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Pre- and Postprandial Expression of the Leptin Receptor Splice Variants OB-Ra and OB-Rb in Murine Peripheral Tissues

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Summary

Leptin receptors (OB-R) are widely distributed in peripheral tissues. However, the RT-PCR data published on the distribution of OB-R are not always consistent. The present study was undertaken in order to test whether the different muscle fiber type profile or the acute nutritional status in which tissue samples were excised from animals may influence OB-R expression. Six 12-week-old male Swiss-Webster mice were killed by decapitation either 1 h after feeding or after a 16-h fast, and the kidneys, testes, brown adipose tissue, gastrocnemius (G), soleus (SOL) and extensor digitorum longus (EDL) muscles were dissected out. In parallel, muscle fibers obtained from other animals were classified on the basis of differences in the staining intensity for myofibrillar adenosinetriphosphatase. The expression of OB-R isoforms was assessed by RT-PCR and ethidium bromide staining. The signal for OB-Ra and OB-Rb was detected in all tissues examined. No differences were observed in samples obtained from either fed or fasted mice. G, SOL and EDL muscles showed the same pattern of OB-R expression. Neither the short-term nutritional changes of the animal as regards to the pre- versus the postprandial-state nor differences in muscle fiber type had any influence on the qualitative expression of the OB-R splice variants a and b in the murine tissues studied. However, quantitative differences cannot be ruled out.

Key words

OB Protein • Fasting • Skeletal muscle • Fiber type composition • Mice

Introduction

The leptin receptor (OB-R) was first cloned from a mouse choroid plexus cDNA expression library (Tartaglia *et al.* 1995). The receptor is a member of the extended class I cytokine-receptor family (Tartaglia *et al.* 1995), having at least 5 splice variants, designated OB-Ra, OB-Rb, OB-Rc, OB-Rd and OB-Re (Lee *et al.* 1996). The OB-Ra variant, possessing a short intracellular domain, was the form originally cloned and it has been postulated that it serves to transport leptin across the blood-brain barrier (Lee *et al.* 1996). Two other splice variants, OB-Rc and OB-Rd, have been implicated in the clearance of leptin from the circulation. The OB-Re variant, having no intracellular domain, is supposed to be a putative soluble receptor. Only the full-length isoform,

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the OB-Rb, has been shown to be involved in leptin signaling, and is, therefore, the functional receptor (Lee *et al.* 1996).

OB-R are widely distributed in the brain, choroid plexus, liver, heart, lungs, kidneys, small intestine, testes, spleen, adipose tissue, skeletal muscle, adrenals, etc. However, the published RT-PCR data are not always in agreement. While some investigators (Lee *et al.* 1996, Cioffi *et al.* 1996) did not find expression of the long form of the receptor in the kidneys, Hoggard *et al.* (1997) clearly showed that OB-Rb is present in renal samples. Conversely, Lee *et al.* (1996) were able to detect OB-Rb expressed in the testes and adipose tissue, while others reported that functional leptin receptors could not be detected in these organs (Cioffi *et al.* 1996, Hoggard *et al.* 1997). No receptor expression was reported in the adrenal glands by Zamorano *et al.* (1997), while the contrary was observed by Hoggard *et al.* (1997).

Controversies regarding the existence of OB-R in skeletal muscle have also been reported. Both absence and presence of OB-R in muscles have been described (Tartaglia *et al.* 1995, Cioffi *et al.* 1996, Hoggard *et al.* 1997, Zamorano *et al.* 1997). However, the authors have not specified the muscles which they had studied. In adult animals, different results are to be expected in muscles possessing wide differences in the proportion of fibers of various metabolic types. Postural muscles, persistently active and composed primarily of slow-contracting and relaxing fatigue-resistant fibers have a high oxidative potential. In contrast, intermittently active muscles are highly fatigable and are composed almost exclusively of fast-contracting fibers with high activities of enzymes involved in glycolysis and low in mitochondrial enzymes. Thus, predominantly slow- or fast-twitch muscles, which have metabolically diverse characteristics, may exhibit differences in the OB-R distribution.

