Regulation of leptin production: a dominant role for the sympathetic nervous system?

Paul Trayhurn*, Jacqueline S. Duncan, Nigel Hoggard and D. Vernon Rayner

Molecular Physiology Group, Division of Biomedical Science, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK

Unravelling the diverse hormonal and neuroendocrine systems which regulate energy balance and body fat has been a long-standing challenge in biology, with obesity as an increasingly important public health focus. A major development in energy balance regulation has come with the discovery in 1994 of a 'new' hormone, leptin, the protein product of the *ob* (obese) gene (Zhang *et al.* 1994). The initial concept was that leptin was synthesized in white adipose tissue, being secreted into the circulation as a signal of the extent of the fat stores, in accordance with the lipostatic model of the regulation of energy balance (Kennedy, 1953). The newly-identified hormone was first viewed as influencing energy balance by acting centrally to inhibit food intake, i.e. leptin was considered to be a satiety factor (Zhang *et al.* 1994).

The original view of the leptin system (where the hormone is produced, the target tissues, and the physiological functions that are regulated), however, has evolved considerably over the past 3 years. In the present article we describe the sites of leptin synthesis and discuss the regulation of the production of the hormone. We consider, in particular, whether physiologically the sympathetic nervous system (SNS) plays a dominant role in controlling the production of leptin in white adipose tissue.

Where is leptin produced?

White adipose tissue is the main site of leptin production, but it is now evident that the hormone is also produced in other tissues. Initial studies on brown adipose tissue were equivocal, with some authors observing little or no evidence for expression of the *ob* gene by Northern blotting (Maffei *et al.* 1995*a*; Trayhurn *et al.* 1995*b*), while others reported high levels of *ob* mRNA in the organ (Moinat *et al.* 1995). In our laboratory we have not generally detected *ob* mRNA on Northern blots of total RNA from brown fat, but *ob* gene expression is evident when the more sensitive technique of reverse transcription–polymerase chain reaction is employed. Definitive evidence for brown adipose tissue as a site of leptin production has now come, however, from several different directions: (1) studies on brown adipocytes in primary culture (Deng *et al.* 1997); (2) studies on brown adipose tissue of newborn rats (Dessolin *et al.* 1997*b*) and Djungarian hamsters (Klingenspor *et al.* 1996); (3) an immunohistochemical study on *db/db* mice (Cinti *et al.* 1997). A central issue is the physiological role of leptin produced by brown fat, one possibility being that it simply adds to the pool of circulating hormone as a reflection of the total combined amount of adipose tissue (white and brown); its contribution would, however, be small relative to that of white fat (Giacobino, 1996). The significance of brown fat as a site of leptin synthesis is made more problematic by the fact that the tissue is neither present in all mammals nor is it necessarily evident throughout life in those species in which it does occur (see Trayhurn, 1995).

An important new dimension to leptin biology has come with the recognition that the placenta expresses the *ob* gene and is a site of production of the hormone; this has now been demonstrated in mice, rats and man (Hassink *et al.* 1997; Hoggard *et al.* 1997; Masuzaki *et al.* 1997). Placental synthesis is particularly intriguing, and suggests that leptin may either be a novel growth factor or act as a signal of energy status between the mother and the fetus. The placenta also expresses the leptin receptor gene, implying that the organ is a target for the action of leptin as well as being a source of the hormone (Hoggard *et al.* 1997). *In situ* hybridization and immunohistochemical studies on pregnant mice have demonstrated leptin synthesis in several regions of the fetus, including the heart, bone and cartilage, and cells of the hair follicle, observations which are discussed in more detail in the accompanying article (Hoggard *et al.* 1998). This diverse picture of where leptin is produced indicates that the functions of the hormone extend considerably beyond the basic lipostatic model originally envisaged.

