

ObRa and ObRe Are Differentially Expressed in Adipose Tissue in Aged Food-Restricted Rats: Effects on Circulating Soluble Leptin Receptor Levels

Nilda Gallardo, Carmen Arribas, Margarita Villar, Manuel Ros, José M. Carrascosa, Carmen Martínez, and Antonio Andrés

Área de Bioquímica, Facultad de Químicas (N.G., M.V., C.M., A.A.), Centro Regional de Investigaciones Biomédicas, Universidad de Castilla-La Mancha, 13071 Ciudad Real, Spain; Facultad de Ciencias del Medio Ambiente (C.A.), Centro Regional de Investigaciones Biomédicas, Universidad de Castilla-La Mancha, 45071 Toledo, Spain; Facultad de Ciencias de la Salud (M.R.), Universidad Rey Juan Carlos, Alcorcón, 28922 Madrid, Spain; and Centro de Biología Molecular Severo Ochoa Universidad Autónoma de Madrid-Consejo Superior de Investigaciones Científicas (J.M.C.), Facultad de Ciencias, Universidad Autónoma, 28049 Madrid, Spain

In rodents, soluble leptin receptor (SLR) may be generated by alternative splicing of ObR mRNA and/or as a cleavage product of ObR membrane-anchored receptors. In this study, we investigated the contribution of both processes on the generation of SLR in 3-, 8-, and 24-month-old Wistar rats fed *ad libitum* (AL) or under food restriction (FR). To this end, we determined serum SLR levels and analyzed ObRa and ObRe mRNA expression under these physiological conditions. Additionally, we studied the cellular distribution of ObRa and the generation of SLR by *N*-ethyl-maleimide-induced shedding from ObRa membrane receptors in isolated adipocytes. Serum SLR levels were significantly increased in 8- and 24-month-old rats under FR, whereas similar amounts were found in rats of different ages fed AL. ObRa and ObRe mRNA

expression in epididymal adipose tissue increased with aging. In contrast, after FR, ObRe mRNA expression decreased, whereas ObRa mRNA expression further increased compared with 8- and 24-month-old rats fed AL. Additionally, FR promoted a change in the distribution of ObRa between internal and plasma membranes in isolated adipocytes, increasing its presence at the cell surface. Finally, the generation of SLR by *N*-ethyl-maleimide-induced shedding from ObRa was also increased under FR. These data suggest that shedding of ObRa membrane-anchored receptors, rather than ObRe expression, might preferentially contribute to the generation of the increased levels of SLR in serum under conditions of FR. (*Endocrinology* 146: 4934–4942, 2005)

LEPTIN, A POLYPEPTIDE hormone mainly secreted by adipocytes to the blood, plays its most relevant physiological functions in the brain (1, 2). Leptin regulates food intake and energy homeostasis modifying not only the expression of hypothalamic orexigenic and anorexigenic neuropeptides but also the action of these neuropeptides after they are secreted (3). Leptin may also have an antisteatotic function in peripheral tissues preventing fatty acid accumulation in circumstances of excess of food intake (4).

Leptin acts by binding to its receptor, structurally related to the cytokine receptor family, which can be alternatively spliced into several isoforms (5). The alternative mRNA splicing of mammal leptin receptor generates four membrane isoforms, which differ in the amino acid length of their cytoplasmic domains that include crucial specific motifs for leptin signaling via the janus kinase/signal transducer and activator of transcription pathway (6, 7). Although ObRb, the long isoform of the leptin receptor, is assumed to be responsible for transmitting the majority of biological signals me-

diating the central effects of leptin on appetite and energy expenditure, other receptor isoforms may still be necessary for leptin to exert its full spectrum of *in vivo* functions. ObRa, a short isoform, appears to be the predominant receptor isoform expressed in most peripheral tissues. However, the function of the ObRa remains to be clearly established, although it has been suggested that it may be involved in the transport of leptin across the blood-brain barrier as well as in leptin internalization and degradation (8, 9).

In rodents, an additional splice variant not found in human tissues, the ObRe, has been reported (6, 7, 10). This isoform lacks the transmembrane and cytoplasmic domains and is predicted to be secreted, being the main source of serum leptin binding protein, at least during late pregnancy in mice (11). Nevertheless, *in vitro* and *in vivo* studies in mice overexpressing ObRa or ObRb indicate that murine soluble leptin receptor (SLR), as reported in humans (12, 13), may also be generated by ectodomain shedding from membrane-anchored receptors (14). Moreover, although the functional significance of SLR is yet to be defined, recent studies have suggested that it may modulate the biological effects of leptin in tissues in which direct action of leptin has been demonstrated (15).

Previously, we and others reported that aged rats develop hyperleptinemia and central leptin resistance and suggested that the elevated levels of circulating leptin might play a key

First Published Online July 21, 2005

Abbreviations: AL, *Ad libitum*; BL, bound leptin; E, epididymal; FL, free leptin; FR, food restriction/food-restricted; NEM, *N*-ethyl-maleimide; SLR, soluble leptin receptor.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

role in the development of insulin resistance in adipose tissue, leading to overall insulin insensitivity (16, 17). In addition, we have also shown that prolonged moderate food restriction (FR) improves central leptin sensitivity, lowers hyperleptinemia, and restores adipocyte insulin sensitivity in aged rats (18, 19). However, studies on the regulation of the circulating free leptin (FL), bound leptin (BL), and SLR levels in aged rats and the effect of FR initiated at different moments of the lifespan on these components have not yet been addressed.

In the present work, we have determined serum FL and BL, as well as SLR levels in 3-, 8-, and 24-month-old rats fed *ad libitum* (AL) or under moderate FR. Additionally, we have evaluated the generation of SLR by alternative splicing of ObR mRNA expression and/or by shedding of ObRa membrane-anchored receptors in isolated rat adipocytes with aging with or without FR.

Materials and Methods

Animals

Three-, 8-, and 24-month-old male Wistar rats were provided by the Center of Molecular Biology Severo Ochoa (Madrid, Spain). Animals were housed in climate-controlled quarters with a 12-h light cycle and fed AL with standard laboratory chow and water. They were handled according to the European Union laws and the National Institutes of Health guidelines for animal care. The institutional committee of bioethics approved the experimental procedures, and special care was taken to minimize animal suffering and to reduce the number of animals used.

FR

FR was undertaken as described earlier (18). Five- and 21-month-old rats from our own colony were randomly placed in individual cages and were fed daily with an amount of chow equivalent to 75–80% of the normal food intake until they reached a body weight equivalent to 85% of that of AL-fed aged mates. Thereafter, the rats were weighed weekly, and the amount of food provided was adjusted individually to maintain their weight. Animals were used at the ages of 8 and 24 months, respectively. This FR protocol is enough to obtain animals of different ages with adiposity index similar to those of young mature 3-month-old animals (18).

Biological samples

Epididymal (E), perirenal, and retroperitoneal fat depots were carefully dissected and weighed. The sum of the weight of these fat pads (visceral fat) divided by body weight $\times 100$ was used as an index of adiposity. Serum samples were obtained by blood centrifugation and stored at -70°C until used.

Gel filtration chromatography

Sephadex G-100 was used to separate the FL and BL forms in serum. In each case, 500 μl serum was applied on a Sephadex G-100 column (30 \times 0.7 cm) equilibrated and eluted with PBS (pH 7.4) containing 0.01% sodium azide at a flow rate of 0.2 ml/min. The fractionation was conducted at 4 $^\circ\text{C}$. Fractions of 600 μl were collected and lyophilized.

