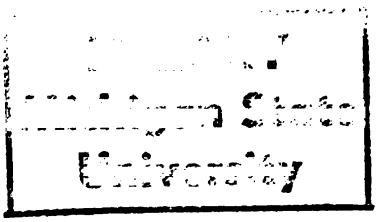

This is to certify that the

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presented by
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EFFECT OF DIFFERENT THERMOGENIC STATES ON SELECTED DETERMINANTS OF THYROID HORMONE ACTION

By

Frank Bradley Hillgartner

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ABSTRACT

EFFECT OF DIFFERENT THERMOGENIC STATES ON SELECTED DETERMINANTS OF THYROID HORMONE ACTION

By

Frank Bradley Hillgartner

Obese (ob/ob) mice and low protein-fed rats exhibit impaired and enhanced adaptive thermogenesis, respectively, relative to corresponding controls that may be mediated, in part, by changes in thyroid hormone action at the level of the nuclear 3,3',5-triiodothyronine (T_3) receptor or the peripheral conversion of thyroxine (T_4) to metabolically active T_3. To investigate this possibility, microsomal iodothyronine 5'-deiodinase activity, nuclear T_3 receptor concentration and affinity and endogenous T_3 content associated with the nuclear receptor were measured in lean and obese mice. Only iodothyronine 5'-deiodination was examined in low protein-fed rats.

Kinetic analysis of hepatic and renal iodothyronine 5'-deiodinase revealed that maximal enzyme activity was lower in obese mice as early as 1-2 wks of age relative to corresponding lean mice while the Km of the enzyme was similar in both phenotypes. The above finding suggests that T_3 availability to thermogenic target tissues may be impaired in obese mice. Scatchard analysis showed that the maximal binding capacity (Bmax) and equilibrium dissociation constant (Kd) of solubilized nuclear T_3 receptors prepared from liver were similar in both 4 and 8-10 wk-old lean and obese mice, indicating that reduced thyroid hormone action in the latter phenotype was not caused by alterations in nuclear T_3 receptor concentration or affinity. In contrast, the

concentration of hepatic endogenous T_3 associated with the specific nuclear receptor was 13 and 26% lower in 4 and 8-10 wk-old obese mice, respectively. These data correlate with reported changes in hepatic thyroid hormone-sensitive enzymes in obese mice, consistent with a diminished nuclear T_3 signal initiating thyroid hormone action. Decreased endogenous nuclear T_3 concentration may be caused by the reduction in iodothyronine 5'-deiodination. Alternately, lowered endogenous nuclear T_3 content may result from reduced transport of T_3 from the plasma to the nuclear compartment.

Maximal (hepatic+renal) iodothyronine 5'-deiodination per 100 g body weight was inversely related to enhanced adaptive heat production in low protein (5% casein)-fed rats. However, a causal relationship between the above parameters is unlikely since similar alterations in maximal 5'-deiodinase activity were observed in 8% casein-fed rats which failed to exhibit changes in adaptive thermogenesis. Hence, control points regulating thyroid hormone action other than peripheral iodothyronine 5'-deiodination may mediate elevated adaptive heat production in rats fed a low protein diet.

To my parents for their loving support

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REVIEW OF LITERATURE

Cold- and diet-induced thermogenesis are defective in obese (ob/ob) mice. Regarding the former process, several studies have shown that the stimulation of heat production resulting from acute cold exposure is severely impaired in obese mice (63). As a result, these animals are unable to survive prolonged periods of cold exposure at 4°C. Focusing on the latter process, it has been reported that the thermogenic response to feeding a highly palatable "cafeteria" diet is reduced in obese mice compared to their lean counterparts (65). It has been postulated that alterations in the above thermogenic mechanisms may at least be partly responsible for the increased energy efficiency in obese mice. This hypothesis is consistent with the findings that maintenance energy requirements are lower in obese mice than in lean mice housed at 33°C (37). Furthermore, the maintenance energy requirement of obese mice remains unchanged as environmental temperature is lowered from 33°C (thermoneutrality) to 25°C-30°C, while in lean mice the maintenance energy requirement is significantly increased by 35 percent (69). The obese mouse has been employed as an experimental model in the study of human obesity since both syndromes share numerous physiological characteristics (7). This animal model may also have utility in more basic studies examining the hormonal regulation of heat production during conditions of impaired thermogenesis.

In contrast to the obese mouse, another experimental model has been developed for the study of thermogenesis during conditions when heat production is enhanced. It has been shown that feeding a low protein diet to either weanling or adult Sprague-Dawley rats results

in increased energy expenditure and reduced energy efficiency (50,62). In general, rats fed a low protein diet will maintain a normal or slightly elevated energy intake (expressed per 100 g body weight) but will gain significantly less body energy than animals fed an adequate protein diet. Feeding animals a variety of palatable foods (cafeteria feeding) has also been shown to stimulate thermogenesis (52). However, the latter feeding procedure has a disadvantage in that dietary composition cannot be strictly controlled. This problem is circumvented by the low protein feeding regimen since semi-purified diets of defined nutrient composition are employed.

Adaptive thermogenesis induced by either cold exposure or diet appears to be regulated by a number of factors. The sympathetic nervous system is thought to play a prominent role in initiating and maintaining cold- and diet-induced thermogenesis (51). Thyroid hormones also appear to be required for thermogenesis and probably act in concert with the sympathetic nervous system. Evidence that thyroid hormones are necessary for cold-induced thermogenesis is provided by studies with thyroidectomized rats which reveal that these animals are unable to increase metabolic rate in response to cold exposure and hence exhibit poor cold tolerance (55). In addition, thermogenic response to norepinephrine administration, commonly used as an index of the maximal capacity of cold-induced thermogenesis, is greatly diminished in thyroidectomized animals (61). Both cold- and catecholamine-induced thermogenesis can be fully restored by thyroid hormone replacement. Regarding the role of thyroid hormones in regulating diet-induced thermogenesis, several studies with lean rats

have shown that increased heat production resulting from feeding either a "cafeteria" or low protein diet is associated with a marked elevation in serum 3,3',5-triiodothyronine (T_3) concentration (67). In obese rats, which have an impaired thermogenic response to diet, the rise in serum T_3 is absent (79). It has been suggested that the elevation in serum T_3 concentration may be important in the development of diet-induced thermogenesis (67).

The above findings raise the possibility that altered thyroid hormone action may be responsible, at least in part, for changes in cold- and diet-induced thermogenesis. In support of this hypothesis, several biochemical processes which have been proposed as mechanisms for adaptive heat production have been shown to be sensitive to thyroid status. They include Na^+, K^+-ATPase activity (36), protein turnover (15,48), futile cycling in carbohydrate metabolism (23,25), mitochondrial α-glycerolphosphate shuttle activity (68), and proton conductance pathway activity in brown adipose tissue (66). Obese mice exhibit reductions in Na^+,K^+-ATPase (36), α-glycerolphosphate dehydrogenase (26,47) and proton conductance pathway activity (22) indicating impaired thyroid hormone expression. Conversely, low protein-fed rats exhibit elevations in α-glycerolphosphate dehydrogenase and proton conductance pathway activity (50) suggesting enhanced thyroid hormone action.

The expression of thyroid hormone action at the cellular level appears to be initiated by the interaction of T_3 with specific low capacity, high affinity receptors in the nucleus (43). These receptors have been isolated in the non histone protein fraction and shown to be

present in a variety of thyroid hormone-sensitive tissues. T_3 is considered the main metabolically active substance since approximately 85 percent of the nuclear bound iodothyronine in liver and kidney has been estimated to be T_3 and the remaining 15 percent thyroxine (T_4). A positive relationship has been demonstrated between nuclear T_3 receptor occupancy in vivo and induction of thyroid hormone responsive enzymes, indicating that the latter process may play a role in regulating thyroid hormone action (44,60). Currently, there are no data available for nuclear T_3 receptor occupancy in either lean or obese mice or animals fed a low protein diet.

There is evidence that thyroid hormone expression may also be influenced by nuclear T_3 receptor concentrations. For example, starvation has been shown to reduce maximal nuclear T_3 binding capacity in rat liver which is associated with an impaired induction of hepatic malic enzyme by T_3 administration (13). Guernsey and Morishige (19) have reported that nuclear T_3 receptor concentrations are also lower in liver and lung of obese mice than in lean mice. However, the above study did not account for leaching of T_3 receptors from chromatin during the incubation procedure. The extent of leaching may have varied in lean and obese mice and therefore may have introduced significant error in estimating phenotypic differences in nuclear T_3 receptor concentration. In addition, the age of the animals was not reported. It would be of interest to know how early in the obese syndrome changes in nuclear T_3 receptor concentration occur. More detailed studies employing improved methodology are clearly needed to confirm the above findings. Recent studies employing solubilized T_3

receptors from liver revealed that neither nuclear T_3 receptor concentration nor receptor binding affinity was affected by low protein feeding in weanling rats (59). Since extraction of T_3 receptors from chromatin may have varied in different dietary groups, the above findings await confirmation by T_3 binding studies in which efficiency of extraction of the nuclear T_3 receptor is taken into account.

Another control point which may regulate thyroid hormone action is extrathyroidal 5'-monodeiodination of T_4 to T_3. Approximately 80 percent of the circulating T_3 is derived from intracellular 5'-monodeiodination of T_4 in the peripheral tissues (32). This reaction, which is catalyzed by the enzyme T_4 5'-deiodinase, may be of regulatory importance in whole animal energy metabolism since the activity of this enzyme can influence intracellular and circulating T_3 concentrations and hence, the level of nuclear T_3 receptor occupancy in target tissues mediating adaptive thermogenesis. Although the quantitative contribution of various tissues to T_3 production from T_4 in vivo is unknown, T_4 5'-deiodinase specific activity assayed in vitro is greatest in liver and kidney (10). This microsomal enzyme has an apparent Michaelis constant (Km) of $\sim$5-10 uM for T_4, is activated by thiols in a two-step transfer (ping-pong) mechanism, is inhibited by propylthiouracil and can use reverse T_3 (3,3',5'-triiodothyronine, rT_3) as an alternate substrate (Km $\sim$0.5 uM) (72). To date, very little is known regarding T_4 5'-deiodination during conditions of altered adaptive thermogenesis. Information on iodothyronine metabolism in lean and obese mice is completely lacking.

Recently, Smallridge et al. (59) have reported that hepatic and renal 5'-deiodinase activities were not altered by feeding a low

protein diet to rats even though plasma T_3 concentrations were markedly elevated in these animals. The above enzyme assays were performed with tissue homogenates in which significant non-specific substrate binding to non-enzyme protein occurs. Under such conditions, no relationship between protein concentration in the homogenate enzyme preparation and enzyme activity can be demonstrated. Therefore, the specific activity of 5'-deiodinase will vary depending on the protein content of the homogenate. Measuring enzyme activity under these conditions is invalid and may lead to spurious results when making comparisons between treatment groups. Studies incorporating improved methodology are needed to determine the effects of low protein feeding on 5'-deiodination.

The purpose of the following studies was to examine whether alterations in thyroid hormone action during conditions of impaired or enhanced thermogenesis are mediated by changes in either nuclear T_3 receptor occupancy, nuclear T_3 receptor concentration, peripheral iodothyronine metabolism or combination of the above. To study the regulation of thyroid hormone action during conditions of enhanced thermogenesis, comparisons were made between rats fed low protein or adequate protein diets. During conditions of impaired thermogenesis, comparisons were made between lean and obese (ob/ob) mice.

REGULATION OF IODOTHYRONINE 5'-DEIODINATION IN LEAN AND OBESE (Ob/Ob) MICE

Introduction

Obese (ob/ob) mice exhibit increased energy efficiency relative to their lean counterparts which may be attributed to a defect in non-shivering thermogenesis (63,64) and diet-induced thermogenesis (65). Recently, work has focused on the regulatory role of the sympathetic nervous system in mediating reduced adaptive thermogenesis in obese mice. This has lead to the proposal that sympathetic activation of the proton conductance pathway in brown adipose tissue is markedly impaired in obese mice (31). There is also evidence that thyroid hormones may play a role in mediating reduced energy expenditure in obese mice. For example, thyroid hormones are necessary for the expression of non-shivering thermogenesis since thyroidectomized rats exhibit poor cold tolerance (55), a condition also observed in obese mice (63). Thermogenic response to norepinephrine administration, commonly used as an index of maximal capacity of non-shivering thermogenesis, is also greatly diminished in both thyroidectomized rats (61) and obese mice (64). Furthermore, stimulation of thermogenesis by feeding a low protein diet is associated with an elevation in serum 3,3',5-triiodothyronine (T_3) concentration in lean rats (77); whereas, in genetically obese rats, which have an impaired thermogenic response to diet, the rise in serum T_3 is absent (79). Finally, several biochemical processes which have been proposed as mechanisms for reduced heat production in obese mice are sensitive to thyroid status. They include Na^+,K^+ATPase activity (36), protein turnover (15,48) futile cycling in carbohydrate metabolism (23) and proton conductance pathway activity in brown adipose tissue (66).

Extrathyroidal 5'-monodeiodination of thyroxine (T_4) to the metabolically active form T_3 has been identified as one of several control points regulating thyroid hormone action (32). Approximately 80% of the circulating T_3 is derived from intracellular 5'-monodeiodination of T_4 in peripheral tissues. This reaction, which is catalyzed by T_4 5'-deiodinase, may be of regulatory importance in whole animal energy metabolism since it influences intracellular and circulating T_3 concentrations and hence, the level of T_3 nuclear occupancy in target tissues mediating thermogenesis. The quantitative contribution of various tissues to T_3 production from T_4 in vivo is unknown, but T_4 5'-deiodinase activity assayed in vitro is greatest in liver and kidney (10).

In both liver and kidney, T_4 5'-deiodinase is isolated in the microsomal fraction (14,33) and requires the presence of thiol reducing agents such as dithiothreitol for maximal activity (34,72); reduced glutathione may serve as a cofactor in vivo. Liver and kidney are also active in monodeiodinating 3,3',5'-triiodothyronine (rT_3), an inactive intermediate product of T_4, in the 5' position to yield $3,3'T_2$. Similarities in the subcellular distribution of the T_4 and rT_3 5'-deiodinases and in the effects of inhibitors on deiodination suggest that a single enzyme catalyzes 5'-deiodination of both T_4 and rT_3 in liver and kidney (27,35).

It is evident that altered thyroid hormone metabolism may be an important factor contributing to the defective expression of adaptive thermogenesis in the obese mouse. Since little is known about thyroid hormone metabolism in lean and obese (ob/ob) mice, we have examined the

effects of age, environmental temperature and T_3 administration on the kinetic parameters of 5'-deiodinase in hepatic and renal microsomes of each phenotype.

Materials and Methods

Materials. The ^{125}I-labeled iodothyronines, (3' or 5') L-T_4 (60-80 uCi/ug) and (3' or 5') L-rT_3 (600-800 uCi/ug) were purchased from Abbott Laboratories (N. Chicago, IL). ^{125}I-labeled L-T_3 (150 uCi/ug) was purchased from New England Nuclear Corp. (Boston, MA). Dithiothreitol (DTT) and unlabeled L-T_4 and L-T_3 were obtained from Sigma Chemical Co. (St. Louis, MO). Unlabeled L-rT_3 was purchased from Calbiochem (LaJolla, CA). All other chemicals were reagent grade.

Animals. Male C57 BL/6J lean (ob/+ or +/+) and obese (ob/ob) littermates obtained from our breeding colony were weaned at 3 weeks of age and housed individually in plastic solid-bottom cages at 25^{o}C. All animals were provided a stock diet (Wayne Rodent Blox from Continental Grain Co., Chicago, IL) and water ad libitum. A 12 h;12 h light-dark cycle was maintained throughout the studies.

Experiments. In experiment 1, iodothyronine metabolism was assessed in 5 age groups (1, 2, 4, 6 and 8-10 weeks of age) of lean and obese mice to compare the pattern of enzyme development between phenotypes before and after the onset of gross obesity in ob/ob mice. Phenotypes of pre-weanling mice were identified based on differences in oxygen consumption (6). Body energy content of these mice was also measured (69). Oxygen consumption and body energy content of 1 week old lean and ob/ob mice were 3768±186 and 2472±83 ul O_2/h/g body weight ($p<0.05$) and 10.8±0.3 and 12.2±0.9 kcal/animal (NS), respectively. Data

for 2 week old lean and ob/ob mice were 3579±114 and 2451±106 ul O_2/h/g body weight ($p<0.05$) and 16.9±0.7 and 24.5±0.8 kcal/animal ($p<0.05$), respectively. Thus, there was a detectable increase in body energy of the ob/ob mice examined at 2 weeks of age, but not in mice examined at 1 week of age. Phenotypes of post-weanling mice were identified visually.

Because the adaptive response in heat production to changes in environmental temperature is different in lean and obese mice (63) the effect of environmental temperature (14^{o}, 25^{o}, and $33^{o}C$) on iodothyronine metabolism was determined in lean and obese mice (experiment 2). Four week old mice were placed in temperature-controlled chambers maintained at either $14^{o}C$ or $33^{o}C$ for 2 weeks. Animals maintained at $25^{o}C$ were housed in the animal room.

