Hyperleptinaemia in mice induced by administration of the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine

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Abstract α-methyl-*p*-tyrosine (αMPT), an inhibitor of tyrosine hydroxylase, was administered to mice to block noradrenaline synthesis. Ten hours after injection of αMPT there was a 6-fold increase in plasma leptin. The level of *ob* mRNA in epididymal white adipose tissue was also increased, but UCP1 mRNA in brown fat fell. In contrast to lean mice, *ob* mRNA in white fat of *ob/ob* mice was not increased by αMPT. αMPT raised plasma leptin in fasted as well as fed mice. Hyperleptinaemia was attenuated by treatment with a β3-adrenoceptor agonist. Inhibition of noradrenaline synthesis leads to the rapid induction of hyperleptinaemia; it is suggested that sympathetic tone plays a pivotal role in regulating leptin production.
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Key words: α-Methyl-*p*-tyrosine; β3-Adrenoceptor; Leptin; *ob* gene; Sympathetic nervous system; White adipose tissue

1. Introduction

Leptin, the protein product of the *ob* (obese) gene, is a 16 000 M_r hormone, whose principle site of synthesis is white adipose tissue [1], although it is now evident that it is also produced in brown fat [2–4] and the placenta [5,6]. Leptin is considered to be a signalling molecule to the brain in the regulation of whole-body energy balance [1,7–9] and in conveying the extent of the adipose tissue stores for the initiation of reproduction [10,11]. It is also increasingly recognised that leptin is an important metabolic hormone, influencing processes such as insulin secretion and glucose utilisation [12,13]. A key issue is how the production of leptin is regulated, and a number of factors which influence the synthesis of the hormone by white adipose tissue have been identified; these include fasting, catecholamines, insulin, dexamethasone and thiazolidiones [14–20].

Noradrenaline, isoprenaline and β3-adrenoceptor agonists have a powerful inhibitory effect on *ob* gene expression and induce a rapid fall in circulating leptin [15,21–23]. From this it is suggested that the sympathetic nervous system is involved in a negative feedback loop to adipose tissue regulating *ob* gene transcription through β3-adrenoceptors [23–25], and we have recently hypothesised that physiologically the sympathetic system may be the pivotal regulator of leptin production [25]. This proposition has parallels with the central role long attributed to the sympathetic system in the regulation of lipolysis in white fat [26,27].

In the present report we show that treatment with α-methyl-*p*-tyrosine (αMPT), a specific inhibitor of tyrosine hydroxylase [28], the rate limiting enzyme in the synthesis of noradrenaline, leads to a rapid and substantial increase in circulating leptin. This appears to be due to an increase in *ob* gene transcription, resulting from the inhibition of normal sympathetic tone.

2. Materials and methods

2.1. Animals

Male mice, aged 8 weeks, were used in this study. They were either of the Aston variety, both lean (+/?) and obese (*ob/ob*), from a colony bred at the Rowett Research Institute, or of the C57BL/6J strain (Harlan Olac). They were housed at 22°C in a room with a 12 h light/12 h dark cycle (lights on at 07:00 h) and fed a commercial rodent diet (Biosure; Special Diet Services) containing 18% protein and 2.4% fat (w/w). The animals were given ad libitum access to both food and water.

In the majority of experiments mice were injected intraperitoneally with αMPT (methyl ester, Sigma; 300 mg/kg body wt) [29] in saline (0.9% NaCl) at 0 and 5 h, with the control animals receiving injections of saline alone. Blood was taken and the animals killed by cervical dislocation at 10 h after the first injection. Epididymal (and subcutaneous) white adipose tissue, together with the interscapular brown fat pads, were rapidly removed and frozen in liquid nitrogen. Blood was collected into heparinised tubes and centrifuged to obtain the plasma. The plasma was stored at −80°C until analysed for leptin.

In some experiments, mice were treated with BRL 35135A (a gift from SmithKline Beecham Pharmaceuticals) as well as with αMPT. The β3-adrenoceptor agonist was injected subcutaneously at a dose of 750 μg/kg body wt at 0 and 5 h (at the same time as αMPT) and tissues removed at 10 h. Control mice received injections of saline alone.

In one experiment mice were fasted for 24 h in cages with wire-mesh floors, and αMPT (or saline) injected at 6, 14 and 19 h after the removal of food. Control mice were given free access to food and received αMPT or saline. At 24 h after the start of the experiment, tissues and blood were removed.