Leptin and insulin measurements

Leptin levels, in serum, in the incubation medium, and in the fractions obtained by gel filtration chromatography, were measured using a specific rat leptin RIA kit (LINCO Research, Inc., St. Charles, MO) with a detection limit of 0.5 ng/ml. Serum insulin levels were determined with a specific rat RIA kit (LINCO Research, Inc.) with a detection limit of 0.02 ng/ml (0.5 $\mu\text{U}/\text{ml}$).

Partial affinity purification of the SLR

Purification of SLR was performed as described by Li *et al.* (20). Briefly, affinity leptin beads were obtained by coupling rat leptin to Affigel 15 (Bio-Rad, Hercules, CA), 1.5 mg rat leptin/ml Affi-Gel 15, following manufacturer's instructions. To purify the SLR, 0.5 ml rat serum was diluted 1:2 with PBS (pH 7.4) and mixed with 30 μl leptin beads. After overnight incubation at 4 $^\circ\text{C}$, the beads were washed twice in cold PBS and twice in cold PBS [10 mM (pH 7.4)] with 200 mM NaCl, reconstituted in 2 \times sodium dodecyl sulfate sample buffer, boiled for 5 min and applied onto a 7.5% SDS-PAGE. Bound protein (SLR) was detected by Western-blot analysis with specific leptin receptor antibodies as described below.

Isolation of rat adipocytes and membrane fraction preparations

Adipocytes were obtained by collagenase digestion of E adipose tissue in Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 3% BSA according to Rodbell's method (21). Plasma membrane of fat cells was purified in the presence of 1 mM phenylmethylsulfonyl fluoride and 1 mM Na_3VO_4 as described by Massag  and Czech (22), and the internal membrane (light and heavy microsomes) was obtained by centrifugation of the infranantant at 200,000 $\times g$ for 1 h. The presence of ObRa in both membrane fractions was detected by Western-blot analysis.

Effect of *N*-ethyl-maleimide (NEM) on the SLR production

Adipocytes from 8- and 24-month-old rats fed AL and/or under FR were used for the generation of SLR by NEM-induced shedding from ObRa membrane receptors. Fat cells were incubated (1 ml cells per 2 ml medium) at 37 $^\circ\text{C}$ in Krebs-Ringer-Phosphate buffer (pH 7.4) containing 2 mM glucose, 1.2 mM CaCl_2 , and 0.01% BSA in the presence of 5 mM NEM or vehicle (0.1% ethanol) for 5 min (13). After the treatment, the incubation media was collected, concentrated with Centricon 3 (Millipore Corp., Billerica, MA), and used for affinity purification of the SLR by AffiGel 15-leptin chromatography as described before. SLR and ObRa at the plasma membrane, in the absence or presence of NEM treatment, were detected by Western-blot analysis.

Western blot

For SDS-PAGE electrophoresis, protein samples were boiled for 5 min in 2 \times Laemmli buffer under reducing conditions and electrophoresed in 7.5% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose sheets (0.2 μm , Whatman Schleicher and Schuell, Keene, NH) for 2 h with a constant current of 300 mA. The transfer buffer used was 48 mM Tris, 39 mM glycine, 0.01% sodium dodecyl sulfate, and 10% methanol. Blots were then blocked with 5% (wt/vol) nonfat skim milk in PBS for 1 h at room temperature. Once blocked, the blots were incubated during 16 h at 4 $^\circ\text{C}$ with a polyclonal antibody (Affinity BioReagents, Golden, CO) that had been raised against the amino acids 577–594 of the extracellular domains of rat ObR. The nitrocellulose membranes were then washed five times with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with the second antibody (rabbit antimouse IgG coupled to horseradish peroxidase, diluted 1/5000 in rinse buffer). The blots were again washed as detailed above. The immunocomplexes formed were visualized using the ECL Western-blotting detection kit (Amersham Biosciences, Inc., Piscataway, NJ). Bands corresponding to the SLR or membrane leptin receptor were subjected to a densitometric analysis.

RNA extraction and RT-PCR

Total RNA from E adipose tissue was isolated using RN-Easy Mini kit (QIAGEN, Valencia, CA). Five micrograms total RNA was reverse-transcribed into cDNA in a total reaction volume of 20 μl and then diluted to 100 μl as described by Peralta *et al.* (23). PCRs were performed basically as described previously (23), except for RT cDNA template: 5 μl for actin, 10 μl for leptin and ObRa, and 20 μl for ObRe; and the amplification cycle numbers: 25 for actin, 30 for ObRa and leptin, and 35 for ObRe amplification, respectively. The primers used for Ob-Re cDNA PCR amplification were 5'-CCTTACGTTCTCATGGCCCAT-

GAG-3' as forward and 5'-GGTTGACATCCTTGCTTGAGGTTGTC-3' as reverse one. The primers used for ObRa cDNA PCR amplification were 5'-GAGAACGGACACTCTTGAAGTC-3' as forward and 5'-AGTCATTCAAACCATAGTTTAGG-3' as reverse one. These primers were synthesized according to the published sequences for the rat leptin receptors and flank a 310-bp fragment (nucleotides 509–819, National Center for Biotechnology Information accession no. AF007819) for ObRe receptor and a 138-bp fragment (nucleotides 2727–2865, accession no. U42467) for ObRa receptor. The primers used for leptin cDNA PCR amplification were: 5'-TCACCCCATCTGAGTTTTGTC-3' as forward and 5'-CGCCATCCAGGCTCTCT-3' as reverse one. Both flank a 203-bp fragment for the described rat leptin sequences (nucleotides 257–460, National Center for Biotechnology Information accession no. NM013076). The rat actin primers were 5'-GGTATGGAATCCTGTGCGATCCATGAAA-3' for the 5' end and 5'-GTGTAACACGCAGCTCAGTAACAGTCC-3' for the 3' end. Both primers flank a 356-bp fragment (24). After reactions, samples were separated by agarose gel (2.5%) electrophoresis, and the amplified bands were analyzed as described by Peralta *et al.* (23). Control reactions with different number of cycles were performed previously to select conditions for amplification in the linearity range.

Leptin secretion

Adipocytes from 3- and 24-month-old rats fed AL were prepared by the collagenase method using DMEM containing 25 mmol/liter HEPES (pH 7.4) and 4% BSA (19). Cells were washed in HEPES-buffered balanced salt solution (pH 7.4) containing 20 mmol/liter HEPES, 120 mmol/liter NaCl, 1.2 mmol/liter MgSO₄, 2 mmol/liter CaCl₂, 2.5 mmol/liter KCl, 1.0 mmol/liter NaH₂PO₄, 1% BSA, and 5 mmol/liter glucose and incubated in the same buffer (1 ml cells per 3 ml medium) at 37°C for 4.5 h. At times 0, 1.5, 3, and 4.5 h, aliquots of the medium were removed for the determination of the leptin released.

Other methods

Protein concentration was determined by Lowry's method with BSA as standard. All values shown are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA (GraphPad Prism 3.03 software, GraphPad Software, Inc., San Diego, CA). When the main effect was significant, the Bonferroni post hoc test was applied to determine individual differences between means. For comparisons between FR and AL aged mates, the unpaired Student's *t* test was used. *P* values less than 0.05 were considered to be statistically significant.