In experiment 3, the effect of T_3 administration on iodothyronine metabolism was determined. This experiment was conducted so that a comparison could be made with other hepatic enzymes which have been shown to give a different maximal response to thyroid hormone administration in lean and obese mice. Four week old mice were employed since most of the physiological changes associated with the obese syndrome have occurred by this age without the appearance of massive obesity which may have secondary effects on T_3-induced enzyme response. Lean mice were injected intraperitoneally with 5 ug T_3/100 g body weight twice daily for 7 days. Obese mice were injected with the same amount of T_3 as their lean littermates. Based on the data of Kaplan and Utiger (28), this dosage was sufficient to produce a maximal induction in 5'-deiodinase activity. Control animals received an

equal volume of vehicle solution (155 mM NaCl, 2 mM NaOH). On day 7, mice were killed 4 h after their last injection.

Tissue Preparation. All iodothyronine deiodinase assays were conducted using a washed, crude microsomal fraction obtained from liver or kidney. To prepare this fraction mice were killed by cervical dislocation and the liver and kidneys quickly excised, washed with homogenization buffer and placed on ice. Each tissue was homogenized at $0^{o}C$ in 4 vol of 0.25 M sucrose, 0.05 M potassium phosphate, 0.01 M EDTA buffer, pH 7.4 using a Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 15,000 x g for 10 min at $4^{o}C$. The resulting supernatant was passed through 3 layers of cheesecloth and centrifuged at 100,000 x g for 65 min yielding the crude microsomal pellet. The microsomal pellet was washed twice before being resuspended in the homogenization buffer to produce a protein suspension of 5-6 mg/ml and 2-3 mg/ml for liver and kidney, respectively. After taking a sample for protein determination (39) 2 mM DTT was added to the suspension before it was rapidly frozen in acetone and dry ice. Thereafter, the microsomal preparation was stored at $-80^{o}C$ until deiodinase assay.

Enzyme Assay.

Incubation Procedure. Microsomal T_4 5'-deiodinase ($T_4$5'-D) and $rT_3$5'-deiodinase ($rT_3$5'-D) were measured using a radiolabeled tracer procedure. The generation of radiolabeled T_3 and iodide from T_4 and rT_3, respectively, was monitored in each reaction. Both $T_4$5'-D and $rT_3$5'-D were assayed separately in hepatic microsomes. Only $rT_3$5'-D was assayed in renal microsomes since the activity of $T_4$5'-D was very low in this preparation.

Based on preliminary studies, optimal conditions were established for $T_4$5'-D activity. The results indicated that $T_4$5'-deiodination was maximal at pH 6.8, 37°C and 2 mM DTT using a 0.05 M potassium phosphate, 0.01 M EDTA incubation buffer. In addition, $T_4$5'-deiodination was linearly related to microsomal protein concentration from 0.1 mg/ml to 0.4 mg/ml during a 1 h incubation at a T_4 concentration of 2 uM. Hence, $T_4$5'-D activity was assayed using the above reaction conditions and a microsomal protein concentration of 0.35 mg/ml. Enzyme reactions were carried out in polystyrene culture tubes (75 x 12 mm) with various amounts of unlabeled T_4 added to ^{125}I-T_4 (1.25 uCi/tube) to give T_4 concentrations ranging from 2 uM to 13 uM in a total incubation volume of 285 ul. Incubations proceeded for 30-120 min at 37°C under N_2 gassing. Reaction times were adjusted so that less than 12% T_4 was converted to T_3. Reactions were terminated by plunging the assay tubes in cracked ice and immediately adding 0.5 ml ice-cold ethanol.

rT_3 5'-deiodination in hepatic microsomes was measured in 0.05 M potassium phosphate, 0.01 M EDTA, 5 mM DTT, pH 8.0 at protein concentrations ranging from 0.01 mg/ml to 0.10 mg/ml. The above pH for the incubation buffer was chosen based on the findings of Visser et al. (71) showing that a pH 8.0 phosphate buffer gave the highest V_{max} for $rT_3$5'-D in hepatic microsomes compared to pH 6.5 and 7.2. Our own observations revealed that $rT_3$5'-D required a slightly higher DTT concentration (5 mM vs 2 mM) than $T_4$5'-D for maximal enzyme activity. The reaction tubes contained ^{125}I-rT_3 (1.0 uCi/tube) and unlabeled rT_3 to provide final rT_3 concentrations ranging from 0.125 uM to 3.0 uM. Enzyme reactions were carried out for 7-20 min at 37°C under N_2

gassing. The assay for $rT_3$5'-deiodination in renal microsomes was similar to that in hepatic microsomes except for two alterations in the incubation buffer; 0.25 M sucrose was added to the 0.05 M potassium phosphate, 0.01 M EDTA and 5 mM DTT buffer, and the pH was adjusted to 7.4 rather than 8.0. These alterations of the incubation buffer were made because the small amount of protein recovered from kidneys of the mice required that a relatively large volume of original suspension buffer be added to the incubation buffer to provide adequate amounts of enzyme for assay. To minimize effects of adding relatively large and varying amounts of suspension buffer to the incubation buffer we employed an incubation buffer similar in composition to that of the original suspension buffer. The range of microsomal protein concentrations and incubation times for the renal rT_3 5'-D assay were 0.015 mg/ml to 0.10 mg/ml and 7 min to 15 min, respectively. Reaction times and/or protein concentrations for all $rT_3$5'-D assays were adjusted so that less than 16% of the substrate was consumed during the incubation. $rT_3$5'-D reactions were terminated by plunging the assay tubes in cracked ice and adding either 0.5 ml ice-cold ethanol or 0.1 ml 2% bovine serum albumin followed by 0.5 ml 10% trichloroacetic acid depending on which method of enzyme product analysis was employed.

Since T_3 is a reaction product of $T_4$5'-D, alterations in T_3 degradation are a potential source of error when comparing hepatic $T_4$5'-D among treatment groups. Therefore, T_3 degradation was measured in hepatic microsomes by monitoring the disappearance of ^{125}I-T_3. Details of the procedure and reaction conditions for this assay are the same as those for the $T_4$5'-D determination, except that T_3

degradation was measured at only one substrate concentration (0.35 uM T_3).

Analysis of Incubation Products. To determine the percentage generation of T_3 and iodide from T_4 and rT_3, respectively, and the percentage degradation of T_3, enzyme reactants and products were separated by descending paper chromatography employing the solvent system of hexane-tertiary amyl alcohol-2 N ammonia (1:10:11) (2). Briefly, ethanolic extracts of reaction mixtures were spotted on Whatman no. 1 paper strips (Whatman Inc., Clifton, NJ) along with appropriate carrier compounds. After development, T_3 and T_4 spots were visualized with 0.5% ninhydrin while a 0.1% palladium chloride solution was used to identify the iodide spot. Next, the paper strips were cut into sections which were then measured for radioactivity using a gamma counter. Values for the fractional generation of reaction products or the fractional degradation of substrate obtained from tissue-free control incubations were subtracted from the observed experimental values of tissue-containing incubations to correct for any non-enzymatic reactions and for ^{125}I-labeled reaction contaminants.

In some experimental groups, iodide release from rT_3 was measured using the ion exchange method of Leonard and Rosenberg (35) rather than paper chromatography. In this procedure, trichloroacetic acid extracts of reaction mixtures were applied to Dowex 50W-X8 columns (BioRad Laboratories, Richmond, CA) equilibrated with 1.74 M acetic acid. Free iodide was eluted from the column with three column volumes of 1.74 M acetic acid divided into two aliquots. Results of the above method were similar to those obtained using the paper chromatography procedure.

Treatment of Enzyme Data. Kinetic analysis was performed on data derived from the $T_4$5'-D and $rT_3$5'-D assays. Enzyme activity (pmol product formed/mg microsomal protein/min) was determined at each substrate concentration and double reciprocal plots were constructed. Slopes and intercepts of the linear plots were determined using unweighted least squares analysis. In calculating 5'-deiodinase activity, it was assumed that ^{125}I-T_4 was randomly distributed between the 3' and 5' positions of ^{125}I-T_4 and ^{125}I-rT_3. Hence the observed fractional generation of T_3 and iodide from T_4 and rT_3, respectively, was doubled to obtain the actual values.

Plasma T_4 and T_3 Determinations. Immediately prior to killing the mice, blood samples were obtained from the orbital sinus for plasma T_4 and T_3 analysis. Plasma T_4 was measured using a solid phase RIA system supplied by Becton Dickinson Immunodiagnostics (Orangeburg, NY). Plasma T_3 was assayed based on the method of Nejad et al. (41) which uses charcoal-dextran to separate bound from free hormone.

Statistical Analysis. The data were subjected to analysis of variance. Statistical comparisons were made with the Bonferroni t test or Student's t test as noted in table footnotes and figure legends.

Results

Effect of Age on iodothyronine Metabolism. Effects of age and phenotype on the kinetic parameters of 5'-deiodinase in liver and kidney derived from Lineweaver-Burke plots are summarized in Table 1. Sample double reciprocal plots are presented in Figure 1 for 4 week old mice. In examining the pattern of development of hepatic 5'-deiodinase activity in mice, a large increase in $T_4$5'-D activity was observed between 1 and 2 weeks of age. V_{max} values could not be compared at

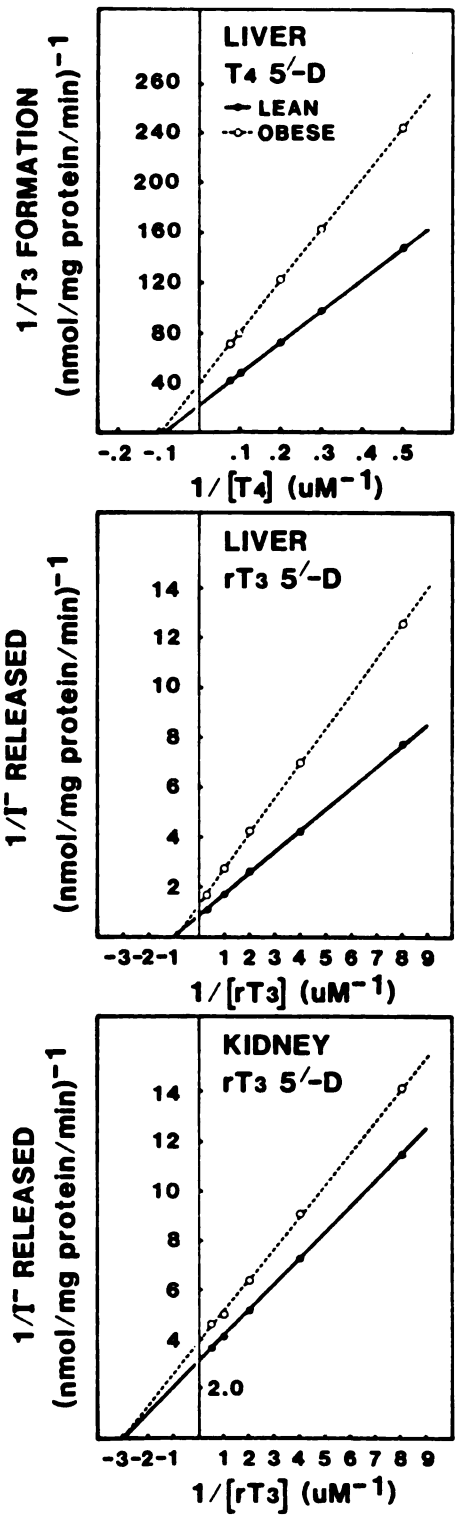

Figure 1. Representative Lineweaver-Burk plots of hepatic and renal iodothyronine 5'-deiodinase in 4 week old lean and obese mice. Each line is derived from data of 5 animals.

Table 1. Effect of age on iodothyronine metabolism in lean and obese mice

Phenotype	Age, wk				
	1	2	4	6	8-10
			-Liver $T_4$5'D Vmax-		
Lean	†	32.9 ± 4.6^{a}	46.6 ± 4.0^{b}	44.7 ± 2.6^{b}	ND
Obese	†	24.0 ± 2.3^{a}	$26.3\pm4.2^{a*}$	$21.1\pm1.5^{a*}$	ND
			-Liver $T_4$5'-D Km-		
Lean	ND	9.8 ± 1.0^{a}	11.7 ± 0.6^{a}	11.6 ± 0.5^{a}	ND
Obese	ND	9.2 ± 0.7^{a}	10.6 ± 1.2^{a}	11.5 ± 0.9^{a}	ND
			-Liver $rT_3$5'-D Vmax-		
Lean	345 ± 20^{a}	974 ± 70^{b}	1173 ± 100^{b}	1004 ± 20^{b}	1032 ± 71^{b}
Obese	$282\pm15^{a*}$	$777\pm51^{b*}$	$803\pm68^{b*}$	$547\pm52^{c*}$	$628\pm36^{c*}$
			-Liver $rT_3$5'-D Km-		
Lean	1.10 ± 0.08^{a}	1.12 ± 0.09^{a}	0.99 ± 0.05^{ab}	0.98 ± 0.10^{ab}	0.85 ± 0.04^{b}
Obese	1.14 ± 0.06^{a}	1.02 ± 0.06^{ab}	1.16 ± 0.06^{a}	1.11 ± 0.03^{a}	0.95 ± 0.04^{b}
			-Liver T_3 Degradation-		
Lean	51.0 ± 8.5^{a}	122.9 ± 8.2^{b}	141.7 ± 15.8^{b}	146.2 ± 10.1^{b}	150.9 ± 14.7^{b}
Obese	51.4 ± 4.1^{a}	104.3 ± 8.8^{b}	$86.2\pm8.2^{b*}$	$68.3\pm3.7^{c*}$	$74.7\pm10.3^{bc*}$
			-Kidney $rT_3$5'-D Vmax-		
Lean	ND	355 ± 44^{a}	327 ± 19^{a}	267 ± 21^{b}	169 ± 10^{c}
Obese	ND	$235\pm20^{ab*}$	$260\pm8^{a*}$	$200\pm13^{bc*}$	174 ± 14^{c}
			-Kidney $rT_3$5'-D Km-		
Lean	ND	0.41 ± 0.05^{ab}	0.35 ± 0.02^{b}	0.51 ± 0.03^{a}	0.49 ± 0.04^{a}
Obese	ND	0.35 ± 0.02^{a}	0.34 ± 0.01^{a}	0.43 ± 0.03^{b}	0.49 ± 0.04^{b}

Values represent the mean±SEM of 5-6 mice. The Vmax and Km of both $T_4$5'-D and $rT_3$5'-D were determined by Lineweaver-Burke analysis and are expressed as pmols/mg protein/min and uM, respectively. T_3 degradation assayed at a single substrate concentration (0.35 uMT_3) is expressed as fmols/mg protein/min. Means on the same line with different superscript lower case letters are different ($p<0.05$) as determined by analysis of variance. *Means are different ($p<0.05$) from those of lean mice of the same age as determined by Student's t-test. †$T_4$5'-D, assayed at a single substrate concentration (2.0 uMT_4), was 1.63±0.07 and 1.13±0.04 pmol/mg protein/min ($p<0.05$) for lean and obese mice respectively. ND, not determined.

the above ages since the fractional conversion of ^{125}I-T_4 to ^{125}I-T_3 at 1 week of age was too low to monitor at substrate concentrations greater than 2.0 uM T_4. However, if $T_4$5'-D activity at 2.0 uM T_4 is considered, 2 week old lean and obese mice had 237% and 222% higher enzyme activity, respectively, than respective 1 week old animals. After 2 weeks of age, maximal $T_4$5'-D activity in lean mice continued to increase until a plateau was reached at 4 weeks of age, while in obese mice maximal activity was already evident at 2 weeks of age.

In general, the developmental profile of hepatic $rT_3$5'-D was similar to that of $T_4$5'-D in lean and obese mice. Hepatic $rT_3$5'-D in lean and obese mice peaked at 2 weeks of age and remained high in the former phenotype for the remainder of the experimental period but declined significantly in the latter phenotype by 6 weeks of age. In all age groups, hepatic 5'-deiodination was significantly lower in obese mice than in respective lean mice. Furthermore, differences in both $T_4$5'-D and $rT_3$5'-D activity between phenotypes were greater in post-weanling mice than in pre-weanling mice.

Developmental alterations in the velocity of renal $rT_3$5'-D followed a general trend of declining maximal activity with increasing age in both lean and obese mice (Table 1). Renal $rT_3$5'-D activity was significantly lower in obese mice at 2, 4 and 6 weeks of age relative to their lean littermates. These differences in renal $rT_3$5'-D activity between lean and obese mice were greatest at 2 weeks of age and became smaller with increasing age. No difference in enzyme activity was observed at 8-10 weeks of age.

K_m values for hepatic and renal 5'-deiodinase were similar between phenotypes in each age group (Table 1). However, in a few instances, significant differences in the K_m of hepatic and renal $rT_3$5'-D were observed in different age groups of a given phenotype. Since the magnitude of the above differences was small, it is probable that they are of little regulatory importance.