White adipose tissue is a heterogeneous organ located in a number of distinct sites. Although each of the major white fat depots appears to express the *ob* gene, there are considerable differences between sites in the relative level of *ob* mRNA (Maffei *et al.* 1995*a*; Trayhurn *et al.* 1995*b*). In adult rodents, *ob* mRNA level is much higher in the gonadal and perirenal sites than in subcutaneous tissue (Trayhurn *et al.* 1995*b*). In contrast, in sucking rats during the first 1–2 weeks after birth the subcutaneous fat is the main site of *ob* gene expression (Rayner *et al.* 1997). If relative levels of *ob* mRNA can be taken to reflect the pattern of leptin production, it would seem that there are major differences between depots in the extent to which they contribute to the total circulating pool of the hormone. Furthermore, the rodent data also imply that there are developmental shifts in the relative

Abbreviations: SNS, sympathetic nervous system.

***Corresponding author:** Professor Paul Trayhurn, fax +44 (0)1224 716622, email pt@rri.sari.ac.uk

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importance of different adipose tissue sites to leptin production. In sucking animals the subcutaneous fat is of greatest importance, whilst in the mature animal the internal depots are more significant.

There are also distinct species variations, in that in adult human subjects the subcutaneous adipose tissue has much higher levels of *ob* mRNA, and is likely, therefore, to be more important in leptin production than the omental tissue (Hube *et al.* 1996; Montague *et al.* 1997). The production of a hormone at varying levels from a number of different sites raises questions in terms of co-ordination, particularly given the evidence that leptin is secreted in a pulsatile manner (Sinha *et al.* 1996; Licinio *et al.* 1997).

Regulation of leptin production

The level of *ob* mRNA in white adipose tissue and the circulating leptin concentration are increased markedly in obesity, as shown in both human studies and in studies on several types of obese animal (Frederich *et al.* 1995; Funahashi *et al.* 1995; Lönnqvist *et al.* 1995*a*; Maffei *et al.* 1995*b*; Ogawa *et al.* 1995; Trayhurn *et al.* 1995*b*; Considine *et al.* 1996; Hardie *et al.* 1996*b*; Ostlund *et al.* 1996). Indeed, in human subjects there is a high correlation between BMI and circulating leptin (Considine *et al.* 1996; Ostlund *et al.* 1996). Thus, the greater the amount of adipose tissue, the higher the level of the hormone. However, acute (and chronic?) regulation of leptin synthesis is superimposed on the endogenous production associated with the extent of the adipose tissue mass.

Both *in vivo* and *in vitro* studies have been undertaken to examine the factors which influence expression of the *ob* gene and the production of leptin. In *in vitro* studies direct rather than indirect effects of a regulatory factor can, of course, be defined. With white adipose tissue three different *in vitro* systems are available: (1) incubation of isolated mature adipocytes (Murakami *et al.* 1995; Gettys *et al.* 1996; Hardie *et al.* 1996*a*; Rentsch & Chiesi, 1996; Slieker *et al.* 1996); (2) adipocyte clonal cell lines, e.g. 3T3L1 cells (MacDougald *et al.* 1995; LeRoy *et al.* 1996; Dessolin *et al.* 1997*a*); (3) adipocytes differentiated in primary culture from fibroblastic pre-adipocytes (Wabitsch *et al.* 1996; Mitchell *et al.* 1997). Isolated mature adipocytes will secrete leptin during incubation for a period of 24–48 h (Hardie *et al.* 1996*a*), while white adipocytes differentiated in primary culture (both human and rat) have been shown to secrete the hormone over a period of about 2 weeks following differentiation (Wabitsch *et al.* 1996; Mitchell *et al.* 1997).

Adipocyte cell culture studies and studies *in vivo* have shown that both insulin and glucocorticoids stimulate *ob* gene expression (Becker *et al.* 1995; De Vos *et al.* 1995; Saladin *et al.* 1995; Leroy *et al.* 1996; Wabitsch *et al.* 1996). Testosterone (or dihydrotestosterone), on the other hand, inhibits leptin production in primary cell culture (Wabitsch *et al.* 1997). Since there is a negative relationship between serum testosterone and BMI in men (Elbers *et al.* 1997; Wabitsch *et al.* 1997), this is consistent with the concept that a suppressive effect of androgens is the primary reason for the lower circulating levels of leptin in males compared with females, even when adjustments for body fatness are made.