Results

Characteristics of the animals

The biological characteristics of the animals used in this study are summarized in Table 1. As we expected, body weight, visceral fat, and serum leptin of rats fed AL significantly increased with advancing age, whereas insulin levels remained constant. Nevertheless, FR prevented the weight gain and the increment of adiposity in 8-month-old rats;

consequently, leptin and insulin levels acutely fell to values below those found in 3-month-old rats. In contrast, in 24-month-old animals under FR, there was a decrease in serum leptin levels, but never beyond the values observed in 8-month-old rats fed AL, and no change was observed in their serum insulin levels. These data agree with previously published results (23). When the leptin to adiposity ratio was calculated (Table 1), no statistically significant differences could be found between 3- and 8-month-old rats fed AL. However, we observed a marked increase of this ratio at 24 months of age in both AL and FR rats.

Effects of aging and FR on leptin mRNA expression in E adipose tissue

Because factors, like changes in leptin synthesis and/or secretion, may contribute to alter the leptin to adiposity ratio with aging, we studied leptin mRNA expression in E adipose tissue from 3-, 8-, and 24-month-old AL- and FR-fed rats. As shown in Fig. 1, leptin mRNA expression only decreases at 24 months of age in AL-fed rats, whereas in aged FR rats, a further significant decrease was observed. Thus, we next examined the effect of aging on leptin secretion in isolated adipocytes from 3- and 24-month-old rats fed AL. To this end, a time course of leptin release was done, and it was found that leptin secretion across all time points measured was about 1.5- to 2-fold higher in adipocytes from old compared with young rats (data not shown).

Changes in serum FL and BL levels with age and nutritional status

Serum from 3-, 8-, and 24-month-old AL- and FR-fed rats were fractionated in a Sephadex G-100 gel filtration column. Figure 2 shows a representative chromatographic fractionation of serum obtained from rats fed AL at different ages. In agreement with previously published results (11, 25–27), we observed two different leptin immunoreactive peaks after the chromatographic fractionation. As can be seen, FL and BL levels increase with age (Fig. 2). Nevertheless, the major differences were found in the values of the FL form.

Moreover, when the data are analyzed in terms of percent respect total leptin, the differences between young and aged rats were even more clearly seen. As shown in Table 2, at 3 months of age, BL represents 39% and FL 61%, whereas in 8- and 24-month-old rats fed AL, BL form was decreased to 20

TABLE 1. Biological characteristics of the animals

	3-Month-old AL	8-Month-old AL	8-Month-old FR	24-Month-old AL	24-Month-old FR
Body weight (g)	385 ± 17	519 ± 15 ^a	438 ± 19 ^b	699 ± 44 ^a	567 ± 29 ^b
Visceral fat (g) (E + PR + RP)	9.9 ± 2.7	20.8 ± 4.2 ^a	10.3 ± 4.4 ^d	26.7 ± 2.8 ^a	15.9 ± 3.2 ^b
Adiposity index (% fat pad weight)	2.4 ± 0.6	3.9 ± 0.5 ^c	2.3 ± 0.8 ^d	3.8 ± 0.2 ^c	2.7 ± 0.5 ^d
Serum leptin (ng/ml)	4.7 ± 0.6	6.5 ± 1.4	1.8 ± 0.4 ^b	16.9 ± 2.4 ^a	7.04 ± 1.2 ^d
Leptin to adiposity ratio	1.6 ± 0.1	1.5 ± 0.5	0.7 ± 0.1 ^e	3.9 ± 0.5 ^a	2.5 ± 0.5 ^b
Serum insulin (μU/ml)	33 ± 5	34 ± 6	14 ± 1 ^a	32 ± 5	34 ± 3

Data are the mean ± SEM of six to eight separate determinations.

^a *P* < 0.005 compared with 3-month-old rats.

^b *P* < 0.005 compared with AL age-mate rats.

^c *P* < 0.01 compared with 3-month-old rats.

^d *P* < 0.01 compared with AL age-mate rats.

^e *P* < 0.05 compared with AL age-mate rats.

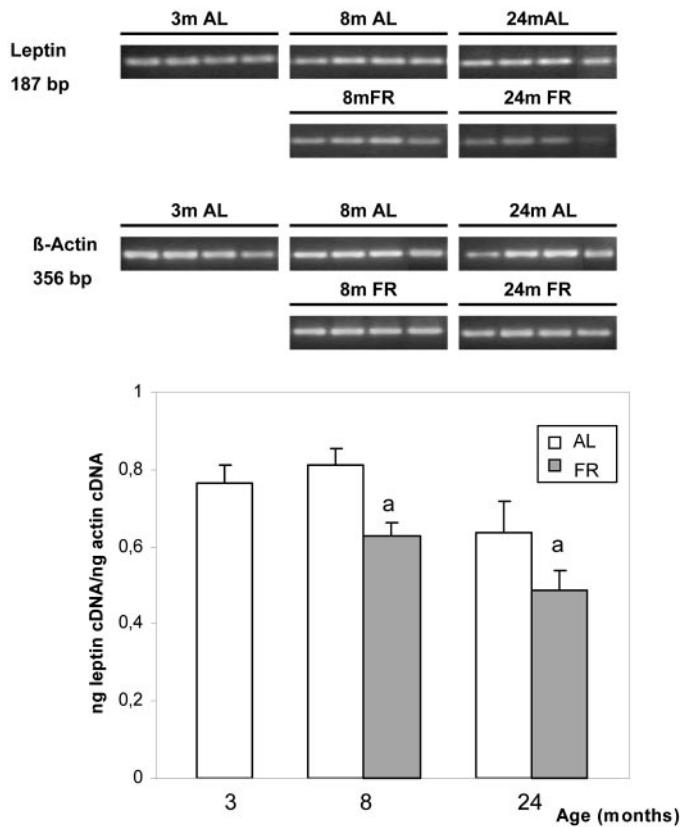


FIG. 1. Leptin mRNA expression in E adipose tissue from aged and FR rats. Expression of leptin in E adipose tissue from 3-, 8-, and 24-month-old rats fed AL as well as their nutritionally restricted age-mates (FR) were determined by RT-PCR as indicated in *Materials and Methods* and quantified as the ratio of nanograms of leptin cDNA amplified per nanogram of cDNA of actin, under the reaction conditions used. Quantification of leptin expression in AL (white bar) and FR (gray bar) rats. a, $P < 0.01$ vs. same age fed AL. Values are means \pm SEM of five separate determinations per group of animals.

and 15%, respectively. At the same time, the FL levels were increased to 80 and 85% in 8- and 24-month-old rats, respectively, probably due to the higher serum leptin concentration in these rats. The similar distribution pattern observed in 8- and 24-month-old rats fed AL changes after moderate FR. Thus, although the distribution between BL and FL became approximately 50% in 8-month-old FR rats, no significant change in the distribution of BL and FL was observed in 24-month-old rats after FR compared with controls.

Effects of aging and FR on SLR and ObRa protein expression

SLRs isolated from serum by affinity purification with leptin-agarose beads were analyzed by Western blot and the bands quantified by scanning densitometry. The results showed that SLR levels were comparable in rats of different ages fed AL. However, after FR, SLR levels were markedly increased in both 8- and 24-month-old rats (Fig. 3A).

