Triiodothyronine Treatment Attenuates the Induction of Hepatic Glucose N-Methyltransferase by Retinoic Acid and Elevates Plasma Homocysteine Concentrations in Rats1,2

Kelly A. Tanghe, Tim A. Garrow,* and Kevin L. Schalinske3

Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011 and *Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL 61801

ABSTRACT Recent studies indicated that hormonal imbalances have a role in modulating the metabolism of methyl groups and homocysteine, interrelated pathways that when disrupted, are associated with a number of pathologies. Retinoic acid (RA) was shown to induce hepatic glucose N-methyltransferase (GNMT), a key regulatory protein in methyl group metabolism, and to reduce circulating homocysteine levels. Because thyroid status influences the hepatic folate-dependent one-carbon pool and retinoids can alter thyroid hormone levels, the aim of this study was to examine the interaction between retinoids and thyroid function. For hypothyroid studies, rats were administered 0.5 g/L propylthiouracil in the drinking water for 15 d, and RA [30 μmol/(kg · d)] for the final 5 d. For hypothyroid studies, rats were treated with RA [30 μmol/(kg · d)] for 8 d and triiodothyronine [T3; 50 μg/(100 g · d)] the last 4 d. T3 treatment prevented the RA-mediated increase in GNMT activity. However, GNMT abundance remained elevated, indicating that GNMT regulation by T3 in RA-treated rats may be, at least in part, at the post-translational level. In addition, T3 treatment elevated plasma levels of homocysteine 177%, an elevation that was prevented by RA. T3-mediated hyperhomocysteinemia may be due to a 70% decrease in hepatic betaine-homocysteine S-methyltransferase, the enzyme that catalyzes folate-independent remethylation of homocysteine, whereas the RA-mediated stimulation of hepatic homocysteine remethylation by folate-dependent methionine synthase may contribute to lowering plasma homocysteine levels. These findings indicate that thyroid hormones, alone and in conjunction with RA, play an important role in the regulation of methyl group and homocysteine metabolism. J. Nutr. 134: 2913–2918, 2004.

KEY WORDS: • thyroid • glycine N-methyltransferase • homocysteine • retinoic acid • rats


1 Presented in part at Experimental Biology 04, April 2004, Washington, DC.
2 Supported in part by the Iowa Agriculture and Home Economics Experiment Station; the Iowa State University Office of Biotechnology; the United States Department of Agriculture NRI 01–35200-09854 (K.L.S.); the American Institute for Cancer Research 00B078REV (K.L.S.); the Cancer Research and Prevention Foundation (K.L.S.); and the National Institutes of Health DK52501 (T.A.G.).
3 To whom correspondence should be addressed.
4 Abbreviations used: BHMT, betaine-homocysteine S-methyltransferase; BSA, bovine serum albumin; CBS, cystathionine β-synthase; DEX, dexamethasone; GNMT, glycine N-methyltransferase; MS, methionine synthase, MTHFR, 5,10-methylene tetrahydrofolate reductase; 5-methyl-THF, 5-methyltetrahydrofolate; PMSF, phenylmethylsulfonyl fluoride; PTU, propylthiouracil; RA, all-trans-retinoic acid; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; T3, triiodothyronine; T4, thyroxine; TTBS, Tween Tris-buffered saline.

methyl group supply from the one-carbon pool through its allosteric inhibition of the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) (7,8). Glycine N-methyltransferase (GNMT) catalyzes the conversion of glycine to sarcosine, along with the associated conversion of SAM to SAH. Sarcosine has no known physiologic function, suggesting that GNMT functions to regulate the SAM/SAH ratio. Moreover, GNMT is allosterically inhibited by 5-methyltetrahydrofolate (5-methyl-THF), the product of the reaction catalyzed by MTHFR (9,10). It follows that high levels of SAM inhibit MTHFR activity and the subsequent synthesis of 5-methyl-THF, thereby increasing GNMT activity and allowing excess methyl groups to be disposed of as sarcosine until the SAM/SAH ratio is restored. Conversely, if methyl groups and subsequent SAM levels are low, 5-methyl-THF synthesis will increase, GNMT activity will be inhibited, and methyl groups will be conserved for transmethylation reactions of biological importance.

