

# Caloric restriction reverses the deficits in leptin receptor protein and leptin signaling capacity associated with diet-induced obesity: role of leptin in the regulation of hypothalamic long-form leptin receptor expression

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## Abstract

The objectives of this study were to determine if reduced long-form leptin receptor (ObRb) expression in diet-induced obese (DIO) animals is associated with deficits in maximal leptin signaling and, secondly, to establish the effects of short-term caloric restriction (CR) on ObRb expression and function. Groups of DIO and life-long chow-fed (CHOW) F344 × BN male rats, aged 6 months, were given an i.c.v. injection containing 2 µg leptin or artificial cerebrospinal fluid (ACSF) vehicle. Leptin induced a >6-fold increase in STAT3 phosphorylation in CHOW rats, but less than 2-fold increase in DIO. Reduced maximal leptin-stimulated STAT3 phosphorylation in DIO rats was coupled with a decline in both ObRb expression and protein. At this point, subgroups of

DIO and CHOW animals underwent CR for 30 days and were then tested for acute leptin responsiveness. CR resulted in a 45 and 85% increase respectively in leptin-stimulated STAT3 phosphorylation in CHOW and DIO animals. Similarly, CR increased ObRb expression and protein in both CHOW and DIO animals. To explore the role of leptin in regulating ObRb expression, we reversibly overexpressed leptin in the hypothalamus and found that ObRb mRNA inversely follows central leptin expression. By enhancing both ObRb expression and signaling capacity, CR may enhance leptin responsiveness in leptin-resistant DIO animals.

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## Introduction

Common human obesity is associated with hyperleptinemia and leptin resistance. Clinical trials with leptin have been disappointing due to this phenomenon of leptin resistance in the obese state (Gura 1999) and interest in leptin to treat obesity has waned. Although it is a subject of rigorous investigation, the precise mechanism of leptin resistance in obesity is unknown. While transport of leptin across the blood–brain barrier (BBB) appears to be saturated at serum leptin levels observed in obesity (Van Heek *et al.* 1997, Banks *et al.* 1999), deficient BBB transport is clearly not the only factor contributing to leptin insensitivity. Both we and others have reported convincing evidence for a central component to leptin resistance in obese animal models including obese A<sup>y</sup> mice (Halaas *et al.* 1997), diet-induced obese (DIO) C57BL/6J mice (El-Haschimi *et al.* 2000), DIO Sprague–Dawley rats (Levin & Dunn-Meynell 2002), and aged-obese F344 × BN rats (Shek & Scarpace 2000, Scarpace & Tumer 2001, Scarpace *et al.* 2001). Recent data from our

laboratory demonstrated that DIO F344 × BN rats are completely unresponsive to an i.c.v. injection of recombinant adeno-associated virus-encoding-leptin (rAAV-leptin) (Wilsey *et al.* 2003). A similar dose of rAAV-leptin administered to age-matched chow-fed (CHOW) animals causes potent anorexia sustained for at least 150 days, thermogenesis, and a near complete loss of visceral white adipose tissue (Scarpace *et al.* 2002, 2003, Wilsey *et al.* 2003).

It has been previously demonstrated that various obese animal models have reduced leptin receptor (ObRb) expression and/or protein in the hypothalamus. This includes high fat-fed Osborne–Mendel rats (Madiehe *et al.* 2000), DIO C57 mice (Lin *et al.* 2000), and aged-obese Wistar and F344 × BN rats (Scarpace *et al.* 2001, Fernandez-Galaz *et al.* 2002). Similarly, we recently demonstrated that our DIO F344 × BN rat model also exhibits reduced ObRb expression (Wilsey *et al.* 2003). It is unknown whether these reductions in ObRb expression and protein are associated with reduced maximal leptin signaling. Our objective was to determine if DIO animals

have reduced ObRb-mediated JAK/STAT3 signaling capacity as measured by maximal leptin-induced STAT3 phosphorylation in the hypothalamus. We reasoned that if DIO animals had both reduced ObRb expression and reduced ObRb-mediated STAT3 phosphorylation capacity, then reduced ObRb expression may directly contribute to the reduced responsiveness to pharmacological leptin observed in these animals. A second objective was to test whether 30 days of caloric restriction (CR) could reverse any potential deficits in ObRb expression and leptin signaling observed in DIO rats. Our final objective was to determine what effect, if any, chronically elevated leptin in the hypothalamus had on ObRb expression in lean rats.

## Methods and Materials

### Animals

Three-month-old male Fischer 344 × Brown Norway rats were obtained from Harlan Sprague–Dawley (Indianapolis, IN, USA). Upon arrival, rats were examined and remained quarantined for 1 week. Animals were individually caged with a 12 h light:12 h darkness cycle (0700–1900 h). Animals were cared for in accordance with the principles of the NIH Guide to the Care and Use of Experimental Animals.

### Experimental design 1: leptin signaling in DIO rats

All animals were maintained on standard rat chow (Diet 2018; Harlan Teklad, Madison, WI, USA) from weaning until 2 weeks after arriving in our laboratory, at which point animals were approximately 3 months old. This CHOW diet provides 3.3 kcal/g of digestible energy and 15% of energy as fat. At this point, 65 animals were switched to a high fat/high sucrose (HF) diet (F3282; BioServ, Frenchtown, NJ, USA). This HF diet provides 5.3 kcal/g and 59.4% of energy as fat. The top 40% of weight gainers on the HF diet were designated as DIO and the rest were removed from the study. This is similar to the designation system used previously by Levin & Keesey (1998), who defined the top 37.5% of weight gainers on a high energy diet as DIO. An additional group of animals were continuously maintained on CHOW to serve as the 'lean' control group. DIO and CHOW animals remained on their respective diets through to the conclusion of the study (when acute leptin sensitivity was evaluated) or until the start of CR (when remaining DIO animals were switched to a restricted CHOW diet). After approximately 105 days of HF feeding in DIO rats, groups of DIO and CHOW animals were prepared for stereotaxic surgery as described below. Animals were given a single injection into the third cerebroventricle containing 2 µg leptin peptide or 4 µl artificial cerebrospinal fluid (ACSF)

vehicle. Sixty minutes after leptin delivery, animals were killed for the measurement of hypothalamic STAT3 phosphorylation and ObRb expression. This time frame was chosen because we have previously demonstrated that leptin-induced STAT3 phosphorylation peaks at ~60 min (Scarpace *et al.* 2001).

