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Dietary vitamin A supplementation in rats: suppression of leptin and induction of UCP1 mRNA

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Abstract All-*trans*-retinoic acid (RA), an active metabolite of vitamin A, induces the gene expression of uncoupling protein 1 (UCP1) in brown adipose tissue (BAT) and suppresses leptin gene expression in white adipose tissue (WAT) when given as an acute dose. These contrasting effects of RA leave in doubt the overall effect of chronic RA or vitamin A supplementation on energy homeostasis. To investigate the effects of dietary vitamin A supplementation on leptin and UCP1 gene expression, rats were fed either a normal diet (2.6 retinol/kg diet) or a vitamin A-supplemented diet (129 mg retinol/kg diet) for 8 weeks, and adiposity, serum leptin levels, leptin mRNA levels in perirenal WAT, UCP1 and UCP2 mRNA levels in BAT, and β_3-adrenergic receptor mRNA levels in BAT and WAT were examined. Rats on both diets gained a similar amount of weight, but there was a small 9% decrease in the adiposity index in the vitamin A-supplemented rats. Dietary vitamin A supplementation increased UCP1 gene expression in BAT by 31%, but suppressed leptin gene expression by 44% and serum leptin levels by 65%. UCP2 and β_3-adrenergic receptor gene expression in BAT and perirenal WAT were unchanged by the vitamin A diet. These data suggest that dietary vitamin A has a role in regulating energy homeostasis by enhancing UCP1 gene expression and decreasing serum leptin levels.—Kumar, M. V., G. D. Sunvold, and P. J. Scarpace. **Dietary vitamin A supplementation in rats: suppression of leptin and induction of UCP1 mRNA.** ***J. Lipid Res.*** **1999.** 40: **824–829.**

Supplementary key words retinoic acid • β3-adrenergic receptors • brown adipose tissue

Obesity is the most prevalent nutritional disorder in Western societies. More than three in ten adult Americans weigh at least 20% in excess of their ideal body weight (1). Because available treatments are minimally effective, novel methods to reduce adiposity would be beneficial. We and others have demonstrated that all-*trans*-retinoic acid (RA), one of the active metabolites of vitamin A, can induce the gene expression of uncoupling protein 1 (UCP1) in brown adipose tissue (BAT) both in vitro and in vivo in rats and mice (2–4).

Thermogenesis in BAT serves not only as a regulator of body temperature but also as a means for the dissipation of large quantities of energy as heat. Due to its function in energy expenditure and energy balance, BAT has been implicated to play an important role in the control of obesity (5). The key element in BAT thermogenesis is the unique expression of the mitochondrial protein, UCP1. UCP1 is a proton carrier that, upon activation, uncouples respiration from oxidative phosphorylation, thus facilitating dissipation of energy as heat. The UCP1 gene is under strict transcriptional control in relation to BAT development and heat demand. The observation that RA up-regulates UCP1 gene expression (2–4) suggests that RA or vitamin A, by increasing energy expenditure, may have a potential role for energy homeostasis.

Another gene that has been recently identified to play an important role in energy homeostasis is the ob gene (6, 7). The product of the ob gene, leptin, is primarily produced in white adipose tissue (WAT), and leptin is believed to be the signal for the level of adiposity (8). We previously reported that acute administration of RA, besides increasing UCP1 expression, paradoxically suppresses leptin gene mRNA levels (3). Leptin is an anti-obesity agent that both decreases food intake and increases energy expenditure (7–9). Thus, RA may both promote obesity by reducing leptin gene expression or reduce obesity by inducing UCP1 gene expression. The overall effect of chronic administration of RA or its parent compound, vitamin A, on adiposity is difficult to predict based on the contrasting effects on leptin and UCP1 gene expression. However, β_3-adrenergic agonists also both suppress leptin gene expression and increase UCP1 gene expression (10–12), and these compounds are anti-obesity agents in rats (13). To determine the chronic effect of dietary vitamin A on leptin and UCP1 mRNA levels, rats were fed either a

Abbreviations: BAT, brown adipose tissue; WAT, white adipose tissue; PWAT, perirenal WAT; RA, retinoic acid; UCP1, uncoupling protein 1; UCP2, uncoupling protein 2.

