A Thyroid Hormone Receptor α Gene Mutation (P398H) Is Associated with Visceral Adiposity and Impaired Catecholamine-stimulated Lipolysis in Mice*

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Thyroid hormone has profound effects on metabolic homeostasis, regulating both lipogenesis and lipolysis, primarily by modulating adrenergic activity. We generated mice with a point mutation in the thyroid hormone receptor α (TRα) gene producing a dominant-negative TRα mutant receptor with a proline to histidine substitution (P398H). The heterozygous P398H mutant mice had a 3.4-fold (p < 0.02) increase in serum thyrotropin (TSH) levels. Serum triiodothyronine (T3) and thyroxine (T4) concentrations were slightly elevated compared with wild-type mice. The P398H mice had a 4.4-fold increase in body fat (as a fraction of total body weight) (p < 0.001) and a 5-fold increase in serum leptin levels (p < 0.005) compared with wild-type mice. A 3-fold increase in serum fasting insulin levels (p < 0.002) and a 55% increase in fasting glucose levels (p < 0.01) were observed in P398H compared with wild-type mice. There was a marked reduction in norepinephrine-induced lipolysis, as reflected in reduced glycerol release from white adipose tissue isolated from P398H mice. Heart rate and cold-induced adaptive thermogenesis, mediated by thyroid hormone-catecholamine interaction, were also reduced in P398H mice. In conclusion, the TRα P398H mutation is associated with visceral adiposity and insulin resistance primarily due to a marked reduction in catecholamine-stimulated lipolysis. The observed phenotype in the TRα P398H mouse is likely due to interference with TRα action as well as influence on other metabolic signaling pathways. The physiologic significance of these findings will ultimately depend on understanding the full range of actions of this mutation.

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1 The abbreviations used are: HSL, hormone-sensitive lipase; BAT, brown adipose tissue; TR, thyroid hormone receptor; RTH, resistance to thyroid hormone; T3, triiodothyronine; T4, thyroxine; TSH, thyrotropin; RIA, radioimmunoassay; α-GPD, α-glycerol phosphate dehydrogenase.
increased body fat, elevated serum levels of leptin, glucose, and insulin. Expression of T3-dependent genes in the liver mediating lipolysis and lipogenesis were reduced. We identified a marked impairment in sympathetic-mediated lipolysis in white adipose tissue. The phenotype of the TRα P398H mice likely reflects direct interference with TRα signaling, as well as additional influences of the mutation on other metabolic signaling pathways.

**MATERIALS AND METHODS**

**Generation of TRα (P398H) Mutant Mice**—The TRα gene targeting vector and the embryonic stem cell targeting strategy were previously described (30). Briefly, a two-vector “hit and replace” approach was used, leaving only the cytosine to adenine mutation, changing codon 398 in the TRα gene from proline to histidine. No vector sequence was left in the targeted locus with this approach. Two positive clones, as determined by direct sequencing, were injected into C57BL/6 blastocysts to generate chimeras that were identified by coat color. Two chimeras were bred to C57BL/6 females. Germ line transmission of the P398H mutation was detected by direct sequencing of tail DNA from F1 offspring. The sequencing primers used were nucleotides 1438–1468 (sense primer) and nucleotides 1674–1701 (antisense primer). All animals studied were 3-month-old males, unless otherwise indicated. The experimental protocol was approved by the institutional committee for animal protection.

**Hormone Assays—**Serum—T4 and T3 concentration were determined by radioimmunoassay (RIA) (Diagnostic Products Co, Los Angeles, CA). The T4 assay required 25 μl of serum and the T3 assay 100 μl. TSH was analyzed by Dr. Roy Weiss (University of Chicago) using previously described methods (33). Leptin levels in plasma were determined by RIA (Linco Research, St. Charles, MO) using frozen sera (50 μl serum per assay). The intraassay variation was < 4% and lower-limits of detection was 0.2 ng/ml. Insulin levels were determined by sensitive RIA (Linco, Research, St. Charles, MO). The intra-assay variability was 2% and the lower limit of detection was 0.1 ng/ml.

**Glucose Assay—**Plasma glucose level was determined using the method of glucose oxidation (Accu-Check Glucose Monitor, Roche Diagnostics Co). Mice were anesthetized (halothane inhalation) and blood samples (~5 μl) were taken from the tail vein. The assay detects glucose concentrations from 10 – 600 mg/dL. The intra-assay variability was <10%. Mice were fasted for 18 h for the fasting glucose measurement.