### Experimental design 2: leptin signaling following CR

After more than 100 days of high-fat feeding in DIO rats, subgroups of both DIO and CHOW rats were switched to a CR diet. This CR diet provided 60% of mean, basal freely available (ad lib) caloric intake in the form of standard chow diet. Ad lib caloric intake did not differ between CHOW and DIO and averaged approximately 62.4 kcal/day in both groups in the week prior to commencing CR. Sixty percent of this is 37.4 kcal/day, or 11.34 g of chow diet per day. Thus, approximately 11.34 g of chow were provided to each CR DIO and CHOW animal (DIO-CR and CHOW-CR) for 30 days. We chose to switch the DIO animals to chow diet for the CR phase of the study because of concerns about excessive ketosis and related side effects if animals were restricted on the high fat, lower carbohydrate diet. Food was placed in the stainless steel food baskets in the cage of each rat in the early evening (between 1700 and 1800 h). After 30 days of CR, these animals were tested for acute responsiveness to 2 µg i.c.v. leptin exactly as were the DIO-ad lib and CHOW-ad lib animals (see Experimental design 1).

### Experimental design 3: effect of central leptin overexpression on ObRb expression

Animals were given a single hypothalamic injection of rAAV-leptin under the control of a tetracycline responsive element promoter (TET-*Ob*) or control vector encoding green fluorescent protein (GFP). The physiological responses to this TET-*Ob* gene therapy system, as well as details about its production and delivery, have been previously described in detail (Wilsey *et al.* 2002). All rats received doxycycline hydrochloride (Sigma, St Louis, MO, USA) (400 µg/ml) in their drinking water for 34 days, activating the leptin transgene. At this point, doxycycline was withdrawn from half of the TET-*Ob*-treated animals for 32 days to de-activate the leptin transgene (Ob-OFF) while half continued to receive doxycycline (Ob-ON). At this end point, animals were killed for the evaluation of hypothalamic ObRb expression as well as leptin transgene expression.

### Leptin administration

Rats were anesthetized with 60 mg/kg pentobarbital and their heads were prepared for surgery. Animals were placed into a stereotaxic frame and a small incision (1.5 cm) was made over the midline of the skull to expose

the landmarks of the cranium (Bregma and Lamda). The following coordinates were used for injection into the third cerebroventricle: 1.3 mm posterior to Bregma and 9.4 mm ventral from the skull surface on the midline (medial fissure), with the nose bar set at 3.3 mm below the ear bars (below zero) and the cannula set at 20° posterior from vertical. A small hole was drilled through the skull and a 23-gauge stainless steel guide cannula was lowered to the third cerebroventricle. This was followed by an injection cannula attached to a 10 µl syringe. Two micrograms of leptin dissolved in 4 µl ACSF (or ACSF vehicle alone) were slowly injected (approximately 0.5 µl/min) to minimize tissue damage.

#### Vector administration

Rats were anesthetized with 60 mg/kg pentobarbital and their heads were prepared for surgery. Animals were placed into a stereotaxic frame and a small incision (1.5 cm) was made over the midline of the skull to expose the landmarks of the cranium (Bregma and Lamda). The following coordinates were used for third ventricle injection: 1.8 mm posterior to Bregma, 1.3 mm anterior to Bregma, 9.2 mm ventral from skull surface, on the midline (medial fissure). The nose bar was set 3.3 mm below zero (on same plane with ear bars) and the cannula was set 20° posterior from vertical. A small hole was drilled through the skull and a 23-gauge stainless steel guide cannula was lowered to the hypothalamus. This was followed by an injection cannula attached to a 10 µl syringe. We injected 5 µl of viral particles in Ringer's solution at approximately 0.25 µl/min. Animals received either the two-vector TET-*Ob* system ( $n=14$ ) or control virus encoding GFP ( $n=6$ ).

#### Tissue harvesting

Anesthetized rats were killed by cervical dislocation. Blood was collected by cardiac puncture and serum was harvested by a 10 min centrifugation in serum separator tubes. The circulatory system was perfused with 20 ml cold saline. Perirenal white adipose tissue (PWAT) and retroperitoneal white adipose tissue (RTWAT) and hypothalami were excised, weighed, and immediately frozen in liquid nitrogen. The hypothalamus was removed by making an incision medial to piriform lobes, caudal to the optic chiasm, and anterior to the cerebral crus to a depth of 2–3 mm. Tissues were stored at –80 °C until analysis.