Some modest inhibition of leptin production by growth hormone has been observed in human adipocytes in primary culture (Wabitsch *et al.* 1997), although not with mature rat adipocytes (Hardie *et al.* 1996*a*). Hypophysectomy results in a fall in *ob* mRNA levels, but this is not reversed by the administration of growth hormone (Boni-Schnetzler *et al.* 1996). Other agents which influence expression of the *ob* gene in white fat include thiazolidinediones (drugs that activate peroxisome proliferator-activated receptor γ), which are inhibitory (De Vos *et al.* 1996; Kallen & Lazar, 1996). There is conflicting evidence as to the effects of thyroid hormones on leptin production, with some suggesting that triiodothyronine is inhibitory (Fain *et al.* 1997), while others suggest that it is stimulatory (Yoshida *et al.* 1997). Our own work using adipocytes differentiated in primary culture has found, however, no effect of triiodothyronine on leptin production, but thyroxine was markedly inhibitory (SE Mitchell and P Trayhurn, unpublished results). Circulating leptin levels in human subjects appear not to be under the control of thyroid hormones, at least in the short-term (Mantzoros *et al.* 1997).

Physiological influences on leptin production include fasting and acute exposure to cold, both resulting in a reduction in *ob* gene expression and a fall in circulating leptin (Becker *et al.* 1995; MacDougald *et al.* 1995; Trayhurn *et al.* 1995*a*,*b*; Hardie *et al.* 1996*b*). Refeeding after fasting leads to a rapid restoration of gene expression and plasma leptin (Becker *et al.* 1995; Trayhurn *et al.* 1995*b*; Hardie *et al.* 1996*b*). Thus, leptin production is subject to nutritional regulation in a manner consistent with a central role for the hormone in the control of energy balance. Cold exposure induces a substantive rise in energy expenditure, through shivering and non-shivering thermogenesis, and this is fuelled by a major increase in substrate flux. Since food intake gradually rises during prolonged exposure to cold, inhibition of the synthesis of a hormone which acts as a satiety factor is an appropriate response. Interestingly, transfer of animals from a cold to a warm environment quickly leads to a restoration of *ob* mRNA levels in white adipose tissue, and to the normalization of circulating leptin (Trayhurn *et al.* 1995*a*; Hardie *et al.* 1996*b*).

In a study on fasted golden hamsters, fever induced by lipopolysaccharide was found to result in a stimulation of *ob* gene expression, an effect which was mimicked by administration of the cytokines tumour necrosis factor-α or interleukin-1 (Grunfeld *et al.* 1996).

β-Agonists and the β3-adrenoceptor

The SNS plays a pivotal role in the regulation of the increased energy expenditure associated with the adaptive response to cold, particularly in relation to the stimulation of thermogenesis in brown adipose tissue (see Landsberg & Young, 1984). Administration of noradrenaline or the non-specific β-adrenoceptor agonist, isoprenaline, inhibits *ob* gene expression in white fat (Trayhurn *et al.* 1995*a*), and we have recently observed that acute treatment with noradrenaline and isoprenaline leads to a reduction in circulating leptin levels (A Mostyn, DV Rayner and P Trayhurn, unpublished results). These results suggest that the cold-induced fall in leptin production may be mediated by the SNS. Additionally,

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isoprenaline has been shown to induce an acute suppression of plasma leptin in human subjects (Pinkney *et al.* 1998).

The general effects of catecholamines and β-agonists obtained in whole-animal studies are paralled in studies on mature adipocytes, on adipocytes from clonal cell lines and with differentiated adipocytes in primary culture (Gettys *et al.* 1996; Hardie *et al.* 1996*a*; Dessolin *et al.* 1997*a*; Mitchell *et al.* 1997). This indicates that there is a direct interaction between catecholamines and white adipose tissue in the control of leptin production. The inhibition appears to occur primarily through β3-adrenoceptors, since both *in vivo* and *in vitro* studies have shown that selective β3-agonists (e.g. BRL 35153A, CL316243, Ro 16-8714) have a powerful suppressive effect on *ob* gene expression with a rapid reduction in circulating leptin (Moinat *et al.* 1995; Gettys *et al.* 1996; Mantzoros *et al.* 1996; Slieker *et al.* 1996; Trayhurn *et al.* 1996; Dessolin *et al.* 1997*a*).