The mentioned inconsistencies in tissue OB-R distribution may reflect species-specific differences as well as sensitivity problems of the techniques applied as detection limits of RT-PCR can vary over a very wide range. On the other hand, the apparent discrepancies may be attributed to differences in the nutritional state of the experimental animals as authors do not document whether tissue samples were obtained from fasted or fed mice. Due to the fact that leptin is thought to inform the CNS about the state of body fat, thereby regulating feeding behavior, metabolism, and endocrine physiology to be coupled to the nutritional state of the organism, this may be of relevance in OB-R determinations.

Consequently, the present study was undertaken with three objectives: 1) to investigate OB-R expression in tissue samples obtained from animals in the satiated state or after an overnight fasting; 2) to examine the possible differences in OB-R isoforms expression of predominantly fast- and slow-twitch hindlimb muscles, and 3) to study the possible relationship between receptor isoform expression and muscle fiber type composition.

Method

Animal procedures

Six twelve-week-old male Swiss-Webster mice weighing about 25 g were used. Half of the animals were killed 1 h after they had been fed while the other half was sacrificed after a 16-h overnight fast with free access to water. The kidneys, testes, brown adipose tissue (BAT) and skeletal muscles were quickly dissected out. The following hindlimb muscles were excised for the chemical estimations: the lateral and medial heads of the gastrocnemius (G) representing a mixed muscle, the extensor digitorum longus (EDL) as a predominantly fast-twitch muscle and the soleus (SOL) as a predominantly slow-twitch muscle. Muscles weighing less than 10 mg were pooled with those of the contralateral hindlimb of the same animal. All experimental procedures were performed according to institutional guidelines for Animal Care and Use at the University of Navarra.

Reverse transcription-polymerase chain reaction (RT-PCR) amplification of leptin receptor

Total RNA was isolated by the ULTRASPEC™-II RNA ISOLATION SYSTEM (Biotecx Laboratories, Houston, TX, USA) from 100-150 mg of tissue. After 30 min at 37 °C treatment with 10 units of RNase free DNase I (Boehringer Mannheim, Barcelona, Spain), 1 μg of RNA was used to synthesize first-strand cDNA. The RT reaction was carried out in a volume of 20 μl containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiotreitol, 100 ng of random hexamers (Boehringer Mannheim), 1 mM each dNTP (Bioline, London, UK), 20 units of RNase inhibitor (Promega, Madison, WI, USA), 200 units of M-MLV RT (GIBCO BRL, Life Technologies, Gaithersburg, MD, USA) and incubated at 37 °C for 60 min. The enzyme was inactivated by heating at 95 °C for 5 min. Four μl from the RT reaction were amplified in a 50 μl reaction mixture containing 40 ng of each primer, 16 mM $(NH_4)_2SO_4$, 67 mM Tris HCl (pH 8.8), 2 mM $MgCl_2$,

0.1 % Tween-20, 0.2 mM each dNTP and 1 unit of BIOTAQ™ polymerase (Bioline). Primers used to amplify OB-Ra cDNA (GenBank U49106) were 5'-ACACTGTTAATTTCACACCAGAG-3' (sense, 2569-2591) and 5'-AGTCATTCAAACCATAGTTT AGG-3' (antisense, 2783-2805) and OB-Rb (GenBank U49107) 5'-ACACTGTTAATTTCACACCAGAG-3' (sense, 582-604) and 5'-TGGATAAACCCTTGCTCT TCA-3' (antisense, 1007-1027). As a control, 'mock' amplifications were carried out in the presence of the RN A template and Taq polymerase, but in the absence of reverse transcriptase. As a control for RNA quality and quantity, β-actin mRNA was amplified from all RNA samples using primers 5'-TCTACAATGAGCTGCGT GTG-3' (sense, 1599-1618) and 5'-GGTCAGGATCTT CATGAGGT-3' (antisense, 2357-2376), based upon the sequence of rat β-actin (GenBank J00691). cDNA was amplified for 35 (OB-Ra), 45 (OB-Rb) and 30 cycles (β-actin) in muscles, and 40 (OB-Ra), 40 (OB-Rb) and 30 cycles (β-actin) in BAT, kidney and testes, using the following parameters: 94 °C for 30 s, 59 °C (OB-Ra), 61 °C (OB-Rb) and 60 °C (β-actin) for 30 s and 72 °C for 30 s, with a final extension step at 72 °C for 7 min. Amplifications were carried out in a GeneAmp® PCR System 2400 (Perkin Elmer, Norwalk, CT, USA). The amplified products were resolved in a 1.5 % agarose gel with ethidium bromide. Identity of the PCR product amplifications was demonstrated after digestion with BspH I (New England Biolabs, Beverly, MA, USA) and Mnl I (New England Biolabs) for OB-Ra and Sph I (Amersham, Buckinghamshire, UK) and Taq I (New England Biolabs) for OB-Rb. All restriction enzymes yielded the predicted fragments.

