Pancreatic one-carbon metabolism in early folate deficiency in rats 

Mesbaheddin Balaghi, Donald W Horne, Stephen C Woodward, and Conrad Wagner

ABSTRACT  An amino acid–defined, folate-deficient diet was used to investigate the regulation of pancreatic glycine N-methyltransferase in vivo. This enzyme modulates the ratio of S-adenosylmethionine to S-adenosylhomocysteine and is inhibited by bound folate in vitro. Rats were fed either a folate-deficient diet, a folate-supplemented diet (pair-fed to the deficient group), or a folate supplemented diet ad libitum and measurements were made after 2, 3, and 4 wk. Folate concentrations were greatly reduced in the folate-deficient pancreas after only 2 wk and pancreatic glycine N-methyltransferase activity was elevated but the amount of immunologically measured enzyme protein was the same. The ratio of S-adenosylmethionine to S-adenosylhomocysteine was rapidly reduced in the deficient pancreas. This ratio was also reduced with age in the ad libitum control rats. The pancreas of deficient rats had more immature secretory granules and the ducts were devoid of secreted material.  


KEY WORDS  S-adenosylmethionine, methylation, glycine N-methyltransferase

Introduction

Many recent studies have suggested that one-carbon compounds may play an important role in the function of the exocrine pancreas. The major function of this organ is the synthesis and secretion of enzymes that are used for the digestion of a variety of nutrients. Ethionine, an analogue of methionine that blocks cellular methylation reactions, has a specific toxic effect on the pancreas (1). An experimental mouse model for pancreatitis has been developed that involves the feeding of a choline-deficient diet containing 0.5% ethionine (2). Gilliland and Steer (3) showed that this defect was a failure to discharge the zymogen granules under conditions of methyl group deficiency. S-adenosylmethionine (SAM) is the universal physiologic methyl donor for both small and macromolecules. The ratio of SAM to S-adenosylhomocysteine (SAH) is thought to play a key role in such methylations (4). Glycine N-methyltransferase (GNMT) (EC2.1.1.20) carries out the SAM-dependent methylation of glycine to form sarcosine and SAH. GNMT is very abundant in liver, comprising 1–3% of the soluble protein (5) and it has been suggested that the physiological role of the enzyme is to regulate the ratio of SAM to SAH (SAM:SAH) (6). More recently we showed that a major folate-binding protein of rat liver was GNMT (7) and that the activity of GNMT was inhibited by the natural polyglutamated form of 5-methyltetrahydrofolate (5-CH_{2}-THF) (8). It was proposed that this provides a mechanism for the tissue concentration of SAM to regulate the folate-dependent de novo synthesis of methyl groups. Unpublished immunohistochemical studies (E-J Yeo, C Wagner, 1990) show that GNMT is very abundant in pancreatic acini, which is consistent with the high amount of enzyme activity and messenger RNA found in the pancreas (6, 9). This suggests that GNMT may play a similar role in regulating SAM-SAH ratios in the pancreas as it does in the liver. We have also shown that the concentration of folate in the pancreas is second only to that of the liver among the major organs of rats and that folate deficiency resulted in a major change in the ratio of SAM to SAH of the pancreas after rats were fed an amino acid–defined folate-deficient diet (10) for 4 wk. The purpose of this study was to investigate the development of folate deficiency on the sequence of changes in the ratio of SAM to SAH and to determine the early effects of folate deficiency on the activity of GNMT in the pancreas.

Materials and methods

Animals and diets

Three groups of male weanling Sprague-Dawley rats (Harlan Laboratories, Indianapolis) with mean body weights of ~47 g were used. These animals were housed in identical plastic cages and kept in an environmentally controlled animal laboratory with a 12-h light-dark cycle and fed the following diets: 1) The first group (D) received an amino acid–defined folate-deficient diet (11) containing 396 g dextrin, 197.28 g sucrose, 100 g corn oil, 50 g cellulose, a complete amino acid mixture (175 g/kg body wt), and minerals and vitamins essentially in amounts recommended by the American Institute of Nutrition (AIN) ad hoc committee (12). 2) The second group (ALC) received the same diet, supplemented with 8 mg folate/kg diet ad libitum. 3) The third group (PFC) received the folate-supplemented diet but was individually pair-fed to the average daily food intake of the first group.

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Diets were obtained from Dyets Inc (Bethlehem, PA). All the animals had free access to drinking water and all diets contained 1% succinylsulfathiazole to repress intestinal microfloral folate production. Animals were weighed twice a week. The protocol for animal use was approved by the Vanderbilt University and VA Medical Center Animal Committees.