2.2. Northern blotting

Total RNA was extracted from adipose tissue using a guanidium isothiocyanate-phenol method, and separated according to size by agarose gel electrophoresis [14,15]. The RNA was blotted onto a nylon membrane (Boehringer Mannheim) by vacuum blotting and fixed with UV light. *ob* mRNA was detected by a chemiluminescence procedure, utilising a 33-mer antisense oligonucleotide (5′-GGTCTGAGGCAGGGAGCAGCTCTTGGAGAAGGC′) probe end-labelled (5′) with digoxigenin (Boehringer Mannheim), as described previously [14,15]. A similar procedure was used for the detection of UCP1 mRNA, using a 32-mer antisense oligonucleotide (5′-CGGACTTTGGCGGTGTCCAGCGGGAAGGTGAT) [15]. The oligonucleotides were synthesised commercially (Oswel DNA Services).

Hybridisation was performed overnight at 42°C in pre-hybridisation buffer containing the antisense oligonucleotide (25 ng/ml). Post-hybridisation washes were performed as previously [14,15], and the membranes then incubated with an anti-digoxigenin Fab/alkaline phosphatase conjugate (Boehringer Mannheim). CDP-*Star* (Tropix) was used as the chemiluminescence substrate. Signals were detected

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Abbreviations: αMPT, α-methyl-*p*-tyrosine

on film and quantified by densitometry. After exposure to film (5–60 min) the membranes were stripped and re-probed for 18S rRNA using a 31-mer digoxigenin-labelled antisense oligonucleotide (10 pg/ml) [15].

2.3. Immunoassay for leptin

Plasma leptin was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) [20] employing a rabbit anti-murine leptin IgG prepared at the Rowett. A detection limit of 100 pg of leptin/ml was achieved by utilising a biotin-avidin sensitivity enhancing system [20]. A recombinant murine leptin standard (R and D Systems Europe) was used in the assay.

2.4. Data analysis

The statistical significance of differences between groups was assessed by Student's unpaired t-test. Densitometric data from Northern blots were normalised to control mice, which were assigned an arbitrary value of 1. mRNA levels were corrected for differences in gel loading or blotting by reference to the level of 18S rRNA.

3. Results

αMPT was administered at dose levels and in a protocol similar to that employed in studies on the turnover of noradrenaline in tissues [28–30]. Administration of αMPT to mice led to a 6-fold increase in the level of plasma leptin by 10 h after the first of the two injections (Fig. 1). The level of *ob* mRNA was then examined in the epididymal white adipose tissue to determine whether treatment with αMPT led to changes in *ob* gene expression. The treated mice exhibited a 3-fold increase in the level of *ob* mRNA compared with the untreated animals (Fig. 2a). There was also an increase (1.4-fold) in *ob* mRNA in the subcutaneous adipose tissue of the treated animals, but the level of the mRNA in this fat depot was very low and the increase was not statistically significant ($P > 0.05$; results not shown).

UCP1 mRNA in interscapular brown adipose tissue was also examined following the administration of αMPT. Treatment with the drug led to a substantial fall in UCP1 mRNA level, to one-third of that in untreated animals (Fig. 2b). When αMPT was administered to obese (*ob/ob*) mice, there was no increase in *ob* mRNA in epididymal white fat, in contrast to lean animals; indeed, there was a small, though not statistically significant, decrease (Fig. 3).

In the next experiment, mice treated with αMPT were also injected with a selective β3-adrenoceptor agonist, BRL 35135A, to determine whether this would counter the hyperleptinaemia induced by αMPT. The administration of BRL
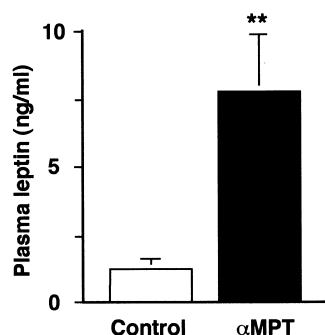
Fig. 2. Effect of αMPT on (a) *ob* mRNA level in epididymal white adipose tissue and (b) UCP1 mRNA level in interscapular brown fat of mice. Mice were treated as in Fig. 1. Representative Northern blots are shown in the upper panel of (a) and (b). The lower panels show the quantification of mRNA levels expressed relative to the controls, with the results given as mean values ± S.E. (bars) for six mice in each group. **$P < 0.01$, ***$P < 0.001$, compared with control mice.