#### Real-time RT-PCR

We designed primers and a Taqman probe specific for ObRb using Primer Express software, version 1.5 (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA, USA). The sequences for the ObRb primers were for-

ward primer: 5'-GGGAACCTGTGAGGATGAGTGT-3', reverse primer: 5'-TTTCCACTGTTTTACGTTGCT-3'. The fluorescent probe sequence was: 6 FAM-AGAGTCAACCCTCAGTTAAATATGCAACGCTG-TAMRA. Optimization experiments showed that 300 nM of forward primer, 900 nM of reverse primer and 50 nM Taqman probe gave the most reproducible results and maximally efficient PCR (i.e. lowest threshold cycle ( $C_T$ ) values). Total RNA (6 µg) was treated with RNase-free DNase using a DNA-free kit (Ambion, Inc., Austin, TX, USA). First-strand cDNA was generated from 1.6 µg RNA in a 40 µl volume using random primers (Gibco BRL, Gaithersburg, MD, USA) containing 200 U of M-MLV reverse transcriptase (Gibco BRL). Real-time PCR for ObRb was performed on 100 ng cDNA template in a 50 µl total volume including Taqman RT-PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using an ABI Prism GeneAmp 5700 Sequence Detection System (Applied Biosystems). ObRb expression was quantified using an 18S rRNA standard (Applied Biosystems) and the  $\Delta\Delta C_T$  method (Bustin 2000). Briefly, a  $\Delta C_T$  value was calculated for each sample by subtracting the  $C_T$  for 18S rRNA amplification from the  $C_T$  for ObRb. A calibrator is then chosen and subtracted from each  $\Delta C_T$  value to yield  $\Delta\Delta C_T$ . The mean  $\Delta C_T$  in the control group (CHOW) was chosen as the calibrator for  $\Delta\Delta C_T$  calculation. Finally, a  $2^{-\Delta\Delta C_T}$  value was calculated for each sample to yield a quantitative measure of ObRb expression relative to 18S rRNA. Units are arbitrary.

#### STAT3/phosphorylated-STAT3 assay

These methods were described in detail previously (Scarpace *et al.* 2000). Briefly, hypothalamus was sonicated in 10 mM Tris-HCl, pH 6.8, 2% SDS, and 0.08 µg/ml okadaic acid plus protease inhibitors (polymethylsulfonyl fluoride, benzamidine and leupeptin) (an aliquot of this sonicate was frozen for RNA analysis). Sonicate was diluted and quantified for protein using a detergent-compatible Bradford assay. Samples were boiled and separated on a 7.5% agarose, Tris-HCl gel (BioRad, Hercules, CA, USA) and electrotransferred to nitrocellulose membrane. Immunoreactivity was assessed with an antibody specific to phosphorylated-STAT3 (antibody kit from New England Biolabs, Beverly, MA, USA). Immunoreactivity was visualized by chemiluminescence detection (Amersham Life Sciences, Piscataway, NJ, USA) and quantified by video densitometry (BioRad). Following phosphorylated-STAT3 quantification, membranes were stripped of antibody with Immunopure (Pierce, Rockford, IL, USA) and immunoreactivity was reassessed using a total STAT3 antibody. Since total STAT3 was not affected by dietary group or any treatment, STAT3 phosphorylation is expressed as phosphorylated-STAT3/total STAT3 in each sample.

### ObRb protein in hypothalamus

ObRb protein was evaluated by Western analysis on the same hypothalamic tissue used for the phosphorylated-STAT3/STAT3 assay. Samples were sonicated in a protease-inhibiting buffer as described above. Forty micrograms of protein were loaded into 15  $\mu$ l wells of a 5% agarose, Tris-HCl gel (BioRad) and then separated at  $\sim$ 90 V for 70 min. Samples were electrotransferred to a nitrocellulose membrane and then incubated with an ObRb primary antibody (Linco Research, St Louis, MO, USA). Immunoreactivity was visualized by chemiluminescence detection (Amersham Life Sciences) and quantified by video densitometry (BioRad).

### Leptin mRNA levels in white adipose tissue

RTWAT (300 mg/sample) was sonicated in guanidine buffer, phenol extracted, and isopropanol precipitated using a modification of the method of Chomczynski & Sacchi (1987). Isolated RNA was resuspended in ribonuclease-free water and quantified by spectrophotometry. Integrity was verified using 1% agarose gels stained with ethidium bromide. For dot blot analysis, multiple concentrations of RNA were immobilized on nylon membranes using a dot blot apparatus (BioRad). Membranes were baked in a UV crosslinking apparatus. Membranes were then prehybridized in 10 ml Quickhyb (Stratagene, La Jolla, CA, USA) for 30 min followed by hybridization in the presence of a labeled probe for leptin mRNA and 100  $\mu$ g salmon sperm DNA. After hybridization for 2 h at 65 °C, the membranes were washed and exposed to a phosphor imaging screen for 72 h. The screen was then scanned using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA) and analyzed by Image Quant Software (Molecular Dynamics). Data are expressed as ObRb mRNA per total RTWAT pad.

### Serum leptin

Serum leptin was measured using a mouse leptin ELISA Kit from Crystal Chem. Inc. (Chicago, IL, USA) on blood harvested at killing by cardiac puncture.

### Statistical analysis

All data are expressed as means  $\pm$  S.E.M. The  $\alpha$  level was set at 0.05 for all analyses. Comparisons of mean food intake and body weight gain in DIO vs CHOW rats were made by a repeated-measures two-way ANOVA with time and diet as factors. Comparisons of absolute body weights of CHOW vs DIO rats were made by Student's *t*-test. Comparison in absolute body weights of CHOW-ad lib, DIO-CR, and CHOW-CR during the CR period were made by a repeated-measures two-way ANOVA with