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normal diet or a vitamin A-supplemented diet for 8 weeks. Different parameters of energy homeostasis including leptin mRNA levels in perirenal white adipose tissue (PWAT), serum leptin levels, UCP1 mRNA levels in BAT, as well as adiposity were assessed in rats fed either a normal diet or a vitamin A-supplemented diet for 8 weeks.

MATERIALS AND METHODS

Animals

Male F-344 x BN rats 5 months of age were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Upon arrival, rats were examined and remained in quarantine for 1 week. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals. Rats were housed individually with a 12:12 h light–dark cycle (07:00 to 19:00 h). Ambient temperature was 26°C, thermoneutrality for these rats (14).

Experimental design

Diets formulated to contain vitamin A (as retinyl acetate) with either 2.6 mg retinol/kg diet (normal diet) or 129 mg retinol/kg diet (vitamin A-supplemented diet) were provided by the IAMS Company (Lewisburg, OH). Rats were fed the diets for a period of 8 weeks. Food and water were provided ad libitum. Body weights and food intake were recorded weekly. At the end of 8 weeks, half the rats from each group were challenged with a single dose of the β_3-adrenergic specific agonist, CGP-12177 (0.75 mg/kg, i.p.), or saline.

Chemicals

CGP-12177 was a gift of Ciba-Geigy (Summit, NJ) and was prepared in pyrogen-free saline. Diets were provided by the IAMS Company (Lewisburg, OH).

Tissue harvesting

Rats were killed by cervical dislocation under 90 mg/kg pentobarbital anesthetic. Blood was collected in Vacutainer SST tubes (Becton Dickinson, Franklin Lakes, NJ) via cardiac puncture using an 18-gauge needle followed by perfusion with 60 ml of 0.9% saline. Interscapular brown adipose tissue (BAT), perirenal white adipose tissue (PWAT), retroperitoneal white adipose tissue, epididymal white adipose tissue, and liver were excised, weighed, and rapidly stored in liquid nitrogen. The tissues were stored at −70°C until analysis.

Determination of adiposity levels

Adiposity was determined by the adiposity index (the sum of the weights of perirenal WAT, retroperitoneal WAT, and epididymal WAT divided by body weight × 100) (15). This adiposity measure is highly correlated with the percentage of body fat (15).

Leptin radioimmunoassay

Serum leptin levels were measured with a rat leptin radioimmunoassay kit (Linco Research, St. Charles, MO).

Liver retinol

Total retinol from liver (500 μg) was extracted by the addition of 1 ml hexane with 0.01% butylated hydroxy toluene in the cold. The hexane extraction was repeated twice and the extracts were pooled and evaporated to dryness under nitrogen. Extracted lipids were resuspended in hexane–methanol 1:9 solution containing 0.01% butylated hydroxy toluene and analyzed for retinol by HPLC (16).

mRNA levels

Total cellular RNA was extracted using a modification of the method of Chomczynski and Sacchi (17). The integrity of the isolated RNA was verified using 1% agarose gels stained with ethidium bromide. The RNA was quantified by spectrophotometric absorption at 260 nm using multiple dilutions of each sample.

The probe to detect leptin mRNA was a 33-mer antisense oligonucleotide (5′-GGTCTGAGGCAGGGAGCAGCTCTTGGAGA AGGC) (18), end-labeled using terminal deoxynucleotidyl transferase (Promega, Madison, WI). The oligonucleotide was based on a region of the mRNA downstream from the site of the primary mutation in ob/ob mice and synthesized at the University of Florida core facility and verified by Northern analysis as previously described (10). The full-length cDNA clone for uncoupling protein-1 (UCP1) was kindly provided by Dr. Leslie Kozak, Jackson Laboratory, Bar Harbor, ME (19) and verified by Northern analysis, as previously described (14). The UPC2 cDNA (IMAGE 389584) was provided by Dr. Craig Warden (20) and the β_3AR cDNA was provided by Dr. James Granneman (21). The cDNA probes and the full-length human β-actin cDNA probe (Clontech, Palo Alto, CA) were random prime labeled using Prime-a-Gene (Promega).