Also presented in Table 1 are the effect of age and phenotype on hepatic T_3 degradation. In general, hepatic T_3 degradation in lean and obese mice followed a similar pattern of development as that observed for hepatic 5'-deiodination. In addition, T_3 degradation was lower in obese mice than in lean mice in each age group, but only to a significant extent in post-weanling mice.

Data for microsomal protein content (mg protein/organ) for liver and kidney of each age group are presented in Table 2. In both lean and obese mice, microsomal protein content in liver and kidney progressively rose with increasing age until peaking at 6 weeks of age. Between 6 and 8-10 weeks of age a slight decline in hepatic protein content was observed in both phenotypes while renal protein content remained constant. Hepatic protein levels were similar between lean and obese mice at 1, 2 and 4 weeks of age. However, at 6 and 8-10 weeks of age when gross hypertrophy of the obese liver was readily apparent, microsomal protein content was greater in obese mice than in lean counterparts. No significant differences in renal protein content were observed between phenotypes at any age investigated.

Employing the data in Table 2, the calculation of maximal 5'-deiodinative capacity per organ (V_{max} x microsomal protein/organ)

Table 2. Microsomal protein content of tissues from lean and obese mice

Phenotype	Age, wk				
	1	2	4	6	8-10
			-Liver-		
Lean	2.2±0.1[a]	3.8±0.1[b]	14.2±0.5[c]	25.1±0.5[d]	20.7±0.8[e]
Obese	2.1±0.1[a]	4.2±0.2[b]	13.3±0.9[c]	30.1±1.1[d*]	24.8±1.0[e*]
			-Kidney-		
Lean	ND	0.90±0.06[a]	2.16±0.07[b]	3.33±0.26[c]	3.50±0.19[c]
Obese	ND	0.86±0.04[a]	1.99±0.07[b]	3.01±0.16[c]	3.16±0.19[c]

Values for microsomal protein are expressed as mg protein/organ.

Abbreviations and superscript letters and symbols are defined in Table 1.

could be performed. In general, maximal 5'-deiodination per liver was significantly lower in obese mice than in lean mice at all age groups studied with differences between phenotypes being greater in older animals than in younger animals (results not shown). 5'-deiodinative capacity per kidney was also significantly lower in obese mice at all age groups except 8-10 weeks. The above differences in maximal 5'-deiodinative capacity per liver or kidney between lean and obese mice were mainly the result of phenotypic alterations in 5'-deiodinase specific activity in these respective organs.

In Table 3, plasma T_4 and T_3 concentrations for 4, 6 and 8-10 week old mice in experiment 1 are presented. Relative to lean mice, plasma T_4 concentrations in obese mice were significantly lower at 4 and 6 weeks of age, but were not different in 8-10 wk old mice. Plasma T_3 concentrations were similar in lean and obese mice of each age group.

Effect of Environmental Temperature on Iodothyronine Metabolism. Kinetic analysis of $T_4$5'-D and $rT_3$5'-D activity was performed in mice housed at 14°C, 25°C and 33°C (Figure 2). Exposing lean and obese mice to 14°C for 2 weeks stimulated maximal hepatic $T_4$5'-D activity by 89% and 80%, respectively, relative to mice housed at 25°C. Compared to their lean littermates, hepatic $T_4$5'-D activity in obese mice was similarly lower (55% and 53%) at 14°C and 25°C, respectively. Housing lean mice at 33°C produced a 83% decline in hepatic $T_4$5'-D activity compared to lean mice housed at 25°C. No data for obese mice housed at 33°C are shown since hepatic $T_4$5'-D activity in obese mice at 33°C was too low to accurately measure.

Table 3. Plasma T_4 and T_3 concentrations in lean and obese mice

	Concentration	
	T_4(ug/dl)	T_3(ng/dl)
4 wk age	Experiment 1	
Lean	3.75±0.23[a]	105±8[a]
Obese	2.67±0.18[a*]	85±6[a]
8 wk age		
Lean	4.13±0.20[a]	131±12[a]
Obese	3.57±0.27[b]	127±10[b]
14°C	Experiment 2	
Lean	3.52±0.15[a]	128±13[a]
Obese	1.91±0.19[a*]	108±11[a]
25°C		
Lean	3.90±0.13[a]	103±6[a]
Obese	2.89±0.34[b*]	116±9[a]
33°C		
Lean	3.58±0.18[a]	.95±9[a]
Obese	1.72±0.11[a*]	68±6[b*]
Control	Experiment 3	
Lean	3.22±0.13	120±10[a]
Obese	2.81±0.23	116±9[a]
T_3-treated		
Lean	†	871±34[b]
Obese	†	1410 65[b*]

Plasma T_4 and T_3 concentrations are presented for Experiment 1 (effect of age), Experiment 2 (effect of environmental temperature) and Experiment 3 (effect of T_3 administration). Values are means±SEM of 5-6 animals. Hormone concentrations for 6 week old mice in Experiment 1 are the same as those presented for mice housed at 25°C in Experiment 2. Means with different lower case superscript letters within the same phenotype are different ($p<0.05$) as determined by analysis of variance. *Means are different ($p<0.05$) from those of lean mice of the same treatment group as determined by Student's t-test. †Hormone concentrations were too low to be detected.

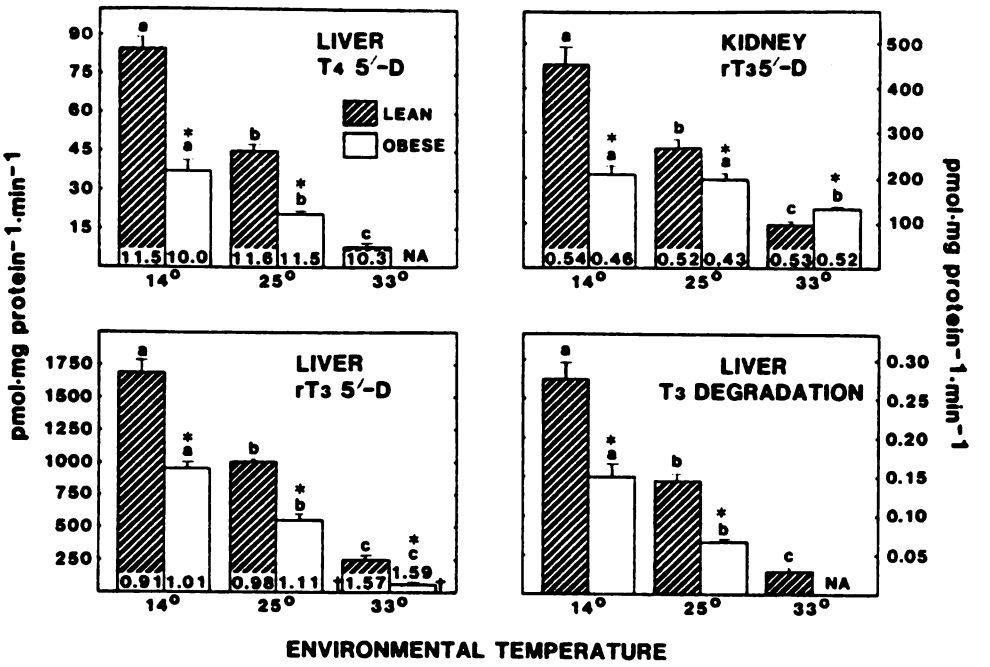

Figure 2. Effect of environmental temperature on iodothyronine 5'-deiodination in lean and obese mice. Vertical bars represent mean values for Vmax (pmol/mg protein/min) whereas the numerals at the base of each bar denote Km values (uM T_4 or rT_3). In the case of T_3 degradation vertical bars represent enzyme activity assayed at a single substrate concentration of 0.35 uM T_3. Bars within phenotype with different letters are different ($P < 0.05$) as determined by analysis of variance. *Means are different ($P < 0.05$) from those of lean mice housed at same temperature as determined by Student's t test. † Km was significantly different ($P < 0.05$) from values observed in mice of same phenotype housed at 14 and 25°C. There were no significant differences in Km between phenotypes at a given environmental temperature. NA, data were not available because enzyme activity was too low to accurately measure.

The response of hepatic $rT_3$5'-D to environmental temperature followed a similar pattern as $T_4$5'-D in both phenotypes housed at 14° and 25°C (Figure 2). Housing lean and obese mice at 33°C produced a marked decrease in hepatic rT_3 5'-deiodination of 76% and 88%, respectively, relative to mice housed at 25°C. Since the magnitude of the above temperature-induced responses was different in lean and obese mice, the percentage difference in hepatic $rT_3$5'-D activity of obese mice housed at 33°C (-71%) relative to their lean littermates was much larger than the differences between lean and obese animals housed at 14°C (-44%) or 25°C (-46%). The effect of environmental temperature on hepatic T_3 degradation followed a similar pattern of regulation as hepatic 5'-deiodination.

Exposure to 14°C stimulated maximal renal $rT_3$5'-D activity in lean mice (69%), but had no significant effect in obese mice when compared to respective animals housed at 25°C (Figure 2). Housing lean and obese mice at 33°C caused a decline in renal $rT_3$5'-D activity of 63% and 39%, respectively, relative to animals housed at 25°C. Due to the above quantitatively dissimilar temperature-induced responses in maximal renal $rT_3$5'-D activity observed in lean and obese mice, the percentage difference in $rT_3$5'-D in obese mice relative to their lean littermates was greater at 14°C (-54%) than at 25°C (-25%). At 33°C, maximal renal $rT_3$5'-D activity was actually higher (+26%) in obese mice than in lean mice.

In experiment 2, no differences in the Km for hepatic and renal 5'-deiodinase were observed between phenotypes housed at a given environmental temperature. However, comparisons within a given

phenotype across different environmental temperatures revealed that the Km of hepatic $rT_3$5'-D was significantly higher in lean and obese mice housed at 33°C than in respective mice housed at 14°C and 25°C.

Plasma T_4 and T_3 concentrations for mice housed at 14°C, 25°C and 33°C are presented in Table 3. In all temperature groups, plasma T_4 concentrations were significantly lower in obese mice than in their lean littermates. Environmental temperature had no effect on plasma T_4 concentrations in lean mice, while in obese mice T_4 concentrations were significantly higher at 25°C relative to 14°C and 33°C. Plasma T_3 concentrations were significantly lower in obese mice housed at 33°C compared to their lean littermates. No significant differences in plasma T_3 concentrations were observed between phenotypes at 14°C and 25°C. In addition, there was no effect of environmental temperature on plasma T_3 levels in lean mice. However, in obese mice plasma T_3 concentrations were significantly lower at 33°C relative to 14°C and 25°C.

Effects of T_3 Administration on Iodothyronine Metabolism. Results from the kinetic analysis of $T_4$5'-D and $rT_3$5'-D in mice injected with either T_3 or vehicle are presented in Figure 3. T_3 administration increased maximal hepatic $T_4$5'-D activity by 327% and 695% in lean and obese mice respectively. In addition, the maximum velocity of hepatic $rT_3$5'-D was stimulated 191% and 424% by T_3 treatment in lean and obese mice, respectively. In control animals, both $T_4$5'-D and $rT_3$5'-D were significantly lower (50% and 44%, respectively) in obese mice than in lean mice. However, no difference in either $T_4$5'-D or $rT_3$5'-D was observed between T_3-treated lean and obese mice, due to the greater

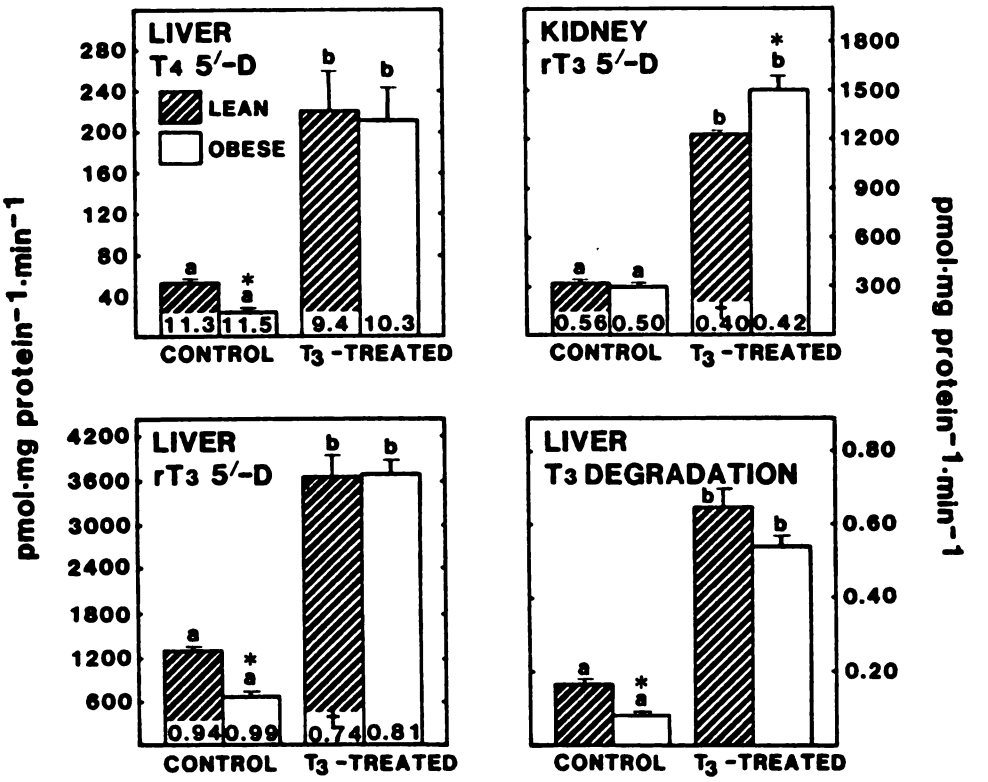

Figure 3. Effect of thyroid hormone administration on iodothyronine 5'-deiodination in lean and obese mice. Vertical bars represent mean values for Vmax (pmol/mg protein/min), whereas numerals at base of each bar denote Km values (uM T_4 or rT_3). In the case of T_3 degradation vertical bars represent enzyme activity at a single substrate concentration of 0.35 uM T_3. Bars within phenotype with different letters are different ($P < 0.05$) as determined by analysis of variance. *Means are different ($P < 0.05$) from those of lean mice of same treatment group as determined by Student's t test. † Km was significantly different ($P < 0.05$) from values observed in control mice of same phenotype. There were no significant differences in Km between phenotypes of a given treatment group.

T_3-induced response in enzyme activity in the latter phenotype. T_3 administration also produced a greater response in renal $rT_3$5'-D of obese mice (+418%) than in lean mice (+285%). Lean and obese controls had similar activities of $rT_3$5'-D, while T_3-treated obese mice had significantly higher renal $rT_3$5'-D activity than T_3-treated lean mice. No differences in the Km for hepatic and renal 5'-deiodinase were observed between phenotypes of a given treatment group. However, Km values for hepatic $rT_3$5'-D and renal $rT_3$5'-D were, in some instances, significantly lower in T_3-treated mice than in controls.

T_3 degradation appeared to be regulated by T_3 administration in a similar manner as that observed for hepatic 5'-deiodination (Figure 3). Compared to control animals, T_3 administration stimulated T_3 degradation in lean and obese mice by 296% and 544%, respectively. As observed in the previous experiments, hepatic T_3 degradation was significantly lower in obese controls than in lean controls. However, no difference in T_3 degradation was observed between T_3-treated lean and obese mice.

Effect of T_3 administration on plasma T_4 and T_3 concentrations are presented in Table 3. No significant difference in either plasma T_4 or T_3 concentrations was observed between lean and obese controls. Compared to control mice, T_3 levels 4 h after the last T_3 injection were 7-fold and 12-fold higher in lean and obese mice, respectively. T_4 levels in T_3-treated mice were too low to be detected.

Discussion

In the present study, hepatic and renal 5'-deiodinase activity was assayed in a washed, microsomal fraction under optimal conditions in a

range of protein concentrations that were linearly related to reaction velocity. Using this assay system, kinetic analysis of hepatic and renal 5'-deiodinase revealed that the substantial variations in V_{max} arising from different physiological states or phenotypes in the study were not accompanied by alterations in Km, except in a few cases where small differences were noted. Hence, it may be assumed that the above alterations in V_{max} were probably the results of changes in enzyme amount.

Both $T_4$5'-D and $rT_3$5'-D in livers of lean and obese (ob/ob) mice responded similarly to the various physiological manipulations examined in the current study. This finding is consistent with the hypothesis that a single enzyme is responsible for 5'-monodeiodination of both T_4 and rT_3 in liver (27). The above hypothesis has also been proposed for kidney (35); therefore, changes in renal $rT_3$5'-deiodinase which was assayed in this study may reflect changes in renal $T_4$5'-deiodinase as well.