35135A alone to untreated mice led to a reduction in circulating leptin (Fig. 4). In the mice treated simultaneously with αMPT, BRL 35135A produced a significant attenuation in the circulating level of leptin, although it was not restored to normal (Fig. 4).

Fasting is associated with a fall in circulating leptin [20], and this was also observed in the present study (Fig. 5). However, the administration of αMPT reversed the fasting-induced decrease in plasma leptin. Indeed, the leptin levels of the fasted animals treated with αMPT were elevated to values similar to those of the treated fed mice (Fig. 5).

Fig. 3. Effect of αMPT on *ob* mRNA level in epididymal white adipose tissue of *ob/ob* mice. Mice were treated as in Fig. 1. Representative Northern blots are shown in the upper panel. The lower panel shows the quantification of mRNA levels expressed relative to the controls, with the results given as mean values ± S.E. (bars) for eight mice in each group.

Fig. 1. Effect of αMPT on plasma leptin in mice. Mice were injected with αMPT at 0 and 5 h and blood taken at 10 h. The results are given as mean values ± S.E. (bars) for eight mice in each group. **$P < 0.01$, compared with control mice.
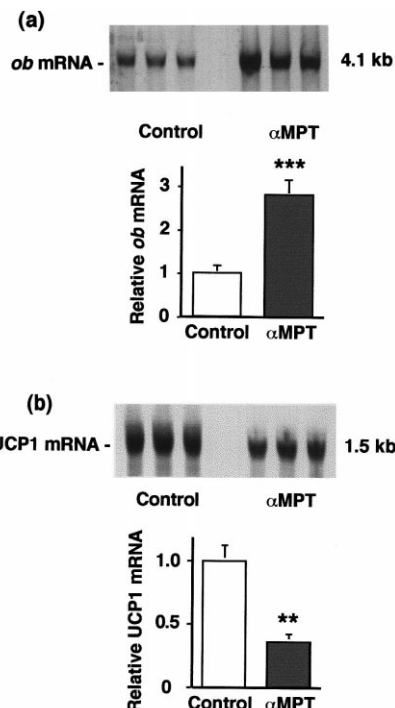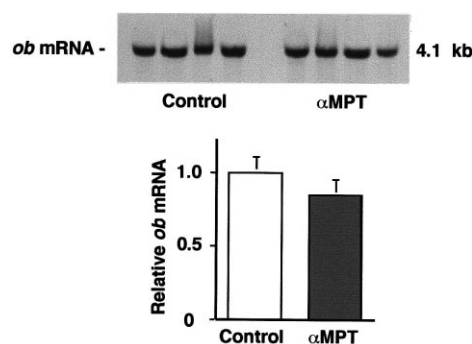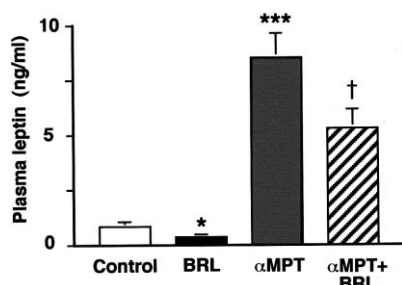
Fig. 4. Effect of the β3-adrenoceptor agonist BRL 35135A on plasma leptin in mice treated with αMPT. Mice were injected with either BRL 35135A, αMPT, or BRL 35135A plus αMPT, at 0 and 5 h and blood taken at 10 h. Control mice received saline injections. The results are given as mean values ± S.E. (bars) for seven mice in each group. *$P < 0.05$, ***$P < 0.001$, compared with control mice; †$P < 0.05$, compared with the αMPT treated group.

4. Discussion

The present study demonstrates that administration of αMPT to mice leads to the rapid induction of a substantial hyperleptinaemia, with plasma leptin levels being increased several-fold a few hours after the injection of the compound. Increases in circulating leptin could, in principle, be due to a reduction in clearance or to a stimulation of the production of the hormone. Since there was a marked elevation in the level of *ob* mRNA in the epididymal white adipose tissue of mice treated with αMPT, it is apparent that increases in production underlie the induction of the hyperleptinaemia.