For dot-blot analysis, multiple concentrations of RNA were immobilized on nylon membranes using a dot-blot apparatus (Bio-Rad, Richmond, CA). The membranes were baked at 80°C for 2 h. The baked membranes were prehybridized using 25 mm potassium phosphate, 750 mm NaCl, 75 mm Na citrate, 5× Denhardt's solution, 50 μg/ml denatured salmon sperm DNA, and 50% formamide. After incubation for 14–16 h at 42°C, the membranes were hybridized with ^{32}P-labeled probes in the prehybridization buffer plus 10% dextran sulfate. After hybridization for 14–16 h at 42°C, the membranes were washed and exposed to a phosphor imaging screen for 48 h. The latent image was scanned using a Phosphor Imager (Molecular Dynamic, Sunnyvale, CA) and analyzed by Image Quant Software (Molecular Dynamics). Intensities per μg total cellular RNA were calculated by comparison to internal laboratory standards of WAT or BAT total RNA present on each nylon membrane.

Data analysis

One-way or two-way analysis of variance was applied where appropriate. When the main effect was significant, Scheffe's post hoc comparison was applied.

RESULTS

Weight gain, food intake, body fat, and vitamin A status

At the end of the 8-week period on the normal or vitamin A-supplemented diet, the vitamin A status of the animals was evaluated by determining the concentration of retinol in the liver. The liver retinol concentration was 10.8-fold greater in the vitamin A-supplemented group, compared with controls (498 ± 28 vs. 5406 ± 163 μg retinol/g liver, $P = 0.0001$). The vitamin A-supplemented rats, however, did not show any of the known signs of vitamin A toxicity, including depressed growth, occasional bleeding from the nose, or partial paralysis of the legs (22).

Food intake was unchanged by dietary vitamin A supplementation (**Table 1**). In addition, pre-diet and post-diet body weights as well as the gain in body weight in the control and in the vitamin A-supplemented groups was

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TABLE 1. Food intake, body weight parameters, and adiposity index in control and dietary vitamin A-supplemented rats

	Diet	
	Normal	Vitamin A-Supplemented
Daily food intake (g/day)	18.1 ± 0.3	18.1 ± 0.4
Pre diet body weight (g)	328 ± 6	326 ± 5
Post diet body weight (g)	390 ± 8	386 ± 7
Body weight gain (g)	61.8 ± 5.1	59.3 ± 5.2
BAT weight (mg)	411 ± 12	430 ± 20
PWAT weight (g)	1.49 ± 0.06	1.23 ± 0.07[a]
RTWAT weight (g)	3.80 ± 0.10	3.53 ± 0.13
EWAT weight (g)	2.98 ± 0.10	2.62 ± 0.09[a]
Adiposity Index	2.09 ± 0.04	1.91 ± 0.04[a]

Data represent mean ± SE of 15 (control) or 16 (vitamin A-supplemented) rats.

[a] $P = 0.005$ (PWAT), $P = 0.013$ (EWAT), or $P = 0.004$ (Adiposity Index) for difference from rats fed the normal diet by one-way ANOVA.

similar (Table 1). In contrast, there was a significant reduction in weights of two WAT depots; PWAT and epididymal WAT weight decreased in the rats fed the vitamin A-supplemented compared with the normal diet (Table 1). In rats on the vitamin A-supplemented diet, there was a 17% reduction in the weight of PWAT and a 12% reduction in the weight of epididymal WAT. The weight of the retroperitoneal WAT, a third WAT depot was unchanged (Table 1). Overall, there was a small but significant decrease in the adiposity index [(sum of three WAT depots divided by the body weight) × 100], in the rats fed the vitamin A-supplemented diet (Table 1). In contrast, there were no changes in BAT weights upon vitamin A supplementation (Table 1).