These results have led to the proposition that the SNS is involved in a negative feedback loop to white adipose tissue, regulating leptin production by inhibiting *ob* gene transcription through β3-adrenoceptors (Trayhurn *et al.* 1995*a*,1996; Giacobino, 1996). The regulatory effects of the SNS are mediated by the cAMP second messenger system since dibutyryl cAMP, bromo-cAMP and forskolin strongly suppress leptin production in adipocyte cell culture systems (Slieker *et al.* 1996; SE Mitchell and P Trayhurn, unpublished results). In this regard, it is pertinent to note that a cAMP response element has been reported in the promoter region of the human *ob* gene (Gong *et al.* 1995).

The importance of the β3-adrenoceptor in modulating *ob* gene expression is also indicated by the results of studies on the effects of β3-agonists on *ob* mRNA levels in obese (*ob/ob*) mice. In contrast to lean animals, there is only a limited fall in *ob* mRNA in white fat of *ob/ob* mice following treatment with BRL 35153A (Trayhurn *et al.* 1996), the obese mutants having a greatly reduced expression of β3-adrenoceptors in their adipose tissue (Collins *et al.* 1994), with the stimulation of adenyl cyclase (*EC* 4.6.1.1) by β3-agonists being blunted (Bégin-Heick, 1996). Similarly, obese *fa/fa* rats also show down-regulation of β3-adrenoceptor expression in white fat (Muzzin *et al.* 1991), and unlike their lean siblings there is no reduction in circulating leptin levels on cold exposure (Hardie *et al.* 1996*b*).

The significance of β3-adrenoceptors in the control of leptin production in human subjects is uncertain, although there is evidence that the receptor may play an important role in the control of lipolysis in human omental and subcutaneous adipose tissue (Enocksson *et al.* 1995; Lönnqvist *et al.* 1995*b*).

A key regulatory role for the sympathetic nervous system?

Sympathetic system and lipolysis

In the preceding section the major factors so far shown to regulate the production of leptin have been described. An important question is whether physiologically any one particular factor plays a predominant role, and whether a specific agent that modulates leptin production *in vitro* in isolation from other factors may have significance *in vivo*. There is a parallel with the regulation of lipolysis in white fat, which *in vitro* studies have indicated may involve an array of hormones, including glucagon, catecholamines, growth hormone and adrenocorticotrophin (see Hales *et al.* 1978). However, it has long been considered probable that *in vivo* the SNS is the key regulator of lipolysis. Evidence in support of this view includes the following observations: (1) denervation leads to an increase in adipose tissue mass; (2) electrical stimulation of the nerves to white adipose tissue results in fatty acid release; (3) pharmacological abolition of sympathetic activity inhibits the mobilization of lipids (Hales *et al.* 1978).

One of the long-standing issues in assessing whether the key functions of white adipose tissue may be regulated by the SNS is the extent to which the tissue is innervated. There is, however, general agreement that white fat does receive a sympathetic innervation, which, although primarily linked to the vasculature, also involves the adipocytes themselves (Fredholm, 1985; Slavin, 1985; Youngström & Bartness, 1995). Apart from any direct effects of the SNS within white fat, there is also the possibility of an indirect interaction via the sympathetic innervation of the adrenals, with the release of adrenaline into the circulation.