On the other hand, the densitometric analysis of ObRa protein present in the adipocyte plasma membrane showed that in rats fed AL, the levels were significantly increased with aging ($P < 0.005$ when compared 3- vs. 8- and 24-

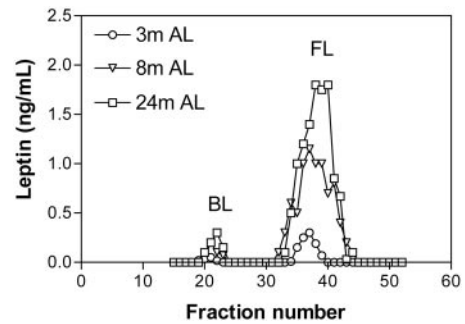


FIG. 2. FL and BL levels with age and FR. Total serum leptin from 3-, 8-, and 24-month-old rats fed AL and their FR age-matched rats was fractionated in a size exclusion chromatography. A representative Sephadex G-100 elution profile of serum from 3-, 8-, and 24-month-old fed AL rats. The two peaks correspond to BL and FL. Pooled fractions, 15–25 and 29–42 for BL and FL respectively, were used for measuring leptin immunoreactivity using a rat RIA kit. Data are presented in Table 2.

month-old rats; Fig. 3B). In addition, FR caused a further significant increase in the ObRa protein levels at the plasma membrane when compared with their AL age mates ($P < 0.01$, 8-month-old AL vs. 8-month-old FR; $P < 0.05$, 24-month-old AL vs. 24-month-old FR).

Effects of aging and FR on mRNA expression of ObRa and ObRe in E adipose tissue

We next analyzed ObRa and ObRe mRNA expression in E adipose tissue from 3-, 8-, and 24-month-old AL and FR fed rats. We performed semiquantitative RT-PCR analyses using ObR isoform-specific primers. As shown in Fig. 4, the expression of both leptin receptor isoforms, ObRa and ObRe, was increased with aging, although to a lesser extent in the case of ObRe (Fig. 4). Interestingly, during FR, the expression of ObRa and ObRe in E adipose tissue was modulated in an opposite manner. In aged FR rats, the expression of ObRe was significantly decreased, reaching values similar to those found in 3-month-old rats (Fig. 4C), whereas the expression of ObRa was further increased respect to the AL age mates (Fig. 4D).

Effect of NEM on SLR production in isolated adipocytes from aged and FR rats

To study whether isolated rat adipocytes could generate SLR by shedding of ObRa membrane-anchored receptors, we incubated adipocytes from 8- and 24-month-old fed AL and FR rats with and without NEM, a sulfhydryl alkylating reagent, that has been used to enhance shedding of membrane-anchored receptors like GHR (28) and ObR (13) through a mechanism that involves a metalloprotease activity. As shown in Fig. 5A, NEM induced a significant loss of ObRa plasma membrane-anchored receptors in adipocytes from 8-month-old rats and to a lesser degree in adipocytes from older rats. Although the content of ObRa at the plasma membrane is quite similar in both groups of aged and FR rats, it is interesting to note that the effect of NEM is higher in rats of 8 months of age (Fig. 5B). The loss of ObRa at the plasma membrane was parallel to the increment of SLR in the ex-

TABLE 2. BL and FL concentrations in serum from 3-, 8-, and 24-month-old rats fed AL and FL

	BL (ng/ml)	FL (ng/ml)	TL (ng/ml)
3 Month	1.58 ± 0.1 (39 ± 3%)	2.46 ± 0.2 (61 ± 4%)	4.04 ± 0.6
8-Month-old AL	1.27 ± 0.4 (20 ± 4%)	5.02 ± 1.8 ^a (80 ± 5%)	6.29 ± 1.4 ^a
8-Month-old FR	0.63 ± 0.1 ^b (45 ± 8%)	0.78 ± 0.05 ^b (55 ± 9%)	1.41 ± 0.4 ^b
24-Month-old AL	2.54 ± 0.5 ^a (15 ± 1%)	14.20 ± 1.3 ^a (85 ± 2%)	16.72 ± 2.4 ^a
24-Month-old FR	1.18 ± 0.08 ^b (18 ± 3%)	5.35 ± 0.3 ^b (82 ± 5%)	6.53 ± 1.2 ^b

Pooled fractions obtained in Fig. 2, 15–25 and 29–42 for BL and FL, respectively, were used for measuring leptin immunoreactivity using a rat RIA kit. Data are the mean ± SEM of six to eight separate determinations.

^a $P < 0.05$ vs. 3-month-old rats. ^b $P < 0.05$ vs. AL age-mate rats. Percentages with respect to total leptin are also indicated.

tracellular medium, being highest in 8-month-old rats under FR (Fig. 5B).

Effect of FR on the subcellular distribution of ObRa in adipocytes from aged rats

Because the availability of ObRa receptor at the plasma membrane may, in part, determine the generation of SLR, we decided to analyze whether the subcellular distribution of ObRa in the adipocytes could be affected in aged rats under FR. As can be seen in Fig. 6, the distribution of ObRa within the subcellular compartments in isolated adipocytes from 8-

and 24-month-old rats changes significantly after FR. In adipocytes from aged rats fed AL, 70% of ObRa receptors were located at the plasma membrane and 30% in the internal membranes. After FR, 90% of ObRa receptors were at the plasma membrane and 10% in the internal membranes.

Discussion

Leptin circulates in serum both as a free biologically active form and as a form bound to a SLR and possibly also to other as yet unidentified binding proteins (25). The balance between FL and BL, and the SLR levels are potential regulators of leptin bioavailability and bioactivity (15, 29). Thus, studies addressing the regulation of circulating SLR and FL under different physiological conditions may contribute to the understanding of the effects of leptin in central and peripheral tissues. The results in the present study confirmed that serum leptin levels were related to increased adiposity. However, at 24 months of age, the leptin to adiposity ratio was higher than expected, suggesting that the leptin resistance associated with aging, in addition to the increased adiposity, might contribute to the hyperleptinemia. In this sense, our data on leptin mRNA expression could not explain the higher serum leptin levels observed in aged rats, although they agree with recent results reporting that aging-related obesity in mice is associated with a decreased leptin gene expression per unit of fat mass (30) and with a decrease in leptin mRNA level in cultured aged 3T3-L1 adipocytes (31). However, as reported in rats with diet-induced obesity (32), we found a higher leptin secretion in adipocytes from old compared with young rats that might contribute to increase serum leptin levels with aging. Nevertheless, because we have not analyzed other fat depots implicated in the synthesis and secretion of leptin, further work has to be done before a conclusion may be drawn.

FL is the predominant form reported herein in serum in rats of different ages fed AL, but the percentage of FL increases with advancing age and a parallel decrease in the BL form is observed. Because aged rats present an increase in adiposity, our results agree with previous findings in normal weight and obese humans, where similar percentage values of FL and BL have been reported (33, 34). Additionally, serum SLR levels in aged rats fed AL were not different from the levels found in 3-month-old rats, despite the differences in serum leptin concentrations. These data are in agreement with previous results reporting similar amounts of circulating SLR in advanced age in humans, as well as in obese and normal-weight individuals (34–36). Nevertheless, other studies have reported that obesity is associated with decreasing levels of the circulating SLR in humans (27, 37).