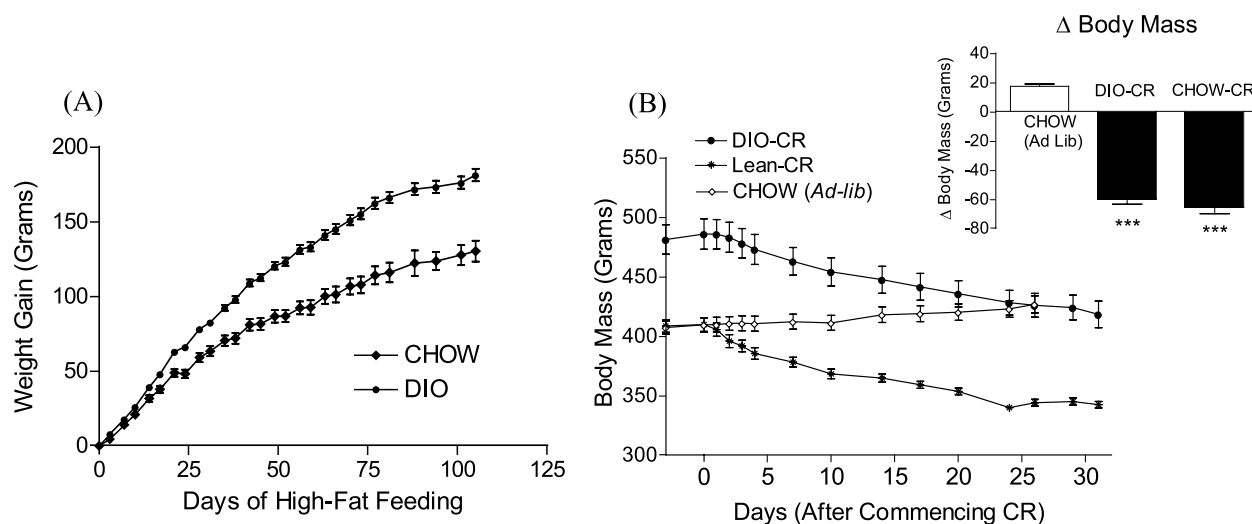
time and diet as factors. Comparisons in  $\Delta$  body mass during the CR experiment (CHOW-ad lib, DIO-CR and CHOW-CR groups) and ObRb expression in the TET-*Ob* experiment were made by one-way ANOVA with Tukey's post-hoc test. All other comparisons were made by two-way ANOVA with dietary group (DIO vs CHOW) and treatment (leptin vs ACSF or ad lib vs CR) as factors. Note that the effects of leptin and CR on STAT3 phosphorylation in DIO and CHOW were analyzed with separate two-way ANOVAs, although the data are represented in a single graph. When only main effects were significant, relevant pairwise comparisons were made using the Bonferroni multiple comparison method with the error rate corrected for the number of contrasts (Rao 1998). When there was an interaction, factors were separated and a further one-way ANOVA was applied with a Bonferroni multiple comparison post-hoc. When separation of factors resulted in only two population means to compare, the one-way ANOVA was replaced with Student's *t*-test. GraphPad Prism software version 4.0 (San Diego, CA, USA) was used for all statistical analysis and graphing. GraphPad QuickCalc (graphpad.com) was used for post-hoc analysis of two-way ANOVAs. Unless otherwise noted: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

## Results

### Food intake body weight

At the start of HF-feeding, there was no difference in body mass between DIO and CHOW rats ( $286 \pm 4.4$  vs  $286 \pm 6.0$  g respectively). Through 105 days of HF-feeding, DIO animals gained 39.3% more mass than CHOW controls (Fig. 1A), translating to 12% greater weight at this point ( $467 \pm 5.2$  vs  $415 \pm 8.9$  g respectively,  $P < 0.0001$ ). Although there was a transient increase in caloric intake immediately after switching pre-DIO animals to the HF diet (data not shown), energy intake returned to that of CHOW animals within 5 days, after which point caloric intake did not differ between CHOW and DIO through to the conclusion of the experiment ( $59.7 \pm 1.40$  kcal/day in CHOW,  $61.1 \pm 0.65$  kcal/day in DIO). This is consistent with earlier reports that DIO animals have increased feed efficiency and increased rate of adipose deposition on a high-fat diet without hyperphagia (Levin & Dunn-Meynell 2000).

After approximately 115 days of HF-feeding in DIO rats, subgroups of DIO and CHOW animals were put on a CR diet, providing 60% of ad lib caloric intake. Note that the CR diet that was provided to both CHOW-CR and DIO-CR was in the form of standard chow as described in Methods. By the end of the 30 day CR period, DIO-CR lost  $62 \pm 3.5$  g, eliminating the difference in body weights between DIO-CR and CHOW-ad



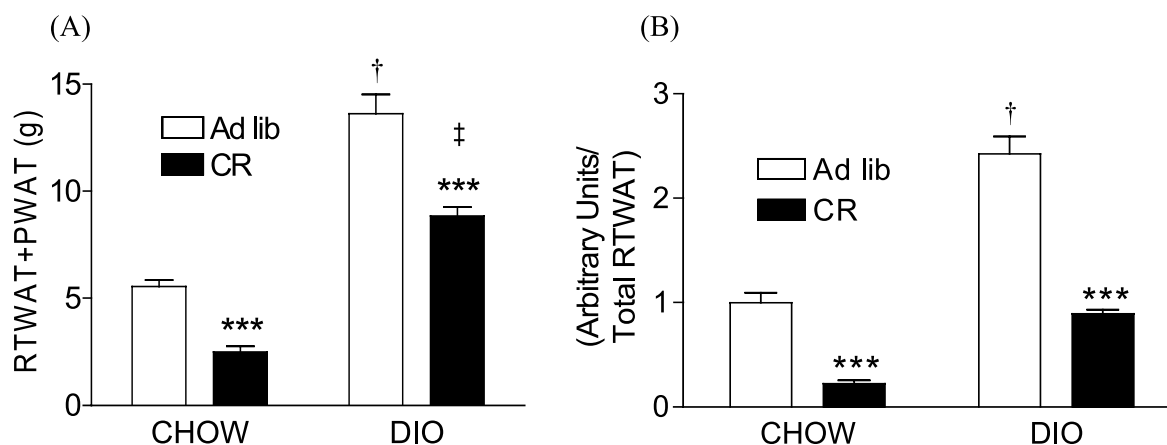
**Figure 1** (A)  $\Delta$  body mass during HF feeding. Values represent means  $\pm$  S.E.M. of CHOW ( $n=9$ ) and DIO ( $n=27$ ) rats. By repeated-measures two-way ANOVA, significance was found for both the time ( $F=1115.8$ ,  $P<0.0001$ ) and dietary group ( $F=44.39$ ,  $P<0.0001$ ) main effects. The interaction between time and diet was also significant ( $F=36.92$ ,  $P<0.0001$ ). (B) Body mass during CR. By repeated-measures two-way ANOVA, significance was found for both the time ( $F=292.96$ ,  $P<0.0001$ ) and CR ( $F=28.95$ ,  $P<0.0001$ ) main effects. The interaction between time and CR was also significant ( $F=154.1$ ,  $P<0.0001$ ). Inset:  $\Delta$  body mass during the CR period. By one-way ANOVA and Tukey post-hoc analysis,  $***P<0.0001$  for difference in total  $\Delta$  body mass between CHOW-CR and CHOW-ad lib group,  $***P<0.0001$  for difference in  $\Delta$  body mass between DIO-CR and CHOW-ad lib.