Based on the results of experiment 1, slight differences in the developmental profile of hepatic and renal 5'-deiodinase were observed between lean and obese mice (Table 1). These differences are illustrated by the fact that the percentage reduction in hepatic or renal 5'-D in obese mice relative to their lean counterparts varied as a function of age; for example, differences in hepatic 5'-deiodinase between lean and obese mice were greater in the post-weaning age groups than in the pre-weaning age groups. An opposite trend was observed for renal 5'-deiodinase where the greatest reduction in enzyme activity in obese mice relative to lean littermates occurred in younger animals rather than older animals. Hence, the developmental

pattern of 5'-deiodinase appeared to be a function of both phenotype and tissue type.

The reduction in hepatic 5'-deiodinase in ob/ob mice relative to their lean counterparts in experiment 1 was not a secondary result of overt obesity since significant differences between phenotypes were observed as early as 1 week of age. There are no data confirming the above findings although it has been reported that 5'-deiodination in 10% liver homogenates is similar in 5 mo old lean and obese mice (16); these data conflict with our results derived from microsomal preparations. The above discrepancy may be attributed to possible experimental error associated with measuring 5'-deiodinase specific activity in crude homogenates where a significant amount of non-specific substrate binding occurs. For example, we have been unable to demonstrate any relationship between homogenate protein concentrations of the magnitude seen in 5-20% liver homogenates and 5'-deiodinase activity (unpublished observations). Dilution of a 20% liver homogenate by 1/2 or 1/4 resulted in no apparent change in enzyme activity. Similar studies by others (33) employing 18% kidney homogenates revealed that $T_4$5'-D activity actually increased with homogenate dilution. It was concluded that homogenate dilution greatly increased free substrate (T_4) concentrations which apparently more than offset the decrease in enzyme protein in the system. Without a linear relationship existing between homogenate protein concentration and enzyme velocity, the assay of 5'-deiodinase specific activity (product formed/min/mg protein) is not valid because the above measurement will vary depending on the protein content of the

enzyme preparation. This could lead to spurious results when making physiological comparisons in 5'-deiodinase specific activity since different treatment groups may yield homogenates of different protein concentration as is the case in lean and obese mice.

Accompanying reduced $T_4$5'-D activity in obese mice relative to respective lean mice are lowered rates of T_3 degradation in each age group of experiment 1. Since T_3 is a product of $T_4$5'-D, the difference in activity of $T_4$5'-D in lean and obese mice should be theoretically underestimated. However, the rate of T_3 degradation in hepatic microsomes is much lower than the enzymatic production of T_3 so that the amount of error is diminished.

Results from experiment 2 revealed that the response of 5'-deiodinase to environmental temperature was a function of both phenotype and tissue type (Figure 2). Regarding the former determinant, it was shown that in a given tissue enzyme response to environmental temperature was quantitatively different in lean and obese mice. The latter determinant was indicated by the fact that as environmental temperature shifted from one extreme to another, changes in the relative levels of 5'-deiodinase in lean and obese mice followed opposite trends in liver and kidney. The latter finding and the earlier observations showing that developmental changes in 5'-deiodinase were dependent on tissue type suggest that different isoenzymes for 5'-deiodinase may exist in liver and kidney. To confirm the above hypothesis, additional studies are needed which directly compare the kinetic properties of the renal and hepatic enzyme in a purified system.

In experiment 3, T_3 administration was shown to produce a greater maximal induction in both hepatic and renal 5'-deiodinase in obese mice than in lean mice (Figure 3). Although T_3 administration resulted in higher plasma T_3 levels in obese mice than in lean mice, it is unlikely that this is responsible for the greater enzyme induction in the former phenotype since the T_3 dosage used probably produced a maximal enzyme response. The latter supposition is supported by studies showing that the thyroid hormone dosage required for maximal 5'-deiodinase induction in lean rats (5 ug T_4/100 g body weight/day for 5 days) (28) is lower than the hormone dosage employed in the present study (10 ug T_3/100 g body weight/day for 7 days). Furthermore, we injected T_3 which is a more potent inducer of 5'-deiodinase than T_4 (18). The differential response of hepatic 5'-deiodinase to T_3 administration between lean and obese mice was qualitatively similar to that reported for other hepatic, T_3-regulated enzymes such as mitochondrial glycerol-3-phosphate dehydrogenase (26,47) and Na^+,K^+-ATPase (36). In addition, the basal activity of all 3 enzymes is lower in untreated obese mice housed at 25°C than in their lean counterparts (26,36,47). The lower basal enzyme activity in obese mice does not appear to be the result of lowered circulating T_3 concentrations since the preponderance of the data from this study (Table 3) and other reports (40,76,78) show that blood T_3 concentrations are similar in lean and obese mice. An alternative mechanism for the lowered basal enzyme activities in obese mice may be reduced tissue sensitivity to T_3. Indeed, nuclear T_3 receptor concentration in liver has been reported to be lower in obese mice than in lean mice (19). However, the above mechanism does

not account for the greater enzyme response to T_3 administration in obese mice. This discrepancy may be explained based on the proposal that thyroid hormone action can be modulated by other hormonal and metabolic factors at a level distal to the nuclear receptor (43). It has also been postulated that T_3 by itself may generate unidentified regulatory factors which amplify the nuclear T_3 signal at the post-receptor level (38). One may therefore speculate that T_3 administration results in a greater production of such intracellular activation factors in obese mice than in lean mice. Thus, alterations in the post-receptor modulation of thyroid hormone action between lean and obese mice may explain the augmented enzyme response to exogenous T_3 in the latter phenotype in the face of lowered nuclear T_3 receptor concentration.

In an attempt to extrapolate the in vitro results of the present study to the in vivo situation, maximal 5'-deiodinase activity determined in vitro and expressed on a per organ basis (V_{max} x microsomal protein/organ) may be compared among lean and obese mice to estimate phenotypic differences in whole animal T_4 to T_3 conversion. The above exercise assumes that all other factors regulating enzyme activity besides enzyme amount are equal in lean and obese mice and that liver and kidney are responsible for a large fraction of the T_3 production in vivo. In general, maximal 5'-deiodinative capacity per liver or kidney was found to be lower in obese mice than in respective lean mice in most of the age groups examined in experiment 1. Based on these results, one would expect serum T_3 concentrations to be lower in obese mice than in lean mice, but as discussed earlier circulating

T_3 concentrations appear to be similar in both phenotypes. Possibly T_3 metabolism in vivo is lowered to a similar extent as T_4 to T_3 conversion in obese mice. Another possibility is that other regulatory factors, such as cytosolic cofactor concentrations are operating in vivo to maintain normal 5'-deiodination in obese mice in spite of lowered enzyme capacity. In vivo kinetic studies with thyroid hormones would be useful in resolving this issue.

In conclusion, the present study demonstrated that hepatic and renal 5'-deiodinase activity is adaptive to changes in age, environmental temperature and thyroid hormone status in both lean and obese mice. The magnitude of enzyme response to the above physiological alterations, however, was shown to vary between phenotypes. Under basal conditions at an environmental temperature of 25^{o}C maximal 5'-deiodination per mg microsomal protein or per organ was lower in obese mice than in lean mice. Furthermore, significant differences between phenotypes were observed as early as 1 week of age before external signs of obesity were apparent. These findings suggest that altered 5'-deiodination may play a role in the expression of reduced energy expenditure in obese mice which is also observed as early as 1 week of age (6). Conceivably, the reduced conversion of T_4 to T_3 in obese mice would result in lowered T_3 availability and consequently, impaired thyroid hormone action in tissues mediating adaptive thermogenesis. In conflict with the above hypothesis is the observation that plasma T_3 concentrations are similar in lean and obese mice. However, in the case of liver, intracellular T_3 concentrations in obese mice may be reduced since local T_4 monodeiodination in euthyroid rats accounts for approximately 28% of

this pool (58). Also, plasma T_3 concentrations may not accurately reflect intracellular nuclear T_3 receptor occupancy if there are alterations in T_3 transport across the cell membrane or changes in nuclear T_3 receptor concentrations (19). The above situation suggests that coordinate alterations at several metabolic control points may collectively be responsible for impaired thyroid hormone action in obese mice. Further studies to examine T_3 transport across the cell membrane and nuclear T_3 receptor occupancy are needed to explore this possibility.

IODOTHYRONINE 5'-DEIODINATION IN RATS FED LOW PROTEIN DIETS: LACK OF CORRELATION WITH ENERGY BALANCE

Introduction

Rats fed low-protein diets exhibit increased heat production and reduced efficiency of energy retention (30,62,67). Recently, efforts have been made to identify the mechanisms responsible for adaptive thermogenesis induced by feeding low protein diets. It has been proposed that increased heat production observed in rats consuming a low protein diet results from sympathetic activation of brown adipose tissue (50). This is supported by data showing that norepinephrine turnover in brown adipose tissue is accelerated in rats fed a low protein diet (30). There is also evidence that thyroid hormones may play a role in mediating the thermogenic response to low protein diets. Consumption of a low protein diet is associated with a marked rise in serum 3,3',5-triiodothyronine (T_3) concentration (50,67) as well as an elevation in hepatic mitochondrial α-glycerol phosphate dehydrogenase activity (50,68). In addition, several biochemical processes which have been proposed as mechanisms of adaptive heat production are sensitive to thyroid hormone status. They include Na^+,K^+-ATPase activity (36), protein turnover (15,48), futile cycling in carbohydrate metabolism (23) and proton conductance pathway activity in brown adipose tissue (66).

Extrathyroidal 5'-monodeiodination of thyroxine (T_4) to the metabolically active form T_3 has been identified as one of several control points regulating thyroid hormone action (32). This enzymatic reaction is most active in liver and kidney and accounts for approximately 80% of the circulating T_3 in the body. Enhanced thyroid hormone action in rats fed a low protein diet may be mediated by a

stimulation in iodothyronine 5'-deiodination since the latter process would augment the supply of T_3 to thermogenic target tissues.

In the present study, we examined the kinetic parameters of iodothyronine 5'-deiodinase assayed in hepatic and renal microsomes of young rats fed low protein (5 or 8% casein) or normal protein (22% casein) diets. In addition, the above enzyme data were related to energy balance measurements performed in the respective dietary treatment groups.

Materials and Methods

Materials. ^{125}I-labeled (3' or 5') L-3,3',5'-triiodothyronine (rT_3) (600-800 uCi/ug) was purchased from Abbott Laboratories (N. Chicago, IL). Unlabeled L-rT_3 was obtained from Calbiochem (LaJolla, CA). Dithiothreitol (DTT) was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Animals.

Experiment 1. Twenty-four 3 week old male Sprague-Dawley rats, obtained from Harlan Industries, Inc. (Indianapolis, IN), were individually housed in wire-bottomed cages in a room maintained at 23-25^{o}C with a 12-hour light-dark cycle. After an initial adaptation period of 5 days in which rats were provided a stock diet ad libitum (Wayne Rodent Blox from Continental Grain Co., Chicago, IL), animals were segregated into 3 groups of equal number and similar average body weight. One group of animals was killed immediately for determination of initial body energy content. The remaining two groups were fed ad libitum either an 8% casein (low protein, LP-8) or 22% casein (normal protein, NP) semi-purified diet for 17-20 days. Composition of the

diets is given in Table 4. Animals were provided water ad libitum. Food intake and body weights were monitored every 3 days during dietary treatment. At the end of the feeding trial, rats were decapitated and the liver and kidneys were rapidly removed for subsequent enzyme assay. Carcasses were saved for determination of final body energy content.

Experiment 2. The experimental design and conditions of this experiment were similar to that described for experiment 1 except that 4 groups of 8 rats were employed. One group was again killed at the start of the experiment for determination of initial body energy. The remaining 3 groups were fed ad libitum either a 5% (LP-5) or 6% (LP-6) or 22% (NP) casein diet for 17-20 days.

Measurement of Iodothyronine 5'-deiodination. Iodothyronine 5'-deiodinase activity was determined in hepatic and renal microsomes using the tracer technique of Leonard and Rosenberg (35) where generation of radiolabeled product, $^{125}I^-$ from ^{125}I labeled (3' or 5') substrate, L-rT_3, was monitored. $rT_3$5'-deiodinase was assayed rather than $T_4$5'-deiodinase since iodide release by the former reaction is easier to quantitate (ion exchange chromatography) than is T_3 production by the latter reaction (paper chromatography). Differences in $rT_3$5'-deiodination observed between treatment groups should reflect differences in $T_4$5'-deiodination as well since it has been proposed that a single enzyme is responsible for 5'-monodeiodination of both T_4 and rT_3 in liver (27) and kidney (35). Previous studies have shown that both enzymes respond similarly to various physiological manipulations (20,27). Details of the assay procedure have been previously

Table 4. Composition of Diets

	Low Protein			Normal Protein
Casein	5	6	8	22
Glucose	76	75	73	59
Basal mix	19	19	19	19
	100	100	100	100

Basal mix contained in g/100g; corn oil, 10; cellulose, 3.6; mineral mix, 4 (4); vitamin mix, 1 (4); choline chloride, 0.2; and methionine, 0.2.

reported (27). Briefly, hepatic and renal microsomes were prepared by differential centrifugation in 0.25 M sucrose, 0.05 M potassium phosphate, 0.01 M EDTA, (pH 7.4). Microsomal suspensions (5-7 mg protein/ml) were frozen in acetone and dry ice and stored at -80^{o}C after adding 2 mM DTT. Microsomal protein concentration was determined according to the method of Markwell et al. (39). Prior to enzyme assay, aliquots of hepatic and renal microsomes were thawed and then diluted with incubation buffer consisting of 0.05 M potassium phosphate, 0.01 M EDTA, 5 mM DTT, pH 8.0 to yield final protein concentrations of 6-8 ug/ml and 12-14 ug/ml, respectively. The above protein concentrations were within the range where $rT_3$5'-deiodinase activity was linearly related to microsomal protein concentration. Diluted microsomes were incubated with ^{125}I-rT_3 (1.0 uCi/tube) and unlabeled rT_3 to provide final rT_3 concentrations ranging from 0.125 uM to 2.0 uM. Enzyme reactions were carried out for 10 min at 37^{o}C under N_2 gassing. Reaction times and microsomal protein concentrations for the $rT_3$5'-deiodinase assay were adjusted so that less than 16% of the substrate was consumed during the incubation. Enzyme reactions were terminated by plunging the assay tubes in cracked ice and adding 0.1 ml 2% bovine serum albumin followed by 0.5 ml 10% trichloroacetic acid.

To determine the fractional generation of iodide from rT_3, enzyme reactants and products were separated using the ion exchange method of Leonard and Rosenberg (35). In this procedure, trichloroacetic acid extracts of the reaction mixtures were applied to Dowex 50W-X8 columns (BioRad Laboratories, Richmond, CA) equilibrated with 1.74 M acetic acid. Free iodide was eluted from the column with three column

volumes of 1.74 M acetic acid divided into two aliquots and counted in a gamma counter. Values for the fractional generation of iodide from rT_3 obtained from tissue-free control incubations were subtracted from the observed experimental values of tissue-containing incubations to correct for any non-enzymatic reactions and for ^{125}I-labeled contaminants.

Treatment of Enzyme Data. Kinetic analysis was performed on data derived from the $rT_3$5'-deiodinase assay. Enzyme activity (pmol product formed/mg microsomal protein/min) was determined at each substrate concentration and double reciprocal plots were constructed. Slopes and intercepts of the linear plots were determined using unweighted least squares analysis. In calculating 5'-deiodinase activity, it was assumed that ^{125}I was randomly distributed between the 3' and 5' positions of ^{125}I-rT_3. Hence the observed fractional generation of iodide from rT_3 was doubled to obtain the actual values.

Determination of Efficiency of Energy Retention. Efficiency of energy retention (%) was calculated as body energy gain (kilocalories) divided by metabolizable energy intake (kilocalories) times 100. Body energy gain during the dietary treatment period was determined as the final energy content of each carcass minus that estimated at the beginning of the experiment. Initial body energy was estimated using a linear regression equation developed by relating body energy to body weight of rats killed at the start of the experiment. Body energy content was determined by homogenizing carcasses 1:1 (w/v) in water and drying aliquots at 55°C. A sample of the dried aliquot was then combusted in a bomb calorimeter (Parr Instrument Co., Moline, IL).

The metabolizable energy content of the diets was calculated based on the assumption that the metabolizable energy values of casein, corn oil and glucose were 4, 9 and 3.64 kcal/g, respectively. Kevonian et al. (30) demonstrated in feeding trials that the low and normal protein diets of this study have equivalent metabolizable energy content.

Serum Total T_4, Total T_3 and Free T_3 Determinations. Blood samples obtained at the time of killing were analyzed for serum total T_4, total T_3 and free T_3 by radioimmunoassay. Serum total T_4 and free T_3 were measured by using a solid phase system supplied by Becton Dickinson Immunodiagnostics (Orangeburg, NY). Serum total T_3 was measured based on the method of Nejad et al. (41) which uses charcoal-dextran to separate bound from free hormone.

Statistical Analysis. All values are presented as means ± SEM. The data were subjected to analysis of variance and statistical comparisons were made using the Bonferroni t test (17), or Student's t test as noted in Table footnotes.

