Biochemical and Molecular Actions of Nutrients

All-trans-Retinoic Acid Rapidly Induces Glycine N-methyltransferase in a Dose-Dependent Manner and Reduces Circulating Methionine and Homocysteine Levels in Rats1,2

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ABSTRACT Glycine N-methyltransferase (GNMT) regulates the methyl group supply for S-adenosylmethionine-dependent transmethylation reactions. Retinoids have been shown to perturb methyl group metabolism by increasing the abundance and activity of GNMT, thereby leading to the loss of methyl groups. Previous studies used pharmacologic doses (30 μmol/kg body weight) of various retinoids administered daily for a total of 10 d. Here, we examined the dose- and time-dependent relationships between all-trans-retinoic acid (ATRA) administration and induction of GNMT, as well as determining additional indices of methyl group and folate metabolism. For the dose-response study, rats were administered 0, 1, 5, 10, 15 or 30 μmol ATRA/kg body weight for 10 d. For the time-course study, rats were given 30 μmol ATRA/kg body weight for 0, 1, 2, 4, or 8 d. A significant increase (105%) in GNMT activity was observed with doses as low as 5 μmol/kg body weight, whereas maximal induction (231%) of GNMT activity was achieved at 30 μmol/kg body weight. Induction of hepatic GNMT by ATRA was rapid, exhibiting a 31% increase after a single dose (1 d) and achieving maximal induction (95%) after 4 d. Plasma methionine and homocysteine concentrations were decreased 42 and 53%, respectively, in ATRA-treated rats compared with controls. In support of this finding, the hepatic activity of methionine synthase, the folate-dependent enzyme required for homocysteine remethylation, was elevated 40% in ATRA-treated rats. This work demonstrates that ATRA administration exerts a rapid effect on hepatic methyl group, folate and homocysteine metabolism at doses that are within the therapeutic range used by humans. J. Nutr. 133: 4090–4094, 2003.

KEY WORDS: • retinoids • glycine N-methyltransferase • dose-dependent • rats • homocysteine

Methyl group and folate-dependent one-carbon metabolism play an important role in the methylation of phospholipids, neurotransmitters, proteins and nucleic acids (1,2). These interrelated pathways provide the needed methyl groups for numerous transmethylation reactions that ultimately affect human health. Transmethylation reactions in methyl group metabolism result in the production of S-adenosylhomocysteine (SAH)4 from S-adenosylmethionine (SAM). After hydrolysis of SAH, the resulting homocysteine can undergo transsulfuration to produce cystathionine, which can eventually be metabolized to cysteine and glutathione, as well as other important compounds. Alternatively, remethylation of homocysteine and the concomitant conversion of 5-methyltetrahydrofolate (5-methyl-THF) to tetrahydrofolate (THF) from the folate-dependent one-carbon pool results in the regeneration of methionine, the precursor for SAM (2). An inability to metabolize homocysteine by either transsulfuration and/or transmethylation leads to increased plasma levels, a characteristic associated with thromboembolic diseases and disruption of vascular wall maintenance (3,4).

A number of regulatory mechanisms function to maintain optimal metabolism of folate, methyl groups and homocysteine. SAM allosterically inhibits 5,10-methylenetetrahydrofolate reductase (MTHFR), the enzyme that irreversibly produces 5-methyl-THF for the subsequent remethylation of homocysteine, a reaction that requires the vitamin B-12–dependent enzyme methionine synthase (MS) (5,6). SAM also serves as a positive modulator of cystathionine β-synthase, the enzyme that catalyzes the formation of cystathionine from homocysteine as the initial step in the transsulfuration pathway (7). Regulation is also maintained by the action of glycine N-methyltransferase (GNMT), a key cytosolic enzyme that converts glycine to sarcosine to regulate the utilization of methyl groups and optimize the ratio of SAM/SAH (2,8). Because SAH is a potent inhibitor of most methyltransferases (9), the ratio of SAM/SAH and its regulation by GNMT control transmethylation processes. GNMT is also a folate-binding protein, which is allosterically inhibited by the folate 4 Abbreviations used: ATRA, all-trans-retinoic acid; GNMT, glycine N-methyltransferase; 5-methyl-THF, 5-methyltetrahydrofolate; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

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coenzyme 5-methyl-THF (10,11). In addition to allosteric regulation, phosphorylation of GNMT represents additional post-translational control of the protein (12). Thus, modulation of any of these regulatory mechanisms has the potential to disrupt the metabolism of folate, methyl groups or homocysteine.