Serum leptin levels and leptin mRNA levels in PWAT

In general, serum leptin levels reflect body fat content, thus the decrease in adiposity in the dietary vitamin A-supplemented rats should result in a corresponding decrease in serum leptin. However, the decrease in serum leptin in the vitamin A-supplemented rats was 65%, surprisingly, much greater than predicted from the 9% decrease in the adiposity index (**Fig. 1**). This dramatic decrease in serum leptin suggests leptin synthesis was inhibited.

To assess whether there was a suppression of leptin mRNA levels in PWAT upon vitamin A supplementation, leptin mRNA levels were compared in the control animals on the vitamin A-supplemented and normal diet. There was a 44% suppression of leptin mRNA levels per unit of total RNA in rats on the vitamin A-supplemented compared with the normal diet (**Fig. 2**). β_3-adrenergic agonists also inhibit leptin gene expression. To determine whether the suppression of leptin gene expression by the β_3-adrenergic agonist, CGP-12177, and that by dietary vitamin A supplementation were additive, at the end of the 8-week dietary regimen, half the animals from each dietary group were administered either saline or CGP-12177 and leptin mRNA levels were determined in PWAT 4 h later. As expected, the β_3-adrenergic agonist suppressed leptin mRNA levels by 27% in the rats on the normal diet (Fig. 2). In contrast, in the vitamin A-supplemented group, administration of CGP-12177 had no further effect on leptin mRNA levels, compared with the saline-treated controls on the same diet (Fig. 2). There were no changes in the

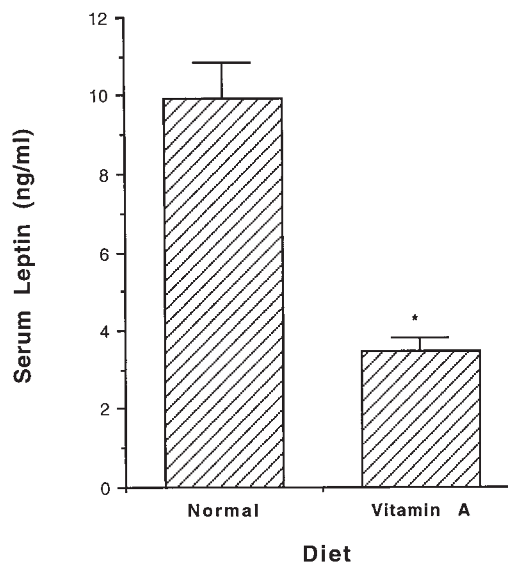

Fig. 1. Serum leptin levels in rats fed a normal diet and a vitamin A-supplemented diet for 8 weeks. Data represent the mean ± SE of 6–7 rats per group. $^*P = 0.0001$ for difference from rats on a normal diet by one-way ANOVA.

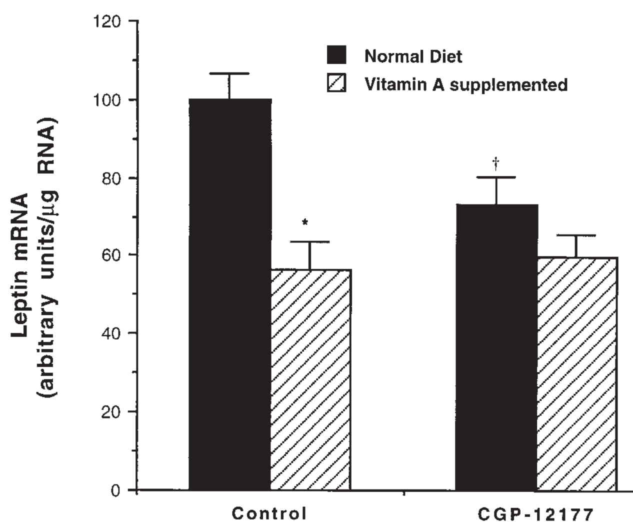

Fig. 2. Leptin mRNA levels in PWAT from rats fed a normal diet or a vitamin A-supplemented diet (left two bars). Some rats on each diet were administered CGP 12177 (0.75 mg/kg) and the animals were killed 4 h post-injection (right two bars). Data represent mean ± SE of 7–8 rats in each treatment group. Leptin mRNA levels are expressed in arbitrary units/μg RNA, with the levels of leptin mRNA in control rats fed a normal diet set to 100 with SE adjusted proportionally. $^*P = 0.0002$ for difference between rats fed normal and vitamin A-supplemented diets by two-way ANOVA; $P = 0.0006$ for difference between normal and vitamin A diets among control rats; $^{\dagger}P = 0.03$ for interaction between diet and CGP-12177 administration; $P = 0.016$ for difference between CGP-12177 administration and control rats on the normal diet.