Results

Experiment 1. Effects of feeding an 8% casein (low protein) diet on the kinetic parameters of hepatic and renal $rT_3$5'-deiodinase are presented in Table 5. The Lineweaver-Burk plots from which the Vmax and Km of 5'-deiodinase were derived are shown in Figure 4. Consumption of an 8% casein diet had no effect on maximal activity of hepatic 5'-deiodinase expressed per mg microsomal protein, but stimulated Vmax of the renal enzyme by 40%. In both tissues, the Km of the reaction was not affected by dietary treatment.

Table 5. Effects of low protein diets on kinetic parameters of iodothyronine 5'-deiodinase

	Liver		Kidney	
	Km- uM rT_3	Vmax- pmol/min/ mg protein	Km- uM rT_3	Vmax- pmol/min/ mg protein
		Experiment 1		
8% Casein	0.57 ±0.03	2097 ±154	0.70 ±0.03	1016* ±55
22% Casein	0.55 ±0.03	1850 ±100	0.68 0.04	726 ±51
		Experiment 2		
5% Casein	0.68 ±0.02	2125 ±160	0.77 ±0.05	1036* ±46
22% Casein	0.64 ±0.03	1952 ±75	0.73 ±0.02	860 ±50

Values represent the means ± SEM of 8 animals. Kinetic parameters for hepatic and renal iodothyronine 5'-deiodinase were derived from Lineweaver-Burk plots. *Means are different ($p<0.05$) from those of the corresponding 22% casein-fed group within an experiment as determined by Student's t test.

Figure 4. Lineweaver-Burk analysis of iodothyronine 5'-deiodinase in rats fed low protein diets. Each point represents the mean reciprocal enzyme velocity of 8 animals at a given substrate concentration. Lines were calculated by linear least squares regression.

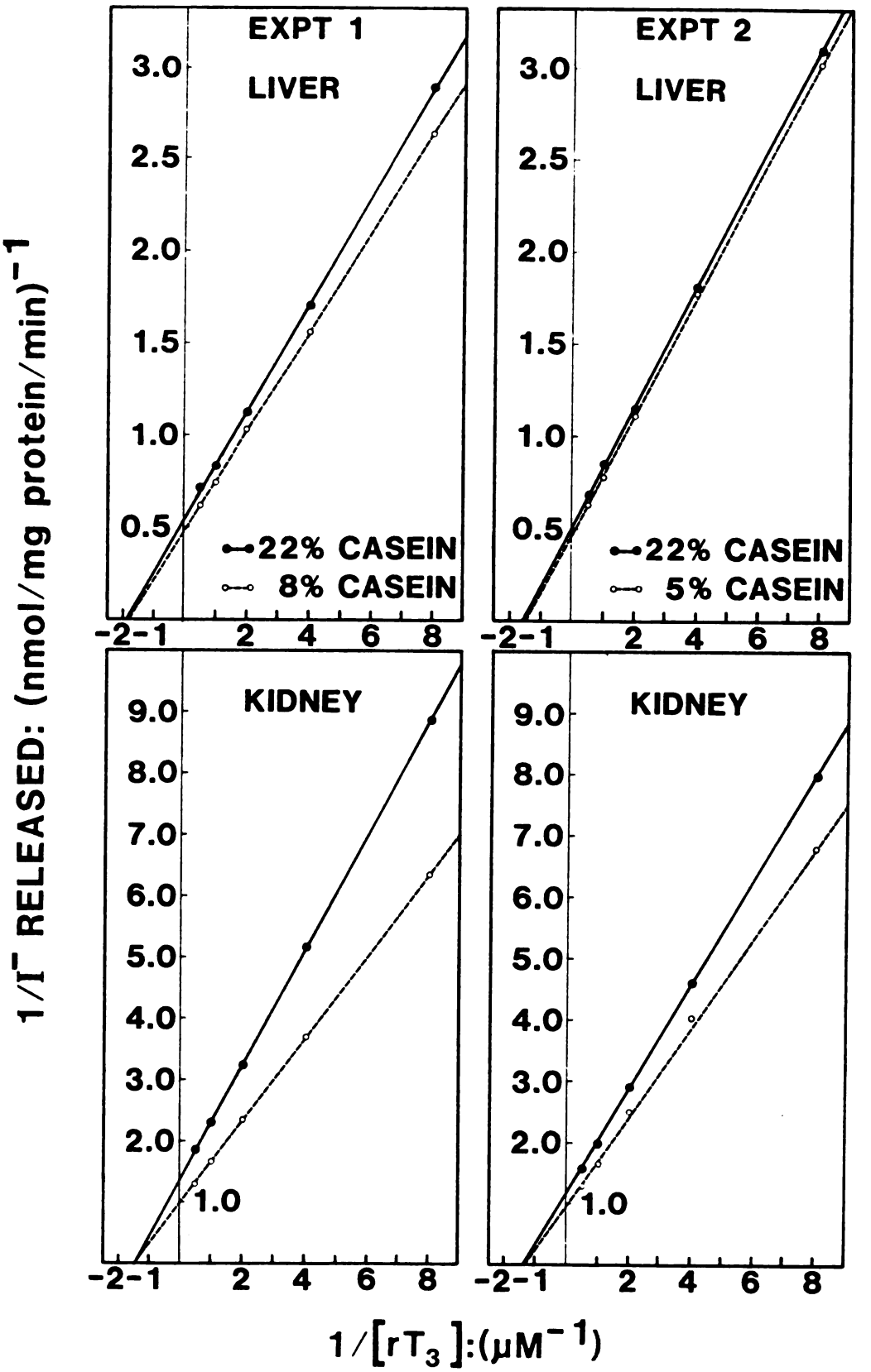
EXPT 1
LIVER
22% CASEIN
8% CASEIN
EXPT 2
LIVER
22% CASEIN
5% CASEIN
KIDNEY
1.0
KIDNEY
1.0
1/I⁻ RELEASED: (nmol/mg protein/min)⁻¹
1/[rT3]:(μM⁻¹)

Figure 4.

Hepatic and renal tissue weight, microsomal protein content and maximal iodothyronine 5'-deiodinase activity expressed on either a unit tissue or body weight basis are presented in Table 6. Liver weight per 100 g body weight was similar in LP-8 and NP animals while kidney weight was reduced 18% in the former group. Hepatic microsomal protein content expressed per g tissue and per 100 g body weight was 30 and 36% lower in LP-8 rats, respectively, relative to NP controls. Renal microsomal protein content was reduced 21% when expressed per 100 g body weight, but was unchanged when expressed per g tissue. In calculating maximal 5'-deiodinase activity on either a g tissue or 100 g body weight basis, Vmax (Table 5) was multiplied by the appropriate expression of microsomal protein content. Maximal hepatic 5'-deiodinase activity per g liver and per 100 g body weight was 22 and 27% lower in LP-8 rats than in NP animals. Maximal renal 5'-deiodinase activity per g kidney was slightly elevated (38%) in LP-8 rats while enzyme activity per 100 g body weight was unchanged. The sum of hepatic and renal maximal 5'-deiodination per 100 g body weight was reduced by 26% in LP-8 rats.

Energy balance measurements for experiment 1 are summarized in Table 7. Metabolizable energy intake expressed either on an absolute basis or per unit body weight basis was higher, 9 and 27% respectively, in LP-8 rats than in NP animals. Body energy gain in the LP-8 group was also increased (18%); hence, the calculated efficiency of energy retention was similar in both the LP-8 and NP groups.

Experiment 2. Because consumption of an 8% casein diet failed to stimulate adaptive thermogenesis in experiment 1, one could not

Table 6. Effects of low protein diets on tissue weight, microsomal protein content and maximal iodothyronine 5'-deiodinase activity expressed on a unit tissue weight and body weight basis

	Liver					Kidney					Combined Hepatic & Renal Maximal 5'-Deiodinase Activity
	Tissue Weight	Microsomal Protein		Maximal Hepatic 5'-Deiodinase Activity		Tissue Weight	Microsomal Protein		Maximal Renal 5'-Deiodinase Activity		
	g tissue/ 100 g body weight	mg protein/ g tissue	mg protein/ 100 g body weight	nmol/min/ g tissue	nmol/min/ 100 g body weight	g tissue/ 100 g body weight	mg protein/ g tissue	mg protein/ 100 g body weight	nmol/min/ g tissue	nmol/min/ 100 g body weight	nmol/min/ 100 g body weight
					Experiment 1						
8% Casein	4.0 ±0.2	11.2* ±0.5	44.9* ±1.8	23.3* ±1.8	95* ±9	0.67* ±0.02	10.9 ±0.3	7.2* ±0.2	11.1* ±0.6	7.4 ±0.5	102* ±9
22% Casein	4.4 ±0.2	16.1 ±0.4	70.2 ±2.6	29.9 ±1.6	131 ±10	0.82 ±0.03	10.9 ±0.3	9.1 ±0.3	8.0 ±0.5	6.7 ±0.6	137 ±10
					Experiment 2						
5% Casein	4.3 ±0.3	10.1* ±0.4	43.6* ±2.6	21.5* ±1.7	91* ±6	0.80 ±0.03	11.2 ±0.3	9.1 ±0.3	11.7 ±0.5	9.4 ±0.4	101* ±6
22% Casein	4.4 ±0.2	16.6 ±0.5	72.8 ±3.1	32.3 ±1.2	143 ±9	0.83 ±0.03	11.8 ±0.3	9.7 ±0.3	10.0 ±0.6	8.4 ±0.7	151 ±10

Values represent the means ± SEM of 8 animals. Maximal hepatic and renal 5'-deiodinase activity per g tissue and per 100 g body weight was calculated as the product of the Vmax of the reaction (table 2) multiplied by microsomal protein content (mg protein/g tissue and mg protein/100 g body weight, respectively). *Means are different ($p<0.05$) from those of the corresponding 22% casein-fed group within an experiment as determined by Student's t test.

Table 7. Energy intake and retention in rats fed low protein diets

Measurement	Percentage casein in the diet				
	Exp 1		Exp 2		
	8%	22%	5%	6%	22%
Initial body wt - g	59 ± 2[a]	58 ± 2[a]	65 ± 1[a]	64 ± 1[a]	65 ± 1[a]
Final body wt - g	140 ± 2[a]	174 ± 3[b]	87 ± 1[a]	98 ± 1[b]	173 ± 3[c]
ME intake - kcal/expt	1076 ±21[a]	986 ±20[b]	723 ±18[a]	815 ± 8[b]	990 ±13[c]
ME intake - kcal/100g body wt/day	57 ± 1[a]	45 ± 1[b]	49 ± 1[a]	51 ± 1[a]	43 ±0.2[b]
Body energy gain - kcal/expt	254 ±12[a]	215 ± 8[b]	94 ± 6[a]	132 ± 7[b]	214 ± 6[c]
Efficiency of energy retention - %	23.7 ±1.2[a]	21.8±0.8[a]	13.1±0.8[a]	16.5±0.8[b]	21.7±0.6[c]

Values are the means ± SEM for 8 rats fed for 17-20 days. ME (metabolizable energy) intake and body energy gain were determined as described in the Materials and Methods. Efficiency of energy retention was calculated as body energy gain (kilocalories) divided by ME intake (kilocalories) times 100. Means with different lower case superscript letters within each experiment were different ($p<0.05$) as determined by either Student's t test (expt 1) or analysis of variance followed by the Bonferroni t test (expt 2).

determine whether iodothyronine 5'-deiodination is altered during conditions when the former process is enhanced. Therefore, protein content of the diet was further lowered to either 5% or 6% casein in an effort to activate adaptive heat production. This modification succeeded in stimulating adaptive thermogenesis since efficiency of energy retention of LP-5 and LP-6 rats was reduced by 40% and 24%, respectively, relative to NP animals (Table 7). Although absolute energy intake was lower (27% and 18%, respectively) in the LP-5 and LP-6 groups than in the NP group, energy intake expressed on a per unit body weight basis was higher in LP-5 and LP-6 animals. Body energy gain was markedly reduced in LP-5 and LP-6 rats by 56% and 38%, respectively.

Iodothyronine 5'-deiodination was examined in rats fed LP-5 because this group exhibited a greater stimulation in adaptive heat production than did LP-6 animals. In general, the alterations in enzyme data observed in the LP-5 group relative to NP animals were very similar to those described for the LP-8 group in experiment 1 (Table 5, Table 6, Figure 4). Consumption of a 5% casein diet increased maximal activity of renal 5'-deiodinase by 21%, but had no effect on Vmax of the hepatic enzyme (Table 5). In both tissues, the Km of the reaction was unaffected by dietary treatment. Hepatic microsomal protein content and maximal hepatic 5'-deiodinase activity were reduced 39 and 33% when expressed per g liver and 40 and 36% when expressed per 100 g body weight, respectively, in LP-5 rats compared to NP animals. The above measurements for kidney were similar in both dietary groups. Combined hepatic and renal maximal 5'-deiodination per 100 g body

Table 8. Effects of low-protein diets on serum thyroid hormones

	Total T_4- ng/ml	Total T_3- ng/ml	Free T_3- pg/ml
		Experiment 1	
8% Casein	46.3±1.7[a]	2.34±0.12[a]	6.70±0.28[a]
22% Casein	49.0±1.7[a]	1.56±0.04[b]	5.95±0.23[a]
		Experiment 2	
5% Casein	44.8±1.5[a]	2.41±0.10[a]	5.72±0.29[a]
6% Casein	49.1±2.6[a]	2.15±0.09[a]	6.31±0.31[a]
22% Casein	46.5±2.0[a]	1.42±0.06[b]	5.87±0.28[a]

Values are the means ± SEM for 8 rats. Serum was obtained from animals at the end of each experiment and analyzed for thyroid hormones as described in the Materials and Methods. Means with different lower case superscript letters within each experiment were different ($p<0.05$) as determined by either Student's t test (expt 1) or analysis of variance followed by the Bonferroni t test (expt 2).

weight was reduced by 33% in LP-5 animals. Liver and kidney weights per 100 g body weight were unchanged in LP-5 rats.

Serum Thyroid Hormones. Effects of dietary treatments in experiments 1 and 2 on serum thyroid hormone concentrations are presented in Table 8. Total serum T_3 was markedly increased in LP-5, LP-6 and LP-8 rats by 70%, 51% and 50%, respectively, relative to corresponding NP animals. However, serum free T_3 concentrations were unaffected by the dietary treatments. Total serum T_4 concentrations were also not altered by dietary manipulation.

Discussion

We previously reported (20) that iodothyronine 5'-deiodination is reduced in obese (ob/ob) mice, a genetic model where adaptive thermogenesis is impaired. In the present study, rats were fed low protein diets in an effort to examine iodothyronine metabolism during conditions of enhanced adaptive heat production. However, in experiment 1, we were unable to accomplish this objective because rats fed an 8% casein diet failed to exhibit an elevation in energy expenditure (Table 7). Subsequently, in experiment 2, the protein content of the diet was further reduced to either 5% or 6% casein which finally succeeded in stimulating adaptive thermogenesis. Energy intake (per 100 g body weight) was also elevated in the latter dietary groups. It is possible that the reduced efficiency of energy retention in rats fed the 5% or 6% casein diets was a secondary response to an elevation in energy intake. But, this is unlikely because rats fed 8% casein (experiment 1) exhibited an increase in energy intake as well, but without a concommitant change in heat production.

Kinetic analysis of microsomal $rT_3$5'-deiodinase revealed that consumption of a low protein (5% or 8% casein) diet had no effect on the Vmax (expressed per mg microsomal protein) of the hepatic enzyme, but increased the maximal activity of the renal enzyme (Table 5). Alterations in Vmax, if any, probably represented changes in enzyme amount since the Km of hepatic and renal 5'-deiodinase was not affected by dietary treatment, and the enzymes were assayed under optimal conditions. The above findings partially conflict with the data of Smallridge et al. (59) where consumption of a low-protein diet (9% casein) had no effect on 5'-deiodination in either liver or kidney homogenates. The discrepancy regarding effects of low protein feeding on renal 5'-deiodination may be attributed to the different assay systems used to measure this enzyme. We have discussed earlier (20) the problem caused by substrate binding to non-enzyme protein in measuring 5'-deiodinase in homogenate systems which may lead to spurious results. In the present study, this problem was circumvented by assaying 5'-deiodination in an isolated subcellular fraction under conditions where enzyme activity was linearly related to protein concentration.

In an attempt to extrapolate the in vitro results of the current study to the in vivo situation, maximal 5'-deiodinase activity determined in vitro was expressed per 100 g body weight for each tissue (Vmax x mg microsomal protein/100 g body weight) (Table 6) to account for differences in microsomal protein content and body weight among dietary treatment groups. When making comparisons of maximal 5'-deiodinase activity/100 g body weight, it is assumed that all other

factors regulating enzyme activity besides enzyme amount are constant among treatment groups. The possibility that availability of either substrate or cofactor may have differentially influenced enzyme activity in rats fed low and normal protein diets cannot be ruled out, but there is currently no evidence indicating that this occurs in rats fed low protein diets or in any other dietary manipulation.