Our research to date indicates that retinoids disrupt the interrelation between the one-carbon pool and methyl group metabolism (11–14). We demonstrated that retinoid compounds are capable of decreasing the hepatic concentration of SAM and the SAM/SAH ratio (15,16). More recently, we found that the mechanistic basis for altering methyl group metabolism resides in elevated GNMT activity and induction of GNMT protein abundance. All-trans-retinoic acid (RA) was the most potent inducer of active GNMT protein, and this activation was found to be biologically important as shown by the subsequent hypomethylation of DNA (12). Moreover, RA treatment reduced circulating levels of methionine and homocysteine (11). Because the induction of GNMT does not appear to be mediated directly by RA, research efforts have focused on identifying other physiologic or cellular signals that may be involved.

A number of studies showed that thyroid status can perturb hepatic one-carbon metabolism (17–23), and thus may function to regulate the activity of GNMT post-translationally. For example, hypothyroidism decreases the activity of MTHFR. It would be expected that this would lead to diminished synthesis of 5-methyl-THF and, consequently, increased GNMT activity. Conversely, hyperthyroidism increases the enzymatic activity of MTHFR, thereby resulting in 5-methyl-THF accumulation and potentially inhibiting GNMT activity. However, there have been no previous investigations into the direct effects of thyroid status on the regulation of GNMT.

Interestingly, it was observed that large doses of retinol and RA resulted in a sharp decrease in serum thyroid hormone concentrations, a decrease in the half-life of thyroxine (T4) and T3, and an increased concentration of triiodothyronine (T3) and T4 in the tissues of rats (24). Thus, it is plausible that treatment with T3 may prevent or reverse some of the anomalies resulting from retinoid administration. Our focus in this study was to determine the following: 1) whether a hypothyroid condition resulted in similar disruption of hepatic methyl group metabolism as RA; and 2) whether treatment with T3 could prevent the effects of RA on methyl group and homocysteine metabolism.

**MATERIALS AND METHODS**

**Chemicals and reagents.** Reagents were obtained as follows: S-adenosyl-L-[methyl-3H]methionine, New England Nuclear; protease inhibitors, RA, and phenylmethylsulfonyl fluoride (PMSF), Calbiochem; Coomassie Plus Protein Reagent, Pierce Chemical; enhanced chemiluminescence Western blotting detection reagents and 5-fluoro-L-[methyl-THF], Amersham Pharmacia; goat anti-mouse IgG horseradish peroxidase, Southern Biotechnology; [14C]-methyl-betaine was obtained from Moraveck; tissue culture reagents, Life Technologies; and dexamethasone (DEX), dl-homocysteine thiolactone, propylthiouracil (PTU) and T3, Sigma-Aldrich Chemical. GNMT antibody was provided by Y.-M.A. Chen (National Yang-Ming University, Taipei, Taiwan) (25) and cystathionine β-synthase (CBS) antibody provided by J. Kraus (University of Colorado Health Sciences Center). All other reagents were of analytical grade.

**Animals and diets.** All animal experiments were approved by and conducted in accordance with guidelines established by Iowa State University Laboratory Animal Resources. Male Sprague-Dawley (Harlan Sprague Dawley) rats (50–74 g) were housed in plastic cages in a room with a 12-h light:dark cycle and consumed water and a control diet (14) ad libitum. The control diet consisted of (g/kg diet): cornstarch, 402; glucose monohydrate, 393; casein, 100; AIN 93 mineral mix, 40; AIN 93 vitamin mix, 10; corn oil, 50; choline bitartrate, 2; and l-methionine, 3. For experiment series 1, rats were randomly assigned to 1 of 4 treatment groups (n = 6): control, RA, PTU, and PTU + RA. PTU was used to induce a hypothyroid state and was administered at 0.5 g/L drinking water for 15 d (18). Rats were administered a daily dose of either corn oil vehicle or corn oil 3 containing RA (30 μmol/kg body weight) for a total of 5 d beginning on d 10. For experiment series 2, rats were randomly assigned to 1 of 4 treatment groups (n = 6): control, RA, T3, and T3 + RA. Corn oil or corn oil containing RA (30 μmol/kg body weight) was administered for a total of 7 d. For the hyperthyroid treatment, rats were injected daily (i.p.) with either 50 μg T3/100 g body weight or vehicle (0.01 mol/L sodium hydroxide) for a total of 4 d beginning on d 3. This experimental design was based on previous studies designed to provide saturating levels of T3 and RA (11,14,16,19,26). After the treatment period in each of the experimental series, rats were anesthetized with ketamine:xylazine (90:10 mg/kg body weight) 4–5 h into the light cycle, and blood and liver samples were immediately collected. Rats were killed by exsanguination.