Histochemical assessment of muscle fiber types

Five millimeter transverse sections were taken from the midbelly of SOL, EDL and G muscles of 10 mice. Care was taken to ensure that the same region in each muscle type was taken from all animals. The muscle pieces were oriented for transverse sectioning, mounted on cork, covered with Tissue-Tek® (Miles Inc., Diagnostics Division), immediately immersed in isopentane (2-methyl butane) cooled by liquid nitrogen, and stored at –80 °C until assayed. Serial cross sections of the muscle samples were cut in 10 μm slices with a cryostat (Reichert-Jung, Slough, UK) kept at –20 °C and stained for Ca^{2+}-activated myofibrillar adenosine-triphosphatase (mATPase) after preincubation at various pH levels (Johnson 1990, Hämäläinen and Pette 1993). Muscle fibers were classified on the basis of differences in staining intensity for mATPase after alkaline (pH 9.4) preincubation. Type I (slow-twitch oxidative) fibers stain lightly for mATPase, whereas type II (fast-twitch) fibers stain darkly. By acid preincubation at pH 4.6 and 4.2 the type II fibers were further subclassified according to their staining reaction into IIa, IIb and IIc. At least 200 fibers per muscle were examined and the percentage of frequency of each fiber type was calculated.

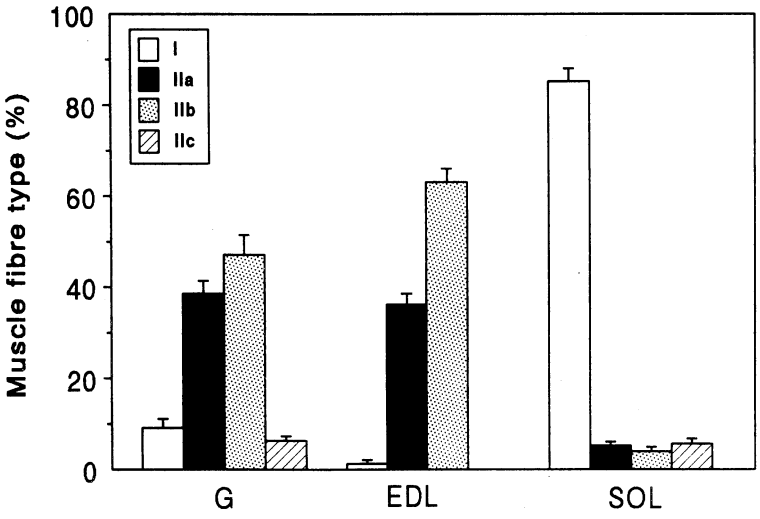

Fig. 1. *Fiber type composition of gastrocnemius (G), soleus (SOL) and extensor digitorum longus (EDL) muscles of Swiss-Webster mice. Values are means ± S.E.M. (n = 10).*

Results

The muscle fiber type composition of the different muscles studied is shown in Figure 1. It clearly indicates how the muscles differ in their mean percentage composition of each fiber type. While the SOL muscle is composed mainly of type I fibers (85±3%), the EDL muscle has only a small percentage of type I fibers

(1±1%). Despite these evident differences in the metabolic characteristics between the muscles studied, no qualitative differences were observed in the expression of the short and long isoforms of OB-R (Fig. 2). Moreover, the nutritional state of the mice did not play a role in the expression of the short and long isoforms of the receptor as OB-Ra and OB-Rb were expressed in muscle samples obtained from animals fasted overnight for 16 h or mice fed 1 h before tissue sampling (Fig. 2).