**Metabolic Balance—**Male mice were housed in “metabolic” cages (one animal per cage) with collecting devices for urine and feces. A fixed quantity of diet (Prolab RMH 2500) was supplied to each cage for a 24-h period. Urine, feces, and unconsumed diet were measured every 24 h for 72 h. Mice were weighed on a balance (sensitivity to 1:1000 g) at baseline and after 72 h.

**Adipose Tissue Mass—**Adipose tissue mass was determined by weighing fat pads dissected from specific deposits (epididymal, inguinal, and interscapular fat pads) in 3-month-old male mice. The fat mass was expressed as a fraction of total body weight of each mouse.

**Heart Rate—**Electrocardiogram (ECG) was recorded under pentobarbital anesthesia (50 mg/kg body weight, intraperitoneal) with mice placed on a heating pad maintained at 37 °C. The electrode probes were attached to front and back feet and ECG conductive cream applied. The heart rate was monitored using a portable ECGRespiration monitor (Silogic International Ltd, UK). Each wild-type and mutant mouse was tested for 30 min. The heart rate and ECG were recorded by computer and the average rate analyzed.

**Cold Exposure—**The mice were placed in a cold room with water, but without food, and were exposed to 4 °C for 8 h. The body temperature was measured at 8 h using the thermosonde system (4000 series, YSI Inc, Yellow Spring, OH). The body weight was determined before and after cold exposure.

**Real-Time PCR—**Total RNA was isolated using Trizol (Invitrogen Inc., Carlsbad, CA), digested with DNaseI (Ambion Inc, Austin, TX) and further cleaned using RNaseasy (Qiagen Biotech, Valencia, CA). Before reverse transcription, RNA was analyzed on an agarose gel to determine the quality of RNA. Each sample was tested. RNA (5 μg) was reverse-transcribed using Superscript II (Invitrogen). The quantitative real-time PCR was performed on Opticon DNA Engine (M. J. Research, Incline Village, NV). The cDNA was diluted 1:5, 1:25, 1:125, and 1:625 to generate a standard curve. The samples to be tested were diluted 1:10 and were tested in duplicate. A standard curve was generated for each pair of primers. The mRNA level of β-actin was significantly greater than the tested genes, therefore, samples for β-actin were diluted at 1:10, 1:50, 1:250, and 1:1250 for the standard curve. The samples to be tested were diluted 1:25 and were tested in a group of four replicates. The dilution strategy was designed to perform quantitative measurements in the early linear portion of the standard curve. The data were normalized to β-actin mRNA level and expressed in arbitrary units.

**UCP1 Western blot—**Brown fat was isolated as described previously (23). Mitochondrial membrane protein (40 μg/lane) was electrophoresed on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane for Western blot analysis. The membrane was incubated with anti-UCP1 antibody (1:100 dilution) and the conjugated protein was detected by chemiluminescence using a Western blotting kit (Calbiochem-Novabiochem, San Diego, CA).

**α-Glycerol Phosphate Dehydrogenase (α-GPD) Activity—**Liver tissue was homogenized in phosphate-buffered saline containing 250 mM su-

| Table I: Serum hormone levels in male wild-type and TRα P398H mice |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | 3-Month-old     | 10–18-Month-old |
|--------------------------------|-----------------|-----------------|-----------------|-----------------|
| T3 (ng/dL)                     | T4 (μg/dL)      | TSH (mU/L)      | T3 (ng/dL)      | T4 (μg/dL)      | TSH (mU/L)      |
| Wild-type                      | 84 ± 12         | 3.6 ± 0.9       | 58 ± 12         | 87 ± 12         | 4.5 ± 0.6       | 53 ± 22         |
| P398H                          | 96 ± 9          | 4.0 ± 1.0       | 197 ± 92        | 2.2 ± 1.1       | 94 ± 43         |

*a p < 0.05 compared to wild-type.
Isolation of White Fat Cells—Epipidymal adipose tissue was removed and washed in Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4). Fat cells were isolated according to a previously described method (34). In brief, adipose tissue fragments (0.5–1.0 g) were incubated with 2 mg/ml collagenase in KRB buffer containing 40 mg/ml bovine serum albumin and 3 μmol glucose (pH 7.4). The same buffer was used for washing the fat cells. Fat cells were separated from stromal debris by centrifugation at 1,000 × g for 30 min. After the incubation, the reactions were run in duplicate. Glycerol release was determined by a standard kit assay (Sigma-Aldrich Co). The dose-response curve was analyzed for each lipolytic agent.

