Formation of S-Adenosylethionine in Liver of Rats Chronically Fed with DL-Ethionine

Zbynek Brada, Ingrid Hrstka, and Stefan Bulba

ABSTRACT

The early changes in the metabolism of L-ethionine were examined in rats preexposed to chronic administration of DL-ethionine. The capacity of liver to accumulate S-adenosylmethionine after a single injection of L-ethionine decreases rapidly from the onset of the carcinogenic regimen. This drop is caused by diminished S-adenosylmethionine synthesis, a consequence of lower activity of the ATP-L-methionine adenosyltransferase. This change is accompanied by the rapid increase of the concentration of free ethionine and ethionine sulfoxide. The concentration of hepatic ATP depends in the control animals on the L-ethionine dose and is inversely related to the S-adenosylmethionine concentration, but in DL-ethionine-pretreated rats it becomes gradually independent of the L-ethionine dose. The alterations in L-ethionine metabolism observed are not attributed to the change in the ratio of hepatocytes to oval cells but rather to the functional alterations of hepatocytes.

INTRODUCTION

The administration of many hepatocarcinogenic substances, including DL-E, arrests the proliferation of hepatocytes (2-4). With continued administration of the carcinogenic substance, the appearance of new isolated focal hepatocyte populations can be observed (for review, see Ref. 5) that later develop into hyperplastic nodules (see Ref. 6). Hyperplastic areas and nodules in liver, representing groups of slowly proliferating hepatocytes, are considered by many investigators as probable precursors of cancer. These cellular populations are resistant to the toxic effect of the carcinogen.

By studying the early sequential changes in the metabolism of the carcinogenic ethionine in liver before the emergence of new cell populations, it is possible to elucidate the development of the resistance to carcinogens from the beginning of the dietary carcinogenic regimen.

MATERIALS AND METHODS

Female FCN rats were purchased from Carworth, Division of Charles River Breeding Laboratories, Wilmington, MA. The rats in the control group were fed ad libitum C-24, a semisynthetic diet (7). The experimental group was fed the same diet supplemented with 0.30% DL-E (Nutritional Biochemicals Corp., Cleveland, OH). DL-E was added to the basal diet replaced a corresponding amount of sucrose. L-[ethyl-1-14C] Ethionine was purchased from ICN Pharmaceuticals, Inc., Irvine, CA, and cold L-E was from ICN, Cleveland, OH. The rats were housed in stainless steel cages with wire mesh bottoms at 22°C and 50% relative humidity. The windowless room was illuminated from 6 a.m. to 6 p.m. Each group of rats was weighed weekly and sacrificed at different stages during the DL-E treatment. L-E was administered i.p. to the rats after 16 h of fasting.

At the end of each experiment, the rats were lightly anesthetized with ether, and a blood sample was obtained by cardiac puncture. A portion of the liver or other organs was quickly removed, frozen in liquid nitrogen, and homogenized in a 10-fold volume (w/v) of cold 3% perchloric acid in a Waring blender. After the homogenates were centrifuged in the cold acid for 10 min at 2000 rpm, the resulting supernatant was used for the SAE assay. In the case of radioactive material, an aliquot of the supernatant was used for determining the total radioactivity. The assay of SAE was done by the method of Schlenk and DePalma (8) modified by Stekol (9). When needed, the resulting sediment after the centrifugation was washed twice with 3% perchloric acid, once with 95% ethanol, twice with a mixture of ethanol/diethyl ether (3:1) once at 60°C and once at room temperature, and again twice with diethyl ether; they were then dried in a vacuum to constant weight (dry fat-free substance).

The analysis of L-E and its metabolites was performed on 5% trichloroacetic acid extracts from liver (perchloric acid treatment increases the amount of EO). An aliquot of liver trichloroacetic acid extract, after removal of trichloroacetic acid by repeated ether extraction, was applied on an AG 50W-X12 column, 200 to 400 mesh (Bio-Rad Laboratories, Richmond, CA), according to Brada et al. (10). The eluates from the column were collected in 5-ml fractions, and aliquots were mixed with Tritosol scintillation cocktail (11) and counted on a Hewlett-Packard scintillation spectrometer.

The aliquot of perchloric acid extract was neutralized by 5 N KOH. ATP was determined by the method proposed by Cohn (12) and modified by Siekevitz and Potter (13) and Stekol. Liver ATP was separated on an AG 1-X8 column (Bio-Rad Laboratories) and hydrolyzed. The liberated adenosine was isolated on the same exchange material; the adenosine concentration was subsequently determined spectrophotometrically (method details and limitations in Ref. 10).