Maximal hepatic 5'-deiodinase activity/100 g body weight was lower in rats fed a 5% casein (low protein) diet than in animals fed a 22% casein (normal protein) diet; while in kidney, maximal enzyme activity/100 g body weight was similar in both treatment groups. Combined hepatic and renal maximal 5'-deiodination/100 g body weight was lower in 5% casein-fed rats than in 22% casein-fed controls because the contribution of the hepatic enzyme to total 5'-deiodinase activity/100 g body weight was much greater than the contribution of the renal enzyme. Since increased energy expenditure caused by feeding a 5% casein diet is associated with a reduction in iodothyronine 5'-deiodination/100 g body weight, an inverse correlation between the above parameters may be indicated. However, a causal relationship is unlikely because similar alterations in maximal hepatic and renal 5'-deiodinase activity/100 g body weight occurred in both the 5% casein-fed rats where reduced efficiency of energy retention was observed and in the 8% casein-fed group where adaptive thermogenesis was not stimulated. Since iodothyronine 5'-deiodinase activity was measured only in the microsomal fraction, the validity of the above comparisons of maximal enzyme activity/100 g body weight depends on the degree of variation between low and normal protein-fed animals in the

fractional recovery of total tissue enzyme activity in this subcellular fraction during differential centrifugation. Recovery of total tissue 5'-deiodinase in the microsomal fraction was not measured in the present study; nevertheless, maximal possible differences in this variable between low and normal protein-fed animals can be determined from dietary treatment differences in microsomal protein content expressed per g tissue. If one assumes the worst case scenario that the decrease in hepatic microsomal protein content per g liver (Table 6) in 5 and 8% casein-fed rats is entirely the result of reduced efficiency of recovery of the microsomal fraction during differential centrifugation and considers the fact that hepatic and renal iodothyronine 5'-deiodinase is localized in the microsomal fraction (14,33), then maximal hepatic 5'-deiodinase activity/100 g body weight and combined maximal hepatic and renal enzyme activity/100 g body weight corrected to account for differences in the above parameter would be similar in low and normal protein-fed animals, rather than decreased in the former group if these measurements were left uncorrected. In either case, the same conclusion would be made: that iodothyronine 5'-deiodination does not mediate changes in energy balance in low protein-fed rats. A similar conclusion may be deduced separately from the kinetic data for 5'-deiodinase (Table 5), which are independent of changes in efficiency of recovery of the microsomal fraction, since Vmax and Km of hepatic and renal 5'-deiodinase varied in a similar manner in both 5 and 8% casein-fed rats even though these two groups exhibited different responses in efficiency of energy retention relative to normal protein-fed controls. The above conclusion

contrasts with our initial hypothesis suggesting that increased adaptive thermogenesis resulting from consumption of a low protein diet is at least partly mediated by an increase in peripheral iodothyronine 5'-deiodination. Additional studies examining in vivo thyroid hormone kinetics in animals fed low protein diets are needed to confirm the above results.

In agreement with earlier reports (11,59,77), rats fed either a 5%, 6% or 8% casein diet had higher serum total T_3 concentrations than normal protein-fed controls without corresponding changes in serum free T_3 concentrations (Table 8). These changes in circulating T_3 levels do not correlate well with the observed alterations in energy expenditure in these dietary groups. As an illustration, feeding rats a 5% casein diet and an 8% casein diet produced different thermogenic responses relative to normal protein-fed animals, yet similar changes in serum total T_3 and free T_3 concentrations. This indicates that changes in circulating thyroid hormone concentrations, whether in the free or bound form, are not directly responsible for activating adaptive thermogenesis in 5% and 6% casein-fed rats. But, one cannot rule out the possibility that the elevation in serum total T_3 in 5% casein-fed rats has a permissive role in reducing efficiency of energy retention.

In summary, it appears that control points regulating thyroid hormone action other than peripheral iodothyronine 5'-deiodination and circulating thyroid hormone concentrations are active in mediating alterations in energy efficiency resulting from consumption of a low protein diet. One such control point might be hepatic nuclear T_3

receptor concentration; however, consumption of a low protein diet (9% casein) was without effect on this parameter (59). It was not reported whether this diet stimulated thermogenesis. Additional studies focusing on other determinants of thyroid hormone action such as thyroid hormone transport from the plasma to the nuclear compartment and nuclear T_3 receptor occupancy are needed to provide a more complete understanding of the mechanisms involved in enhanced heat production after consumption of low protein diets.

NUCLEAR TRIIODOTHYRONINE RECEPTOR BINDING CHARACTERISTICS AND OCCUPANCY IN LEAN AND OBESE (Ob/Ob) MICE

Introduction

Obese (ob/ob) mice gain more body energy than their lean littermates in part as a result of defective non-shivering (63,64) and diet-induced thermogenesis (65). Diminished adaptive heat production in obese mice may be associated with impaired thyroid hormone action since thyroid hormones play a permissive role in conjunction with the sympathetic nervous system in regulating this process (21). The above hypothesis is supported by the findings that activities of the thyroid hormone-sensitive enzymes, α-glycerolphosphate dehydrogenase (26,47) and Na^+,K^+-ATPase (36) are lower in several tissues of obese mice than in lean mice. Furthermore, obese mice exhibit characteristics of hypothyroidism such as cold intolerance (64) that are partially reversed by thyroid hormone administration (22,42).

Recently, efforts have been made to identify the specific mechanisms responsible for reduced thyroid hormone action in obese mice. We reported that one determinant of thyroid hormone action, thyroxine (T_4)5'-deiodination, is decreased in hepatic and renal microsomes of obese mice as early as 1-2 weeks of age (20). Conceivably, reduced conversion of T_4 to metabolically active 3,5,3'-triiodothyronine (T_3) in obese mice would result in lowered T_3 availability, and consequently, impaired thyroid hormone expression in tissues mediating adaptive thermogenesis. However, this hypothesis is not in accord with findings that plasma T_3 concentrations are similar in lean and obese mice (20,40,76), suggesting that diminished $T_4$5'-deiodination in obese mice may not directly mediate reduced thyroid hormone action.

Alternate mechanisms for impaired thyroid hormone expression in obese mice include changes at the level of nuclear T_3 receptors. Binding of T_3 to specific nuclear T_3 receptor sites is generally regarded as the point of initiation of thyroid hormone action (43). Nuclear T_3 receptors have been characterized in several thyroid hormone responsive tissues (46). Possibly diminished thyroid hormone action in tissues of obese mice is caused by a reduction in numbers of nuclear T_3 receptor binding sites. Modulation of some indices of thyroid hormone expression through changes in nuclear T_3 receptor concentration has already been proposed for other physiological states such as starvation (8,12,13) and partial hepatectomy (13). Another possible mechanism for impaired thyroid hormone action in obese mice is a reduction in nuclear T_3 receptor occupancy by endogenous T_3. Previous studies have shown that thyroidal stimulation of both Na^+, K^+-ATPase (60) and α-glycerolphosphate dehydrogenase (44) is correlated with occupancy of specific nuclear T_3 binding sites.

In the present study, we examined the possibility that nuclear T_3 receptor concentration and occupancy by endogenous T_3 are altered in obese (ob/ob) mice. This report focuses only on liver since current concepts of the mechanism of thyroid hormone action are largely derived from studies employing this tissue. Also, data are available comparing the activities of thyroid hormone-sensitive enzymes in livers of lean and obese mice during various physiological states. Analysis of nuclear T_3 binding and endogenous T_3 concentration was conducted in vitro in soluble (0.4 M NaCl) nuclear extracts. This preparation was employed to obviate the problem of T_3 receptor leaching from

chromatin that occurs during incubation of intact nuclei, and is a potential source of error in measuring maximal binding capacity (3). In addition, soluble nuclear extracts exhibit relatively low nonspecific binding and are suitable for the measurement of endogenous T_3 content via direct radioimmunoassay.

Materials and Methods

Animals. Male C57 BL/6J lean (ob/+ or +/+) and obese (ob/ob) littermates obtained from our breeding colony were weaned at 3 wk of age and housed individually in plastic solid-bottom cages at 23-25^{o}C. All animals were provided stock diet (Wayne Rodent Blox from Continental Grain, Chicago, IL) and water ad libitum. A 12 h light-dark cycle was maintained throughout the studies. Experiments were conducted with mice at either 4 wk of age, a time point early in the development of the obese syndrome, or at 8-10 wk of age when the expression of gross obesity is well established.

Preparation of Nuclei and Solubilization of Nuclear Receptor Proteins. The following procedures were performed at 0-4^{o}C. Hepatic nuclei were isolated according to the method of Widnell and Tata (73). Briefly, 7 and 6 livers from 4 wk-old lean and obese mice and 4 and 3 livers from 8-10 wk-old lean and obese mice, respectively, were pooled and homogenized (1:3) in 0.32 M sucrose, 3 mM $MgCl_2$ (25 strokes, 1200 RPM) using a loose fitting teflon-glass homogenizer. The homogenate was filtered through nylon mesh and then diluted with 0.6 volumes of homogenizing medium and 0.25 volumes of water to lower the tissue and sucrose concentrations. Next, 0.32 M sucrose, 3 mM $MgCl_2$ was layered underneath the homogenate followed by centrifugation at 700 x g for

10 min. The pellet of crude nuclei was resuspended in 2.4 M sucrose, 1 mM $MgCl_2$ and centrifuged at 60,000 x g for 60 min. The resulting pellet of nuclei was washed with 0.14 M NaCl, 3 mM $MgCl_2$ and recovered by centrifugation at 1000 x g for 10 min.

Nuclear T_3 receptors were solubilized according to the technique of Silva et al. (57). The washed nuclear pellet was first suspended in TEM buffer (30 mM Tris, 2 mM EDTA, 5 mM $MgCl_2$, 5 mM mercaptoethanol, 10% (v/v) glycerol (pH 8.0 at 25°C)) using a glass rod. An equal volume of TEM buffer containing 0.8 M NaCl was then added to the suspension while gently stirring. To equalize protein concentrations in each extract the total combined volume of solubilization buffer (TEM, 0.4 M NaCl) added to each nuclear pellet was approximately 1.15 and 1.00 mg/g liver homogenized for 4 wk-old lean and obese mice, respectively, and 1.10 and 0.65 ml/g liver for 8-10 wk-old lean and obese mice, respectively. T_3 receptors were extracted while vortexing the nuclear suspension on ice for 15 sec at 5 min intervals for 45 min. The preparation was then centrifuged at 25,000 x g for 30 min; the clear supernatant was decanted and used for subsequent measurement of T_3 binding and endogenous T_3 concentration.

Efficiency of extraction of the nuclear T_3 receptor was estimated by injection of tracer levels of (^{125}I)T_3 (400-600 mCi/mg; Abbott Labs, N. Chicago, IL) in lean and obese mice of each age group 1 h before removal of tissue. The fraction of radioactivity in the washed nuclear pellet that was solubilized with the receptor was determined.

Nuclear T_3 Receptor Binding Assay. Fresh nuclear extract (100 ul) diluted 1:3 with TEM, 0.4 M NaCl was mixed with 100 ul of (^{125}I)T_3 diluted in TEM, 0.025% bovine serum albumin to give final (^{125}I)T_3 concentrations of 0.2×10^{-10} to 10×10^{-10}M. The mixture was first incubated at 30°C for 40 min to dissociate endogenous T_3 from the nuclear receptor followed by incubation at 0°C for 20 h (57). Bound and free (^{125}I)T_3 were separated as described by Seelig et al. (54) using an anion exchange resin. Briefly, 1.0 ml of 10% (w/v) ice cold slurry of Dowex AG 1-X8 resin (200-400 mesh) in TEM, 0.2 M NaCl was added to the T_3 binding assay mixture and incubated on ice for 20 min with mixing at 5 min intervals. The resin, which adsorbs free (^{125}I)T_3 and T_3, was then pelleted by centrifugation at 2000 x g for 5 min. Radioactivity in the bound fraction (supernatant) and free fraction (resin) was quantitated using a gamma counter. Nonspecific binding was determined by addition of 10^{-6} M T_3 to the nuclear extract containing (^{125}I)T_3.

Measurement of Endogenous T_3 Concentration in Nuclear Extracts. Endogenous T_3 concentration was determined by a modification of the direct radioimmunoassay procedure of Yagura and Walfish (74). The assay mixture consisted of 300 ul of undiluted nuclear extract or standard containing 0-200 pg T_3 in TEM, 0.4 M NaCl, 0.1% bovine serum albumin; 50 ul of TEM, 0.4 M NaCl containing 250 ug 8-anilinonaphltalene-1-sulphonic acid (ANS), 25 ug bovine serum albumin and ~25,000 CPM (^{125}I)T_3 (3300 mCi/mg; New England Nuclear, Boston, MA); and 100 ul anti-T_3 antibody (Monoform, Miles Scientific, Naperville, IL) diluted 1:300 in TEM, 0.4 M NaCl. 250 ug ANS/tube was used since this

concentration was sufficient to minimize the binding of (^{125}I)T_3 to the receptor, and at the same time did not significantly interfere with binding of T_3 to antibody. The mixture was incubated at 4°C for 24 h. Bound and free fractions were separated using the anion exchange resin method described for the T_3 binding assay. Nonspecific binding was assessed by omitting antibody from the assay mixture. Values were separately determined for standards and nuclear extract samples and subtracted from corresponding binding data obtained from samples containing antibody.

Treatment of Data. Data from the binding studies were analyzed by the method of Scatchard (53). The endogenous T_3 content of the nuclear extract was taken into account in calculating total ligand binding at a given (^{125}I)T_3 concentration. In expressing maximal binding capacity and endogenous nuclear T_3 concentration on a unit DNA basis, correction for incomplete receptor solubilization was included. Fractional occupancy of the T_3 receptor was calculated as endogenous T_3 content multiplied by the fraction of hormone associated with the receptor as determined by the application of anion exchange resin to the nuclear extract labeled with (^{125}I)T_3 in vivo; the product finally divided by the maximal binding capacity.

Other Analyses. Protein was determined by the method of Hughes et al. (24) which employs N-ethylmaleimide to neutralize interference in the assay produced by mercaptoethanol. DNA was determined by the method of Burton (9) as modified by Richards (49). Statistical comparisons were performed using the Student's t test for paired data.

Results

Results of the nuclear T_3 binding study are summarized in Table 9. In 4 wk-old mice, extraction of the hepatic nuclear pellet with TEM, 0.4 M NaCl solubilized 16% more protein per mg DNA in obese preparations than in lean preparations even though the efficiency of extraction of the nuclear T_3 receptor as measured in the in vivo labeling experiment was similar in both phenotypes. Maximal binding capacity expressed on either a mg protein or mg DNA basis was not different in 4 wk-old lean and obese mice. In addition, maximal binding capacity expressed on a whole organ basis was similar in lean and obese mice since DNA content per liver was not different between phenotypes. The equilibrium dissociation constant (Kd) for specific nuclear T_3 binding was also similar in 4 wk-old lean and obese mice (Table 9 and Figure 5).

In 8-10 wk-old mice, significantly more (26%) hepatic nuclear protein was extracted per mg DNA in obese preparations than in lean, and a small decrease (5%) in efficiency of extraction of the nuclear T_3 receptor was observed in the former group (Table 9). In contrast to the results for 4 wk-old mice, maximal binding capacity (expressed on a mg protein basis) was reduced 30% in 8-10 wk-old obese mice relative to their lean counterparts (Table 9). There was also a small, but significant, reduction (8%) in maximal binding capacity in obese mice when the results were expressed per mg DNA. Conversely, maximal binding capacity expressed on a whole organ basis was elevated 33% in 8-10 wk old obese mice since the DNA content per liver was 45% higher in this phenotype. No difference in the Kd for specific nuclear T_3 binding was observed between phenotypes (Table 9 and Figure 5).

Table 9. Binding characteristics of the hepatic nuclear T_3 receptor

	mg DNA / Organ	mg Nuclear Protein Extracted / mg DNA	Extraction Efficiency (%)	Maximal Binding Capacity: fmol / mg protein	fmol / mg DNA	fmol / organ	Kd ($\times 10^{-10}$ M)
				4-wk-old			
Lean	2.78 ±0.08	0.84 ±0.04	65.6 ±0.9	500 ±21	640 ±45	1770 ± 95	0.25 ±0.02
Obese	2.97 ±0.06	0.97** ±0.03	67.1 ±0.4	450 ±17	650 ±35	1924 ± 99	0.23 ±0.02
				8-10-wk-old			
Lean	4.23 ±0.11	0.85 ±0.02	68.3 ±1.4	473 ±21	590 ±18	2492 ± 87	0.25 ±0.01
Obese	6.12** ±0.11	1.07** ±0.02	65.1* ±0.6	331** ±21	543* ±29	3313** ±144	0.22 ±0.02

Estimates of maximal binding capacity and Kd were obtained from Scatchard Analysis.
Values are the means ± SE of 5 soluble nuclear receptor preparations. * Means are different ($P<0.05$) from those of lean mice of the same age. **($P<0.01$).