At the end of the second, third, and fourth weeks of the experiment, 12 rats (four from each group) were anesthetized by intramuscular injection of 0.08 mg ketamine/g body wt (Ketased; Aveco Co. Fort Dodge, IA) and 0.013 mg xylazine/g body wt (Rompun; Mobay Corporation, Shawnee, KS). The abdominal cavity was opened and the pancreas was removed, rinsed in cold saline, weighed, and quickly divided into three portions. One portion was used for determining SAM and SAH, the second portion was kept at −70 °C for GNMT assay and folate measurement, and the third portion was used for histological studies. A sample of the proximal part of the jejunum was similarly removed for histologic examination.

**Measurements and histology**

SAM and SAH were measured by using the method described by Cook et al (13). Briefly, the tissue was promptly homogenized in cold 10% trichloracetic acid and centrifuged at 12,000 × g for 15 min at 5 °C. The extract was washed with diethyl ether and filtered, and SAM and SAH were separated and estimated by HPLC.

GNMT activity was assayed in duplicate according to the method of Cook and Wagner (8). Briefly, the tissue homogenate was centrifuged at 25,000 × g for 30 min at 5 °C. The supernate was incubated with glycine and S-adenosyl-L-[methyl]³⁴ me-thionine in pH 9.0 Tris buffer. The free SAM was removed by adding charcoal suspension and the radioactivity was measured in the supernate after centrifugation at 12,000 × g for 6 min. The blank was a duplicate incubation containing water instead of glycine. The activity of the enzyme is expressed as µmol s-arosine produced min⁻¹ g protein.

GNMT protein was measured in the pancreatic supernate, prepared as above by an enzyme-linked immunospecific assay (ELISA) described by Cook et al (13). Briefly, the enzyme is reacted with an antisem to GNMT prepared in rabbits, then with biotiniyalted goat antirabbit antisem, and then with alkaline phosphatase streptavidin. The assay is concluded by measuring phosphatase activity by using p-nitrophenylphosphate as substrate.

Pancreatic folate was estimated as described by Horne et al (14). Approximately 0.2-g portions of pancreas were added to 1 mL extraction buffer (20 g sodium ascorbate/L, 0.2 mol 2-mercaptoethanol/L, 50 nmol HEPES (4-[2-hydroxyethyl]-1-piperazinethanesulfonic acid)/L, 50 nmol CHES (2-[N-cyclohexylamino]ethanesulfonic acid)/L, pH 7.85, heated for 10 min in a boiling water bath, cooled in an ice bath, and homogenized at setting 5 with a Polytron (Kinematica Gmbh, Luzern, Switzerland). The suspension was centrifuged for 10 min in the microcentrifuge (Eppendorf model 5413; Brinkmann Instruments, Westbury, NY). The supernate was removed, treated with rat serum conjugase (14) to hydrolyze folypolyglutamates, and stored at −70 °C until assayed for folate coenzyme content by combined HPLC-Lactobacillus casei microbiological assay according to Horne et al (14).

For measurement of protein, 50 µL of diluted extract (1/50-1/100) was added to 3.0 mL Bradford reagent and mixed (15). The absorbance was measured at 595 nm against a blank (50 µL distilled water mixed with 3.0 mL reagent). The concentration of the protein was calculated from a curve constructed with bovine serum albumin.

Specimens of pancreas and jejunum were paraffin-embedded and conventionally stained with hematoxylin-eosin, periodic acid-schiff, and phosphotungstic acid-hematoxylin for light microscopy. Specimens for electron microscopy were conventionally embedded in plastic as described by Spurr (16). Thick sections were stained with toluidine blue and thin sections were stained with uranylacetate-lead citrate. They were evaluated unblinded.

**Statistical methods**

Results are expressed as mean ± SEM, and statistical differences were determined by a two-factor analysis of variance (ANOVA) by using the Superanova program (Abacus Concepts, Berkeley, CA). Post hoc analysis of significance was made by Fisher’s protected least-squares difference test.

**Results**

The effect of feeding the three different diets on growth of the animals for up to 6 wk was reported previously (10). There was no significant difference among the weights of the animals in the three groups after 2 and 3 wk. After 4 wk the weights of the animals in the D and PFC groups were significantly lower than those of the ALC group.