We showed that retinoids such as all-trans-retinoic acid (ATRA) have the ability to perturb methyl group metabolism by increasing the activity and abundance of GNMT, thereby leading to the loss of methyl groups required for other biological processes (13–16). These studies utilized large doses of ATRA (30 μmol/kg body weight) administered for at least 7–10 d. Thus, it is important to examine lower doses that are more relevant to human usage, as well as the length of treatment with ATRA. In the work presented here, we examined the dose- and time-dependent relationship between ATRA and the induction of GNMT as well as parameters associated with folate and homocysteine metabolism.

**MATERIALS AND METHODS**

**Chemicals and reagents.** Reagents were obtained as follows: S-adenosyl-L-[methyl-3H]-methionine, New England Nuclear (Boston, MA); phenylmethylsulfonyl fluoride and ATRA, Calbiochem (La Jolla, CA); goat anti-rabbit IgG horseradish peroxidase, Sigma Chemical, St. Louis, MO. GNMT antibody was provided by Y.-M.A. Chen, National Yang-Ming University, Taipei, Taiwan (17). All other chemicals were of analytical grade.

**Animals and diet.** All animal experiments were approved by and conducted in accordance with Iowa State University Laboratory Animal Resources Guidelines. For all studies, male Sprague-Dawley (Harlan Sprague Dawley, Indianapolis, IN) rats (50–74 g) were housed in plastic cages in a room with a 12-h light:dark cycle and fed a diet and the oral administration of corn oil for 3 d. For the dose-response study, ATRA was administered orally at 0, 1, 5, 10, 15 and 30 μmol/kg body weight using corn oil as the vehicle for a total of 10 d. Thus, it is important to examine lower doses that are more relevant to human usage, as well as the length of treatment with ATRA. In the work presented here, we examined the dose- and time-dependent relationship between ATRA and the induction of GNMT as well as parameters associated with folate and homocysteine metabolism.

**Measurement of GNMT activity.** GNMT enzymatic activity was assayed as described by Cook and Wagner (10) with minor modifications. Portions of liver were homogenized in three volumes of ice-cold phosphate buffer (10 mmol/L, pH 7.0) sucrose (0.25 mol/L) containing 1 mmol/L EDTA, 1 mmol/L sodium azide and 0.1 mmol/L phenylmethylsulfonyl fluoride. After centrifugation (20,000 × g for 30 min), the supernatants were stored at 4°C for 30 min. The reaction was initiated with the protein supernatant and incubated for 30 min at 30°C. The reaction was stopped with 300 μL 0.5 mol/L sodium carbonate buffer (pH 9.5). Sodium hydroxide (1.55 mol/L) and 4-fluoro-7-sulfobenzo[214]aran (1 g/L). Samples were placed in a boiling water bath for 60 min and after centrifugation at 20,000 × g for 10 min, rat serum conjugate was added to an aliquot of the resulting supernatant and incubated in a shaking water bath for 1 h at 37°C. After activation of Sep-Pak NH2 columns with acetonitrile and sodium acetate buffer (16 mmol/L, pH 4.5), samples were applied and washed with acetonitrile and sodium phosphate (0.1 mol/L) containing 50 mmol/L 2-mercaptoethanol. Folate coenzymes were separated on a Phenyl Radial-Pak column (Waters, Milford, MA) and quantified using fluorometric detection (excitation wavelength, 300 nm; emission wavelength, 356 nm). A gradient mobile phase operated at 2.0 mL/min consisted of: 760 mL/L sodium phosphate (0.1 mol/L, pH 7.5) and 240 mL/L acetonitrile for 4 min; a linear gradient (2 min) to 500 mL/L and 500 mL/L and maintained from 6 to 10 min; a linear gradient (2 min) to 100 mL/L and 900 mL/L and maintained from 12 to 16 min; and a linear gradient (2 min) back to initial conditions (760 mL/L and 240 mL/L) for up to 20 min to reequilibrate the column.