Until recently, direct changes in sympathetic activity in white adipose tissue in response to physiological stimuli which result in increased lipolysis had not been demonstrated, and this is pivotal to the concept that the SNS is the principal regulator of lipolysis. However, a study measuring noradrenaline turnover has now demonstrated that sympathetic activity in white adipose tissue is increased on cold exposure (Garofalo *et al.* 1996). This change parallels the well-documented increase in sympathetic stimulation to brown fat and the heart in the cold (see Landsberg & Young, 1984). The same group has gone on to demonstrate that fasting, on the other hand, selectively increases sympathetic activity in white fat (Migliorini *et al.* 1997), in contrast to brown adipose tissue and the heart where there is a marked decrease (Landsberg & Young, 1984).

Sympathetic activity and leptin in fasting

From these observations it is pertinent to ask whether the reduction in circulating leptin during fasting is primarily the result of an inhibitory effect on *ob* gene transcription resulting from increased SNS activity to white fat (Giacobino, 1996), and not to alternative explanations such as falling insulin levels. Some direct evidence in support of such a notion comes from a recent study in which we have examined the effects of administering the β-adrenoceptor antagonist, propranolol, to mice. Treatment with propranolol led to a significant attenuation in the fasting-induced reduction in circulating leptin (Fig. 1). There was, however, no effect of propranolol on the leptin level in fed mice. Although the effects of propranolol are not dramatic, it is recognized that this compound is not a proficient antagonist of β3-adrenoceptors, the key β-receptor subtype through which the SNS appears to regulate *ob* gene transcription.

In contrast to lean animals, fasting has no effect on *ob* mRNA levels in white adipose tissue of *ob/ob* mice (Trayhurn *et al.* 1995*b*) and nor is there any acute effect of food deprivation on the circulating level of leptin in *fa/fa* rats

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(Hardie *et al.* 1996*b*). Given the defects in β3-adrenoceptor expression in both types of obese mutant referred to earlier, a lack of response to fasting would be consistent with a central role for the SNS in the fasting-induced fall in leptin production.

Effects of blockade of noradrenaline synthesis on circulating leptin

An approach that we have utilized in attempting to assess the physiological significance of the SNS in the regulation of leptin production is to examine the effects of inhibiting the synthesis of noradrenaline. The rate-limiting enzyme in noradrenaline synthesis is tyrosine hydroxylase (*EC* 1.14.16.2), and this can be strongly inhibited by α-methyl-*p*-tyrosine. Indeed, one of the standard methods for determining SNS activity is to inhibit the synthesis of noradrenaline with α-methyl-*p*-tyrosine and measure the subsequent rate of fall in the concentration of catecholamine in the tissue(s) of interest. Not only has α-methyl-*p*-tyrosine been employed as a tool for measuring sympathetic activity, but it has also been used clinically to treat patients with phaeochromocytoma (characterized by a hypersecretion of catecholamines).

Administration of α-methyl-*p*-tyrosine to mice at the levels employed in, and with a protocol similar to, studies on noradrenaline turnover induces a rapid increase in the circulating level of leptin (Fig. 2). In subsequent studies, we have found that leptin levels are elevated by up to 8-fold within 6–10 h after the injection of α-methyl-*p*-tyrosine. Thus, blockade of noradrenaline production leads to marked hyperleptinaemia. In principle, hyperleptinaemia could result from either increased production of the hormone or decreased removal, or a combination of the two. That there is an elevation in synthesis is indicated by examination of *ob* mRNA in white adipose tissue; 10 h after treating mice with α-methyl-*p*-tyrosine the level of *ob* mRNA in the epididymal white fat was increased by up to 5-fold (E Simon, JS Duncan, DV Rayner and P Trayhurn, unpublished results).

Further experiments have shown that if a selective β3-adrenoceptor agonist is administered, then the rise in leptin levels induced by treatment with α-methyl-*p*-tyrosine is attenuated (E Simon, DV Rayner and P Trayhurn, unpublished results). This indicates that direct stimulation of β3-adrenoceptors circumvents the effects of the blockade of noradrenaline synthesis on leptin production, as would be predicted.