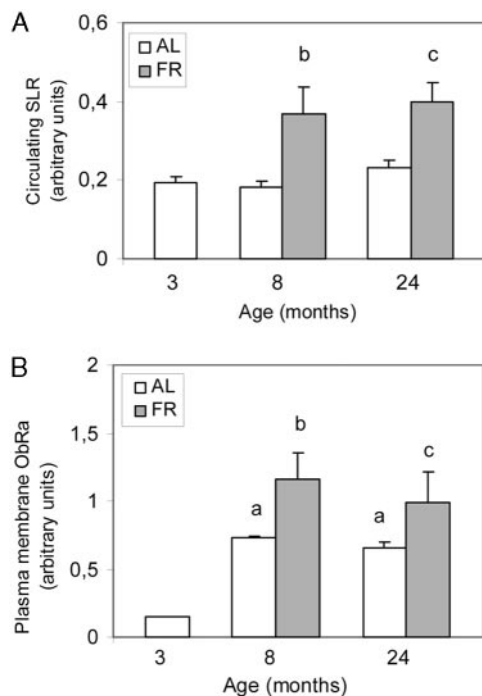


FIG. 3. Effects of aging and FR on the serum SLR and plasma membrane ObRa levels in Wistar rats. In all assays, the blots were incubated with a polyclonal antibody raised against the amino acids 577–594 of the extracellular domain of rat ObR. A, SLR was purified from serum (0.5 ml) of 3-, 8-, and 24-month-old AL and FR fed rats using leptin-agarose beads. After Western-blot analysis, the autoradiographs were quantified by scanning densitometry, and the results are expressed in arbitrary units. b, $P < 0.01$ compared with 8-month-old rats AL; c, $P < 0.05$ compared with 24-month-old rats AL. B, Plasma membrane (60 μ g) from isolated adipocytes from 3-, 8-, and 24-month-old AL- and FR-fed rats was subjected to immunodetection of Ob-Ra. After Western-blot analysis, the levels of Ob-Ra were quantified as described in A. a, $P < 0.005$ compared with 3-month-old rats AL; b, $P < 0.01$ compared with 8-month-old rats AL; c, $P < 0.05$ compared with 24-month-old rats AL. Data are the mean ± SEM of three to five independent experiments.

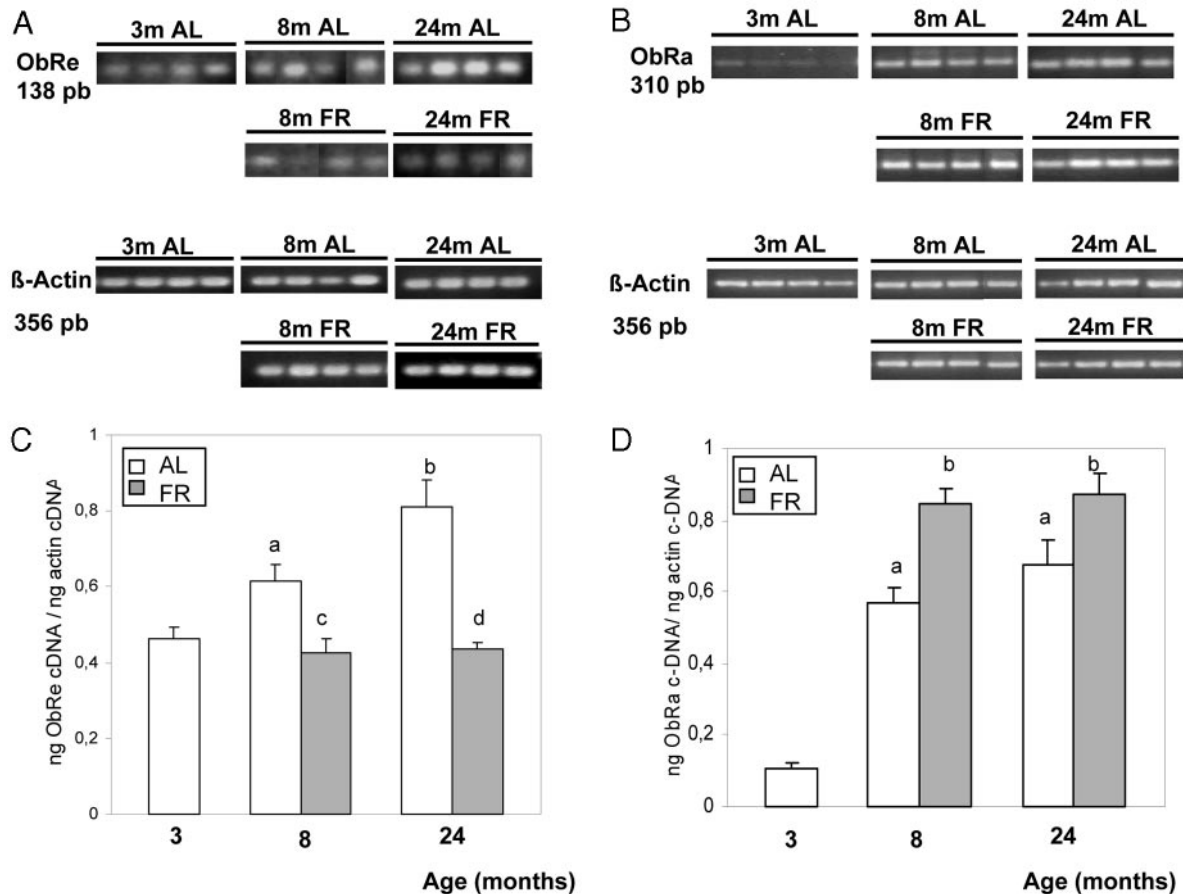


FIG. 4. ObRe and ObRa mRNA expression in E adipose tissue from aged and FR rats. Expression of ObRe (A) and ObRa (B) in E adipose tissue from 3-, 8-, and 24-month-old rats fed AL as well as their nutritionally restricted age-mates (FR) were determined by RT-PCR as indicated in *Materials and Methods* and quantified as the ratio of nanograms of Ob-R cDNA amplified per nanogram of cDNA of actin, under the reaction conditions used. C, Quantification of ObRe expression in AL (white bar) and FR (gray bar) rats. a, $P < 0.025$, and b, $P < 0.005$ vs. 3-month-old animals; c, $P < 0.01$, and d, $P < 0.005$ vs. same age fed AL. D, Quantification of ObRa expression in AL (white bar) and FR (gray bar) rats. a, $P \leq 0.05$ vs. 3-month-old animals; b, $P < 0.05$ vs. same age fed AL. Values are means \pm SEM of five separate determinations per group of animals.

Weight loss in humans is known to induce an increase in the circulating levels of serum SLR and, concomitantly, a decrease in serum leptin levels (27, 38, 39). Because a negative correlation between SLR and leptin levels has been reported in this and other studies (37), these data suggest that leptin, alone or in conjunction with other factors, may play an inhibitory role in the regulation of the serum SLR levels. In fact, it has been reported that administration of recombinant human leptin to fasted lean men for 72 h prevents the significant increase in SLR and even results in a decrease in SLR (38). The results in the present work demonstrate that weight loss in aged rats, after FR, brings about a significant increase in serum SLR and a parallel significant decrease in leptin levels in both 8- and 24-month-old animals. However, despite the similar SLR levels found in aged FR rats, leptin concentration is still significantly much higher in 24- compared with 8-month-old rats. These data support the notion that, in addition to leptin, other unidentified factor(s) may be involved in regulating the circulating concentrations of SLR with aging.