**Homocysteine analysis.** Plasma total homocysteine concentrations were determined according to the method of Ubbink et al. (19). In brief, heparinized blood from the cardiac puncture was immediately centrifuged (3800 × g for 6 min) and plasma samples were stored at –70°C until derivatization. For derivatization, N-acetylcysteine (1 mmol/L) was added as an internal standard to 300 μL of plasma. An equal volume of 100 mL/L tributylphosphine in dimethylformamide was added followed by incubation at 4°C for 30 min. The reaction was stopped with 300 μL trichloroacetic acid (100 mL/L) and 1.0 mmol/L EDTA; after centrifugation at 1000 × g for 5 min, the supernatant was added to a solution containing borate buffer (0.125 mol/L, pH 9.5), sodium hydroxide (1.55 mol/L) and 4-fluoro-7-sulfobenzo[214]aran (1 g/L). Samples were filtered and analyzed by HPLC using fluorometric detection and a mobile phase consisting of 960 mL/L potassium phosphate (0.1 mol/L, pH 2.1) buffer and 40 mL/L acetonitrile operated isocratically.

**Determination of hepatic folate coenzyme concentrations.** THF and 5-methyl-THF were determined using HPLC and fluorometric detection according to Revello (20) with some minor modifications. Briefly, portions of liver were homogenized in 4 volumes of ice-cold sodium phosphate buffer (1.0 mol/L, pH 7.0). Samples were applied and washed with acetate buffer and sodium phosphate (0.1 mol/L) containing 50 mmol/L 2-mercaptoethanol. Folate coenzymes were separated on a Phenyl Radial-Pak column (Waters, Milford, MA) and quantified using fluorometric detection (excitation wavelength, 300 nm; emission wavelength, 356 nm). A gradient mobile phase operated at 2.0 mL/min consisted of: 760 mL/L sodium phosphate (0.1 mol/L, pH 7.5) and 240 mL/L acetonitrile for 4 min; a linear gradient (2 min) to 500 mL/L and 500 mL/L and maintained from 6 to 10 min; a linear gradient (2 min) to 100 mL/L and 900 mL/L and maintained from 12 to 16 min; and a linear gradient (2 min) back to initial conditions (760 mL/L and 240 mL/L) for up to 20 min to reequilibrate the column.

**HTRF determination.** The activity of MTHFR was determined in liver samples previously described (21,22). Briefly, portions of liver were homogenized in 4 volumes of ice-cold potassium phosphate buffer (0.5 mol/L, pH 7.2) containing 0.1 mmol/L dithiothreitol, centrifuged at 40,000 × g for 30 min and stored at –70°C until analysis. The assay mixture contained 1.0 mol/L potassium phosphate (pH 6.7), 0.5 mol/L sodium ascorbate, 0.1 mol/L EDTA, 10 mmol/L menadione, 1.0 mol/L FAD and 25 mmol/L 5-[methyl-14C]-THF (74 Bq/μmol). The reaction was initiated with the protein supernatant and incubated for 30 min at 30°C. The reaction was terminated with dmemedone (3 g/L in 1 mol/L sodium acetate) and samples were placed in a boiling water bath for 5 min after being placed on ice, a 1.0-μL aliquot was extracted with toluene and a 1.0-μL aliquot of the resulting supernatant (500 × g for 5 min) was subjected to liquid scintillation counting.

**MS determination.** The activity of MS was determined as previously described (23). Briefly, liver supernatant samples were incubated at 37°C for 1 h in a reaction mixture containing sodium phosphate buffer (500 mmol/L, pH 7.5), cyanocobalamin (1.5 μmol/L), dithiothreitol (1.0 μmol/L), SAM (10 mmol/L), 2-mercaptoethanol (82.4 μmol/L), homocysteine (100 μmol/L) and 15 mmol/L 5-[methyl-14C]-THF (6.44 kBq/μmol). Reactions were stopped with the addition of ice-cold water and samples were immediately applied to AG 1-X8 (Cl form) resin columns (Bio-Rad, Hercules, CA). Flow through fractions (3 mL total) were collected and subjected to liquid scintillation counting.
Amino acid analysis. Amino acids from plasma samples were prepared by using a EZ:faast GC-MS analysis kit (Phenomenex, Torrance, CA). Derivatized samples were analyzed by GC-MS (6890/5973; Agilent Technologies, Palo Alto, CA).

Statistical analysis. The mean values of each treatment group were subjected to a one-way ANOVA (24). If the ANOVA was significant ($P < 0.05$), the means were compared using Fisher’s least significant difference procedure.