Figure 5. Scatchard plots of binding of T_3 to solubilized hepatic nuclear receptors in 4 and 8-10 week old lean and obese mice. Each point is the mean of 5 preparations. The average protein content per tube was 38 and 37 ug for 4 week old and 32 and 30 ug for 8-10 week old lean and obese mice, respectively.

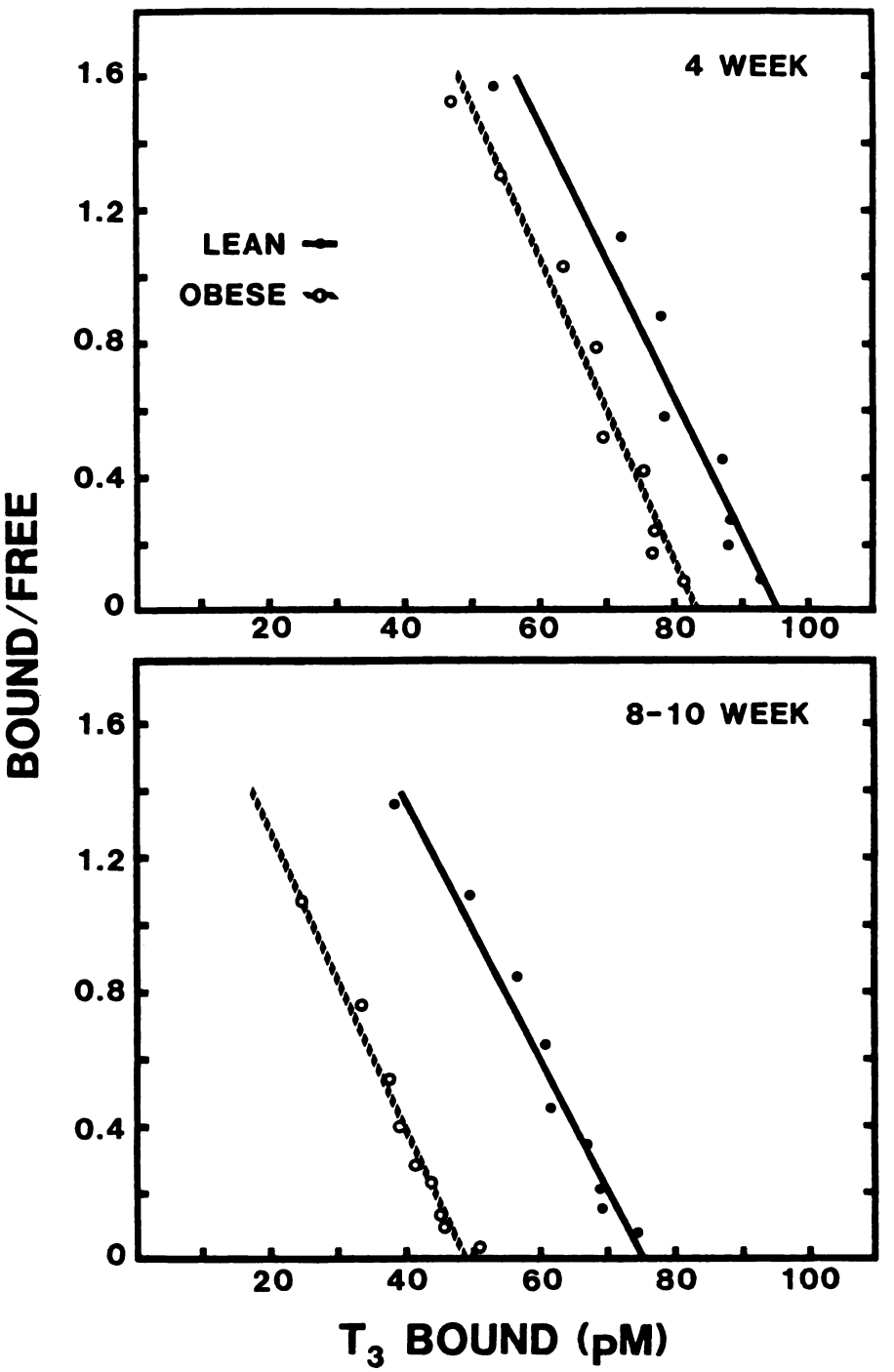
4 WEEK
LEAN
OBESE
8-10 WEEK
BOUND/FREE
1.6
1.2
0.8
0.4
0
20
40
60
80
100
T_3 BOUND (pM)

Figure 5.

Data for endogenous nuclear T_3 concentration and fractional nuclear T_3 receptor occupancy are presented in Table 10. In 4 wk-old animals, hepatic nuclear T_3 concentration expressed on a mg protein basis was 24% lower in obese mice than in lean mice. There was also a small (13%), but significant, reduction in nuclear T_3 concentration expressed on a mg DNA basis in 4 wk-old obese mice. Nuclear T_3 content expressed on a whole organ basis was similar in both phenotypes. The fraction of endogenous nuclear T_3 specifically bound to the receptor, as determined by an ion exchange resin test, was unaffected by phenotype (Table 10). As a result of the above finding and the previous observation that maximal binding capacity per mg DNA was not different in 4 wk-old lean and obese mice, a reduction in fractional nuclear T_3 receptor occupancy (14%) of similar magnitude to that of endogenous nuclear T_3 concentration per mg DNA was calculated for the latter phenotype.

Nuclear T_3 concentrations were depressed even more in 8-10 wk-old obese mice than in 4 wk-old obese mice (Table 10). Nuclear T_3 concentration expressed on either a mg protein or mg DNA basis was 45% and 28% lower, respectively, in 8-10 wk-old obese mice than in lean counterparts. Nuclear T_3 content per liver was unaffected by phenotype (Table 10). The fraction of nuclear T_3 specifically bound to the receptor was also not different between lean and obese mice. Fractional nuclear T_3 occupancy was 23% lower in nuclear extracts of 8-10 wk-old obese mice than in corresponding lean controls.

Table 10. T_3 content of hepatic nuclear extracts.

	Nuclear T_3 Concentration			Fraction of Endogenous T_3 Specifically Bound X100	Fractional Nuclear T_3 Receptor Occupancy X100
	fmol / mg protein	fmol / mg DNA	fmol / organ		
			4-wk-old		
Lean	263 ± 6	336 ±14	929 ±16	80.8 ±0.9	42.6 ±1.5
Obese	200** ± 9	293* ±15	868 ±48	80.9 ±0.9	36.6* ±1.5
			8-10-wk-old		
Lean	227 ± 8	283 ± 3	1193 ± 25	80.8 ±0.8	38.9 ±1.3
Obese	124** ±13	203** ±18	1241 ±110	79.9 ±1.2	29.8** ±2.0

The endogenous T_3 concentration of soluble nuclear extracts was measured using the direct RIA described in the Materials and Methods. Values are the means ± SE of 5 preparations.
* Means are different ($P<0.05$) from those of lean mice of the same age. **($P<0.01$).

Discussion

In determining whether changes in thyroid hormone action are caused by alterations in either nuclear T_3 receptor number or occupancy by endogenous T_3, it is probably most valid to express data for these measurements on a DNA basis since this compound comprises the genome that is transcribed in response to T_3 binding to the specific nuclear receptor. Data stated on a protein basis have little physiological relevance since the amount of nuclear protein extracted per unit DNA can vary between treatment groups as was the case with lean and obese mice at both 4 and 8-10 wks of age (Table 9). Similarly, when correlating the above measurements with indices of thyroid hormone action such as Na^+,K^+-ATPase and α-glycerolphosphate dehydrogenase, the latter should also be expressed in the same units (per unit DNA).

In the present study, hepatic nuclear T_3 receptor concentration (fmol/mg DNA) was similar in 4 wk-old lean and obese mice, and only slightly reduced in obese mice at 8-10 wks of age (Table 9). Theoretically, maximal enzyme response to thyroid hormone administration is limited by the concentration of nuclear T_3 receptors in the tissue. Therefore, the finding that maximal induction of hepatic Na^+,K^+-ATPase (36) and α-glycerolphosphate dehydrogenase (56) by thyroid hormone treatment results in equally high enzyme levels in lean and obese mice is consistent with our observation that nuclear T_3 receptor concentration is not different in the above phenotypes (Table 9).

Our nuclear T_3 binding results are in apparent conflict with the study of Guernsey and Morishige (19) showing a marked reduction (56%) in hepatic nuclear T_3 receptor concentration in obese (ob/ob) mice.

There are several possible explanations for this discrepancy. First, Guernsey and Morishige may have employed older mice (age of the animals not reported) in their experiment than those used in the present study. Possibly a substantial reduction in nuclear T_3 receptor concentration occurs in obese mice after 8-10 wks of age. Second, Guernsey and Morishige conducted their binding studies with intact nuclei isolated by repeated detergent treatment and centrifugation. Bernal and DeGroot (3) have shown that a significant fraction ($\sim$50%) of the T_3 receptors dissociate from detergent treated nuclei incubated at 20^{o}C. Receptor leaching would cause an underestimation of maximal T_3 binding per unit DNA. The rate of receptor dissociation may have been higher in nuclear preparations of obese mice than in lean mice, thus producing an apparent reduction in maximal binding capacity in the former phenotype. In the present study, we employed a solubilized nuclear receptor preparation to overcome this problem as well as to increase the "signal to noise" ratio of the binding assay. We also determined the efficiency of extraction of the nuclear receptor to account for the possibility that this variable might influence the results. Finally, the different nuclear preparations employed by Guernsey and Morishige (19) and this study could produce divergent results by another mechanism if intact isolated nuclei contain inhibiting and/or activating intranuclear factors at sufficient concentrations to affect nuclear T_3 receptor binding in vitro. Indeed, several nuclear components such as certain histone (1) and nonhistone (5) proteins can modulate the activity of partially purified nuclear T_3 receptors derived from rat liver. Possibly intranuclear regulatory

factors differentially influence T_3 binding in isolated nuclei of lean and obese mice. Use of soluble nuclear extracts should reduce the influence of these factors since the intranuclear environment would be greatly diluted.

In contrast to the nuclear T_3 kinetic binding data, fractional nuclear T_3 receptor occupancy was reduced 14 and 23% in 4 and 8-10 wk-old obese mice, respectively, relative to corresponding lean mice (Table 10). This finding was mainly the result of changes in endogenous T_3 concentration associated with the specific nuclear receptor rather than alterations in nuclear T_3 receptor number. Diminished nuclear T_3 receptor occupancy in obese mice is closely paralleled by similar alterations in the concentration of the thyroid hormone-sensitive enzyme, Na^+, K^+-ATPase. The number of hepatic Na^+, K^+-ATPase enzyme units is reported to be decreased 24 and 46% (36) in 4 and 8 wk-old obese mice, respectively; a less pronounced depression in the concentration of enzyme units in obese mice is evident in each age group when the data are expressed on a DNA basis (D.R. Romsos, unpublished results). In addition, Na^+, K^+-ATPase activity expressed on a per organ basis is not different in both 4 and 8 wk-old lean and obese mice (36) which is consistent with data showing a similar nuclear T_3 content per organ (Table 10) in each phenotype. The above correlations between nuclear T_3 receptor occupancy and enzyme expression suggests that the former parameter plays a role in mediating impaired thyroid hormone action in obese mice. This proposal is congruent with data showing a greater percentage stimulation in Na^+, K^+-ATPase activity in thyroid hormone-treated obese mice compared to similarly

treated lean mice (36) since a greater fractional increase in nuclear T_3 receptor occupancy would occur in the former phenotype.

There are several possible mechanisms that can cause a reduction in nuclear T_3 receptor occupancy in obese mice. First, a decrease in plasma T_3 concentration can produce corresponding changes in nuclear T_3 receptor occupancy (45,70). This mechanism, however, may be disregarded since circulating T_3 concentrations are similar in lean and obese mice (20,40,76). Second, a decrease in local T_4 to T_3 conversion in a given tissue could result in a diminished supply of T_3 to the nuclear compartment and hence, occupancy of receptor sites. Indeed, microsomal $T_4$5'-deiodinase activity is 32 and 40% lower in 4 and 8-10 wk-old obese mice, respectively, than in corresponding lean mice (20). But, the importance of this process in influencing hepatic nuclear T_3 receptor occupancy may be minimal since van Doorn et al. (70) have recently shown using a continuous infusion technique that the hepatic nuclear T_3 pool is almost exclusively derived from the plasma T_3 compartment with little contribution coming from local conversion of T_4 to T_3. Third, inhibition of binding of endogenous T_3 to the specific nuclear receptor by intranuclear regulatory factors could also cause a reduction in nuclear T_3 receptor occupancy. Presently, there is no evidence either supporting or refuting this proposal in regards to obese mice. A fourth and final mechanism for lowered nuclear T_3 receptor occupancy in obese mice is a reduction in transport of plasma T_3 to the nuclear compartment. Altered T_3 transport in obese mice is consistent with the hypothesis of York (75) which proposes that changes in membrane fluidity and phospholipid composition in obese mice may

affect the activity of membrane associated proteins, some of which may be involved in transport processes. Further supporting this mechanism, Kaplan et al. (29) have shown that tissue to plasma T_3 ratios following in vivo injection of tracer T_3 are markedly reduced in livers of obese mice relative to lean controls, indicating that transport across the plasma membrane is diminished in the former phenotype. Additional studies directly measuring transport of T_3 from the plasma to the nuclear compartment in livers of lean and obese mice are needed to confirm the above proposal.

In summary, minimal differences in maximal binding capacity expressed on a DNA basis were observed between lean and obese mice, whereas nuclear T_3 receptor occupancy was reduced in obese mice at 4 and 8-10 wk of age, the extent of the decrease being greater in 8-10 wk-old animals. Alterations in nuclear T_3 receptor occupancy in obese mice were correlated with reported changes in the thyroid hormone-sensitive enzyme, Na^+, K^+-ATPase, thus suggesting a possible causal relationship between these two parameters. Finally, it is proposed that reduced hepatic nuclear receptor occupancy by endogenous T_3 in obese mice results primarily from an impairment in the transport of T_3 from the plasma pool to the nuclear compartment.

GENERAL CONCLUSIONS

In the present studies, iodothyronine 5'-deiodination was examined in lean and obese mice and rats fed low protein diets in an effort to elucidate the mechanism(s) of altered thyroid hormone action in these experimental animal models. It was hypothesized that alterations in T_4 conversion to T_3 in liver and kidney would influence the supply of metabolically active T_3 to thermogenic target tissues, resulting in changes in nuclear T_3 receptor occupancy. Reduced thyroid hormone action in obese mice was shown to be paralleled by changes in microsomal $T_4$5'-deiodination; however, the latter parameter was not correlated with increased thyroid hormone expression in low protein-fed rats. Whether impaired thyroid hormone action in obese mice is caused by reduced 5'-deiodination of T_4 remains unclear since plasma T_3 concentrations are not altered in these animals. Increased thyroid hormone action in low protein-fed rats is probably mediated at another level of the thyroid system.

To study an alternate mechanism of impaired thyroid hormone action in obese mice, the binding characteristics and endogenous T_3 occupancy of solubilized hepatic nuclear T_3 receptors were examined in these animals. Maximal binding capacity and K_d were shown to be similar in 4 and 8-10 wk-old lean and obese mice, indicating that changes in nuclear T_3 receptor concentration and affinity do not mediate reduced thyroid hormone expression in the latter phenotype. In contrast, nuclear T_3 receptor occupancy was decreased in obese mice, the extent of the reduction being greater at 8-10 wks of age. Diminished nuclear T_3 receptor occupancy in obese mice was correlated with reported changes in the activities of the thyroid hormone-sensitive

enzymes, Na^+, K^+-ATPase (36) and α-glycerolphosphate dehydrogenase (47), indicating that a decreased nuclear T_3 signal may mediate, in part at least, impaired hepatic thyroid hormone action in this animal model. Whether alterations in nuclear T_3 receptor occupancy are responsible for changes in thyroid hormone action in other thermogenic tissues (i.e. brown adipose tissue) of obese mice remains to be addressed.

The possibility that enhanced thyroid hormone action in low protein-fed rats results from changes in the binding characteristics of the nuclear T_3 receptor has been previously examined by Smallridge et al. (59). They reported that the number and affinity of solubilized hepatic nuclear T_3 receptors were similar in low (9% casein) and normal (18% casein) protein-fed rats. However, in calculating maximal binding capacity, they did not account for incomplete extraction of the specific T_3 receptor from the nuclear pellet. This parameter could have varied between the dietary treatment groups which would have produced corresponding differences in maximal binding capacity expressed on a DNA basis. Nevertheless, assuming that extraction efficiency is similar in low and normal protein-fed rats, it may be concluded that enhanced thyroid hormone action in the former group is not caused by alterations in nuclear T_3 receptor concentration and affinity, the same deduction having been made during conditions of impaired thyroid hormone expression in obese mice.

