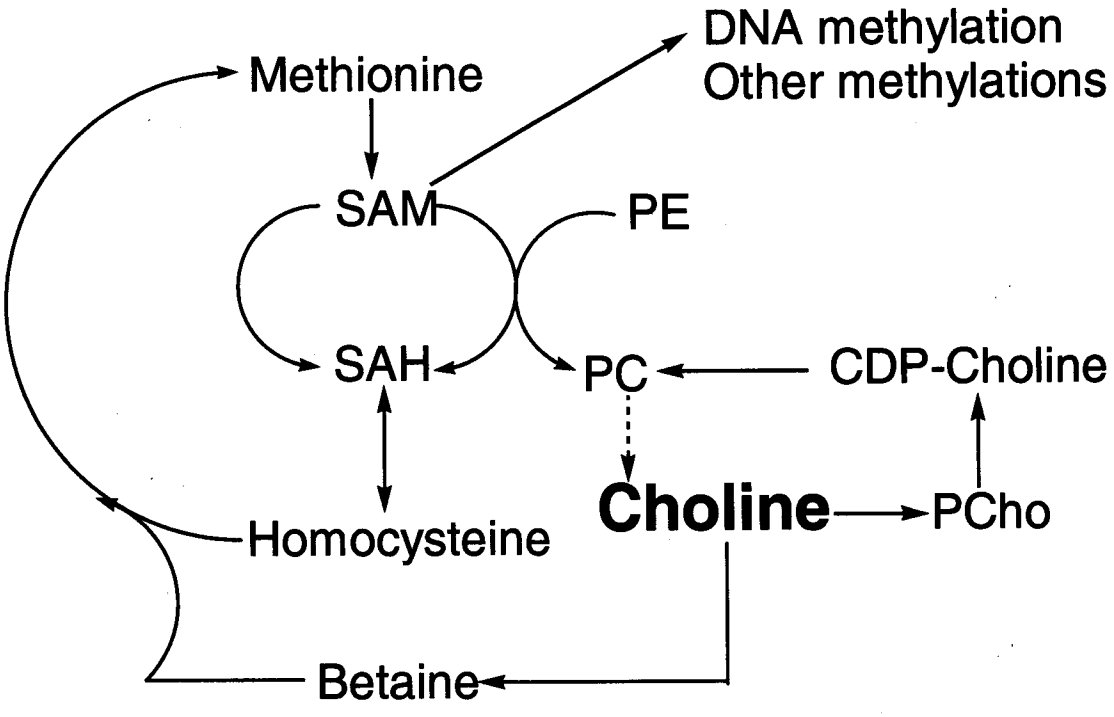
DNA methylation
Other methylations
Methionine
SAM
PE
SAH
PC
CDP-Choline
Choline
PCho
Homocysteine
Betaine

FIGURE 1

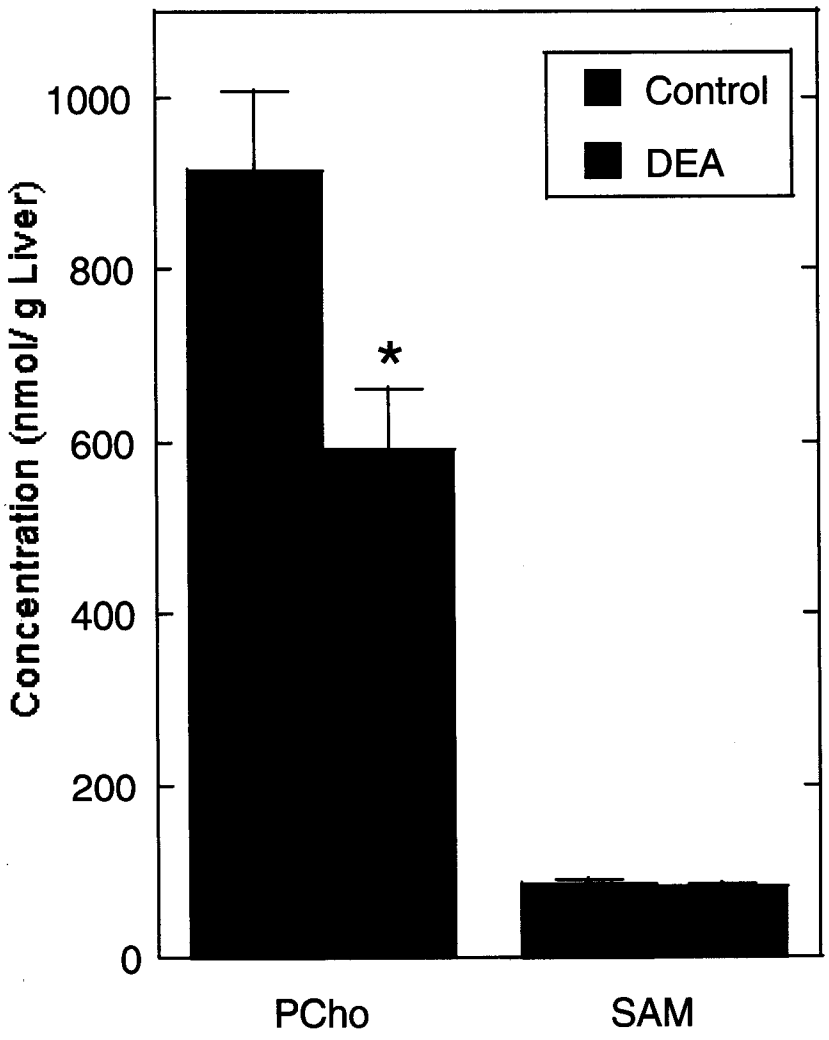
Control
DEA
Concentration (nmol/ g Liver)
1000
800
600
400
200
0
*
PCho
SAM