RESULTS

ATRA induced hepatic GNMT in a dose-dependent manner. Hepatic GNMT activity (Fig. 1) increased in response to graded levels of ATRA. For the 10-d treatment period, 5 μmol/kg body weight was the lowest dose of ATRA that significantly increased (105%) GNMT activity. GNMT activity continued to increase with respect to the dose of ATRA administered, achieving a maximal response (231%) at 30 μmol/kg body weight. Immunoblotting analysis indicated that the changes in activity were also reflected in GNMT abundance (data not shown).

Hepatic GNMT was rapidly induced by administration of ATRA. GNMT activity was significantly increased 31 and 62% after a single dose of ATRA compared with either group of untreated rats on d 0 and 8, respectively (Fig. 2). Maximal induction was achieved on d 4 and 8, exhibiting a 95 and 83% increase, respectively, compared with control values on d 0. Hepatic GNMT protein abundance also increased as a function of treatment time with ATRA (data not shown).

Plasma homocysteine and methionine concentrations were significantly decreased by ATRA treatment. ATRA treatment resulted in a 53% decrease in plasma homocysteine levels (Fig. 3). Similarly, plasma methionine concentrations were decreased 42% from 50.3 to 29.1 μmol/L in control and ATRA-treated rats, respectively.

Hepatic folate coenzyme concentrations and activity of MTHFR were not affected, whereas MS activity was increased by ATRA administration. The hepatic concentrations of THF and 5-methyl-THF were not affected by administration of ATRA at 30 μmol/kg body weight for 10 d (Table 1). Similarly, the hepatic activity of MTHFR was not altered by treatment with ATRA. In contrast, the hepatic activity of the homocysteine remethylation enzyme MS was elevated 40% in ATRA-treated rats compared with control activity values.

DISCUSSION

The availability of methyl groups for SAM-dependent transmethylation reactions is important in the production of vital biological compounds. Thus, regulation of folate and methyl group metabolism is critical to ensure optimal health because disruption of these interrelated pathways is associated with a number of detrimental conditions (4,25,26). We showed here and in our previous studies that the administration of retinoid compounds in large doses for 7–10 d induced...
RETINOIC ACID ALTERS METHYL GROUP METABOLISM

TABLE 1
Administration of all-trans-retinoic acid (ATRA) for 10 d increased the activity of methionine synthase (MS) in rat liver1,2

<table>
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<tr>
<th>ATRA, μmol/kg body weight</th>
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| THF          | 1.74 ± 0.20 | 1.85 ± 0.44 |
| 5-Methyl-THF | 1.67 ± 0.28 | 2.22 ± 0.46 |

| MTHFR        | 226 ± 44    | 357 ± 85    |
| MS           | 31.8 ± 3.0  | 45.5 ± 2.4* |

1 Data are means ± SEM, n = 6. * Different from control (0 μmol/kg body weight) values, P < 0.05.
2 Abbreviations: 5-methyl-THF, 5-methyltetrahydrofolate; MTHFR, 5,10-methylene tetrahydrofolate reductase; THF, tetrahydrofolate.

GNMT, thereby compromising the methylation of important biological compounds. Of particular importance in this study was the observation that the ability of ATRA to modulate GNMT and methyl group metabolism was rapid (i.e., a single dose), and could be demonstrated at a much lower level (5 μmol/kg body weight). These findings have tremendous relevance for humans who use retinoids such as ATRA (tretinoin, Vesanoid) or 13-cis-retinoic acid (isotretinoin, Accutane) in the treatment of certain cancers and skin disorders. Recommended dosages for both of these retinoid compounds typically range from 2 to 7 μmol/kg body weight and are administered for a period of 15–20 wk (27). Although we have found that 5 μmol/kg body weight was the minimum effective dose in a 10-d study, we would expect that even lower levels of retinoid compounds would be effective at perturbing GNMT and methyl group metabolism when administered for a longer time period. Taken together with our previous demonstration (14) that retinyl palmitate induced GNMT, although to a lesser extent than ATRA or 13-cis-retinoic acid, the unsupervised use of vitamin A supplements for a sufficient period of time may have adverse effects on methyl group metabolism as well. We are currently in the early stages of conducting research focused on the effect of chronic retinoid administration.