**GNMT activity.** The enzymatic activity of GNMT was assayed as described by Cook and Wagner (27) with minor modifications. Liver tissue samples were homogenized in 4 volumes of ice-cold buffer containing 10 mmol/L sodium phosphate (pH 7.0), 0.25 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/L sodium azide, and 0.1 mmol/L PMSF. After centrifugation at 20,000 g for 30 min, the resulting supernatant was removed and 2-mercaptoethanol was added to a final concentration of 10 mmol/L. The substrate mixture for the assay consisted of 0.1 mmol/L Tris buffer (pH 9.0), 3 mmol/L dithiothreitol, 1 mmol/L glycine, and 1 mmol/L S-adenosyl-L-[methyl-3H]methionine (4.77 MBq/mmol). The reaction was initiated upon addition of 250 μg of sample protein. The assay was linear with respect to time and protein concentration. Total soluble protein in the tissue extract was determined using a commercial kit (Coomassie Plus, Pierce) according to the method of Bradford (28) with bovine serum albumin (BSA) as a standard.

**GNMT and CBS abundance.** For the measurement of relative protein abundance of GNMT and CBS, 75 μg of total protein was denatured in Laemmli sample buffer, loaded onto a 10–20% gradient SDS polyacrylamide gel and run using a Tris-glycine buffer system. Separated proteins were transferred electrophoretically to nitrocellulose and blocked for 1 h with Tween Tris-buffered saline (TTBS) buffer [20 mmol/L Tris (pH 7.5), 500 mmol/L NaCl, 0.05% Tween 20] containing 50 g/L nonfat dry milk. After subsequent washing with TTBS, membranes were incubated overnight at 4°C with GNMT antibodies (1:3,000) or CBS antibodies (1:2,000) in TTBS containing 10 g/L BSA. GNMT and CBS protein monomer (~32 and 63 kDa, respectively) were detected using a goat anti-mouse (1:5,000) and goat anti-rabbit (1:10,000) secondary antibody, respectively, in TTBS. After chemiluminescence detection and subsequent exposure to Kodak X-omat AR film, the relative density of the protein bands was quantified using SigmaGel software (SPSS).

**Total homocysteine and glutathione.** Plasma total homocysteine and glutathione levels were measured using the method described by Ubbink et al. (29) with minor modifications. Whole-blood samples were collected by cardiac puncture using heparinized syringes. Plasma was immediately obtained by centrifugation at 400 × g for 5 min and stored at −20°C before analysis of homocysteine. The plasma sample was determined using a commercial kit (Coomassie Plus, Pierce) according to the method of Bradford (28) with bovine serum albumin (BSA) as a standard.

**Plasma concentrations of retinoids and metabolites.** Plasma retinoids and metabolites were measured in a previous study as described by Tanghe et al. (9) with minor modifications. Rats were killed by exsanguination, and plasma was immediately obtained by centrifugation at 400 × g for 5 min and stored at −20°C before analysis of retinoids and metabolites.
was thawed and treated with 100 mL tri-n-butyl phosphate in dimethylformamide to release protein-bound homocysteine and reduce mixed disulfides. After incubation at 4°C for 30 min, 100 g/L trichloroacetic acid containing 1 mmol/L EDTA was added to precipitate protein. After centrifugation at 10,000 × g for 5 min, the supernatant was removed followed by the addition of 20 μL of 1.55 mol/L sodium hydroxide, 250 μL of 0.125 mmol/L borate buffer containing 4 mmol/L K2EDTA (pH 9.5), and 100 μL of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. Homocysteine and glutathione were separated by reversed-phase HPLC using a Waters C18 μBondapak column in conjunction with fluorometric detection (excitation: 385 nm; emission: 515 nm). An internal standard of N-acetylcysteine and standard curves were used to quantify plasma homocysteine and glutathione concentrations.