lib (Fig. 1B). CHOW-CR lost  $65 \pm 4.1$  g during CR, leaving this group with 20.4% lower mean body weights than CHOW-ad lib controls (Fig. 1B).

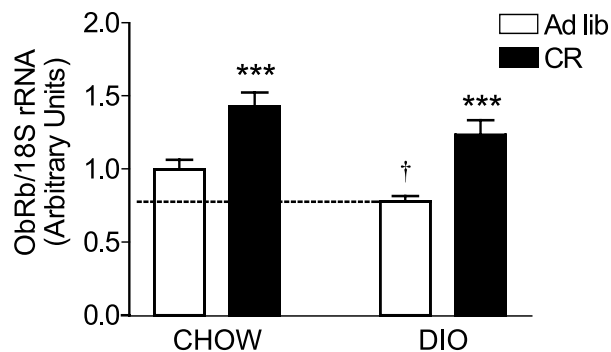
#### Adiposity, white fat leptin expression and serum leptin

Visceral adiposity (as represented by sum of RTWAT and PWAT depots) was elevated 2.46-fold in DIO with

respect to CHOW rats (Fig. 2A). Thirty days of CR reduced visceral adiposity by 55% in CHOW and by 35% in DIO (Fig. 2A). However, visceral adiposity after CR in DIO remained 60% above that of CHOW-ad lib animals (Fig. 2A). Although CHOW experienced a slightly greater loss in visceral adiposity during CR when expressed as a percent of starting adiposity, the magnitude of visceral fat loss was actually greater in DIO (mean



**Figure 2** (A) Visceral adiposity (sum of RTWAT and PWAT) at killing. Values represent means  $\pm$  S.E.M. of CHOW-ad lib ( $n=8$ ), DIO-ad lib ( $n=5$ ), CHOW-CR ( $n=8$ ), and DIO-CR ( $n=5$ ). By two-way ANOVA, significance was found for both the CR ( $F=79.01$ ,  $P<0.0001$ ) and dietary group ( $F=267.42$ ,  $P<0.0001$ ) main effects. By post-hoc analysis, adiposity was significantly greater in DIO compared with CHOW rats ( $\dagger P<0.001$ ). By post-hoc analysis, effect of CR was significant in both CHOW ( $***P<0.001$ ) and DIO ( $***P<0.001$ ) rats. Visceral adiposity in DIO-CR remained above that of CHOW-ad lib ( $\ddagger P<0.001$ ). (B) Leptin expression in RTWAT at killing. By two-way ANOVA, both diet and CR main effects were significant ( $F=126.75$ ,  $P<0.0001$ ;  $F=153.79$ ,  $P<0.0001$  respectively), as was the interaction between the main effects ( $F=16.54$ ,  $P<0.001$ ). By post-hoc analysis,  $\dagger P<0.0001$  for difference in basal RTWAT leptin expression in CHOW-ad lib and DIO-ad lib;  $***P<0.0001$  for effect of CR in both CHOW and DIO.



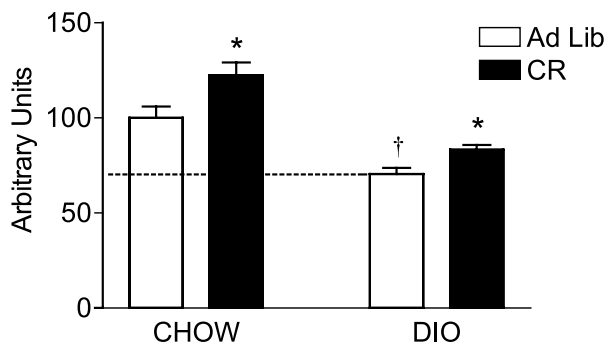
**Figure 3** ObRb expression in the hypothalamus. Values represent means  $\pm$  S.E.M. of CHOW-ad lib ( $n=8$ ), DIO-ad lib ( $n=17$ ), CHOW-CR ( $n=8$ ), and DIO-CR ( $n=5$ ). By two-way ANOVA, significance was found for both the CR ( $F=39.85$ ,  $P<0.0001$ ) and dietary group ( $F=8.81$ ,  $P<0.01$ ) main effects. By post-hoc analysis, basal ObRb expression was significantly reduced in DIO-ad lib vs CHOW-ad lib rats,  $\dagger P<0.05$ . Effect of CR was significant in both CHOW ( $***P<0.001$ ) and DIO ( $***P<0.001$ ) rats.

visceral fat loss was 3.05 g in CHOW and 4.79 g in DIO). Leptin expression per RTWAT pad was elevated nearly 2.5-fold in DIO-ad lib compared with CHOW-ad lib (Fig. 2B). Thirty days of CR caused a 78% decrease in RTWAT leptin expression in CHOW animals and a 63% decrease in DIO (Fig. 2B). The effect of CR in DIO rats brought RTWAT leptin expression to a level comparable with that in CHOW-ad lib (Fig. 2B). Consistent with the adiposity data, serum leptin was elevated approximately 2.4-fold in DIO animals compared with CHOW ( $17.2 \pm 1.37$  and  $7.3 \pm 0.53$  ng/ml respectively,  $P<0.0001$ ). An accurate measure of endogenous serum leptin in both CR groups (CHOW-CR and DIO-CR) could not be obtained due to leakage of the supramaximal leptin injection from the cerebrospinal fluid.