On the other hand, although FR initiated at 20 months of age had a small, insignificant, effect in the distribution of free

and bound forms of serum leptin, this treatment decreased significantly the percentage of FL (~50%) in serum in 8-month-old FR rats. Thus, prevention of fat mass gain at earlier ages during the life span could better regulate the proportion of free bioactive leptin and, consequently, leptin action. In fact, in aged FR rats under euglycemic-hyperinsulinemic clamp conditions, we found that *in vivo* insulin stimulation of glucose use by white adipose tissue is only restored in 8- but not in 24-month-old rats that remain resistant to the action of insulin (Escrivá, F., M. L. Gavete, C. Perez, C. Alvarez, A. Andrés, M. Ros, and J. M. Carrascosa, unpublished observations). Thus, it may be possible that the peripheral inhibitory effect of leptin on insulin action in white adipose tissue in FR rats is only abolished at 8 months of age, when serum leptin concentration is much lower, and the increased SLR levels would better modulate the amount of FL and, therefore, indirectly leptin action on peripheral tissues, as it has recently been suggested (15).

Because the source of SLR *in vivo* remains unknown, we do not know at this point whether a rise in SLR levels could be associated with any change in leptin receptor expression isoforms and/or with shedding of membrane-anchored re-

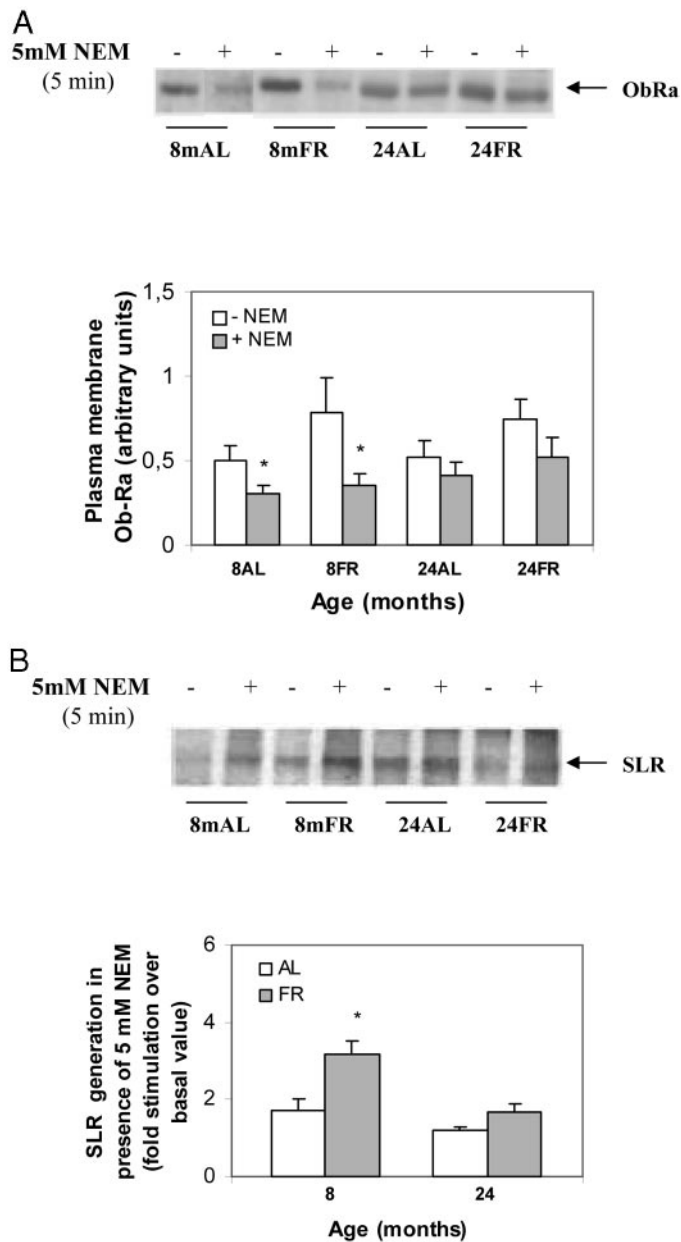


FIG. 5. Effects of NEM on ObRa level at the cell surface and SLR production. Adipocytes from E adipose tissue from 3-, 8-, and 24-month-old AL- and FR-fed rats were incubated with and without 5 mM NEM (5 min). In all assays, the blots were incubated with a polyclonal antibody raised against the amino acids 577–594 of the extracellular domain of rat ObR. After Western-blot analysis, the autoradiographs were quantified by scanning densitometry, and the results are expressed in arbitrary units. A, Plasma membrane ObRa levels in adipocytes incubated in the absence (–) or presence (+) of NEM. B, Autoradiograph shows the SLR levels detected in the concentrated extracellular media, purified by affinity using leptin-agarose beads. The results are expressed in arbitrary units as fold stimulation of SLR production above basal (in the absence of NEM). Data are depicted as mean \pm SEM of four experiments. *, $P < 0.005$ compared with (–NEM); +, $P < 0.005$ compared with their age-mate rats.

ceptors in different tissues. Our results show that ObRe and ObRa mRNA expression in white adipose tissue increase with aging by 2- and 5-fold, respectively, in rats fed AL, but this is not followed by a parallel increase in serum SLR levels.

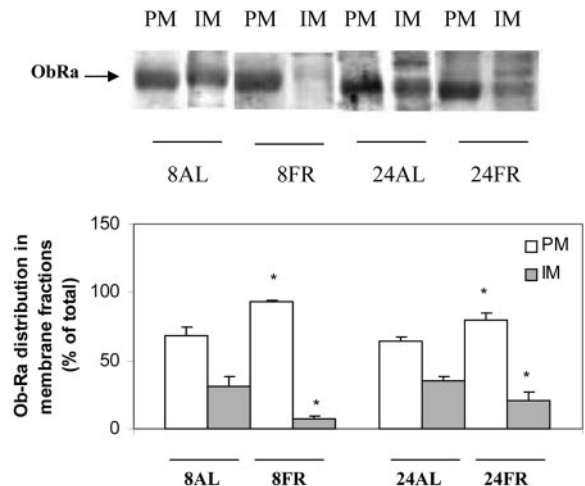


FIG. 6. Subcellular distribution of ObRa in adipocytes from aged FR rats. Fifty micrograms plasma membrane (PM) or internal membrane (IM) fractions purified from adipocytes from E adipose tissue were subjected to immunodetection with a polyclonal antibody raised against the extracellular domain of the ObR. After Western blot analysis, the levels of Ob-Ra in PM (white bars) and in IM (gray bars) were quantified by scanning densitometry, and the results are expressed in arbitrary units. *, $P < 0.05$ compared with their age-mate rats. Data are the mean \pm SEM of three to four independent experiments.