Lipolysis Assay—The lipolysis assay has been described in detail elsewhere (4, 35, 36). Briefly, fat cells (500 cells/100 μl) were incubated in a 96 well microtiter plate for two hours in KRB buffer containing 40 mg/ml bovine serum albumin, 3 μmol of glucose and 0.1 mg/ml ascorbic acid. Pharmacologic agents utilized included norepinephrine, forskolin (activating adenylate cyclase), dibutyryl cAMP (dcAMP, a phosphodiesterase resistant cAMP analogue), terbutaline (β2-selective adrenergic agonist), and dobutamine (β1-selective adrenergic agonist). Incubations were run in duplicate. Glycerol release was determined by a standard kit assay (Sigma-Aldrich Co). The dose-response curve was analyzed for each lipolytic agent.

Data Analysis—All data are expressed as mean ± S.E. Statistical analysis utilized ANOVA, unless otherwise noted, with significance at p < 0.05.

### Table II

**Body fat composition in male wild-type and TRα P398H mice**

|                | 3-Month-old | 10-18-Month-old |
|----------------|-------------|-----------------|
|                | Wild-type   | P398H           | Wild-type   | P398H           |
| Body weight (g)| 25.40 ± 1.65| 30.0 ± 3.35     | 32.40 ± 2.20| 42.90 ± 3.20    |
| Total fat (g)  | 0.43 ± 0.08 | 2.25 ± 0.97     | 1.49 ± 0.21 | 7.11 ± 1.43     |
| Epidypanal fat (g)| 0.36 ± 0.23| 1.44 ± 0.19     | 0.72 ± 0.20 | 4.00 ± 0.72     |
| Interscapular fat (g)| 0.03 ± 0.01| 0.28 ± 0.21     | 0.45 ± 0.26 | 1.43 ± 0.67     |
| Inguinal fat (g)| 0.04 ± 0.01 | 0.53 ± 0.40     | 0.32 ± 0.23 | 1.68 ± 0.60     |
| Total fat (% of total body weight) | 1.69 ± 0.23 | 7.48 ± 2.53     | 4.60 ± 0.21 | 16.57 ± 1.40    |

All data are mean ± S.E. (12 mice in each group). Statistical comparison is between the wild-type and P398H mutant mice.
for each primer pair. The dilution of samples was 1:25 for test samples. The mRNA levels are expressed in arbitrary units divided by the level of $\beta$-actin mRNA. The fold-change is the ratio of expression of wild-type to expression in samples from P398H mice.

Liver mitochondrial protein was isolated from wild-type and P398H mutant mice. For each assay, 80 mg of protein was used. The assay was performed in triplicates. The data shown are the mean values ± S.E. Statistical comparison is between the wild-type and P398H mutant mice.

### Table III

Expression of thyroid hormone-responsive mRNA in liver of male wild-type and TRα P398H mice

mRNA expression was analyzed by quantitative real time PCR (as described in “Materials and Methods”). A standard curve was generated for each primer pair. The dilution of samples was 1:25 for $\beta$-actin and 1:10 for test samples. The mRNA levels are expressed in arbitrary units divided by the level of $\beta$-actin mRNA. The fold-change is the ratio of expression of wild-type to expression in samples from P398H mice.

| Lipogenic enzymes          | Wild-type | P398H | Fold-change (wt/P398H) |
|---------------------------|-----------|-------|-----------------------|
| Acetyl CoA-carboxylase     | 42        | 6.7   | 6.3                   |
| Fatty acid synthase        | 227.5     | 10.7  | 21.3                  |
| Spot 14                   | 72.5      | 21.8  | 3.3                   |
| Glucose-6P-dehydrogenase  | 78.2      | 45.5  | 1.7                   |
| Lipolytic enzymes          |           |       |                       |
| Lipoprotein lipase 1      | 174       | 72.9  | 2.4                   |
| $\alpha$-GPD              | 113.5     | 25.8  | 4.4                   |
| Hepatolipase              | 43.4      | 15    | 2.9                   |
| Glucose utilization       |           |       |                       |
| Hexokinase                | 53        | 7.2   | 7.4                   |
| Glucokinase               | 60.3      | 28.8  | 2.1                   |
| Glucose-6-phosphatase      | 109.5     | 34.2  | 3.2                   |
| Others                    |           |       |                       |
| Malic enzyme              | 60        | 35.4  | 1.7                   |
| IGFFBP2                   | 331       | 76.7  | 4.3                   |
| PGC-1                     | 83.7      | 19.8  | 4.2                   |