In BAT, kidneys and testes, both OB-R isoforms, a and b, were found to be expressed regardless of the nutritional state of the mice. As shown in Fig. 3, no qualitative differences were observed in the expression of OB-Ra and OB-Rb in the renal and adipose samples as well as in the testes obtained from fasted or fed animals.

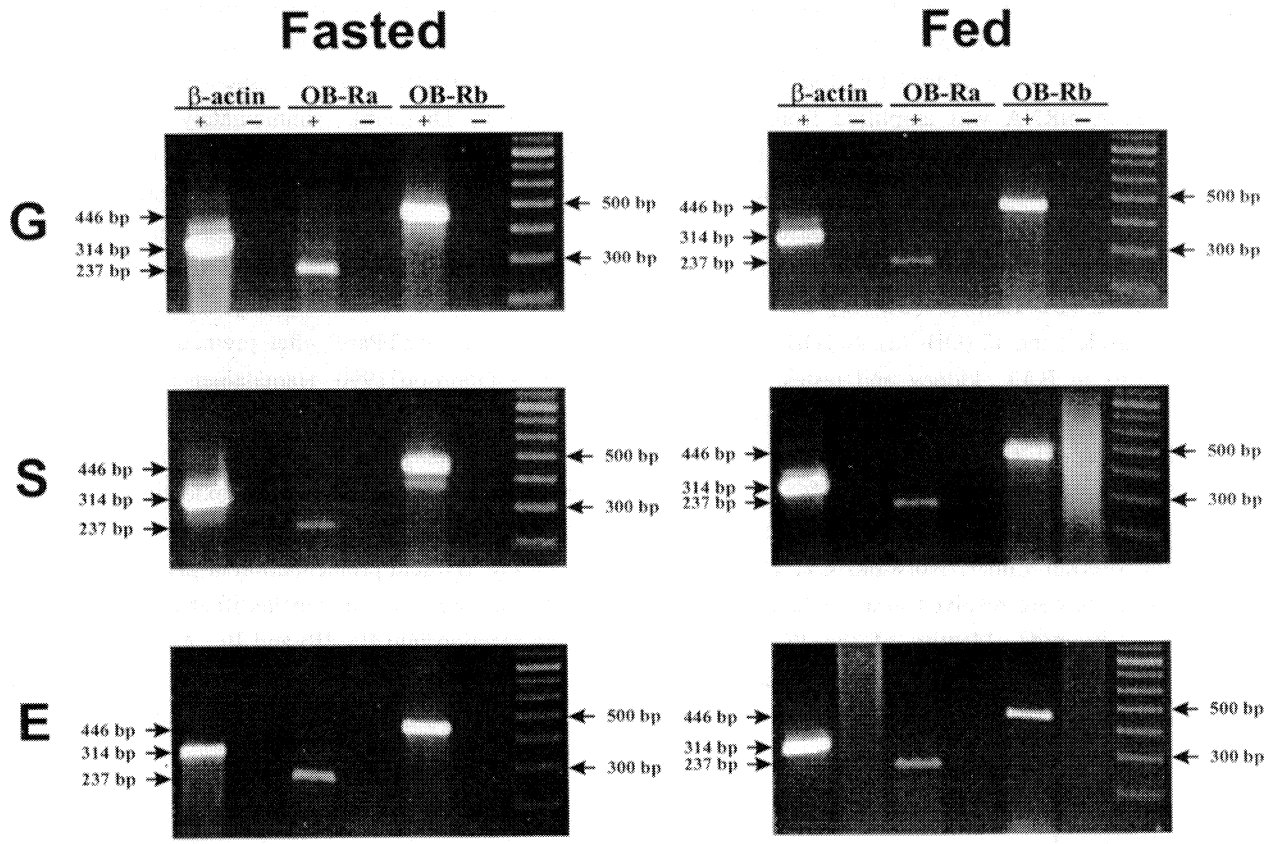

Fig. 2. *RT-PCR expression analysis of mouse leptin receptor isoforms in the presence (+) or absence (-) of reverse transcriptase. Gastrocnemius muscle (G), soleus (S) and extensor digitorum longus (E) were analyzed in the fasted or fed state. Amplified products corresponding to OB-Ra, OB-Rb and β-actin were detected using the primer pairs described in the Methods. The sizes of the PCR products are 237, 446 and 314 for OB-Ra, OB-Rb and β-actin respectively, and were estimated by alignment with a 100 Base-Pair Ladder marker (Pharmacia, Uppsala, Sweden).*