#### ObRb expression and protein levels in the hypothalamus

DIO animals had a 22% reduction in ObRb expression in the hypothalamus with respect to CHOW rats (Fig. 3). CR resulted in a 43% increase in ObRb expression in CHOW-CR vs CHOW and a 58% increase in DIO-CR vs DIO (Fig. 3). The  $C_T$  value for the 18S rRNA control signal was not affected by obesity or CR ( $C_T$  values for 18S rRNA were  $16.60 \pm 0.11$  in CHOW-ad lib,  $16.43 \pm 0.15$  in DIO-ad lib,  $16.78 \pm 0.11$  in CHOW-CR, and  $16.79 \pm 0.14$  in DIO-CR), suggesting that the observed changes in ObRb were due to changes in the absolute level of ObRb mRNA.

Changes in ObRb protein qualitatively mirrored the mRNA data. DIO animals had a 30% reduction in ObRb protein with respect to CHOW (Fig. 4). CR caused modest ( $\sim 20\%$ ), but statistically significant increases in

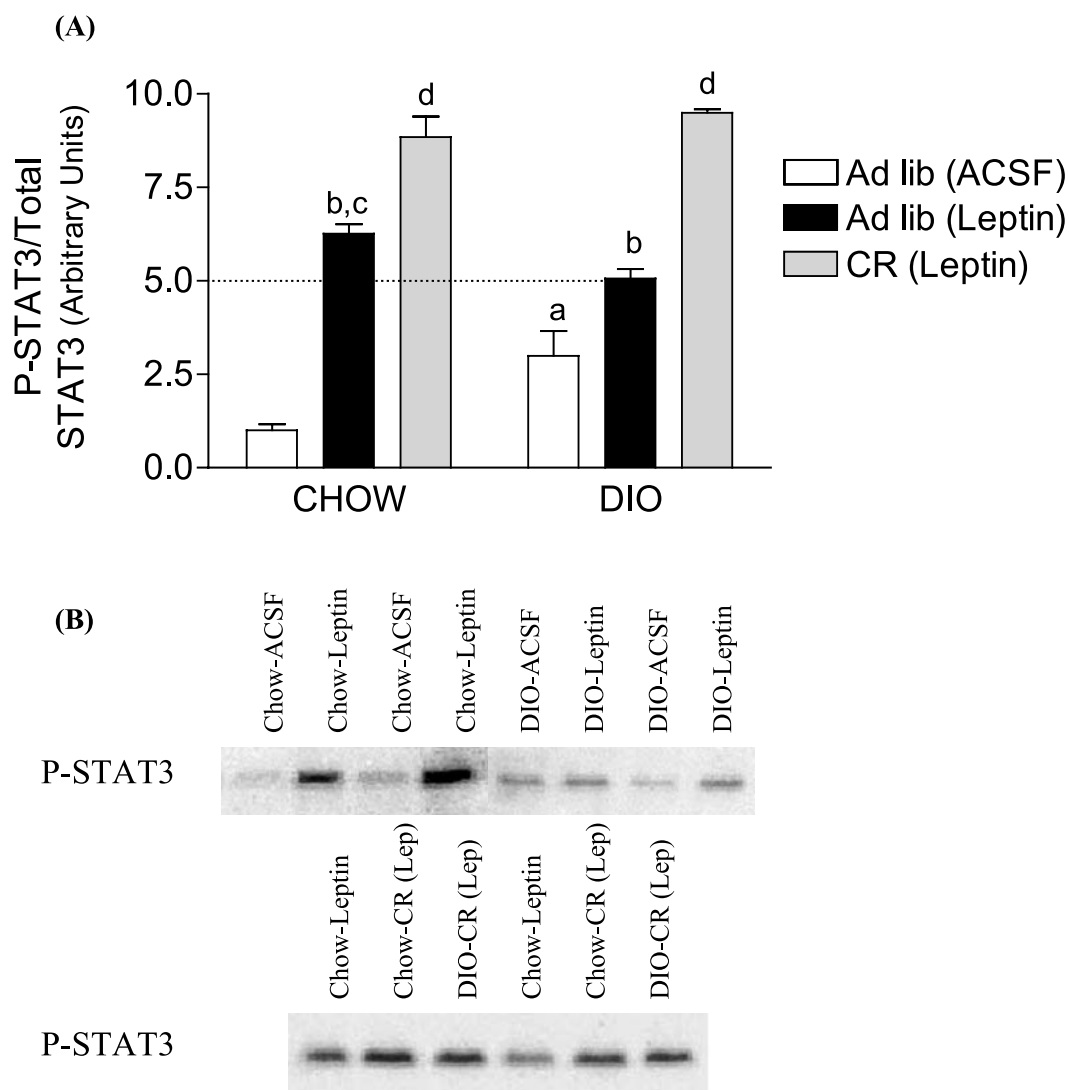


**Figure 4** ObRb protein in the hypothalamus. Values represent means  $\pm$  S.E.M. of CHOW-ad lib ( $n=8$ ), DIO-ad lib ( $n=17$ ), CHOW-CR ( $n=8$ ), and DIO-CR ( $n=5$ ). By two-way ANOVA, significance was found for both the CR ( $F=8.93$ ,  $P<0.01$ ) and dietary group ( $F=34.60$ ,  $P<0.0001$ ) main effects. By post-hoc analysis, basal ObRb protein was significantly reduced in DIO-ad lib vs CHOW-ad lib,  $\dagger P<0.05$ . Effect of CR was significant in both CHOW ( $*P<0.05$ ) and DIO ( $*P<0.05$ ) animals.