### RESULTS

**Generation of P398H Mutant Mice**—The TRα P398H mutation was based on the RTH-associated TRβ P449H mutation. The TRα P398H and TRβ P449H mutations were previously characterized in transient transfection assays and shown to function as dominant negative receptors (38). The heterozygous P398H mutation, introduced into embryonic stem cells, showed a 50% reduction in nuclear T3 binding capacity and a significant reduction in T3-dependent gene expression in in vitro embryonic stem cell differentiation studies (30). The targeting vector contained a single nucleotide mutation (cytosine to adenine), producing a change in amino acid from proline to histidine at codon 398. Germline transmission was achieved in the C57BL/6 strain (see “Materials and Methods”). The offspring carrying the mutation were identified by PCR amplification and direct sequencing of genomic DNA from tail samples. In greater than two years of breeding, homzygous offspring were not detected. Live-born mutant mice, as well as stillborn pups, were genotyped. The homozygous TRα P398H mutation is likely to be an embryonic lethal, as has been reported for the TRα PV mutation (31). Mice homozygous for the TRα R438C mutation died at 3 weeks of age (32). Since no homozygous mice were available for study, the P398H heterozygous mice are referred to as P398H mutant mice.

The P398H mice had a higher mortality rate and reduced fertility, similar to that reported for the TRα R438C mutations (31, 32). The TRα PV mutant mice manifest dwarfism and the TRα R438C mice had delayed development (32). In contrast, the P398H mutant mice were normal in size for the first 2–3 months and then the males showed increased body weight. Female mice were normal in size. We observed that P398H mice frequently had delayed hair growth. In some cases, the mice had no body-hair for up to 8 weeks of age.

**Hormone Levels**—RTH is associated with mutations in the TRβ gene, and is manifest by elevated serum T3 and T4 concentrations and an “inappropriately” elevated TSH, with reduced responsiveness to thyroid hormone in some target tissues. At 3 months of age, the serum T4 concentration was slightly elevated in P398H mice (4.0 ± 1.0 µg/dL), compared with wild-type (3.6 ± 0.9 µg/dL). Serum T3 was increased 14% in P398H (96 ± 9 ng/dL) compared with wild-type mice (84 ± 12 ng/dL) (Table I). The mean TSH level was significantly elevated in P398H mice (197 ± 92 mU/liter) compared with
wild-type mice (58 ± 12 mU/liter) (p < 0.02), indicating impaired feedback of thyroid hormone at the hypothalamic-pituitary axis. These data are consistent with the levels of thyroid hormone in the TRα PV mice (31). The severity of thyroid hormone resistance was reduced with increasing age (>10 months). The TSH level in P398H mice (>10 months) was reduced 46% compared with the levels at 3 months of age (Table I). Similar reductions in serum TSH concentration with age were also reported in the TRα−/−, TRβ−/−, and combined knockout mice (39). Thyroid hormone levels did not change significantly with age in the wild-type mice.

Heart Rate—The chronotropic actions of thyroid hormone are thought to be mediated by TRα. In RTH (associated with TRβ mutations) and in TRβ knockout mice, increased heart rate is observed, indicating that cardiac sensitivity to elevated thyroid hormone levels is retained. In contrast, TRα knockout mice had a 20% reduction in heart rate (18). The heart rate in P398H mice (3-month-old), as determined by an average of a 30-min electrocardiogram (ECG) recording, was 420 ± 45 beats/min, 14% lower than that in wild-type mice (490 ± 41 beats/min) (p < 0.01). The lower heart rate in P398H mice is consistent with reduced cardiac sensitivity to thyroid hormone. Considering P398H mice have elevated serum thyroid hormone, the reduced heart rate reflects reduced sensitivity to both thyroid hormone and sympathetic stimuli.