We extended our previous work by examining the potential alterations expected in homocysteine metabolism as a result of retinoid administration. The concentration of homocysteine can be modulated by changes in its production (i.e., via transmethylation) and metabolism by either the transsulfuration pathway or remethylation by folate-dependent and/or folate-independent mechanisms (28). For plasma homocysteine levels, renal homocysteine metabolism plays an important role as well. In our studies, the marked induction of GNMT would be expected to result in the accumulation of homocysteine, particularly if the capacity of the transsulfuration and/or remethylation pathways were compromised. However, we found that ATRA administration reduced circulating homocysteine levels, indicating that the metabolism of homocysteine may be enhanced. Similarly, we reported in an earlier study that ATRA was effective at reducing elevated homocysteine concentrations to normal levels in adrenalectomized rats; however, the reduction of plasma homocysteine concentrations by ATRA in intact animals was not significant (16). This discrepancy may reflect the difference in the experimen-tal design because the earlier study was for only 5 d and utilized older rats. We found in a number of studies that older rats exhibit increased sensitivity to retinoids, as indicated by further increases in GNMT activity, but are less sensitive to the effect of retinoids on homocysteine metabolism (Knoblock, V. E. and Schalinske, K. L., unpublished data).

In an attempt to begin to address how retinoid administration modulates homocysteine metabolism, we examined the hepatic folate-dependent remethylation of homocysteine as a possible mechanism. Although the hepatic concentration of 5-methyl-THF and the activity of MTHFR were not altered, the elevated activity of MS suggests that remethylation of homocysteine by the one-carbon pool may be enhanced in ATRA-treated rats. Thus, it appears that the folate-dependent one-carbon pool may compensate for the retinoid-mediated loss of methyl groups by increasing their supply. Determination of actual carbon flux in vivo will be required to assess the effect of changes in MS, or any other relevant enzyme, on the metabolism of homocysteine. Although in vitro regulation may contribute to homocysteine remethylation as well. For example, allosteric factors such as the concentration of SAM play an important role in the regulation of MTHFR by inhibiting its enzymatic activity (5,6). We found that retinoids reduce hepatic SAM concentrations (29,30); thus, an elevation in the endogenous activity of MTHFR would be expected to further contribute to homocysteine remethylation.

Similar to homocysteine, it appears that ATRA treatment increased the catabolism of methionine, as indicated by the reduction in circulating methionine levels. This observation supports our earlier work and that of others that retinoid compounds have the ability to enhance methionine catabolism under both normal and excess dietary methionine conditions (29–32). This finding also underscores the implication that the effect of retinoids on hepatic sulfur amino acid metabolism may have an effect on reducing their availability for other tissues and cells.

It is not clear how retinoid administration may alter the metabolism of homocysteine in humans. Schulpis et al. (33) reported an inability to catabolize a methionine load and increased homocysteine levels in adults that were given 0.5 mg/kg body weight (i.e., 1.7 μmol/kg body weight) (13-cis-retinoic acid) for 45 d; however, this may reflect the hepatotoxicity associated with long-term isotretinoin usage exhibited by these patients. In our rat studies using large (30 μmol/kg body weight) doses of ATRA administered for 10 d, serum liver enzymes were not elevated (data not shown). It will be important in future research to determine the potential effect of these findings on humans. Extrapolation of rat studies focused on factors that modulate homocysteine metabolism to the human situation has met with conflicting results. For example, the alterations seen in homocysteine metabolism as a function of diabetes appear to be similar to that observed in humans (34), whereas thyroid status has opposing effects on homocysteine metabolism when comparing the findings from rat studies to those reported from human research (35).

In summary, we showed that the administration of ATRA, in doses equivalent to those used clinically, was effective at disrupting hepatic GNMT activity and potentially perturbing methyl group metabolism. Moreover, this effect was rapid and resulted in increased metabolism of methionine and homocysteine, with the latter due in part to enhanced folate-dependent remethylation. These findings may have a major effect on a number of individuals who may be exposed to retinoid usage. It was reported recently that the use of isotretinoin has increased 2.5-fold in the last 8 yr, averaging 800,000 new prescriptions per year (36). Moreover, it would be anticipated...
that a large percentage of these individuals may have suboptimal nutritional status, polymorphisms of key enzymes or a physiologic condition that further compromises folate function and methyl group metabolism. We showed recently that the combination of glucocorticoid treatment/diabetes and retinoid administration exerts an additive effect on disrupting methyl group metabolism (16).

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LITERATURE CITED