The folate concentration in the pancreas of group D was already significantly lower than group PFC after only 2 wk on the diet and did not decrease significantly thereafter (Table 1). When the percent distribution of the coenzyme forms of folate was examined at the end of the fourth week, 10-formyltetrahydrofolate (10-HCO-THF) and 5-CH₃-THF were dephosphated whereas tetrahydrofolic acid (THF) and 5-formyltetrahydrofolic acid (5-HCO-THF) were elevated (Fig 1).

It has been reported that nutritional folate deficiency is associated with changes in the epithelium of the small intestine in many cases (17-19). Therefore, it was important to examine the jejunum histologically to determine whether there was evidence of any structural abnormality to indicate that folate deficiency produced in these experiments could lead to secondary deficiency of other nutrients. There was no morphological difference between the PFC and D animals’ jejunum; specifically, the deficient state did not result in atrophy of the mucosa (data not shown).

**TABLE 1**

Effect of folate deficiency on total pancreatic folate in rats fed three diets

<table>
<thead>
<tr>
<th>Week</th>
<th>ALC</th>
<th>PFC</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/g pancreas</td>
<td>nmol/g pancreas</td>
<td>nmol/g pancreas</td>
</tr>
<tr>
<td>2</td>
<td>6.8 ± 0.4</td>
<td>7.4 ± 0.3</td>
<td>1.8 ± 0.2†</td>
</tr>
<tr>
<td>3</td>
<td>6.1 ± 0.3</td>
<td>6.4 ± 0.3</td>
<td>1.6 ± 0.0†</td>
</tr>
<tr>
<td>4</td>
<td>8.0 ± 0.6</td>
<td>8.2 ± 0.6</td>
<td>1.4 ± 0.1†</td>
</tr>
</tbody>
</table>

* ± SEM; n = 3. ALC, ad libitum control; PFC, pair-fed control; D, deficient.
† Significantly different from ALC and PFC, P < 0.05.
FIG 1. Distribution of pancreatic folate coenzymes in folate-deficient, pair-fed, and ad libitum control rats after 4 wk on the corresponding diets. x ± SEM; n = 3. Values for pair-fed and ad libitum controls were not significantly different for any folate coenzyme. Values for deficient vs pair-fed control and deficient vs ad libitum controls were significantly different (P < 0.05) for tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-CH3-THF), and 10-formyltetrahydrofolate (10-HCO-THF).

There was also no difference between the PFC and ALC groups (data not shown). By electron microscopy, pancreatic acinar cell zymogenic granules were reduced in number at 4 wk in D animals, and the granules showed a greater variation in size as compared with PFC rats. The pancreatic ducts were not filled and not easily apparent in D animals (Fig 2).

The effect of folate deficiency on the ratio of SAM to SAH is shown in Figure 3. It can be seen that the SAM-SAH ratio in the pancreas of the D group was lower than that in either of the two control groups after only 2 wk of being fed the deficient diet. Food restriction also appeared to decrease the SAM-SAH ratio because the values of the PFC group were lower than those of the ALC group in the second and third weeks. The age of the animals also appeared to affect the SAM-SAH ratio because values in the ALC group decreased steadily from the second to the fourth week of the experiment. By the end of the fourth week there was no difference between the two control groups, but values for the two control groups were significantly higher than those of the deficient group.

Even after the second week of folate deficiency, the activity of pancreatic GNMT was significantly higher in the deficient group than in either of the control groups. This increase was much greater after 4 wk. To determine whether the increase was due to changes in the amount of the enzyme or to decreased inhibition by folate cofactors, an ELISA measurement was carried out by using antiserum to GNMT. This showed that there was no significant difference among the three groups at the respective time periods. A recalculation of the data showing the amount of enzyme activity expressed per milligram of GNMT protein as determined immunologically is presented in Figure 4. The activity for group D was higher than that for the other

FIG 2. Electron micrograph of the pancreatic acini from pair-fed control (A) and folate-deficient (B) animals. The distribution of zymogen granules was as follows: folate deficient: 25% small, 65% medium, 10% large; pair-fed control: 11% small, 79% medium, 10% large. In the folate-deficient animals the ducts are empty and not easily apparent. Arrows show the pancreatic ducts in control animals (original magnification × 3500).
FIG 3. The ratio of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) in the pancreas of folate-deficient, pair-fed (PFC), and ad libitum control (ALC) rats after 2, 3, and 4 wk on corresponding diets. \( \bar{x} \pm \text{SEM}; n = 4 \). The deficient group always has a lower ratio. This difference is significant \( (P < 0.02) \) in all time periods when the deficient group is compared with the ALC group and in weeks 2 and 4 when compared with the PFC group. The difference between the two control groups is not significant.