The cytosol used for determining the activity of MAT was prepared from liver according to a method described by Liaou et al. (14). The homogenate was centrifuged for 10 min at 900 x g and for 1 h at 230,000 x g. The fatty overlayer and the sediment were discarded, and the isolated cytosol was incubated for 30 min at 37°C in a standard reaction mixture containing 0.05 M Tris-Cl (pH 8.2), 0.15 M KCl, 15 mM MgCl2, 5 mM dithiothreitol, 2 mM ATP, and 1 μmol of L-[methyl-3H]methionine. The reaction was terminated by the addition of 4 N perchloric acid (containing 10 mM cold L-M). The resulting precipitate was removed by centrifugation, the SAM produced was isolated on a microcolumn of AG 50W-X12 (Bio-Rad), and the radioactivity of the substance was determined. The method of separation by phosphate chromatography paper, as recommended by Liaou, produced in our hands error above our tolerance.

The isolation of nonparenchymal cells was performed as follows. After a 2-min perfusion of the liver with Gey's balanced salt solution at 37°C, the perfusion was continued with the same solution containing an additional 0.2% Pronase E (Merck) for 1 min. The paste-like substance was then incubated at 37°C in phosphate-buffered saline solution containing 0.2% Pronase E at pH 7.4 for 60 min. The parenchymal cells were destroyed, and only the nonparenchymal cells remained in the suspension and were freed of erythrocytes and cell debris by centrifugation in a Metrizamide solution with a density of 1.089 g/cm³ at 21°C (15). In a parallel experiment the amount of rapidly dividing cells was determined by thymidine incorporation, and it was found extensive. However, the fraction contains, in agreement with a...
SAE; • EM-SAE. Control rats: O, SAE; D, EM-SAE. Bars, SD.

Liver of DL-Ethionine-pretreated Rats as a Function of Injected L-E. The rats which were fed the DL-E-containing diet, and the test rats were fed for 42 days a DL-E-containing diet. Three rats in each group were given, after 16-h starvation, a single L-E dose of graded doses of L-E. The overall concentration of EM-SAE in both control and DL-E preexposed animals is directly related to injected L-E dose. However, the EM-SAE concentration of experimental animals is always much higher than in controls, in agreement with data of Fig. 1. Fig. 2 indicates the analogous relationship between L-E dose and both SAE and ATP concentration. The SAE concentration in liver of normal rats is directly dependent on the amount of injected L-E. However, in animals preexposed to DL-E for 42 days, the hepatic SAE concentration expressed as a function of the L-E doses is significantly lower than in normal animals, a fact confirming our results given in Fig. 1. Whereas in control rats there was a regular increase in SAE concentration over the entire experiment (up to 600 μmol), in the preexposed rats no increased levels were detected after administering 306 or more μmol of L-E/100 g of body weight. The graphic extrapolation of the values obtained shows the possible differences in the slope of the curve at low doses of L-E; however, experimental data are not available owing to a possible contamination by the residual small pool of EM remaining in the body despite the starvation. The main difference is in the concentration of ATP: whereas in normal animals the ATP concentration is inversely related to the SAE concentration, in the DL-E-preexposed rats in which the ATP concentration is below normal level (×26), it is entirely independent of the SAE concentration and the amount of injected L-E.

Concentration of SAE in Liver as a Function of Time after L-Ethionine Injection. The SAE concentration 1 h after L-E injection is twice as great in control rats as in preexposed animals. The difference is maintained throughout the 24 h, even if it has a decreasing tendency (Fig. 3).

Ethionine Metabolites in Kidney. The concentration of SAE

Table 1 Concentration of ethionine and ethionine sulfoxide in liver as a function of i.t.-ethionine feeding time

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Concentration (μmol/g liver)</th>
<th>Ethionine/EO ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.30 ± 0.10</td>
<td>3.02 ± 0.09</td>
</tr>
<tr>
<td>DL-E 1 wk</td>
<td>3.34 ± 0.01</td>
<td>3.25 ± 0.24</td>
</tr>
<tr>
<td>DL-E 3 wk</td>
<td>4.56 ± 0.23</td>
<td>6.93 ± 0.27</td>
</tr>
<tr>
<td>DL-E 30 days</td>
<td>5.12 ± 0.31</td>
<td>6.59 ± 0.55</td>
</tr>
<tr>
<td>DL-E 42 days</td>
<td>6.60 ± 0.20</td>
<td>5.66 ± 0.15</td>
</tr>
</tbody>
</table>

* L-[ethyl-1-3H]ethionine was administered i.p. in the dose of 613 μmol/100 g of body weight to rats after 16 h of fasting. The rats were sacrificed 4 h later.