Discussion

The regulation of the expression of the *ob* gene has been extensively studied (for review, see Trayhurn 1996). As could be predicted for a factor playing a key role in energy balance, the expression of the *ob* gene is subject to nutritional regulation in both man and animals. Fasting induces a fall in the level of *ob* mRNA, which is reversed on refeeding within 4 hours to levels of satiated animals, and circulating leptin levels change in parallel to tissue mRNA (Kolaczynski *et al.* 1996, MacDougald *et al.* 1995, Saladin *et al.* 1995, Trayhurn *et al.* 1995, Ahima *et al.* 1996). Rodents have the highest level of plasma leptin at 04:00 h, which represents the state of normal physiological satiation after *ad libitum* feeding overnight (Saladin *et al.* 1995). After 24 h of food deprivation, which represents a nutritional state of depleted energy supply, the lowest concentration of leptin in the blood stream has been observed by Saladin *et al.* (1995).

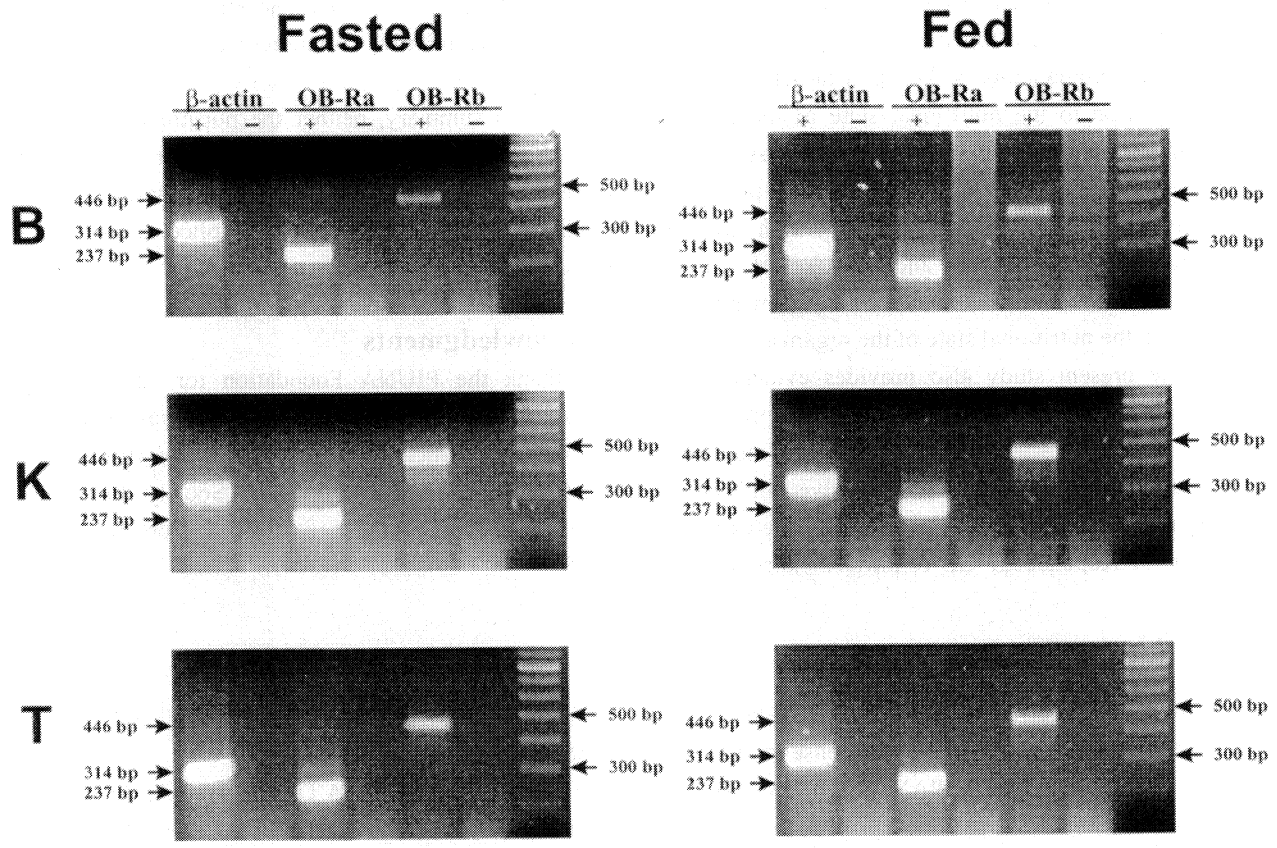

Fig. 3. *RT-PCR expression analysis of mouse leptin receptor isoforms in the presence (+) or absence (-) of reverse transcriptase. Brown adipose tissue (B), kidney (K) and testes (T) were analyzed in the fasted or fed state. Amplified products corresponding to OB-Ra, OB-Rb and β-actin were detected using the primer pairs described in the Methods. The sizes of the PCR products are 237, 446 and 314 for OB-Ra, OB-Rb and β-actin respectively, and were estimated by alignment with a 100 Base-Pair Ladder marker (Pharmacia, Uppsala, Sweden).*

The fluctuation in the expression of OB-R mRNA has also been investigated under different nutritional states in animals (Dyer *et al.* 1997, Lin and Huang 1997). In ewes with restricted food intake for 3 weeks before tissue collection, the mRNA expression of the long OB-R isoform was found to be greater than that observed in well-fed ewes (Dyer *et al.* 1997). In rodents, the levels of OB-R mRNA expression have been shown to be increased in the brain of lean mice fasted for 24 h compared to lean mice in the satiated state (Lin and Huang 1997). Recently, an upregulation of the expression of OB-R mRNA in the *ob/ob* mouse brain has been reported (Huang *et al.* 1997). These findings suggest that OB-R may be an autoreceptor regulated by leptin levels.