In summary, lowered nuclear T_3 receptor occupancy may explain, in part at least, hepatic resistance to normal circulating T_3 concentrations in obese mice. Studies are needed to determine whether this mechanism

is also applicable to the opposite situation of enhanced thyroid hormone action in low protein-fed rats. In addition, the cause(s) for reduced nuclear T_3 occupancy in obese mice should be addressed. Presently, an attractive hypothesis is impaired transport of T_3 from the plasma to the nuclear compartment which is consistent with the theory of a general defect in membrane function proposed by York (75). Direct measurements of hepatic T_3 transport would be required to investigate this possibility.

LIST OF REFERENCES

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1. Anselmet, A., J. Bismuth, M.M. Menezes-Ferreira, and J. Torresani. Triiodothyronine nuclear receptor. Role of histones and DNA in hormone binding. Biochim. Biophys. Acta 739:291-300, 1983.

2. Bellabarba, D., R.E. Peterson and K. Sterling. An improved method for chromatography of iodothyronines. J. Clin. Endocrinol. Metab. 28:305-307, 1968.

3. Bernal, J., and L.J. DeGroot. Thyroid hormone receptors: release of receptor to the medium during in vitro incubation of isolated rat liver nuclei. Endocrinology 100:648-655, 1977.

4. Bieri, J.G., G.S. Stoewsand, and G.M. Briggs. Report of the AIN Ad Hoc Committee on standards for nutritional studies. J. Nutr. 107:1340-1348, 1977.

5. Bismuth, J., A. Anselmet, and J. Torresani. Triiodothyronine nuclear receptor and the role of non-histone protein factors in in vitro triiodothyronine binding. Biochim. Biophys. Acta 840: 271-279, 1985.

6. Boissonneault, G.A., M.J. Hornshuh, J.W. Simons, D.R. Romsos and G.A. Leveille. Oxygen consumption and body fat content of young lean and obese (ob/ob) mice. Proc. Soc. Exp. Biol. Med. 157: 402-406, 1978.

7. Bray, G.A. and D.A. York. Hypothalamic and genetic obesity in experimental animals: an autonomic and endocrine hypothesis. Physiol. Rev. 59:719-809, 1979.

8. Burman, K.D., Y. Lukes, F.D. Wright, and L. Wartofsky. Reduction in hepatic triiodothyronine binding capacity induced by fasting. Endocrinology 101:1331-1338, 1977.

9. Burton, K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315-323, 1956.

10. Chopra, I.J. A study of extrathyroidal conversion of thyroxine (T_4) to 3,3',5-triiodothyronine (T_3) in vitro. Endocronology 101: 453-463,

11. Cox, M.D., S.S. Dalal, C.R.C. Heard, and D.J. Millward. Metabolic rate and thyroid status in rats fed diets of different protein-energy value: the importance of free T_3. J. Nutr. 114:1609-1616, 1984.

12. DeGroot, L.J., A.H. Coleoni, P.A. Rue, H. Seo, E. Martino, and S. Refetoff. Reduced nuclear triiodothyronine receptors in starvation-induced hypothyroidism. Biochem. Biophys. Res. Commun. 79:173-178, 1977.

13. Dillmann, W.H., H.L. Schwartz, and J.H. Oppenheimer. Selective alterations in hepatic enzyme response after reduction of nuclear triiodothyronine receptor sites by partial hepatectomy and starvation. Biochem. Biophys. Res. Commun. 80:259-266, 1978.

14. Fekkes, D., E. van Overmeeren-Kaptein, R. Docter, G. Hennemann and T.J. Visser. Location of rat liver iodothyronine deiodinating enzymes in the endoplasmic reticulum. Biochim. Biophys. Acta 587:12-19, 1979.

15. Flaim, K.E., J.B. Li and L.S. Jefferson. Effects of thyroxine on protein turnover in rat skeletal muscle. Am. J. Physiol. 235(2):E231-E236, 1978.

16. Gambert, S.R. and T.L. Garthwaite. Serum thyroid hormone levels and thyroid hormone metabolosm by 10% liver homogenate from adult lean and obese (ob/ob) mice. Horm. Metab. Res. 13:588-589, 1981.

17. Gill, J.L. Design and Analysis of Experiments in the Animal and Medical Sciences, vol. 1. Iowa State University Press, Ames, IA, 1978.

18. Grussendorf, M. and M. Hufner. Induction of the thyroxine (T_4) to triiodothyronine (T_3) converting enzyme in rat liver by thyroid hormones and analogs. Clin. Chim. Acta 80:61-66, 1977.

19. Guernsey, D.L. and W.K. Morishige. Na^+ pump activity and nuclear T_3 receptors in tissues of genetically obese (ob/ob) mice. Metabolism 28:629-632, 1979.

20. Hillgartner, F.B., and D.R. Romsos. Regulation of iodothyronine 5'-deiodination in lean and obese (ob/ob) mice. Am. J. Physiol. 249 (Endocrinol. Metab. 12):E209-E218, 1985.

21. Himms-Hagen, J. Thyroid hormones and thermogenesis. In: Mammalian Thermogenesis, edited by L. Girardier and M.J. Stock. London: Chapman and Hall, 1983, p. 141-177.

22. Hogan, S. and J. Himms-Hagen. Abnormal brown adipose tissue in genetically obese mice (ob/ob): effect of thyroxine. Am. J. Physiol. 241 (Endocrinol. Metab. 4):E436-E443, 1981.

23. Huang, M.T. and H.A. Lardy. Effects of thyroid states on the cori cycle, glucose-alanine cycle, and futile cycling of glucose metabolism in rats. Arch. Biochem. Biophys. 209:41-51, 1981.

24. Hughes, J., S. Joshi, and D. Ascoli. Elimination of thiol reagent interference during Lowry protein determination. Anal. Biochem. 117:1-5, 1981.

25. James, W.P.T. and P. Trayhurn. An integrated view of the metabolic and genetic basis for obesity. Lancet ii:770-773, 1976.

26. Joosten, H.F.P. and P.H.W. van der Kroon. Role of the thyroid in the development of the obese-hyperglycemic syndrome in mice (ob/ob). Metabolism 23:425-436, 1974.

27. Kaplan, M.M., J.B. Tatro, R. Breitbart and P.R. Larson. Comparison of thyroxine and 3,3',5'-triiodothyronine metabolism in rat kidney and liver homogenates. Metabolism 28:1139-1146, 1979.

28. Kaplan, M.M. and R.D. Utiger. Iodothyronine metabolism in liver and kidney homogenates from hyperthyroid and hypothyroid rats. Endocrinology 103:156-161, 1978.

29. Kaplan, M.M., J.B. Young, E.A. Shaw. Abnormal thyroid hormone binding to serum proteins in ob/ob and db/db genetically obese mice. Endocrinology 117:1858-1863, 1985.

30. Kevonian, A.V., J.G. Vander Tuig, and D.R. Romsos. Consumption of a low protein diet increases norepinephrine turnover in brown adipose tissue of adult rats. J. Nutr. 114:543-549, 1984.

31. Knehans, A.W. and D.R: Romsos. Reduced norepinephrine turnover in brown adipose tissue of ob/ob mice. Am. J. Physiol. 242 (Endocrinol. Metab. 5):E253-E261, 1982.

32. Larsen, P.R., J.E. Silva and M.M. Kaplan. Relationships between circulating and intracellular thyroid hormones: physiological and clinical implications. Endocrine Rev. 2:87-102, 1981.

33. Leonard, J.L. and I.N. Rosenberg. Subcellular distribution of thyroxine 5'-deiodinase in the rat kidney: a plasma membrane location. Endocrinology 103:274-280, 1978.

34. Leonard, J.L. and I.N. Rosenberg. Thyroxine 5'-deiodinase activity of rat kidney: observations on activation by thiols and inhibition by propylthiouracil. Endocrinology 103:2137-2144, 1978.

35. Leonard, J.L. and I.N. Rosenberg. Iodothyronine 5'-deiodinase from rat kidney: substrate specificity and the 5'-deiodination of reverse triiodothyronine. Endocrinology 107:1376-1383, 1980.

36. Lin, M.H., J.G. Vander Tuig, D.R. Romsos, T. Akera and G.A. Leveille. Na^+,K^+-ATPase enzyme units in lean and obese (ob/ob) thyroxine-injected mice. Am. J. Physiol. 237(3):E265-E272, 1979.

37. Lin, P.Y., D.R. Romsos, J.G. Vander Tuig and G.A. Leveille. Maintenance energy requirements, energy retention and heat production of young obese (ob/ob) and lean mice fed a high-fat or high-carbohydrate diet. J. Nutr. 109:1143-1153, 1979.

38. Mariash, C.N. and J.H. Oppenheimer. Thyroid hormone-carbohydrate interaction at the hepatic nuclear level. Federation Proc. 41: 2671-2676, 1982.

39. Markwell, M.A.K., S.M. Haas, L.L. Bieber and N.E. Tolbert. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87: 206-210, 1978.

40. Mobley, P.W. and P.U. Dubec. Thyroid hormone levels in the developing obese-hyperglycemic syndrome. Horm. Metab. Res. 11: 37-39, 1979.

41. Nejad, I., J. Bollinger, M.A. Mitnick, P. Sullivan and S. Reichlin. Measurement of plasma and tissue triiodothyronine concentrations in the rat by radioimmunoassay. Endocrinology 96:773-778, 1975.

42. Ohtake, M., G.A. Bray, and M. Azukizawa. Studies on hypothermia and thyroid function in the obese (ob/ob) mouse. Am. J. Physiol. 233 (Regulatory Integrative Comp. Physiol. 3):R110-R115, 1977.

43. Oppenheimer, J.H. Thyroid hormone action at the cellular level. Science 203:971-979, 1979.

44. Oppenheimer, J.H., P. Coulombe, H.L. Schwartz and N.W. Gutfeld. Non-linear (amplified) relationship between nuclear occupancy by triiodothyronine and the appearance rate of hepatic α-glycerolphosphate dehydrogenase and malic enzyme in the rat. J. Clin. Invest. 61:987-997, 1978.

45. Oppenheimer, J.H., H.L. Schwartz, D. Koerner, and M.I. Surks. Limited binding capacity sites for L-triiodothyronine in rat liver nuclei. Nuclear-cytoplasmic interrelationships, binding constants, and cross reactivity with L-thyroxine. J. Clin. Invest. 53: 768-777, 1974.

46. Oppenheimer, J.H., H.L. Schwartz, and M.I. Surks. Tissue differences in the concentration of triiodothyronine nuclear binding sites in the rat: liver, kidney, pituitary, heart, brain, spleen and testis. Endocrinology 95:897-903, 1974.

47. Otto, W., T.G. Taylor and D.A. York. Glycerol release in vitro from adipose tissue of obese (ob/ob) mice treated with thyroid hormones. J. Endocrinol. 71:143-155, 1976.

48. Peavy, D.E., J.M. Taylor and L.S. Jefferson. Protein synthesis in perfused rat liver following thyroidectomy and hormone treatment. Am. J. Physiol. 240 (Endocrinol. Metab. 3):E18-E23, 1981.

49. Richards, G.M. Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal. Biochem. 57:369-376, 1974.

50. Rothwell, N.J., M.J. Stock and R.S. Tyzbir. Mechanisms of thermogenesis induced by low protein diets. Metabolism 32:257-261, 1983.

51. Rothwell, N.J. and M.J. Stock. Similarities between cold- and diet-induced thermogenesis in the rat. Can. J. Physiol. Pharmacol. 58:842-848, 1980.

52. Rothwell, N.J. and M.J. Stock. A role for brown adipose tissue in diet-induced thermogenesis. Nature 281:31-35, 1979.

53. Scatchard, G. The attraction of proteins to small molecules and ions. Ann. NY Acad. Sci. 51:660-672, 1949.

54. Seelig, S., H.L. Schwartz, and J.H. Oppenheimer. Limitations in the conventional analysis of the interaction of triiodothyronine with solubilized nuclear receptor sites. J. Biol. Chem. 258: 2154-2161, 1981.

55. Sellars, E.A. and S.S. You. Role of the thyroid in metabolic responses to a cold environment. Am. J. Physiol. 163:81-91, 1950.

56. Shimomura, Y., G.A. Bray, and D.A. York. Effects of thyroid hormone and adrenalectomy on Na^+, K^+,-ATPase in the ob/ob mouse. Horm. Metab. Res. 13:578-582, 1981.

57. Silva, E.J., H. Astier, M. Thakare, H.L. Schwartz, and J.H. Oppenheimer. Partial purification of the triiodothyronine receptor from rat liver nuclei. J. Biol. Chem. 252:6799-6805, 1977.

58. Silva, J.E., T.E. Dick and P.R. Larson. The contribution of local tissue thyroxine monodeiodination to the nuclear 3,5,3'-triiodothyronine in pituitary, liver and kidney of euthyroid rats. Endocrinology 103:1196-1207, 1978.

59. Smallridge, R.C., A.R. Glass, L. Wartofsky, K.R. Latham and K.D. Burman. Investigations into the etiology of elevated serum T_3 levels in protein-malnourished rats. Metabolism 31:537-542, 1982.

60. Somjen, D., F. Ismail-Beigi, and I.S. Edelman. Nuclear binding of T_3 and effects on QO_2, Na-K-ATPase, and α-GPDH in liver and kidney. Am. J. Physiol. (Endocrinol. Metab. 3) 250:E146-E154, 1981.

61. Swanson, H.E. Interrelations between thyroxine and adrenalin in the regulation of oxygen consumption in the albino rat. Endocrinology 59:217-225, 1956.

62. Swick, R.W. and C.L. Gribskov. The effect of dietary protein levels on diet-induced thermogenesis in the rat. J. Nutr. 113: 2289-2294, 1983.

63. Thurlby, P.L. and P. Trayhurn. The role of thermoregulatory thermogenesis in the development of obesity in genetically-obese (ob/ob) mice pair-fed with lean siblings. Br. J. Nutr. 42:377-385, 1979.

64. Trayhurn, P. and W.P.T. James. Thermoregulation and non-shivering thermogenesis in the genetically obese (ob/ob) mouse. Pfluegers Arch. 373:189-193, 1978.

65. Trayhurn, P., P.M. Jones, M.M. McHuckin and A.E. Goodbody. Effects of overfeeding on energy balance and brown fat thermogenesis in obese (ob/ob) mice. Nature 295:323-325, 1982.

66. Triandafillou, J., C. Gwilliam, and J. Himms-Hagen. Role of thyroid hormone in cold-induced changes in rat brown adipose tissue mitochondria. Can. J. Biochem. 60:530-536, 1982.

67. Tulp, O.L., P.P. Krupp, E. Danforth and E.S. Horton. Characteristics of thyroid function in experimental protein malnutrition. J. Nutr. 109:1321-1332, 1979.

68. Tyzbir, R.S., A.S. Kunin, N.M. Sims and E. Danforth. Influence of diet composition on serum triiodothyronine (T_3) concentration, hepatic mitochondrial metabolism and shuttle system activity in rats. J. Nutr. 111:252-259, 1981.

69. Vander Tuig, J.G., D.R. Romsos and G.A. Leveille. Maintenance energy requirements and energy retention of young obese (ob/ob) and lean mice housed at 33^{0} and fed a high-carbohydrate or a high-fat diet. J. Nutr. 110:35-41, 1980.

70. van Doorn, J., D. van der Heide, and F. Roelfsema. Sources and quantity of 3,5,3'-triiodothyronine in several tissues of the rat. J. Clin. Invest. 72:1778-1792, 1983.

71. Visser, T.J., D. Fekkes, R. Docter and G. Hennemann. Kinetics of enzymic reductive deiodination of iodothyronines: effect of pH. Biochem. J. 179:489-495, 1979.

72. Visser, T.J., I. van der Does-Tobe, R. Docter and G. Hennemann. Subcellular localization of a rat liver enzyme converting thyroxine into tri-iodothyronine and possible involvement of essential thiol groups. Biochem. J. 157:479-482, 1976.

73. Widnell, C.C., and J.R. Tata. A procedure for the isolation of enzymatically active rat-liver nuclei. Biochem. J. 92:313-317, 1964.

74. Yagura, T., and P.G. Walfish. A new unextracted sample radio-immunoassay method for hepatic endogenous nuclear L-triiodothyronine content. Biochem. J. 208:641-649, 1982.

75. York, D.A. Alterations in membrane function, organization and composition in the obese ob/ob mouse. Proc. Nutr. Soc. 44:189-200, 1985.

76. York, D.A., W. Otto and T.G. Taylor. Thyroid status of obese (ob/ob) mice and its relationship to adipose tissue metabolism. Comp. Biochem. Physiol. 59B:59-65, 1978.

77. Young, R.A., L.E. Braverman and R. Rajatanavin. Low protein-high carbohydrate diet induces alterations in the serum thyronine-binding proteins in the rat. Endocrinology 110:1607-1612, 1982.

78. Young, R.A., S-L Fang, J. Prosky and L.E. Braverman. Hepatic conversion of thyroxine to triiodothyronine in obese and lean Zucker rats. Life Sci. 34:1783-1790, 1984.

79. Young, R.A., O.L. Tulp and E.S. Horton. Thyroid and growth responses of young Zucker obese and lean rats to a low protein-high carbohydrate diet. J. Nutr. 110:1421-1431, 1980.