Fat Accumulation—At age >3 months, the male P398H mice weighed 15–20% more than age-matched wild-type littermates (Fig. 1). The total body adipose tissue (from epididymal, inguinal and interscapular fat pads combined) in male P398H mice expressed as a fraction of total body weight, was 4.4-fold greater than wild-type (1.7% versus 7.5%, p < 0.01) (Fig. 1). The majority of adipose tissue was found in the epididymal fat pad. Only a trace inguinal fat pad, which accounted for 23% of total fat in P398H mice, was found in wild-type mice (Table II). To determine the basis of increased fat mass in P398H mice, we performed a metabolic balance study. The food intake, body weight, feces, and urine were quantified every 24 h for 72 h. The P398H mice consumed significantly less food (65% of wild-type) (p < 0.01) and produced less feces (48% of wild-type) (p < 0.05) and urine (37% of wild-type) (p < 0.05) (Fig. 2). These findings are consistent with increased adiposity from a primary metabolic defect and not related to excess food intake.

Leptin, Glucose, and Insulin Levels—Leptin is secreted by adipose tissue and plays a key role in regulating food intake, energy expenditure, and body weight homeostasis (40). In general, serum leptin levels are proportional to adipose tissue mass. The serum leptin concentration in 3-month-old P398H mice was 5-fold greater than that in wild-type mice, consistent with the increased fat mass (Fig. 3A). Elevated serum leptin levels act as a feedback signal to reduce food intake, as was seen in the P398H mice. Serum leptin levels and fat mass, in both wild-type and P398H mice, increased with age. At 10–18 months of age, serum leptin was increased 1.93-fold in P398H mice (10.1 ± 3.2 ng/ml) and 5.26-fold in wild-type (5.3 ± 2.5 ng/ml) (Fig. 3A) compared with 3-month-old mice. Total fat increased 2.3-fold in P398H mice (16.7% ± 6.0) and 3.1-fold in wild-type mice (4.5% ± 0.2), compared with mice at 3 months of age.

Excess white adipose tissue is associated with impaired insulin action and elevated serum glucose levels. The random glucose level in P398H mice was 179 ± 8 mg/dL, 26% higher than levels in wild-type mice (142 ± 7 mg/dL) (p < 0.01). After an 18-hour fast, glucose levels were 55% higher in P398H mice (142 ± 5 mg/dL) compared with wild-type mice (92 ± 16 mg/dL) (Fig. 3B). Fasting insulin levels in P398H mice (1.24 ± 0.45 ng/ml) were significantly greater than in wild-type mice (0.45 ± 0.25 ng/ml) (Fig. 3C). The elevation of fasting glucose and insulin levels are consistent with insulin resistance in the P398H mice.

Thyroid Hormone Responsive Gene Expression in the Liver—α-Glycerol phosphate dehydrogenase (α-GPD) activity is a well characterized marker of thyroid hormone action on basal metabolic rate and is reduced in hypothyroid rats (41). We measured the enzymatic activity of α-GPD in the liver of P398H mice (Fig. 4). The α-GPD activity was reduced 30% at 3 months and 50% in older mice, compared with wild-type mice, indicating reduced action of thyroid hormone on stimulation of metabolic rate.

Thyroid hormone regulates lipolysis and lipogenesis as well as oxidative processes in the liver. We, therefore, examined the mRNA expression of enzymes involved in lipogenesis, lipolysis, and glucose utilization in the liver (Table III). The mRNA levels of acetyl-CoA carboxylase, a rate-limiting enzyme in fatty acid synthesis, was down regulated 6.3-fold in P398H mice. Fatty acid synthase (FAS), a key lipogenic enzyme, was reduced 21-fold in P398H mice compared with wild-type. Lipolytic enzymes including; lipoprotein lipase I, α-GPD, and hepatoplipase were decreased 2.4-, 4.4-, and 2.9-fold, respectively, in P398H mice compared with wild-type. Hexokinase is abundant in liver and catalyzes the first step of glucose breakdown. The expression of this enzyme in P398H mice was reduced 7.4-fold compared with wild type. In general, all of the T3-regulated genes in the liver tested had reduced mRNA levels.

In Vitro Lipolysis Assays—Excess thyroid hormone enhances
catecholamine-induced lipolysis in adipocytes. Deficiency in thyroid hormone reduces metabolic rate and increases fat storage. P398H mice had markedly enhanced fat storage, slowed metabolism and reduced α-GPD activity in the liver. We hypothesized that the excess fat accumulation in P398H mice was due to reduced sensitivity to catecholamine-simulated lipolysis. Norepinephrine treatment of isolated wild-type white adipocytes showed a dose-dependent increase in lipolysis, as reflected in glycerol production (Fig. 5A). Fat cells isolated from P398H mice had almost no response to norepinephrine. The maximum lipolytic response to norepinephrine was reduced by 73% per 1,000 fat cells in P398H mice compared with wild type. The ED₅₀ calculated from the dose-response curve was 6 × 10⁻¹¹ M for wild-type and 1.5 × 10⁻⁹ M for P398H, a 400-fold reduction in sensitivity. When lipolysis was stimulated at the postreceptor level with forskolin (activating adenylate cyclase) the lipolytic response was diminished in P398H-derived adipocytes compared with wild type. (Fig. 5B). The response to dcAMP (a phosphodiesterase resistant cAMP analogue) was also reduced (data not shown).

