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Detection and Quantification of the Leptin Receptor Splice Variants Ob-Ra, b, and, e in Different Mouse Tissues

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Ob-Ra, b, and e are the major splice forms of the leptin receptor. This study was performed to map the tissue distribution and to quantify the 3 receptor isoforms by heterologous competitive Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and RNase Protection Assay (RNase PA). The mRNA of the truncated, membrane bound isoform Ob-Ra was found to be represented ubiquitously. Messenger RNA for the putative functional isoform Ob-Rb could be detected in brain, hypothalamus and in some peripheral tissues (e.g. heart, lung, lymph nodes). The highest ratio between Ob-Rb and Ob-Ra mRNA was found in the hypothalamus, where leptin probably exerts its satiety action. The fact that Ob-Rb mRNA was found in peripheral tissues could indicate possible additional functions of leptin. Transcripts for the shortest splice variant, Ob-Re, which is expected to encode a soluble form of the receptor, were detected in relatively high amounts in many tissues. The levels were comparable to those of leptin mRNA in fat tissue. It is conceivable, therefore, that Ob-Re might be secreted in sufficient amounts to act as a buffering system for freely circulating leptin.

The adipocyte-derived hormone leptin mediates its effect on energy intake and expenditure mainly through its actions on the hypothalamus (19,18). Several alternatively spliced isoforms of the leptin-receptor (Ob-Ra - Ob-Re) have been identified and could be detected by RT-PCR in a variety of tissues (2,12). All isoforms share the same extracellular domain and, except for Ob-Re, they contain a single membrane-spanning sequence. Only the long isoform, Ob-Rb, contains all the elements which are required for functional leptin signaling. The function of the other shorter isoforms of the Ob-R is not known. Ob-Ra, which is predominant in all peripheral tissues is capable of leptin-mediated signaling but much less effective than the full length receptor (17). It was suggested that this isoform could function as a specific transport system for leptin (12). This possibility is supported by the observation that Ob-Ra mRNA is present in relatively high amounts in the choroid plexus, and that leptin is transported into the cerebrospinal-fluid with the kinetics of a saturable mechanism (20). It has been proposed that the receptor isoform Ob-Re, which is spliced in front of the transmembrane domain, might be a soluble binding protein for leptin (21).

Many recent publications report on the levels of transcription of the Ob-R. The highest relative abundance of the long form of the leptin receptor has been unequivocally detected in the hypothalamus, in accordance with the role of leptin as a satiety factor. On the other hand, information on the distribution and relative amounts of the receptor isoforms in other tissues is poorly documented and controversial. This study was performed to map the tissue distribution and quantify the mRNA expression of the receptor isoforms Ob-Ra, Ob-Rb, and Ob-Re in several relevant mouse tissues by two completely independent methods, i.e., RNase PA and heterologous competitive RT-PCR.

MATERIALS AND METHODS

Tissue and RNA Extraction

Mice (normal bodyweight, strain: Tif:MAG) were anesthetized with CO_2 and decapitated. Organs and tissues were surgically dissected, rapidly removed, snap frozen in liquid nitrogen and stored at −80°C until processed. Total RNA was extracted basically following the method of Chomczynski and Sacchi (3) and estimated by measuring the absorbance at 260 and 280 nm.

Radiolabeled Riboprobes and RNase Protection Assay

The Ob-Rb cDNA ranging from bp 2307 to 2802 (GeneBank accession number: u46135) was cloned into the transcription vector pSPT 19 (SP6/T7 Transcription Kit, Boehringer Mannheim, Cat. No.999 644). After linearization (Pvu II), transcription was performed ac-