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TABLE 2. UCP2, β_3-adrenergic receptor, and β-actin transcript levels with and without CGP-12177 administration in rats fed a normal or vitamin A-supplemented diet

	mRNA Levels (arbitrary units)			
	Normal Diet		Vitamin A-Supplemented Diet	
Transcript	Saline	CGP-12177	Saline	CGP-12177
PWAT				
UCP2	100 ± 11	109 ± 10	110 ± 14	110 ± 10
β_3AR	100 ± 9.2	105 ± 6.0	84 ± 13.1	112 ± 11
β-Actin	100 ± 4.7	107 ± 6.1	117 ± 7.8	114 ± 4.9
BAT				
UCP2	100 ± 13	93.4 ± 12.2	103 ± 5.2	94.0 ± 8.9
β_3AR	100 ± 4	50.9 ± 3.3[a]	104 ± 15.6	59.8 ± 7.9[a]
β-Actin	100 ± 9.9	98.5 ± 7.3	94 ± 6.6	93.8 ± 7.9

Data represent mean ± SE of 6–7 rats per group. UCP2, β_3-adrenergic receptor, and β-actin mRNA levels are expressed in arbitrary units/μg RNA, with the levels of mRNA in control rats fed a normal diet set to 100 with SE adjusted proportionally; β_3AR, β_3-adrenergic receptor.

[a] $P = 0.0001$ for difference between CGP-12177 administration and control rats by two-way ANOVA. $P = 0.0001$ (normal diet) and $P = 0.003$ (vitamin A-supplemented diet) for difference between CGP-12177 administration and corresponding control rats.

levels of β actin mRNA in BAT from the saline- or CGP-12177-treated animals in the two dietary groups (**Table 2**).

UCP1 mRNA levels in BAT

Similar to our previous report on the effects of an acute dose of retinoic acid (3), there was a 31% increase in UCP1 mRNA levels upon dietary vitamin A supplementation (**Fig. 3**). To assess whether there was an increased capacity to respond to β_3-adrenergic agonist stimulation in the dietary vitamin A-supplemented rats, the increases in UCP1 mRNA levels were compared after CGP-12177 administration to animals on the vitamin A-supplemented and normal diets. Upon administration of CGP-12177, there was a greater than 2-fold increase in UCP1 mRNA levels in rats on both the vitamin A-supplemented and normal diets, and the increases with diet and β_3-adrenergic agonist administration were additive (Fig. 3). There were no changes in the levels of β actin mRNA in BAT in the saline- or CGP-12177-treated animals in either dietary group (Table 2).

UCP2 mRNA and β_3-adrenergic receptor mRNA levels in BAT and PWAT

UCP2 mRNA and β_3-adrenergic receptor mRNA levels were measured in PWAT and BAT. There were no changes in the levels of UCP2 mRNA in PWAT between the two dietary groups (Table 2). Furthermore, treatment with CGP12177 had no effect on the levels of UCP2 mRNA in PWAT in either of the two dietary groups of animals. Similar to PWAT, there were no changes in UCP2 mRNA levels in BAT in any of the four experimental groups (Table 2).

Similarly, there were no changes in the levels of β_3-adrenergic receptor mRNA in BAT or PWAT in either the saline- or CGP-12177-treated animals on either the normal or vitamin A-supplemented diet (Table 2). Upon treatment with CGP-12177 for 4 h, there was a similar (50%) down-regulation of β_3-adrenergic receptor mRNA levels in BAT from animals on the vitamin A-supplemented and normal diets compared with the corresponding saline-treated animals from the two dietary groups (Table 2).