Overall, these findings are consistent with the concept that the SNS is playing a key role in the regulation of leptin production through modulating transcription of the *ob* gene. Additional approaches to blocking SNS activity are required, however, to substantiate the hypothesis presented here. Irrespective of whether α-methyl-*p*-tyrosine is acting

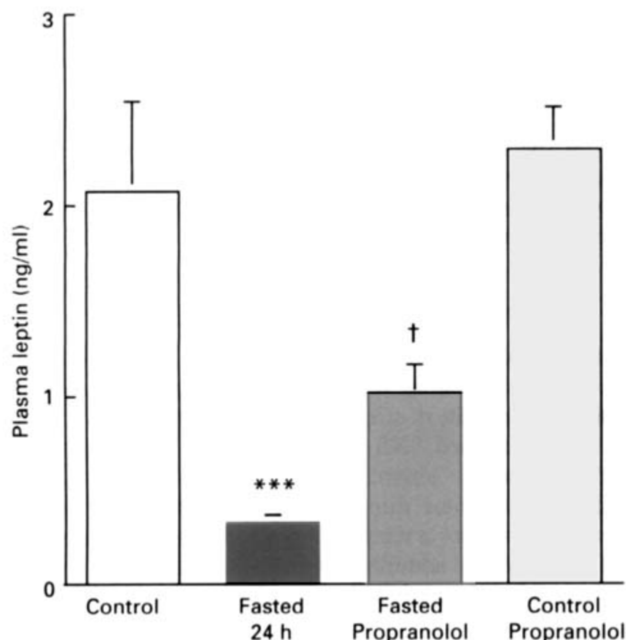

Fig. 1. Effects of the β-adrenoceptor antagonist, propranolol, on the circulating leptin level in fed and fasted mice. Aston strain mice were either fasted for 24 h or allowed to feed *ad libitum*. Animals in each group were injected intraperitoneally with 20 mg (–)propranolol/kg or saline (9 g NaCl/l) at 0, 16 and 20 h after the removal of food. Plasma leptin was measured by ELISA using a murine recombinant leptin standard (Hardie *et al.* 1996*b*). Results are given as means with their standard errors represented by vertical bars for six to eight mice in each group. Mean value was significantly different from that for the fed control group: ***$P < 0.001$. Mean value was significantly different from that for fasted group (Student's *t* test): †$P < 0.05$.

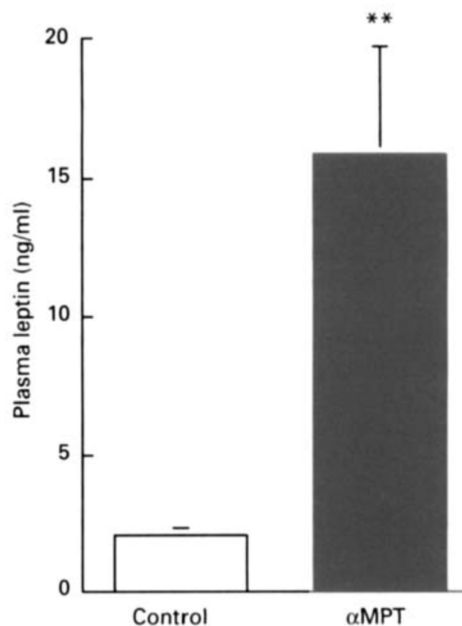

Fig. 2. Effect of tyrosine hydroxylase (*EC* 1.14.16.2) inhibitor α-methyl-*p*-tyrosine (αMPT) on circulating leptin levels in mice. Aston strain mice were injected with either αMPT (methyl ester; 300 μg/kg body weight) or saline (9 g NaCl/l) at 0, 8 and 13 h and blood taken at 18 h after the first injection. Plasma leptin was measured by ELISA using a murine recombinant leptin standard (Hardie *et al.* 1996*b*). Results are given as means with their standard errors represented by vertical bars for eight mice in each group. Mean value was significantly different from that for the control group (Student's *t* test): **$P < 0.01$.