So far, no study has investigated the short-term responses of OB-R expression in the fed and fasted state. The present study clearly shows that the kidneys, testes, BAT and skeletal muscles do not exhibit qualitative differences in the pre- and postprandial expression of the OB-R splice variants a and b. The lack of effect on the expression of OB-R observed in our study may be explained by methodological, metabolic and anatomical factors. Thus, it is possible that the changes in the expression of OB-R mRNA cannot be identified by qualitative RT-PCR and that quantitative techniques are needed to detect more subtle differences. Furthermore, OB-R expression may not concern short-term regulation, which is capable of reacting acutely to differences between the pre- and postprandial state; more prolonged periods are perhaps necessary to trigger a response in the expression of the receptor. A third plausible explanation may involve tissue-dependent differences in the expression of OB-R mRNA. In this sense, Lin and Huang (1997) reported an increase in OB-R mRNA expression in the arcuate and ventromedial hypothalamic nuclei of fasted mice compared to samples obtained in the fed state. On the other hand, the piriform cortex showed no differences in the expression of OB-R between fasted and non-fasted animals. In our study, no changes in OB-R expression were observed in the kidneys, testes, BAT and

skeletal muscles obtained from mice in the pre- or postprandial state. Taken together these data support the idea that the hypothalamus is the main target for leptin action in relation to the nutritional state of the animal. Thus, changes in both *ob* gene and OB-R expression may take place in the hypothalamus within a short period of time, whereas other peripheral tissues, which are not directly involved in food intake and body weight control, may not react in such a sensitive and rapid manner to differences in the nutritional state of the organism.

The present study also provides evidence that both the short and long OB-R isoforms are expressed in skeletal muscles with quite diverse metabolic characteristics. In addition, no qualitative differences were observed in the pre- and postprandial state as far as the pattern of response of OB-R mRNA expression of mainly oxidative or glycolytic muscles is concerned. However, quantitative differences cannot be completely ruled out.

In summary, neither the nutritional state of the animal as regards to the pre- vs the postprandial-state nor differences in muscle fiber type exert an influence on the qualitative expression of the OB-R splice variants a and b in the murine peripheral tissues studied.

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Reprint requests

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