**MS and BHMT analysis.** Liver samples were homogenized in 4 volumes of ice-cold phosphate buffered (10 mmol/L, pH 7.0) sucrose containing 4 mmol/L K2EDTA (pH 9.5), and 100 μL of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. Homocysteine and glutathione were separated by reversed-phase HPLC using a Waters C18 μBondapak column in conjunction with fluorometric detection (excitation: 385 nm; emission: 515 nm). An internal standard of N-acetylcysteine and standard curves were used to quantify plasma homocysteine and glutathione concentrations.

**Cell culture.** Rat hepatoma H4IE cells and pancreatoma AR42J cells (American Type Culture Collection) were grown separately in 10 cm² flasks to 70–75% confluence under 5% CO2 in a humidified incubator at 37°C in DMEM containing 100 mL/mL tretal bovine serum, penicillin (100,000 U/L), and streptomycin (100 mg/L). Cell lines were given fresh media immediately before treatments. Cells were treated with vehicle alone, or with various combinations of DEX (0.1 μmol/L), RA (10 μmol/L), and T3 (2 μmol/L). After a 72-h incubation period in the presence of the various treatment reagents, cells were detached with 2.5 g/L trypsin/1 mmol/L EDTA, washed twice in HBSS, and lysed on ice in a buffer containing 10 mmol/L sodium phosphate buffer (500 mmol/L, pH 7.5), cyanocobalamin (1.3 μmol/L), diethyldithroind (1 mol/L), SAM (10 mmol/L), 2-mercaptoethanol (82.4 mmol/L), homocystine (100 mmol/L), and 5 mL/L Nonidet P-40. Lysates were centrifuged at 16,000 × g for 8 min after which supernatants were removed and stored at −70°C for subsequent analysis of GNMT protein abundance.

**Statistical analysis.** The means of each treatment group were subjected to a two-way ANOVA (P < 0.05) and compared using Fisher’s least significant difference test (31). All statistical analysis were performed using SigmaStat2.0 (SPSS, Chicago, IL).

**RESULTS**

**Altered thyroid status decreased weight gain.** As was reported previously (16,18–23,32), both hypo- and hyperthyroidism decreased weight gain (data not shown). Cumulative weight gains of PTU and PTU + RA groups were 71 and 64% of the control values, respectively. Similarly, cumulative weight gains of T3 and T3 + RA groups were ~70% of the control values. The administration of RA did not affect weight gain.

**GNMT activity was not induced in hypothyroid rats.** The administration of RA increased the hepatic activity of GNMT 72 and 69% in euthyroid and hypothyroid rats, respectively (Fig. 1). PTU treatment had no effect on GNMT activity, indicating that the effect of RA on methyl group metabolism was not mediated by a hypothyroid condition.

**RA-induced GNMT activity, but not abundance, was reversed by T3 treatment.** Hepatic GNMT activity was induced 81% by RA administration in euthyroid rats, but did not affect hyperthyroid rats (Fig. 2). However, the 64% increase in GNMT abundance observed in RA-treated rats was not completely abolished by the subsequent administration of T3. This is also evident in a representative immunoblot displaying these results (Fig. 3). Hyperthyroidism alone had no effect on GNMT activity or abundance. These results suggest that T3 treatment reversed the effects of RA on inducing active GNMT expression in rat liver, and that this regulation, at least in part, may be at a post-translational level.

Potential post-translational regulation of GNMT is further supported by the results from cell culture studies (Fig. 4). Treatment of rat hepatoma H4IE cells with RA, DEX, or both compounds increased GNMT abundance. Although RA was without effect, DEX also induced GNMT abundance in the pancreatoma cell line AR42J. However, for both cell lines, treatment with T3 did not prevent GNMT induction by either RA or DEX.