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cording to the manufacturer's instructions using [α^{32}P]CTP with a specific activity of ~800 Ci/mmol (Amersham, Cat. No. PB 20382). This resulted in a 554 bp transcript complementary to 495 bp of Ob-Rb, 420 bp of Ob-Ra, c, d and 141 bp of Ob-Re. A probe which protected 162 bp of the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT), cloned into pBluescript II SK+ (a kind gift of Dr. R. Skoda, Biocenter, Basle), was used as the internal standard. RNase PA was performed using the Boehringer Mannheim RNase Protection Kit (Cat. No. 1427 580). 150 μg total RNA were coprecipitated with radiolabeled receptor-probe (5×10^5cpm), radiolabeled HPRT-probe (1×10^5 cpm) and 50 μg yeast tRNA as carrier in the presence of 0.3 M NaAc and 2.5 volumes of ice cold ethanol. The pellet was washed with ethanol 70%, dried and dissolved in 30 μl hybridization buffer. After denaturation at 95°C for 3 minutes, the RNA was hybridized overnight in a water bath slowly cooling down from 68°C to room temperature. RNase digestion, extraction and precipitation of the protected fragments was performed following the manufacturer's instructions. The protected RNA fragments were separated on a denaturing 4% polyacrylamide gel containing 7% urea. Radioactive bands were quantified using a PhosphoImager and the ImageQuant Software (Molecular Dynamics Inc.).

Competitive RT-PCR

Approximately 100 ng total RNA were used to quantify β-actin. The amount of RNA used to quantify the receptor splice variants and leptin were normalized to 100 pg β-actin.

Reverse transcription. Total RNA (equivalent to 100 pg β-actin) was mixed with 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.3, 1 mM NTPs, 2.5μM poly dT 15 primer (Boehringer Mannheim), 10 units RNase Inhibitor (Promega), 25 units Reverse Transcriptase SSII (Gibco), in a final volume of 10 μl. The reactions were incubated 10 min at 25°C, 45 min at 42°C and 5 min at 99°C.

Competitive polymerase chain reaction. The reaction was carried out in a total volume of 50 μl, containing 10μl of the reversely transcribed cDNA-reaction mixture, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.2 mM NTPs, 10μM of each forward and reverse primer and 1 unit Taq Polymerase (Gibco). Primer-specific heterologous cDNA was prepared as previously described (13) and added as an internal standard to each reaction in increasing amounts. The RNA was denatured at 94°C for 15 seconds, primers annealed for 60 seconds at primer-specific temperatures and polymerized for 90 seconds at 72°C. 35 cycles were run for β-actin, 40 cycles for the receptor variants and leptin. The reactions were terminated by a final extension for 5 min at 72°C and cooling down to 4°C. The products were separated on agarose gels containing ethidium bromide and quantified using the Wincam 2.1 software (Cybertech).

Primer design. To specifically amplify the cDNA of the three different splice variants, reverse primers were selected which were localized downstream of the translation stop codon of Ob-Ra and Ob-Re. The reverse primer for Ob-Rb was situated in a region unique for the full length receptor. Primer sequences and annealing temperatures were: β-actin (forward: 5′-TGACCCAGATCATGTTTGAGACC-3′, reverse: 5′-CCAT ACCCAAGAAGGAAGGC-3′, 65°C): Ob-Rb (forward: 5′-ACAGTTCTGGCTGTCAATTCCC-3′, reverse: 5′-AGGAGCTGCTAGAGAAAGACTG-3′, 58°C): Ob-Ra (forward: 5′-ACAGTTCTGGCTGTCAATTCCC-3′, reverse: 5′-GTATGGACTGTTGGGAAGTTGG-3′, 61°C): Ob-Re (forward: 5′-GTTGGATGGACTAGGATAT TGG-3′, reverse: 5′-CATTAAATGATTTATTATCAGAATTGC-3′, 57°C): Leptin (forward: 5′-GGTCCTATCT GTCTTATGTTCAAGC-3′, reverse: 5′-TCAGCATTCAGGGCTAACATC-3′, 65°C).

RESULTS

Pancreas RNA was found to be heavily degraded after purification and therefore not used for analysis.