These data indicate that the sensitivity to catecholamine-induced lipolysis in adipocytes isolated from P398H mice was significantly reduced at both the receptor and postreceptor levels. A downstream cAMP activated target of catecholamine is HSL. We measured HSL mRNA expression in adipose tissue and found reduced mRNA levels in P398H mice (1.03 ± 0.42 arbitrary units) compared with wild-type mice (3.30 ± 0.65) (p < 0.001).

We tested adrenergic receptor sensitivity using the β2 selective agonist, terbutaline. Terbutaline stimulation produced only minimal lipolytic activity in P398H-derived fat cells (Fig. 6A). The maximum glycerol release stimulated by terbutaline was reduced more than 80% compared with wild-type-derived cells (p < 0.0001) and the ED₅₀ was shifted significantly to the right. A similar reduction in glycerol release in P398H-derived fat cells compared with wild type was also seen after stimulation with the β1 selective agonist, dobutamine (Fig. 6B).

We also examined the mRNA levels of adrenergic receptors in fat cells by quantitative real-time PCR. Adrenergic receptor β1 and β2 mRNA was not significantly different in fat cells of P398H mice and wild-type mice (Fig. 7, A and B). The α1- and α2-adrenergic receptor mRNA levels were, however, increased 6-fold in older P398H mice compared with wild-type mice (Fig. 7, C and D). These data are consistent with recent studies using human primary adipocyte culture (42). These results indicated that impaired lipolysis in P398H mice was not due to reduced expression of β-adrenergic receptors. Additionally, the increase in α2-adrenergic receptor, known to antagonize β-mediated enhancement of lipolysis, may contribute to the reduced lipolysis seen in P398H mice (43-45).

**Thermal Response to Cold Exposure**—The adaptive thermogenic activity of BAT requires T3, sympathetic input, and UCP1. Hypothyroid mice are intolerant of cold exposure due to reduced UCP1 production and reduced responsiveness to catecholamines in brown fat. We previously showed that augmented catecholamine action by thyroid hormone in brown fat is TRα-mediated (23). We wanted to determine if the TRα P398H mutation influenced adaptive thermogenesis. At baseline, the core temperature was 0.5 °C lower in P398H mice (37.6 ± 0.37 °C) compared with wild type (38.1 ± 0.28 °C). After cold exposure for 8 h, the core temperature of P398H mice dropped 3.9 °C to 33.7 ± 2.3 °C, while the core temperature of wild-type mice only dropped 1.4 °C to 36.7 ± 2.2 °C (Fig. 8A). In brown fat, UCP1 protein levels were not different between wild-type and P398H mice (Fig. 8B), consistent with UCP1 regulation by TRβ.
FIG. 8. Temperature response to cold exposure. Wild-type and P398H mice were exposed to 4°C for 8 h. A, core temperature in response to cold and 8°C, UCP1 protein level in BAT. The data shown are the mean values ± S.E. Statistical comparison is between the wild-type and P398H mutant mice. Brown fat was isolated after cold exposure and 60 mg of mitochondrial protein was loaded in each lane for the Western blot.

DISCUSSION

The TRα P398H mutation disrupted metabolic homeostasis with a significant increase in fat accumulation and associated insulin resistance. The fat accumulation in P398H mice was associated with reduced sympathetic-mediated lipolysis in white adipose tissue. A recent clinical study demonstrated that white fat glycerol release in vitro in response to catecholamines was markedly enhanced in samples from fat biopsies of thyrotoxic patients (4). The catecholamine responsiveness returned to normal in fat samples from the same group of patients once they were euthyroid (4).