ObRb protein in both CHOW-CR and DIO-CR rats (Fig. 4).

#### Hypothalamic STAT3 phosphorylation

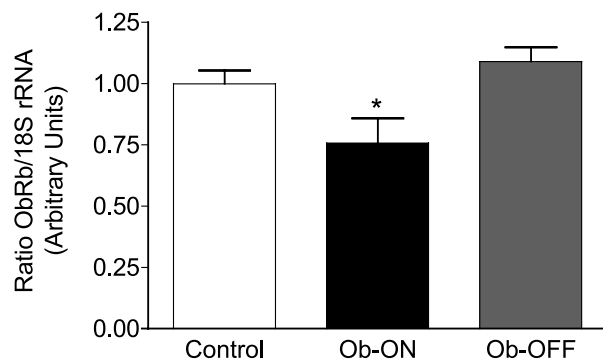
Basal (unstimulated) STAT3 phosphorylation was elevated approximately 3-fold in DIO compared with CHOW rats (Fig. 5). However, we suspected that this high-fat feeding-induced stimulation does not represent maximal STAT3 phosphorylation. Thus, we administered a central dose of leptin that would cause maximal leptin-induced STAT3 phosphorylation at 60 min. The dose of leptin used in this study ( $2 \mu\text{g}$ ) is termed 'supramaximal leptin' because it is  $>8$  times the i.c.v. dose required to achieve maximal STAT3 phosphorylation in both lean and obese F334  $\times$  BN rats in our laboratory (Scarpace *et al.* 2001). A single i.c.v. injection containing supramaximal leptin caused a 6.1-fold increase in STAT3 phosphorylation in CHOW rats, yet caused only a 1.7-fold induction in STAT3 phosphorylation in DIO rats (Fig. 5). However, basal levels of STAT3 phosphorylation were elevated 3-fold in DIO vs CHOW animals. Nevertheless, there was still a small but significant 16% decrease in maximally stimulated STAT3 phosphorylation capacity in DIO vs CHOW rats ( $P<0.05$ , Fig. 5). Maximal STAT3 activation was then examined after 30 days of CR. CR caused a 45% increase in maximal leptin-induced hypothalamic STAT3 phosphorylation capacity in CHOW rats (i.e. CHOW-CR vs CHOW-ad lib) and a dramatic 85% increase in DIO (DIO-CR vs DIO-ad lib), completely reversing the obesity-associated impairment in signaling capacity (Fig. 5). Similar to the pattern observed with ObRb expression, the obesity-associated deficit was completely reversed as DIO-CR rats displayed 56% greater leptin-induced STAT3 phosphorylation capacity



**Figure 5** (A) STAT3 phosphorylation 1 h after i.c.v. leptin (2  $\mu$ g) or ACSF administration in ad lib-fed animals; STAT3 phosphorylation 1 h after i.c.v. leptin in CR animals. Values represent means  $\pm$  s.e.m. of CHOW-ad lib, ACSF ( $n=5$ ), CHOW-ad lib, leptin ( $n=6$ ), CHOW-CR (leptin) ( $n=8$ ), DIO-ad lib, ACSF ( $n=6$ ), DIO-ad lib, leptin ( $n=6$ ), DIO-CR (leptin) ( $n=5$ ). By two-way ANOVA with dietary group and leptin as factors, leptin main effect was significant ( $F=84.40$ ,  $P<0.0001$ ), as was interaction between dietary group and leptin ( $F=14.1$ ,  $P<0.001$ ), but diet main effect was not significant ( $F=1.75$ ). By a second two-way ANOVA with diet and CR as factors, significance was found only for the CR main effect ( $F=60.75$ ,  $P<0.0001$ ). By post-hoc analysis, (a)  $P<0.05$  for difference in basal STAT3 phosphorylation in CHOW vs DIO (white bars); (b)  $P<0.0001$  for effect of leptin in CHOW and  $P<0.01$  for effect of leptin in DIO (solid bars vs white bars); (c)  $P<0.05$  for difference in maximally stimulated STAT3 phosphorylation in CHOW-Lep and DIO-Lep (gray bars); (d)  $P<0.001$  and  $P<0.001$  respectively, for effect of CR on STAT3 phosphorylation capacity in CHOW and DIO (gray vs solid bars). (B) Representative gels of phosphorylated-STAT3 (P-STAT3) in the hypothalamus. Top, first four lanes demonstrate effect of leptin in CHOW rats. Top, last four lanes demonstrate leptin effect in DIO rats. Bottom gel section compares maximal leptin-induced P-STAT3 in CHOW and CHOW-CR and DIO-CR.

compared with the leaner CHOW-ad lib animals (Fig. 5). Total STAT3 in the hypothalamus was not affected by diet or acute leptin (CHOW-ACSF,  $1.00 \pm 0.11$  arbitrary

units; CHOW-Lep,  $1.02 \pm 0.06$ ; CHOW-CR (Lep),  $0.95 \pm 0.03$ ; DIO-ACSF,  $1.03 \pm 0.07$ ; DIO-Lep,  $1.08 \pm 0.04$ ; DIO-CR,  $0.97 \pm 0.06$ ).



**Figure 6** Effect of leptin transgene on ObRb expression in the hypothalamus. Values represent means  $\pm$  S.E.M. of control ( $n=5$ ), Ob-ON ( $n=4$ ) and Ob-OFF ( $n=4$ ). Overall significance was detected by one-way ANOVA ( $F=5.494$ ,  $P<0.05$ ). By Tukey post-hoc analysis, only the difference between Ob-ON and Ob-OFF was significant ( $*P<0.05$ ).