αMPT is a potent and specific inhibitor of tyrosine hydroxylase, the rate limiting enzyme in the synthesis of noradrenaline, and it has been widely used as a tool in the measurement of sympathetic activity [28–30]. By inhibiting synthesis, the rate of noradrenaline depletion provides an index of sympathetic activity in a given tissue. However, depletion of noradrenaline by αMPT also leads to sympathetic blockade. Thus the probable mechanism by which αMPT affects leptin production is through a reduction in noradrenaline synthesis, and hence a consequent decrease in the tonic sympathetic

Fig. 5. Effect of αMPT on plasma leptin in fasted mice. Mice were fasted for 24 h and injected with αMPT at 6, 14 and 19 h after the initiation of the fast, and blood taken at 24 h. Control mice were given free access to food. The results are given as mean values ± S.E. (bars) for eight mice in each group. **$P < 0.01$, ***$P < 0.001$, compared with control mice; †††$P < 0.001$, compared with the fasted group.

stimulation of white adipose tissue. Expression of the UCP1 gene in brown fat is stimulated by the sympathetic system [31], and the concomitant fall in the level of UCP1 mRNA in treated animals indicates that αMPT was having its expected pharmacological effect.

Previous studies have shown that noradrenaline and isoprenaline strongly inhibit leptin production, this occurring through the suppression of *ob* gene expression [15]. These catecholamines operate via β3-adrenoceptors, β3-agonists being potent inhibitors of leptin production [21–25]. Thus it is probable that αMPT leads to an increase in circulating leptin levels by blocking the sympathetically mediated suppression of *ob* gene expression. The increase in plasma leptin induced by αMPT was attenuated by simultaneous treatment with BRL 35135A, a selective β3-adrenoceptor agonist, and this is consistent with the β3-agonist directly substituting for the reduced sympathetic activity consequent upon the inhibition of noradrenaline synthesis.

Fasting leads to a substantial fall in both *ob* gene expression and in circulating leptin levels [14,16,20]. Treatment with αMPT not only reversed the fasting-induced reduction in leptin, but led to a substantial increase, similar to that in the fed mice. It has recently been shown by direct measurements of noradrenaline turnover that fasting results in a selective activation of the sympathetic stimulation to white adipose tissue [32], sympathetic activity in other tissues such as brown fat and the heart being decreased in the fasted animal [30]. It has been proposed subsequently that the inhibitory effects of fasting on leptin production are mediated primarily through the increase in sympathetic activity in white fat [24,25].

Treatment with αMPT did not lead to a rise in the level of *ob* mRNA in obese *ob/ob* mice, which lack functional leptin. These animals exhibit a substantial reduction in β3-adrenoceptor mRNA in white fat relative to lean mice where the level of this mRNA is much higher than that of the mRNAs encoding β1- or β2-adrenoceptors [33]. *ob/ob* mice also show a marked blunting in the stimulation of adenyl cyclase activity by β3-agonists [34]. Thus in these mutants the inhibition of sympathetic stimulation in white fat induced by αMPT would not be expected to lead to the increase in *ob* mRNA level seen in the normal mouse. This is also consistent with the absence of a suppressive effect of fasting on *ob* mRNA levels in *ob/ob* mice [14], if the view is correct that sympathetic activation in white fat is the primary cause of the fall in *ob* gene expression in the fasted animal.

In addition to being a tool for the measurement of sympathetic activity in peripheral tissues, αMPT has been used clinically to treat the hypertension of patients with phaeochromocytoma, a tumour of the adrenal medulla which leads to a hypersecretion of catecholamines. It is conceivable, therefore, that the induction of hyperleptinaemia by αMPT could partly occur through a depletion of circulating adrenaline, with adrenaline playing a role in the catecholamine-induced suppression of *ob* gene expression in white fat.

In conclusion, the central implication of the findings reported here is that expression of the *ob* gene and the resultant production of leptin may normally be constrained by the tonic activity of the sympathetic system – most probably directly through the sympathetic innervation of white adipose tissue. The results suggest that αMPT may be a useful pharmacological tool for investigating the consequences of chronic hyperleptinaemia. They also suggest that the use of αMPT to meas-
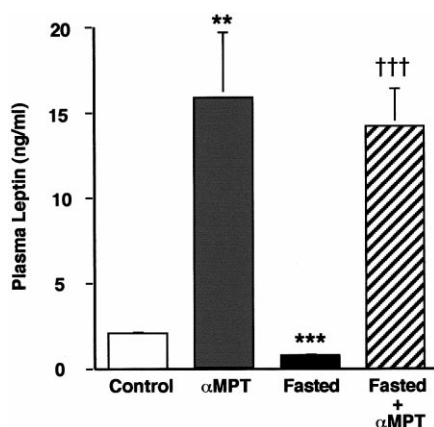
ure sympathetic activity may be problematic since leptin itself is known to stimulate sympathetic activity [35].

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