Fig. 2. The concentration of SAE and ATP in livers of normal and DL-E-fed rats as a function of the dose of the L-E probe. The control rats were fed a basal diet, and the test rats were fed for 42 days a DL-E-containing diet. Three rats were used in each group; the rats were starved for 16 h before being given injections i.p. of an aqueous solution of L-E. They were sacrificed 4 h later, and their livers were analyzed as described in “Materials and Methods.” Control rats: O, SAE; □, ATP. DL-E-fed rats: ●, SAE; ■, EM-SAE. Control rats: O, SAE; □, ATP. DL-E-fed rats: ●, SAE; ■, EM-SAE. Bars, SD.

Fig. 3. The concentration of SAE in the liver of normal rats and rats fed DL-E as a function of time, after the injections of an L-E probe. The control rats were fed a basal diet, and the test rats were fed for 42 days a DL-E-containing diet. Three rats in each group given, 16-h starvation, a single L-E dose (613 μmol/100 g of body weight); then the rats were sacrificed at the times given. The procedures are described in “Materials and Methods.” O, SAE in control rats; ●, SAE in DL-E-fed rats. Bars, SD.
in kidney (determined 4 h after i.p. L-E injection) achieved its maximum with the smallest dose of L-E used (153 µmol/100 g of body weight) for control rats. For rats preexposed to DL-E, the renal SAE concentration at this dose was double that of the control, but did not reach its maximum until the next higher L-E dose (306 µmol/100 g of body weight) where the SAE concentration was triple that of the controls (Fig. 4). As no significant difference was found in the activity of the kidney MAT between the normal and pretreated rats, we assume that the increased amount of SAE is due to higher availability of free ethionine. The concentration of EM-SAE as a function of L-E dose is similar in the kidney of the normal and DL-E-fed rats. However, the ethionine/EO ratio, after an L-E dose of 306 µmol/100 g of body weight, was found to be 0.4 ± 0.05 in the kidney of control rats and 1.36 ± 0.12 in the kidney of rats preexposed to DL-E.

Effect of L-Ethionine Injection on the Drop of Hepatic ATP Concentration as a Function of the Duration of DL-Ethionine Pretreatment. The effect of single injection of L-E on the concentration of ATP in the liver of rats exposed for various periods of time to the carcinogenic regimen of DL-E is illustrated by Fig. 5. The difference in ATP concentration between the rats given injections of water and L-E decreased with the duration of DL-E feeding, and after 3 wk, the difference was no longer present.

Activity of Hepatic ATP-L-Methionine Adenosyltransferase in Rats Fed the DL-E-containing Diet. When comparing Figs. 1 and 5, the observed concentration of liver SAE reached its minimum 1 wk after initiating the feeding. At this point, the concentration of ATP before L-E injection was close to normal, and even after the L-E injection, the drop was smaller than in normal rats. This suggests that ATP is not the limiting factor in SAE synthesis and also that the relatively stable level of ATP in later stages is not a result of a decreased SAE synthesis, unless we consider the presence of a nonreacting ATP pool in cell compartment(s) inaccessible to the SAE synthesis. For these reasons we studied the activity of MAT responsible also for SAE synthesis. It was demonstrated that the activity of this enzyme in cytosol prepared from the liver of rats fed by DL-E was decreased (Table 2). This decrease of enzyme activity was shown also in dialyzed cytosol in which residual free ethionine had been removed.

Metabolism of L-Ethionine in Nonparenchymal Hepatic Cells. Because the ductular (oval) cells in liver of rats being fed hepatocarcinogens proliferate considerably, it is mandatory to determine the extent that these cells contribute to the aforementioned alterations in L-E metabolism. Sells et al. (15) found that these cells can be isolated within the fraction of sinusoidal cells. Rats fed the DL-E-containing diet for more than 2 mo were given injections of L-E after 16 h of fasting; 4 h later, their livers were removed, and nonparenchymal cells were isolated based on their resistance to the Pronase effect. This isolated fraction was analyzed for radioactive substances containing the ethyl group, including SAE. The results given in Table 3 demonstrate that this fraction contains substantially less EM. This means that the increase in EM-SAE in liver cannot be attributed to the presence of the increased population of nonparenchymal cells, since their EM concentration is far less than that in whole liver or hepatocytes.