FIGURE 3

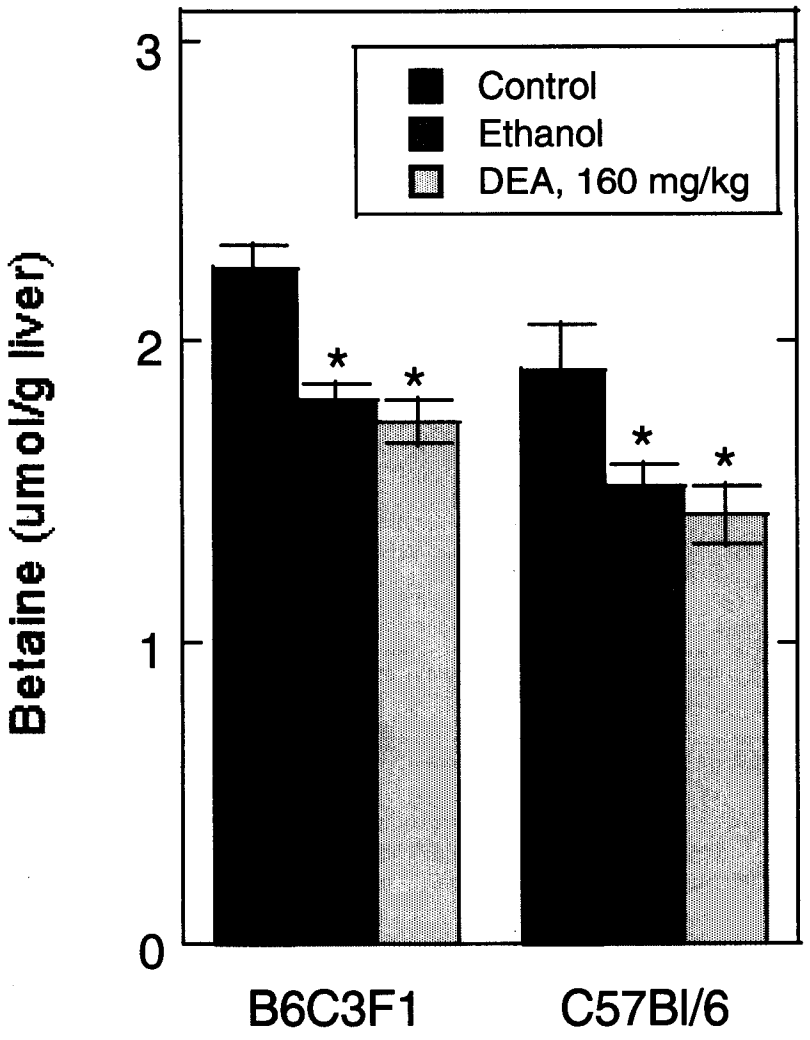
Control
Ethanol
DEA, 160 mg/kg
Betaine (umol/g liver)
0
1
2
3
*
*
*
*
B6C3F1
C57Bl/6

FIGURE 4