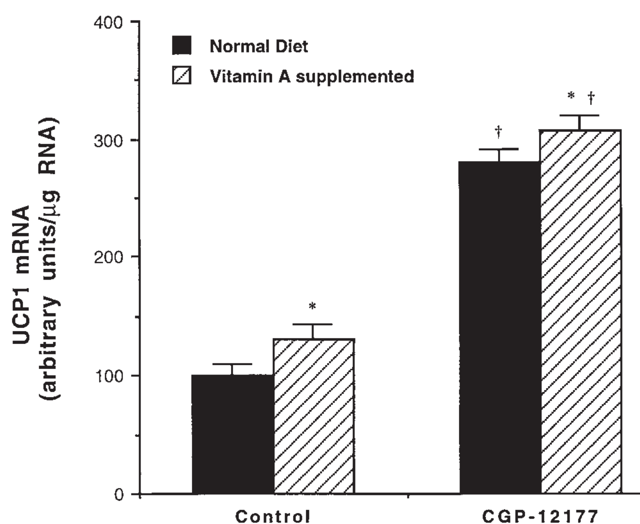

Fig. 3. UCP1 mRNA levels in BAT from rats fed a normal diet or a vitamin A-supplemented diet (left two bars). Some rats on each diet were administered CGP 12177 (0.75 mg/kg) and the animals were killed 4 h post-injection (right two bars). Data represent mean ± SE of 7–8 rats in each treatment group. UCP1 mRNA levels are expressed in arbitrary units/μg RNA, with the levels of UCP1 mRNA in control rats fed a normal diet set to 100 with SE adjusted proportionally. *$P = 0.0175$ for difference between rats fed normal and vitamin A-supplemented diets by two-way ANOVA. Comparison between individual means of vitamin A-supplemented and the corresponding means of rats on the normal diet were not different by post-hoc analysis. †$P = 0.0001$ for difference between CGP-12177 administration and control rats by two-way ANOVA. $P = 0.0001$ for difference between CGP-12177 administration and control rats on the normal or vitamin A-supplemented diet.

DISCUSSION

The present report demonstrates that dietary vitamin A supplementation increases UCP1 mRNA levels but both decreases leptin mRNA levels and reduces serum leptin. Furthermore, the decrease in serum leptin is far greater than the corresponding small decrease in the adiposity index. For the most part, steady state leptin mRNA levels and serum leptin reflect adiposity stores (8). The decrease in serum leptin without a correspondng decrease in body fat suggests inhibition of leptin synthesis, and this suggestion is supported by the decrease in leptin mRNA levels. We previously reported that acute administration of RA suppresses leptin mRNA levels (3). At the present time, retinoic acid response elements have not been identified on the leptin gene promoter. The effect of vitamin A or its metabolite, RA, on leptin mRNA levels may be a direct suppression of leptin gene expression or the suppression may be indirect, mediated by one of the regulators of leptin gene expression. Moreover, despite the greater than

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65% decrease in serum leptin, food intake was unchanged. This suggests that, at least in these rats, leptin may not be the primary regulator of food intake or energy expenditure under these conditions.

In addition to the decrease in leptin mRNA, there was an increase in UCP1 mRNA in BAT after vitamin A supplementation. Thermogenesis in BAT is mediated by norepinephrine activation of sympathetically innervated β_3-adrenergic receptors (14). The β_3-adrenergic signal transduction pathway serves both to activate BAT mitochondrial UCP1 and induce new synthesis of this protein (23). Activated UCP1 uncouples mitochondria, allowing high rates of substrate oxidation and heat production without phosphorylation of adenosine 5′ diphosphate. Our demonstration of an increase in basal UCP1 gene expression after dietary vitamin A supplementation suggests an up-regulation in the capacity for thermogenesis in BAT. It is possible that this increase in UCP1 mRNA may contribute to the small decrease in adiposity after dietary vitamin A supplementation.