Reversibility of the Decreased Capacity of Liver to Accumulate SAE. The reversibility of the diminished capacity of liver to accumulate SAE was examined in rats exposed to the carcinogenic regimen for 3 and 6 wk. The DL-E-containing diet was replaced by a basal diet. In both experiments (Table 4) the

- Table 2 Activity of ATP-L-methionine adenosyltransferase in liver of DL-ethionine-fed rats

<table>
<thead>
<tr>
<th>Experiment</th>
<th>nmol SAM/30 min*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rat</td>
<td>42.7 ± 6.5*</td>
</tr>
<tr>
<td>DL-E 7 days</td>
<td>12.2 ± 2.3*</td>
</tr>
<tr>
<td>DL-E 42 days</td>
<td>14.8 ± 1.8*</td>
</tr>
<tr>
<td>DL-E 42 days (Cytosol dialysed)**</td>
<td>13.7 ± 1.1*</td>
</tr>
</tbody>
</table>

* Three rats from each group were sacrificed after 16-h starvation, cytosol was prepared, and the activity of the synthetase was determined as described in "Materials and Methods."
* Mean ± SD.
** Cytosol dialysed against the homogenization buffer.

- Table 3 Concentration of ethionine and its metabolites in liver, isolated hepatocytes, and nonparenchymal cells of DL-ethionine-fed rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total metabolites (µmol/g DFFS)</th>
<th>SAE (µmol/g DFFS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>53.6 ± 4.8*</td>
<td>11.1 ± 2.5</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>49.6 ± 3.5</td>
<td>12.3 ± 3.1</td>
</tr>
<tr>
<td>Nonparenchymal cells</td>
<td>7.8 ± 1.8*</td>
<td>3.5 ± 0.6*</td>
</tr>
</tbody>
</table>

* Mean ± SD.
* Significant difference compared to liver or isolated hepatocytes.
normal SAE formation was restored on the fourth day of the basal diet. At 3 wk the hematoxylin-eosin sections of liver showed no significant morphological differences in either rats exposed to ethionine until sacrifice or in rats for which the diet had been replaced for 4 days by a basal diet. After 6 wk on the DL-E-containing diet, the rat livers showed distinct proliferation of ductular cells, but replacing DL-E by the basal diet for 4 days was too short a time to produce any significant differences in the morphology. Because the elevated level in EM-SAE concentration persists in rats subsequently placed on the basal diet, the values of SAE and of EM-SAE concentration appear to be mutually independent.

**DISCUSSION**

The alterations induced in liver by chronic administration of most chemical hepatocarcinogens (see Refs. 16 to 18) consist of (a) initial damage to hepatocytes (19, 20) accompanied by progressive proliferation of oval (ductular) cells (21, 22), and (b) progressive modification of proliferating hepatocytes (see Refs. 5, 6, and 18 for review). The hepatocarcinogens are metabolized by liver tissue, and the ensuing metabolites induce changes in the metabolizing cells, resulting in modifying the metabolism of the same carcinogenic molecules. The differences in behavior among the cells initially present, the modified cells, and the newly induced cells may then produce—as a result of the selection pressures—the shift in the population of hepatic cells. The metabolism and some selected toxic effects of the injected L-E were studied and compared with the effect of the same doses of L-E in control rats. Both the acute effects (see Refs. 5, 9, and 23) and the metabolism (1, 24) of L-E studied in normal rats serve as a basis for evaluating the changes observed in the present study of DL-E-ingesting rats.

The experiments discussed in this paper deal with functional alterations in hepatocytes of rats ingesting DL-E, occurring at the very onset of the carcinogenic regimen. These alterations take place in the constant cell population arrested in their proliferation by DL-E feeding (2, 4, 25). This early phase ends when the continuing feeding of DL-E induces progressive proliferation of an increasing number of hepatocytes and of the ductular cells.

The capacity of liver tissue to accumulate SAE rapidly decreases with the continued feeding of the DL-E-containing diet. The concentration of ethionine and other extractable metabolites increases simultaneously, but independently of the SAE formation changes. We have demonstrated in our previous studies that, after administering L-E to normal rats, most of this compound in the liver is immediately oxidized to EO, and that a constant ratio of ethionine to EO is fairly well maintained. We also found previously that the oxidation of ethionine and the reduction of EO followed by acetylation may be important in regulating SAE synthesis (24). In the present experiments, although the livers of DL-E-pretreated rats contain enough ethionine as substrate for SAE synthesis, the ethionine/EO ratio undergoes only a subtle change. This ratio varies directly with the time of exposure from 0.91 for control animals, up to 1.21 for rats fed DL-E, i.e., increasing more than 30%. The modified ratio indicates that either the reduction capacity of liver for EO is increased, or the saturation level of the L-E oxidation system is decreased. The increased concentration of EM-SAE in DL-E-pretreated rats can be caused by altering ethionine metabolism in exposed liver, e.g., by decreasing N-acetylation of EO and diminishing the rate of the elimination of EM from the cells. This explanation is consistent with our earlier observations of the change in ratio between the EO and N-acetyl-EO excreted into urine of rats fed DL-E (26).