There is significant similarity in phenotype among the reported TRα point mutant models (31, 32). The most striking similarity among the models is that the homozygous condition is an embryonic or early neonatal lethal for all TRα mutations (31, 32). This was also found in one of the TRα knockout models (46), but not in others (17, 21). The lethality in the single TRα knockout model is likely a function of the site of TRα disruption, and the influence of other TRα gene products. The thyroid function tests were only mildly disrupted in the TRα mutations. The P398H and PV mutants (31) share the finding of mildly elevated serum T4 and T3 concentrations and the R438C mutation (32) had mildly reduced serum T4 and T3 concentrations. Reduced adrenergic activity is also a commonality as reflected in reduced heart rate compared with wild-type (31, 32). Reduced core body temperature and heart rate, not corrected by T3 administration, was reported in a TRα knockout model (18). The P398H TRα mutation impairs T3-dependent gene expression, demonstrated by both in vitro and in vivo studies, similar to other TR dominant negative mutations.

The metabolic phenotype we report in the P398H mice, however, was not seen in the TRα PV or R438C mutants. The metabolic phenotype does not appear to be related to thyroid hormone status, since the serum levels do not vary significantly among the TRα models. The metabolic phenotype, therefore, is likely due to interference with T3-mediated catecholamine potentiation as well as additional effects of the mutant receptor on metabolic signaling pathways.

There are a number of potential mechanisms for the unique metabolic actions of the P398H TRα mutant. It is well known from in vitro studies, as well as emerging structural studies, that the various TR mutants can differentially influence ligand binding, protein-protein and protein-DNA interactions, and differentially recruit coactivators and corepressors (47-51). A number of nuclear receptors are involved in metabolic regulation, including the PPAR family and liver X receptor (LXR). Both PPAR and LXR heterodimerize with RXR as does TR. LXR has been reported to bind to a direct repeat with a 4-base gap (DR4) element, which may compete with TR binding (52, 53). The P398H TRα mutant may differentially influence PPAR and LXR action by competing with RXR for heterodimerization, or by directly competing for DNA binding sites. This may lead to abnormal regulation of PPAR and LXR target genes (37).

The metabolic phenotype was observed in P398H male, but not female, mice. Several targeted genes that are involved with insulin action or signaling have shown manifestations in male, but not female, rodents. An example is the significant metabolic abnormality in male mice as a result of IRS-2 disruption (54). Possible mechanism mediating the male predominance of the metabolic phenotype include low levels of free testosterone and sex hormone binding globulin associated with obesity and insulin resistance (55-57). Androgens reduce serum levels of adiponectin, which is associated with a high risk of insulin resistance (58). Hepatocyte CYP450, a gene involved in liver oxidative metabolism, is deficient in male RXRα-hepatocyte selective-deficient mice, but not in females (59). Castration increased CYP450 mRNA levels in these mice, indicating that testosterone reduced CYP450 activity (59). It will be important to determine if the male predominance of the metabolic phenotype in the TRα P398H mutant is primarily due to interaction with testosterone, or other gender-influenced factors in fat metabolism, insulin release, or action. It should be possible, by varying sex steroid concentrations, to determine the primary influence on the gender-specific phenotype.

Insulin resistance was observed in the P398H mice. In adipocytes, glucose is transported by insulin-sensitive glucose transporter 4 (GLUT-4). GLUT-4 is activated by both insulin and thrombogenic hormone. Excess thyroid hormone increases GLUT-4 protein concentration in the membrane and enhances glucose uptake by GLUT-4 in adipocytes (57). In the case of P398H mice, both insulin-resistance and adipose tissue resistance to thyroid hormone action likely account for the high level of glucose in the serum. Hyperleptinemia in P398H mice may secondarily influence thyroid axis regulation at the level of the TRH neuron in the paraventricular nucleus of the hypothalamus (60, 61).

Several recent investigations have emphasized the importance of parasympathetic innervation of subcutaneous and intra-abdominal fat (37, 62). Parasympathetic input may counteract catecholamine action in fat and promote energy storage. Reduced thyroid hormone action may augment parasympathetic action in addition to reducing catecholamine stimulation. We identified increased α1- and α2-adrenergic receptor mRNA levels in white fat of P398H mice.

The TRα P398H mutation disrupted thyroid hormone signaling and had a significant impact on metabolic homeostasis. These findings point to the central importance of thyroid hormone augmentation of catecholamine-stimulated lipolysis in balancing regulation of lipogenesis and lipolysis. The metabolic phenotype in the P398H mice is distinct from the previously reported TRα point mutants. It is likely that this mutant will have unique interactions with TR partners and related nuclear receptors involved in metabolic regulation.