**Homocysteine levels increased with hyperthyroidism.** Plasma homocysteine levels were increased by 177% in the hyperthyroid rats (Fig. 5A). Moreover, the T3-mediated increase in plasma homocysteine was not apparent in rats administered RA. Administration of RA alone reduced plasma homocysteine concentrations by 38% in comparison with only the control value. RA was also effective at decreasing (~25%) the circulating concentrations of total glutathione, regardless of thyroid status (Fig. 5A). The marked changes in plasma homocysteine concentrations resulting from T3 and/or RA treatment were not associated with similar alterations in the abundance of CBS, a key enzyme in homocysteine catabolism (Fig. 5B).

**MS activity was increased by RA, whereas T3 reduced the activity of BHMT.** The treatment of euthyroid and hyperthyroid rats with RA resulted in a 39 and 57% increase in the circulating concentrations of total glutathione, regardless of thyroid status (Fig. 5A). Although the hyperthyroid group showed the lowest MS activity level, this reduction was not significant (P = 0.18). In contrast, T3 treatment decreased BHMT activity ~70% for both hyperthyroid groups irrespective of RA administration.

![FIGURE 1](https://academic.oup.com/jn/article-abstract/134/11/2913/4688472/fig1) **Hepatic GNMT activity in rats treated with RA and/or PTU. Data are means ± SEM, n = 6; bars with different letters differ, P < 0.05.**
Proper regulation of methyl group and homocysteine metabolism is a critical determinant in optimizing health and preventing disease. Thus, it is vitally important to elucidate and understand how various physiologic and nutritional factors may play a role in this process. Previous work from our laboratory showed that retinoid compounds function to control the expression of GNMT, a key hepatic regulatory protein in methyl group metabolism (11–14). Here we demonstrated that thyroid status also has a profound effect on these pathways and interacts with RA to modulate its action.

On the basis of earlier reports demonstrating that retinoid administration lowered thyroid hormone levels and thyroid status influenced the hepatic folate-dependent one-carbon pool (16,17,20,21,24,32,33), we initially hypothesized that a mediating signal in the ability of RA to alter GNMT may be a reduction in the circulating levels of thyroid hormones, and subsequent treatment with T3 would restore the anomalies associated with RA administration. It was evident from our earlier work that induction of GNMT protein by RA and/or DEX could be accomplished directly in a cell culture model, indicating that at least part of this regulation was not dependent on hormone action (14). Although hypothyroidism did not modulate GNMT activity, the RA-mediated increase in hepatic GNMT activity was completely abolished by subsequent treatment with T3. To date, T3 treatment represents the first known factor, nutritional or hormonal, that can abrogate the effects of RA on GNMT regulation.

An interesting finding in our studies was that although T3 prevented the RA-mediated increase in GNMT activity, the abundance of GNMT protein remained partially elevated, thus indicating that the restoration of normal GNMT activity by T3 may be accomplished, at least in part, by a posttranslational mechanism. This was supported by cell culture studies demonstrating that T3 had no effect on modulating GNMT protein abundance. GNMT was shown to be posttranslationally regulated by phosphorylation and the allosteric binding of 5-methyl-THF, which function to activate and inhibit the enzymatic activity of the protein, respectively (9,10). This latter inhibitory mechanism represents a plausible explanation for our findings with RA- and T3-treated rats. A hyperthyroid condition was reported to result in an increase in the hepatic activity of MTHFR, whereas the activity of MS was reduced (17,21,32–34). It would be predicted that these changes in one-carbon pool enzymes may result in the accumulation of 5-methyl THF, thereby potentially inhibiting the activity of GNMT. However, it is also clear from our studies that the abundance of GNMT in RA-treated rats administered T3 was not elevated to the same extent as in those administered RA alone, indicating that there are likely multiple mechanisms involved, such as transcriptional control. It is well...
documented that T3 and RA can compete for heterologous regulation of specific transcription factors and subsequent response elements (26,35).