The decrease in the SAE formation is achieved through the reduced activity of MAT which is responsible for the SAE synthesis (Table 2). The synthesis of SAM by hepatic cytosol is decreased in the presence of a sufficient amount of ATP added to the incubation mixture. We therefore conclude that the SAE synthesis is diminished by the decreased activity of this enzyme and not by lack of both substrates. The mechanism of the decline in the activity of MAT is so far unknown. The observed decline in the MAT activity may participate in the mechanism of the decreased methylation of cellular macromolecules. The undermethylation of nucleic acids and proteins by ethionine has already been observed (18, 26–29) and considered to be a factor in the mechanism of transformation to malignancy.

In agreement with the observation of Shull et al. (30) we found in normal rats a direct relationship between SAE concentration and L-E doses and an inverse relationship between ATP concentration and L-E doses. As shown in Fig. 2, the concentration of ATP, i.e., the second substrate in the SAE synthesis, does not correlate in DL-E-preexposed rats to either the amount of injected L-E or the concentration of the hepatic SAE found. The independence of ATP concentration from L-E dosage develops as a function of the duration of DL-E ingestion (Fig. 5) and is proof of the adaptation of the ATP generation in hepatocytes to the toxic milieu caused by DL-E. We demonstrated recently (31) that this acquired ATP independence (achieved during the first 3 wk of DL-E ingestion) is caused by a significantly increased ATP turnover. The stabilizing of a sufficient concentration of the hepatic ATP improves the survival chance of the hepatic cells experiencing an abrupt drop in available energy.

SAE is formed also in kidney, but, in contrast with liver, no formation of tumors was recorded in this organ. However, a paradoxical situation was observed in the SAE concentration. As stated before (24) and confirmed here (Fig. 4), the maximum renal SAE concentration in control rats was obtained after the administration of 153 μmol/100 g of body weight. The highest renal SAE concentration in DL-E-pretreated rats is 3 times greater and was produced by a dose of 460 μmol/100 g of body weight. Characterized by the observed ethionine/EO ratio, the concentration of free ethionine in normal kidney is, therefore,
half of the free ethionine concentration in the kidney of DL-E-preexposed rats. This elevated concentration of free ethionine in the DL-E-preexposed rats is related to the increased capacity of kidney to form SAE. It is suggested that the cause of such a significant change of ethionine/EO ratio is caused by the decreased capacity of the kidney of DL-E-fed rats to acetylate EO into N-acetyl-EO. The fact that DL-E feeding does not produce an inhibitory effect on the activity of kidney MAT (see “Results”) may be related to a possible preservation of an intact SAM formation and transmethylation in the presence of ethionine. This can be part of the mechanism of resistance of this organ to the tumorigenic effect of ethionine.

The alterations in cell populations in liver exposed to the chronic effect of carcinogenic substances may be a complicating factor in interpreting the results described in this study. However, the largest population of nonparenchymal cells in mammalian liver is made up of the sinusoidal lining cells which constitute about one-third of all liver cells. The volume of nonparenchymal cells is, on the average, one order of magnitude lower than that of the parenchymal cells (32, 33) and, therefore, the cells comprise 6% of the volume of a liver lobule. These data indicate that, in normal liver, the results of analyses of many cytoplasmic components may be considered an accurate picture of parenchymal liver cells. The concentration of the total ethionine metabolites, and specifically of SAE in nonparenchymal cells, is lower than that of isolated hepatocytes (Table 3). The number of the ductular cells after a 7-wk exposure to DL-E is increased from 42% (in the normal rat liver) to 77% (34). However, the mass corresponding to the ductular cells does not exceed 10 to 15%; i.e., it is not sufficient to explain the quantitative changes in the concentration of the various metabolites described in this study. This conclusion is also supported by the rapid decrease in SAE concentration during the first week after initiating the carcinogenic regimen, in the absence of significant proliferation of ductular cells.

Sidransky (35) observed that the inhibition of the hepatic protein synthesis after a single injection of L-E is retarded in rats preexposed to hepatocarcinogens. Some of the observed alterations in L-E and ATP metabolism in this study help to clarify his finding.

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