Discussion

Most physiological methylation reactions utilize SAM as the methyl donor and produce SAH. Cantoni et al (4) pointed out that methyltransferase reactions are product inhibited by SAH and suggested that they might be regulated by the SAM-SA\( \text{H} \) ratio. As described in Figure 5, this ratio may be decreased when the amount of methionine, needed for SAM synthesis, is limited. Because the amount of methyl groups needed by the body generally exceeds the amount of dietary methionine, which must also be used for protein synthesis, a methionine cycle exists in the liver whereby SAH is converted to homocysteine and is remethylated to methionine. Decreased ratios of SAM to SAH in the liver have been noted as a result of choline deficiency (21), or a combined choline and methionine (methyl group) deficiency (22). The lowered SAM-SA\( \text{H} \) ratio has been implicated in the increased incidence of spontaneous liver cancer that is found after rats were fed a methyl-deficient diet for prolonged periods (23, 24). It was shown that liver DNA was undermethylated (25), resulting in altered expression of the messenger RNAs for the c-myc and the c-fos oncogenes (26). Exposure of pigs to nitrous oxide for prolonged periods inactivated the vitamin B-12-

dependent methionine synthase (27). They developed a neuropathy characterized by myelin degeneration of the spinal cord. The SAM-SA\( \text{H} \) ratio in the neural tissue of these animals was markedly diminished, and decreased methylation in the central nervous system has been suggested as a reason for the neuropathy (28). We recently showed that a nutritional folate deficiency also produces a decreased ratio of SAM to SAH in the pancreas (10) as well as in the liver (29). Our earlier studies showed that the ratio of SAM to SAH in the pancreas was much lower in the folate-deficient group than in either the pair-fed control or the ad libitum–fed control groups after 4 wk of being fed the experimental diets (10). The results presented here (Fig 3) show that, although the SAM-SA\( \text{H} \) ratio of the D group was lower than the ratio of the PFC group after only 2 wk, it did not become significantly lower until 4 wk. Figure 3 also shows that there is an effect of food restriction on the SAM-SA\( \text{H} \) ratio during the early weeks of the experiment. In both weeks 2 and 3, the SAM-SA\( \text{H} \) ratio of the ALC group was higher than that of the PFC group (NS). The difference between the ALC group and the D group was highly significant, however, and in weeks 2 and 3 was partly due to the effect of food restriction.

GNMT is a key enzyme in the regulation of methyl group metabolism in the liver. Its inhibition by 5-CH\(_3\)-THF provides a mechanism for linking the de novo synthesis of methyl groups to the ratio of SAM to SAH (8). We have shown that GNMT is also abundant in the pancreas and is also inhibited by 5-CH\(_3\)-THF (30). This suggests that GNMT plays a similar role in the pancreas. Decreased concentrations of 5-CH\(_3\)-THF should
We examined the jejunum and pancreas for changes in morphology after 4 wk of feeding the respective diets. The morphologic studies indicated that after 4 wk of feeding the deficient diet there was no apparent difference in appearance of the jejunum between the deficient animals and those of the two control groups. Therefore, as far as these conditions are concerned, it is unlikely that the metabolic effects of feeding the Walzem-Clifford diet (11) are due to a secondary nutrient deficiency caused by damage to the intestinal mucosa (17–19). Note, however, that Halsted et al (32) found changes in absorption of folic acid, d-xylose, glucose, fluid, and sodium in patients that were fed a low-folate diet with ethanol, without changes in jejunal morphology.

Morphologic examination of the pancreas after 4 wk of deficiency revealed changes in ultrastructure. Figure 2 shows representative fields from PFC and D animals. In general, in the deficient pancreas zymogen granules were fewer per cell and were generally smaller in size. In the control pancreas, the zymogen granules are present throughout the body of the acinar cell and are present adjacent to the nuclei in the basal region; this is not so in the deficient acini. Figure 2 also shows that the acinar ducts were clearly visible and filled with secreted material in the control pancreas, but were not apparent in the folate-deficient pancreas. Although not apparent in Figure 2, the central lumen of the acini was generally enlarged in the folate-deficient pancreas. No morphologic examination was made before 4 wk, but on the basis of the extent of the changes, we would expect to see changes at earlier times during development of the deficiency. These observations suggest that folate deficiency alone can cause pancreatic damage that may or may not be related to the changes produced in the SAM-SAH ratio.

References