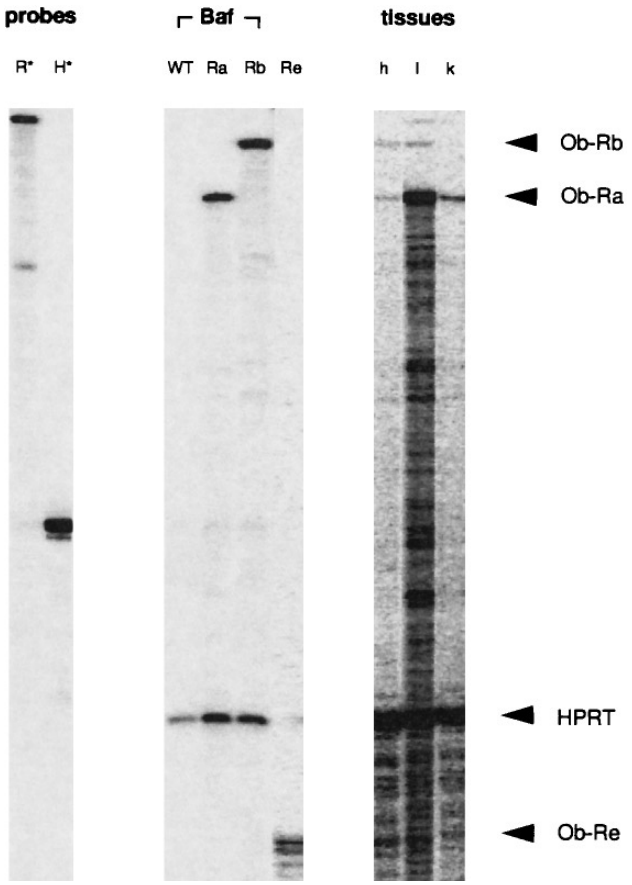

FIG. 1. RNase protection assay. Details on the procedure are given in Materials and Methods. *Left panel:* Undigested riboprobes (R*: antisense probe for the Ob-receptor and H*: antisense probe for HPRT). *Center panel:* Validation of RNase-PA (Baf WT: Baf 3 mouse lymphocytes. Baf Ra, Baf Rb: stably transfected cells expressing the Ob-Ra or Ob-Rb receptor, respectively. Re: in vitro transcribed Ob-Re cRNA). *Right panel:* Examples of determinations in tissue samples (h: hypothalamus; l: lung and k: kidney).

A single RNA probe was designed for RNase-PA which protects fragments of different sizes of the mRNA from the three leptin receptor splice variants (see Figure 1, center panel). The signal for Ob-Ra could be detected and quantified in all tissues examined (see Table 1). In the lung, the levels were considerably higher than in any other tissue. Spleen, heart, lymph nodes, perirenal fat and bladder contained moderate amounts, while brain and all other tissues examined contained the lowest levels of Ob-Ra mRNA. The signal for Ob-Rb mRNA in many tissues was close or below the detection limit of the method. Otherwise a similar pattern as for Ob-Ra was found although levels were much lower (normally below 7-8% of Ob-Ra). The only exceptions were the hypothalamus and other regions of the brain, where the Ob-Rb mRNA levels were similar to or about half of those of Ob-Ra, respectively. Interestingly, however, the highest absolute levels of Ob-Rb were found in the lung. The signal for Ob-Re, unfortunately, migrated

TABLE 1

Summary of the Quantification of the Various Splice Forms in Different Tissues by RNase PA and RT-PCR