However, although ObRe mRNA expression significantly decreased in white adipose tissue of aged FR rats, a further increment in the ObRa mRNA expression is observed parallel to a 2-fold increase in serum SLR levels. These results agree with previous observations in men reporting that the expression of the shortest membrane anchored leptin receptor mRNA in lymphocytes is induced after a 72-h fast, a situation where the SLR levels are also increased (38). Recent *in vitro* and *in vivo* studies in mice overexpressing ObRa or ObRb have shown, as reported in humans (12, 13), that SLR may also be generated by ectodomain shedding from membrane-anchored receptors (14). Our results suggest that changes in leptin receptor gene expression in white adipose tissue, as indicated by ObRa mRNA expression data presented herein, might contribute to generate SLR after ectodomain shedding, either to increase serum SLR levels or, alternatively, to abrogate leptin paracrine actions on adipose cells to avoid leptin-mediated depletion of the major energy storage under moderate FR. Our data do not rule out the possibility that other as yet unidentified tissues, under physiological conditions in which the expression of ObRa and/or ObRe is induced, could also participate in the generation of SLR, as in the case of placenta in mice and rats during pregnancy (11, 40). In fact, it has been very recently reported that in mice, ObR mRNA expression in the liver is induced under conditions of negative balance, and, in parallel, this was associated with increased levels of plasma SLR (41). Because fasting in mice had no effect on levels of ObRe mRNA, these authors suggest that the increased levels of plasma SLR appear to be the result of cleavage and shedding of membrane-anchored leptin receptor (41).

Previous studies have analyzed the generation of human and mice SLR in transient or stable cultured cell lines overexpressing ObRa and/or ObRb leptin receptors (13, 14). Be-

sides this, it has recently been reported that SLR can be generated from human subcutaneous adipose tissue explants (35). Thus, assuming that in rat tissues SLR might also be generated by shedding from membrane-anchored leptin receptors, this work demonstrates that in primary cells, as adipocytes, the constitutive release of SLR into the medium is increased after NEM-induced shedding from ObRa receptors. Although NEM-induced shedding it is not considered a physiological process, our data agree with previous findings in cultured cell lines reporting that NEM enhance the constitutive shedding rate of membrane-anchored receptors like GHR (28) and ObR (13). In addition, the amount of SLR released into the incubation medium by NEM-induced shedding is increased depending on the physiological conditions. In fact, it is higher in adipocytes isolated from adult than from older rats in both AL- and/or FR-fed animals, despite expression of similar amounts of ObRa receptors at the plasma membrane at both ages. Although the metalloprotease activity implicated in leptin receptor shedding is unknown (13), our data suggest that the proteolytic cleavage of membrane-anchored leptin receptor could be modified with aging and/or with FR. In this sense, several studies have reported the existence of changes in metalloprotease activities with aging and obesity in different tissues (42, 43).

Finally, although we have not been able to directly assess the relationship between SLR levels in the circulation and the expression of leptin receptor isoforms in white adipose tissue, our results do demonstrate that the level of SLR detected in the incubation medium from isolated adipocytes was closely related to the level of expression of the ObRa receptor at the plasma membrane. It is known that leptin receptors are mainly localized in intracellular compartments in target tissues (17, 44). In this sense, we have seen by Western-blot analysis that the distribution of ObRa between plasma membrane and internal membranes represents 27 and 73%, respectively, in isolated adipocytes from 3-month-old rats fed AL (data not shown). However, the subcellular distribution of ObRa in the adipocytes changes significantly with aging and with FR, increasing the level of ObRa receptors at the plasma membrane, while decreasing them at the internal membranes. Thus, our results suggest that the increased availability of ObRa receptor at the cell surface in adipocytes of aged and FR rats may, at least in part, determine the generation of SLR. The mechanism by which aging and FR triggers the increased sorting of ObRa to the plasma membrane under these circumstances remains to be established.

In conclusion, we have shown that leptin receptor mRNA expression isoforms, ObRa and ObRe, in white adipose tissue are differently regulated by moderate FR, and in this situation, the subcellular localization of ObRa in the adipocytes is mainly at the plasma membrane. Additionally, our study demonstrate that in isolated rat adipocytes, the level of SLR detected in the incubation medium, after NEM-induced shedding, was closely related to the level of expression of ObRa at the plasma membrane. The observed changes in the circulating FL, BL, and SLR levels in aged and FR rats could be implicated in the regulation of central and peripheral leptin actions.

Acknowledgments

We thank Dr. Pablo Blanco for helpful suggestions.

Received February 22, 2005. Accepted July 14, 2005.

Address all correspondence and requests for reprints to: Antonio Andrés, Área de Bioquímica, Facultad de Químicas, Centro Regional de Investigaciones Biomédicas, Universidad de Castilla-La Mancha, Avenida Camilo José Cela, 10, 13071 Ciudad Real, Spain. E-mail: antonio.andres@uclm.es.

N.G. was supported by Junta de Comunidades de Castilla-La Mancha (JCCM) Research Training Grant PAC-02-004 and by a Ministerio de Educación y Ciencia predoctoral fellowship (to M.V.). The Centro de Biología Molecular is the recipient of institutional aid from the Ramón Areces Foundation. This work was supported by grants BFI2002-04030-C03-01 and GC-02-021 from Ministerio de Ciencia y Tecnología and JCCM, respectively (Spain).

References

- Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM 1994 Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425–432
- Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A, McKellar W, Rostek Jr PR, Schonell B, Smith D, Tinsley FC, Zhang X-Y, Heiman M 1995 The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* 377:530–532
- Sahu A 2004 A hypothalamic role in energy balance with special emphasis on leptin. *Endocrinology* 145:2613–2620
- Lee Y, Wang M-Y, Kakuma T, Wang Z-W, Babcock E, McCorkle K, Higa M, Zhou Y-T, Unger RH 2001 Liporegulation in diet-induced obesity. The anti-steatotic role of hyperleptinemia. *J Biol Chem* 276:5629–5635
- Tartaglia LA 1997 The leptin receptor. *J Biol Chem* 272:6093–6096
- Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JJ, Friedman JM 1996 Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379:632–635
- Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RL, Mongenstern JP 1996 Evidence that the diabetes genes encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* 84:491–495
- Kastin AJ, Pan W, Maness LM, Koletsky RJ, Ernsberger P 1999 Decreased transport of leptin across the blood-brain barrier in rats lacking the short form of the leptin receptor. *Peptides* 20:1449–1453
- Uotani S, Bjorbaek C, Tornae J, Flier JS 1999 Functional properties of leptin receptor isoforms. Internalization and degradation of leptin and ligand-induced receptor downregulation. *Diabetes* 48:279–286
- Takaya K, Ogawa Y, Isse N, Okazaki T, Satoh N, Masuzaki H, Mori K, Tamura N, Hosoda K, Nakao K 1996 Molecular cloning of rat leptin receptor isoform complementary cDNAs: identification of a missense mutation in Zucker Fatty (*fa/fa*) rats. *Biochem Biophys Res Commun* 225:75–83
- Gavrilova O, Barr V, Marcus-Samuels B, Reitman M 1997 Hyperleptinemia of pregnancy associated with the appearance of a circulating form of the leptin receptor. *J Biol Chem* 272:30546–30551
- Lammert A, Kiess W, Bottner A, Glasow A, Kratzsch J 2001 Soluble leptin receptor represents the main leptin binding activity in human blood. *Biochem Biophys Res Commun* 283:982–988
- Maamra M, Bidlingmaier M, Postel-Vinay MC, Wu Z, Strasburger CJ, Ross RJ 2001 Generation of human soluble leptin receptor by proteolytic cleavage of membrane-anchored receptors. *Endocrinology* 142:4389–4393
- Ge H, Huang L, Pourbahrami T, Li C 2002 Generation of soluble leptin receptor by ectodomain shedding of membrane-spanning receptors *in vitro* and *in vivo*. *J Biol Chem* 277:45898–45903
- Yang G, Ge H, Boucher A, Yu X, Li C 2004 Modulation of direct leptin signaling by soluble leptin receptor. *Mol Endocrinol* 18:1354–1362
- Li H, Matheny M, Nicholson M, Türmer S, Scarpace PJ 1997 Leptin gene expression increases with age independent of increasing adiposity in rats. *Diabetes* 46:2035–2039
- Fernández-Galaz C, Fernández-Agulló T, Campoy F, Arribas C, Gallardo N, Andrés A, Ros M, Carrascosa JM 2001 Decreased leptin uptake in hypothalamic nuclei with ageing in Wistar rats. *J Endocrinol* 171:23–32
- Fernández-Galaz C, Fernández-Agulló T, Pérez C, Peralta S, Arribas C, Andrés A, Carrascosa JM, Ros M 2002 Long-term food restriction prevents ageing-associated central leptin resistance in Wistar rats. *Diabetologia* 45:997–1003
- Pérez C, Fernández-Galaz C, Fernández-Agulló T, Arribas C, Andrés A, Ros M, Carrascosa JM 2004 Leptin impairs insulin signaling in rat adipocytes. *Diabetes* 53:347–353
- Li C, Ioffe E, Fidathusein N, Connolly E, Friedman JM 1998 Absence of soluble