An unexpected and novel finding in our studies was the marked hyperhomocysteinemia exhibited by hyperthyroid rats. In contrast, rats administered RA before treatment with T3 did not have elevated plasma homocysteine concentrations. This is consistent with previous studies demonstrating that RA treatment lowers the circulating levels of homocysteine as well as methionine (11). We found similar results in preliminary studies that consisted of RA administration after treatment with T3, thus indicating that RA can prevent or reverse the hyperhomocysteinemia associated with hyperthyroidism (K.A. Tanghe and K.L. Schalinske, unpublished observations).

It appears that regulation of homocysteine metabolism by both RA and T3 may be the result of changes in the activity of the key hepatic remethylation enzymes, MS and BHMT. The hyperhomocysteinemia resulting from a hyperthyroid condition may be related to a marked reduction in folate-independent remethylation (i.e., BHMT), whereas lowering normal or high plasma homocysteine levels by RA could be achieved from upregulation of folate-dependent remethylation (i.e., MS). This is in agreement with earlier studies demonstrating that T3 diminished the activity of MS and BHMT (21,34). In contrast, the catabolism of homocysteine via the hepatic transsulfuration pathway, based on the abundance of CBS, does not appear to be a contributing factor. However, because a number of allosteric factors, such as SAM, can regulate both the remethylation and transsulfuration pathways (17,18,36), direct measurements of metabolic flux in vivo are required to arrive at any definitive conclusions about the relative contributions of these pathways to homocysteine homeostasis. In support of this possibility, we showed previously that in the liver, retinoid compounds diminish SAM concentrations and enhance the catabolism of methionine to taurine, at the expense of glutathione synthesis (15,37). Glutathione synthesis is highly dependent on the transsulfuration pathway (6), and in this study, we found that circulating levels of glutathione were also diminished by RA. T3 treatment had no effect on plasma glutathione levels, in contrast to earlier reports that showed significant elevations in hyperthyroid rats (38). Although the liver is a key organ in methyl group and homocysteine metabolism, as well as the synthesis of glutathione, the contribution of extrahepatic tissues to the circulating levels of homocysteine and glutathione cannot be overlooked. For example, the kidney contributes extensively to the metabolism of homocysteine. Hyperhomocysteinemia was reported under diabetic conditions characterized by renal dysfunction, whereas plasma homocysteine concentrations were lowered in the absence of renal insufficiency (39,40). Similarly, the effects of thyroid hormone and RA on folate, methyl group, and homocysteine metabolism differ in liver and kidney (18,41). It will be important in future research to evaluate the contribution of different tissues to homocysteine and glutathione metabolism as a function of thyroid status and RA administration.

In conclusion, we showed that a significant interaction exists between RA and thyroid status with respect to modulating GNMT function and the metabolism of homocysteine. Our findings complement the recent studies of Brosnan and co-workers directed at examining homocysteine metabolism in the hypothyroid rat (18). They reported that hypothyroidism, via PTU treatment or thyroidectomy, resulted in hypohomocysteinemia. They found that the reduction in plasma homocysteine concentrations was due primarily to an increase in hepatic catabolism of homocysteine by transsulfuration (i.e., CBS and γ-cystathionase), and treatment of thyroidectomized rats with T3 restored these metabolic anomalies. Although there was a significant decrease in the hepatic activity of MTHFR, no changes were observed in the hepatic activities of MS, BHMT, or GNMT. Thus, it is clear that both RA and thyroid status have profound effects on the metabolism of methyl groups and homocysteine in rats. Moreover, it appears that these factors have the ability to interact to attenuate their individual effects.

The relevance of these findings for humans is not clear. It was shown in a number of animal models, including humans, that vitamin A alleviates some of the symptoms associated with a hyperthyroid condition (42,43). We reported that the ability of RA to induce GNMT and modulate methyl group homocysteine metabolism can be achieved rapidly and in clinically relevant doses (11). However, it is well documented that humans exhibit an opposing response compared with rats with respect to thyroid status and homocysteine metabolism. For humans, hyperhomocysteinemia is associated with a hypothyroid state (44–46). Future studies are warranted to elucidate and understand this species-specific difference.

**ACKNOWLEDGMENTS**

The authors express their gratitude to David Falk for his technical assistance and Matthew J. Rowling for the cell culture studies.

**LITERATURE CITED**