	RNase protection assay			RT-PCR	
	OB-Rb (Average ± SEM)	Ob-Ra (Average ± SEM)	Ob-Rb (% of Ra)	Ob-Rb (% of Ra ± SEM)	Ob-Re (% of Ra ± SEM)
Hypothalamus	1.65 ± 0.56	1.14 ± 0.86	69.10	156.63 ± 50.8	131.36 ± 77.7
Brain (-hyp)	1.58 ± 0.36	0.76 ± 0.08	47.81	89.27 ± 26	201.92 ± 86.6
Pituitary	0.97 ± 0.43				
Thymus	1.01 ± 0.34	<0.3 ± 0.08			855.24
Lymph nodes	6.12 ± 1.67	0.36	5.81		
Heart	8.35 ± 2.14	0.65 ± 0.33	7.83	8.14	185.37
Lung	31.09 ± 2.66	1.91 ± 0.22	6.15	8.38	229.04
Spleen	11.49 ± 2.65	0.48 ± 0.12	4.22	12.14	144.77
Liver	0.12				369.6
Stomach	2.68 ± 0.15	<0.3			222.14
Small intestine	2.34 ± 0.10	<0.3 ± 0.07			604
Colon	1.4 ± 0.28	<0.3		26.01	707.02
Kidney	3.13 ± 1.32	<0.3 ± 0.07			182.31
Adrenals	3.26 ± 0.74	<0.3 ± 0.14			366.91
Bladder	5.7 ± 1.74	0.40 ± 0.24	6.97	3.11 ± 1.75	
Testis	1.1 ± 0.16	<0.3 ± 0.01			92.3
Sem. vesicles	2.53 ± 0.47	<0.3 ± 0.13		0.25	
P.epidid. fat	1.06 ± 0.39	<0.3 ± 0.06		13.25	
P.renal fat	4.31 ± 0.30	0.32 ± 0.18	7.45		
Subcut. fat	1.53 ± 0.19	0.45 ± 0.26	29.42	5.73	
Sk. muscle	1.68 ± 0.20	<0.3			
Skin	1.22 ± 0.36	<0.3			
Bone	2.7 ± 1.18	<0.3			

Note. Values obtained with RNase protection assay are expressed as arbitrary units, normalized to the protected HPRT signal. Values $\leq$ 0.3 units were not reliable due to poor signal to noise ratio. Corrected values obtained with RT-PCR are expressed as % of Ob-Ra levels. SEM is given for those experiments with n $\geq$ 3. The other values are averages of duplicates.

in a region of the gel containing several degradation products of the RNase reaction. This high level of background signals did not allow a reliable quantification (see Figure 1, right panel).

Heterologous competitive RT-PCR was applied to the quantification of the three leptin receptor splice variants and of leptin mRNA. As the amplification reaction is dependent on many factors, including size and structure of the amplicon, the efficiency was assessed by quantifying given amounts of *in vitro* generated cDNAs. Correction factors of 0.23, 0.30, 0.31 and 2.3 were obtained for Ob-Re, leptin, Ob-Ra and Ob-Rb, respectively and values were corrected accordingly. The lower efficiency of amplification of Ob-Rb probably reflected the larger size of the Ob-Rb amplicon (951bp for Ob-Rb and 611bp for Ob-Ra and Ob-Re). Figure 2 shows a typical result obtained with brain tissue. Transcripts for the soluble splice form Ob-Re were detected in all tissues examined in quantities similar or higher than those of the ubiquitous Ob-Ra (see Table 1). The absolute values for some major tissues such as skeletal muscle, fat and lung were 2.3, 1.4, and 9.6 pg/ng β-actin, respectively. The results on the distribution of Ob-Ra and Ob-Rb confirmed those obtained by RNase Protection Assay, and are summarized in Table 1. Leptin mRNA was abundant in fat tissue (e.g. 15.3 pg/ng β-actin in epididimal fat pads). Even with the high sensitivity of the method (the detection limit was 5-10 fg), leptin mRNA could not be detected in any other tissue.