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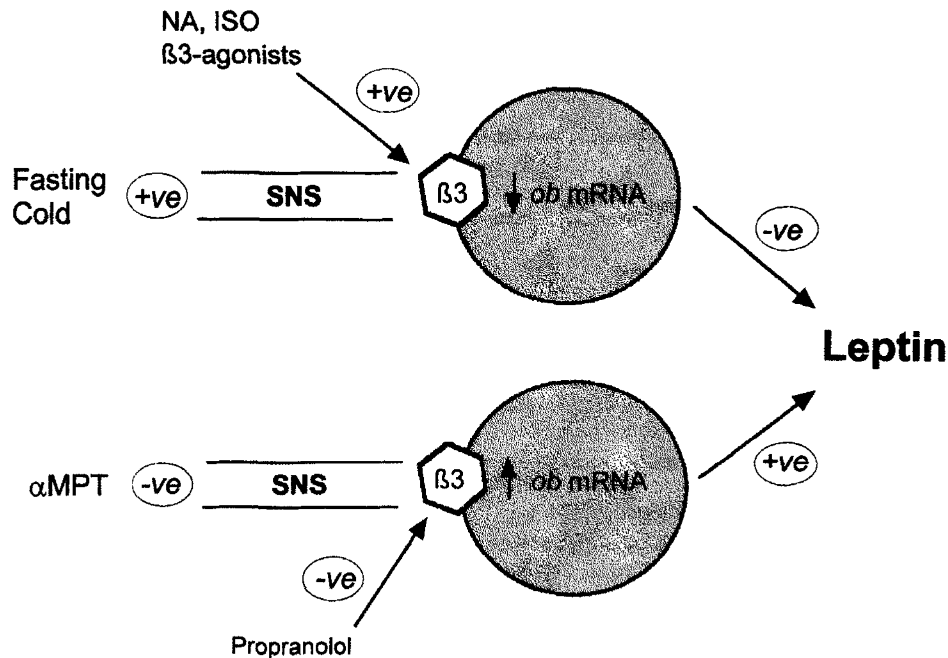

Fig. 3. Schematic view of the regulation of leptin production in white adipose tissue by the sympathetic nervous system (SNS). αMPT, α-methyl-*p*-tyrosine; β3, β3-adrenoceptor; ISO, isoprenaline; NA, noradrenaline; –ve, negative effect; +ve, positive effect.

exclusively through the inhibition of tyrosine hydroxylase, there are certain implications of the results obtained with this. First, α-methyl-*p*-tyrosine provides a pharmacological tool for the up-regulation of circulating leptin levels. Second, since there is evidence that leptin stimulates sympathetic activity to a number of tissues (Collins *et al.* 1996; Haynes *et al.* 1997), it is apparent that using α-methyl-*p*-tyrosine as a tool to measure SNS activity leads to a change in the activity of that which is being measured.

Coda

A number of factors which influence the expression of the *ob* gene and the production of leptin have been identified, and these include hormones such as insulin, glucocorticoids and testosterone. It is proposed that physiologically the SNS plays a dominant role, operating primarily through the β3-adrenoceptor subtype (Fig. 3). Certainly the effects of catecholamines and β-agonists, both non-selective (isoprenaline) and β3-adrenoceptor-selective, on leptin production are rather more pronounced than with other recognized regulatory factors. There is a clear analogy between the problem of identifying the critical physiological regulators of leptin production and the central factors involved in the control of lipolysis.

Our knowledge of the regulation of the expression of the *ob* gene and the production of leptin relates primarily to white adipose tissue. It cannot be assumed, however, that the same factors are involved in regulating the expression of the *ob* gene in other tissues. Nevertheless, β3-adrenoceptor agonists appear to inhibit expression in brown adipose tissue, as in white fat (Moinat *et al.* 1995; Deng *et al.* 1997). That the regulatory systems differ in the placenta is indicated by the presence of a placental-specific enhancer (Bi *et al.* 1997), and by the augmentation of leptin secretion by the placental choriocarcinoma BeWo cell line in the presence of forskolin (Masuzaki *et al.* 1997; N Hoggard, unpublished results); in white adipocytes forskolin strongly inhibits leptin production.

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