In addition to β_3-adrenergic agonists, leptin is another agent that increases thermogenesis in BAT (9). Leptin administration decreases adiposity by reducing food intake and increasing energy expenditure (7, 9). The leptin-induced increase in energy expenditure is a result of an increase in sympathetic nerve activity to BAT (24) resulting in an increase in UCP1 gene expression (9). In the present study, the most dramatic changes observed after dietary vitamin A supplementation were decreases in both serum leptin and leptin gene expression in PWAT. However, in the present study, despite the fall in serum leptin, food intake was unchanged and UCP1 gene expression increased.

In addition to UCP1 gene expression, the present report examined the effect of vitamin A supplementation on another transcript that may be involved in energy balance, UCP2. This uncoupling protein has 59% homology with UCP1 and 73% homology with UCP3 (20, 25). Similar to UCP1, UCP2 can partially uncouple mitochondrial respiration (20). The expression of UCP2, unlike UCP1, is not limited to BAT, and this protein is widely expressed in many tissues, including WAT, heart, and muscle in both rodents and humans (20). The present study found no changes in UCP2 gene expression in PWAT with dietary vitamin A supplementation.

The salient findings of this study, increased UCP1 gene expression and decreased leptin gene expression, are similar to what is observed after administration of a β_3-adrenergic agonist (10,12, 13) with one important difference. β_3-adrenergic agonists suppress leptin gene expression in white adipose tissue (10, 11), greatly enhance UCP1 gene expression in BAT (12), but significantly decrease adiposity in rats (13). Thus, one possibility is that some of the effects of vitamin A supplementation may be mediated by enhanced β_3-adrenergic responsiveness. In BAT, β_3-adrenergic receptor gene expression was unchanged with vitamin A supplementation. UCP1 gene expression was elevated in vitamin A-supplemented rats but this was most likely a direct result of retinoic acid, an active metabolite of vitamin A. We and others have previously demonstrated that retinoic acid administration increases UCP1 gene expression (2–4). This induction is mediated by a retinoic acid response element in the promoter region of the UCP1 gene (2, 26). Moreover, in the present study, the dietary vitamin A- and β_3-adrenergic agonist-induced increases in UCP1 gene expression were additive, i.e., the incremental increase in UCP1 gene expression after CGP-12177 stimulation was the same in rats on both the normal and vitamin A-supplemented diets. In white adipose tissue, there was also no change in β_3-adrenergic receptor mRNA levels with vitamin A supplementation. However, in contrast to BAT, the suppression of leptin gene expression in PWAT by vitamin A supplementation and the suppression by β_3-adrenergic agonist administration were not additive. In fact, in the vitamin A-supplemented rats, there was no further decrease in leptin gene expression with CGP-12177 administration. This suggests that the two treatments share a common mechanism or that with vitamin A supplementation, the leptin gene expression is suppressed to the maximum extent possible and that further suppression by β_3-adrenergic stimulation is ineffective.

Both obesity and the increase in body weight with aging are associated with elevated levels of serum leptin and leptin resistance. Moreover, the increased levels of leptin with obesity may contribute to the diabetes caused by obesity (27). Recent studies have indicated that leptin may impair insulin action. Leptin inhibits the basal and glucose-mediated insulin secretion from isolated pancreatic islets of both normal rats and ob/ob mice (28, 29). Leptin also impairs the metabolic action of insulin in isolated rat adipocytes including glucose transport and lipogenesis (30). A recent study indicated that leptin inhibits insulin signal transduction in human hepatic cells (31). However, this inhibition could not be duplicated in a hepatic cell line transfected with the leptin receptor (32). Thus, the elevated leptin levels in obese individuals may be harmful. Although vitamin A supplementation in humans may be toxic, dietary vitamin A may be a valuable research tool to manipulate elevated serum leptin in animals.

In summary, dietary vitamin A supplementation increases UCP1 mRNA levels in BAT, suppresses leptin mRNA levels in WAT, and reduces serum leptin levels. These data suggest that dietary vitamin A has a contributory role in regulating energy homeostasis.

Supported by the Medical Research Service of the Department of Veterans Affairs and USPS grant AG11465.

Manuscript received 28 September 1998 and in revised form 30 December 1998.

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