DISCUSSION

The tissue distribution of the Ob-R isoforms is still very controversial. Most of the published data were obtained using probes or primers directed against the extracellular domain of the receptor which is common to all splice variants. Using more specific probes (RNase-Protection Assay), Ghilardi et al. (7) detected high levels of the short membrane-bound isoforms of the leptin receptor in lymph nodes, lung and uterus, whereas Ob-Rb accounted only for 3-5% of Ob-R mRNA in these tissues. More recently, Hoggard et al. (9) detected significant levels of Ob-Rb only in the kidneys and the hypothalamus. In contrast to Ghilardi et al. (7), no signals for Ob-Rb were detected in the lung. Contrary to other reports (see for instance Mercer et al. (14), Fei et al. (5) detected Ob-Rb in several regions of the hypothalamus, but nowhere else in the brain. To help clarify these issues, we decided to use two independent sensitive methods (heterologous competitive RT-

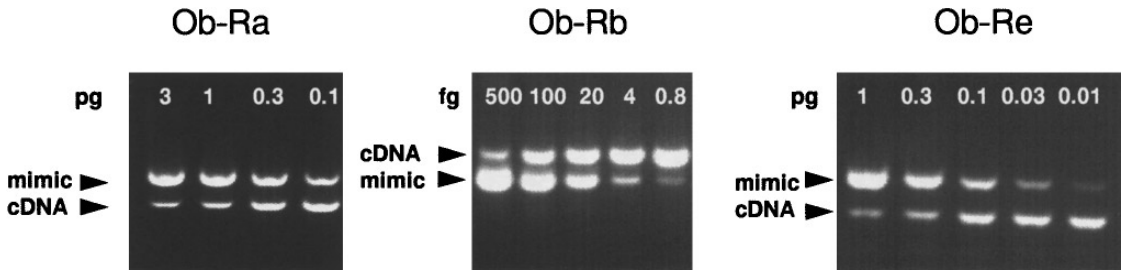

FIG. 2. Heterologous, competitive RT-PCR. Total RNA was extracted from mouse brains after careful dissection of the hypothalamus. cDNA was prepared from total RNA and quantified as described in Materials and Methods by mixing with defined amounts of standard DNA. The values obtained from this example, after correction with the efficiency factors, are: Ob-Ra: 56 fg/ng β-actin, Ob-Rb: 64 fg/ng β-actin, Ob-Re: 29 fg/ng β-actin.

PCR and RNase PA) to measure the distribution of the various receptor subtype in a large number of tissues. Our findings on Ob-Rb and Ob-Ra distribution were consistent with both methods, and basically confirm and extend those of Ghilardi et al. (7). The highest relative level of Ob-Rb was found in the hypothalamus, where leptin is supposed to play its important role in food intake regulation. Interestingly, besides the hypothalamus, other regions of the brain were also found to contain large amounts of the functional receptor. The highest absolute levels of the functional receptor Ob-Rb were found in the lung and were also measurable in a variety of additional peripheral tissues such as the lymph nodes and fat tissue. This might indicate that leptin affects a variety of physiological functions in peripheral organs. In fact, several recent observations have demonstrated a direct effect of leptin in isolated systems such as pancreatic β-cells (11), adipocytes (16) and hematopoietic cells (15,6).

Circulating leptin is specifically and reversibly bound to serum proteins which might modulate its clearance and/or bioactivity (4,10,21). Secreted extracellular domains of cytokine receptors are known to function as specific binding proteins (1,8). It has been proposed that the putative soluble isoform Ob-Re, which is spliced in fronz of the transmembrane domain, functions as a binding protein for leptin (21). Almost nothing is known on the expression levels and distribution of Ob-Re. In a recent publication, Fei et al. (5) stated that Ob-Re is not expressed in significant amounts in mouse tissues since it was undetectable by RT-PCR. These authors, however, did not specify the limits of detection of their RT-PCRs, which can vary over a very wide range. With the primers used in this communication, the detection limits for Ob-Rb and Ob-Re were about 0.2 and 100 fg, respectively, i.e., 500 times more Ob-Re mRNA was needed to be detectable by RT-PCR. While leptin production is restricted to the adipose tissue, we found that Ob-Re expression in mouse is ubiquitous and occurs in rather large amounts. From the data obtained it is conceivable that the soluble receptor Ob-Re is produced at a level sufficiently high to act as a buffering system for free circulating leptin in mice.

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