- leptin receptor in plasma of dbPas/db/Pas and others db/db mice. *J Biol Chem* 273:10078–10082
21. **Rodbell M** 1964 Metabolism of isolated fat cells. *J Biol Chem* 239:375–380
 22. **Massagué J, Czech M** 1982 Role of disulfides in the subunit structure of the insulin receptor. Reduction of class I disulfides does not impair transmembrane signaling. *J Biol Chem* 257:6729–6738
 23. **Peralta S, Carrascosa JM, Gallardo N, Ros M, Arribas C** 2002 Ageing increases SOCS-3 expression in rat hypothalamus: effects of food restriction. *Biochem Biophys Res Commun* 296:425–428
 24. **Tokunaga K, Taniguchi H, Yoda K, Shimizu M, Sakiyama S** 1986 Nucleotide sequence of a full-length cDNA for the mouse cytoskeletal β -actin mRNA. *Nucleic Acids Res* 14:2892
 25. **Sinha, MK, Opentanova I, Obannesian JP, Kolaczynski JW, Heiman ML, Hale J, Becker GW, Bowsher RR, Stephens TW, Caro JF** 1996 Evidence of free and bound leptin in human circulation. Studies in lean and obese subjects and during short-term fasting. *J Clin Invest* 98:1277–1282
 26. **Voegelings S, Fantuzzi G** 2001 Regulation of free and bound leptin and soluble leptin receptors during inflammation in mice. *Cytokine* 14:97–103
 27. **van Dielen FM, van 't Veer C, Buurman WA, Greve JW** 2002 Leptin and soluble leptin receptor levels in obese and weight-losing individuals. *J Clin Endocrinol Metab* 87:1706–1716
 28. **Alele J, Jiang J, Goldsmith JF, Yang X, Maheshwari HG, Black RA, Baumann G, Frank SJ** 1998 Blockade of growth hormone receptor shedding by a metalloprotease inhibitor. *Endocrinology* 139:1927–1935
 29. **Huang L, Wang Z, Li C** 2001 Modulation of circulating leptin levels by its soluble receptor. *J Biol Chem* 276:6343–6349
 30. **Guo K-Y, Halo P, Leibel RL, Zhang Y** 2004 Effects of obesity on the relationship of leptin mRNA expression and adipocyte size in anatomically distinct fat depots in mice. *Am J Physiol* 287:R112–R119
 31. **Yu Y-H, Zhu H** 2004 Chronological changes in metabolism and functions of cultured adipocytes: a hypothesis for cell aging in mature adipocytes. *Am J Physiol* 286:E402–E410
 32. **O'Doherty RM, Nguyen L** 2004 Blunted fasting-induced decreases in plasma and CSF leptin concentrations in obese rats: the role of increased leptin secretion. *Int J Obes* 28:173–175
 33. **Landt M, Horowitz JF, Coppack SW, Klein S** 2001 Effect of short-term fasting on free and bound leptin concentrations in lean and obese women. *J Clin Endocrinol Metab* 86:3768–3771
 34. **Wu Z, Bidlingmaier M, Liu C, De Souza EB, Tschöp M, Morrison KM, Strasburger CJ** 2002 Quantification of the soluble leptin receptor in human blood by ligand-mediated immunofunctional assay. *J Clin Endocrinol Metab* 87:2931–2939
 35. **Brabant G, Nave H, Mayr B, Behrend M, van Harmelen V, Arner P** 2002 Secretion of free and protein-bound leptin from subcutaneous adipose tissue of lean and obese women. *J Clin Endocrinol Metab* 87:3966–3970
 36. **Mann DR, Johnson AOK, Gimpel T, Castracane D** 2003 Changes in circulating leptin, leptin receptor, and gonadal hormones from infancy until advanced age in humans. *J Clin Endocrinol Metab* 88:3339–3345
 37. **Lahlou N, Issad T, Lebouc Y, Carel JC, Camoin L, Roger M, Girard J** 2002 Mutations in the human leptin and leptin receptor genes as models of serum leptin receptor regulation. *Diabetes* 51:1980–1985
 38. **Chan JL, Blüher S, Yiannakouris N, Suchard M, Kratzsch J, Mantzoros CS** 2002 Regulation of circulating soluble leptin receptor levels by gender, adiposity, sex steroids, and leptin. Observational and interventional studies in humans. *Diabetes* 51:2105–2112
 39. **Laimer M, Ebenbichler CF, Kaser S, Sandhofer A, Weiss H, Nehoda H, Aigner F, Patsch JR** 2002 Weight loss increases soluble leptin receptor levels and the soluble receptor bound fraction of leptin. *Obes Res* 10:597–601
 40. **Smith JT, Waddell BJ** 2002 Leptin receptor expression in the rat placenta: changes in Ob-Ra, Ob-Rb, and Ob-Re with gestational age and suppression by glucocorticoids. *Biol Reprod* 67:1204–1210
 41. **Cohen P, Yang G, Yu X, Soukas AA, Wolfish CS, Friedman JM, Li C** 2005 Induction of leptin receptor expression in the liver by leptin and food deprivation. *J Biol Chem* 280:10034–10039
 42. **Maquoi E, Munaut C, Colige A, Collen D, Lijnen HR** 2002 Modulation of adipose tissue expression of murine matrix metalloproteinases and their inhibitors with obesity. *Diabetes* 51:1093–1101
 43. **Moon SK, Cha BY, Lee YC, Nam KS, Runge MS, Patterson C, Kim CH** 2004 Age-related changes in matrix metalloproteinase-9 regulation in cultured mouse aortic smooth muscle cells. *Exp Gerontol* 39:123–131
 44. **Sierra-Honigmann MR, Nath AK, Murakami C, García-Cardeña G, Papatropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR** 1998 Biological action of leptin as an angiogenic factor. *Science* 281:1683–1686