#### Interaction of hypothalamic leptin transgene and ObRb expression

To further explore the role of elevated central leptin in regulating hypothalamic ObRb expression, we administered rAAV-leptin under the control of a tetracycline-responsive promoter (TET-*Ob*) to a subset of normal, CHOW animals. This TET-*Ob* system allows us to regulate leptin transgene expression via doxycycline in the drinking water (Wilsey *et al.* 2002). Animals that had their leptin transgene continuously activated for 2 months (TET-*Ob*-ON) tended to have reduced hypothalamic ObRb expression compared with rats administered a control vector expressing GFP (Fig. 6). Moreover, a subgroup of TET-*Ob*-treated animals that had their leptin transgene activated for 1 month and then silenced for 1 month had significantly greater hypothalamic ObRb expression compared with the TET-*Ob*-ON group (Fig. 6). Our ability to activate and silence the leptin transgene in the hypothalamus was confirmed by RT-PCR (Wilsey *et al.* 2002).

#### Discussion

We previously demonstrated that the DIO F344  $\times$  BN model is non-responsive to the normally potent anorectic and lipopenic effects of hypothalamic leptin over-expression, and that these DIO animals also have reduced ObRb expression in the hypothalamus (Wilsey *et al.* 2003). The major objective of the present study was to determine if the physiological leptin resistance and reduced ObRb expression in this DIO model is associated with deficits in leptin signal transduction capacity. More specifically, we wanted to test the hypothesis that DIO animals have a lower ceiling of leptin-induced hypothalamic STAT3 phosphorylation. Our second objective was

to determine if such a deficit in leptin signaling could be reversed by CR.

Here, we report that DIO animals have a significant reduction in hypothalamic ObRb expression ( $-22\%$ ) and protein ( $-30\%$ ) coupled with a 3-fold elevation in basal STAT3 phosphorylation. While we suspect that the elevation in basal STAT3 phosphorylation in DIO rats is a product of their hyperleptinemia, it is possible that one or more other cytokines contribute to this phenomenon. Nonetheless, when fully stimulated by leptin, maximal STAT3 phosphorylation capacity in DIO rats is diminished by 16% compared with CHOW rats. Moreover, we observed a 58% increase in hypothalamic ObRb expression, an 18% increase in ObRb protein, and an impressive 85% increase in maximal leptin-induced STAT3 phosphorylation capacity in DIO rats following 30 days of CR. CR caused similar increases in hypothalamic ObRb expression and STAT3 phosphorylation capacity in lean CHOW animals.

Changes in ObRb expression, including the decrease in DIO rats and increase with CR, were accompanied by qualitatively similar changes in STAT3 phosphorylation capacity. The significance of this relationship has yet to be established. It has been well documented that the ObRb catalyzes STAT3 phosphorylation via receptor-bound JAK2, the latter of which docks on the intracellular domain of ObRb homodimers upon leptin activation of the receptor complex (Sweeney 2002). We believe that a decrease in ObRb expression and receptor number may decrease the available amount of ObRb for JAK2 binding and activation. This, in turn, may decrease maximal leptin-induced STAT3 phosphorylation capacity. Since ObRb protein and leptin-induced STAT3 phosphorylation capacity were reduced by a similar magnitude in DIO rats, we do not believe that the coupling of the ObRb to JAK2-STAT3 signaling is impaired in obesity. However, coupling of the ObRb to STAT3 signaling may be improved by CR as signaling capacity is increased to a much greater extent than ObRb protein following CR. This pattern of a disproportionate increase in leptin signaling capacity subsequent to CR is especially evident in DIO rats. DIO-CR rats nearly doubled their ceiling of leptin-induced STAT3 phosphorylation despite the relatively meager induction of ObRb.

The mechanism behind reduced ObRb expression in DIO animals and enhanced ObRb expression following CR remains in question. However, it may involve the ability of leptin itself to regulate ObRb expression. Martin *et al.* (2000) reported a decrease in both ObRb expression and protein following 28 days of peripheral leptin infusion. Moreover, Fernandez-Galaz *et al.* (2002) showed that approximately 1 month of food restriction (leading to a 40% reduction in serum leptin) reverses the reduction in ObRb expression observed in hyperleptinemic aged-obese Wistar rats. However, neither of these studies attempted to differentiate between the effects of peripheral and central



leptin. To further explore the ability of leptin to regulate ObRb expression, we overexpressed leptin in the hypothalami of a separate group of normal CHOW animals. Consistent with negative regulation of hypothalamic ObRb expression by leptin, ObRb expression was reduced by ~25% after 2 months of continuous transgene activation compared with animals treated with a control vector (Ob-ON vs control). More impressively, ObRb expression was ~44% greater in a group of animals in which we silenced the leptin transgene for 1 month following a month of activation (Ob-OFF) compared with the group with the continuously activated leptin transgene (Ob-ON). The Ob-ON animals, which had high central leptin expression and presumably high leptin levels at the level of the hypothalamic receptors, had ~70–80% less serum leptin than Ob-OFF and controls (Wilsey *et al.* 2002). This shows that the decrease in ObRb expression in Ob-ON is a specific effect of central leptin as opposed to an indirect effect of peripheral hyperleptinemia.

Short- and long-term CR has been shown to dramatically reduce serum leptin, usually to a much greater extent than would be predicted based on the loss of adiposity (Shimokawa & Higami 2001, Miyawaki *et al.* 2002). In the present study, we report significant reductions in leptin expression in white fat following 30 days of CR (–78% in CHOW and –63% in DIO rats). Although we were unable to obtain a reliable measure of endogenous serum leptin in our CR rats, the diminished leptin expression in the white fat of CR animals is consistent with reduced serum leptin following food restriction. Taken together, the changes in peripheral leptin expression following high-fat feeding and CR along with the TET-*Ob* data discussed in the previous paragraph support our hypothesis that leptin is regulating hypothalamic ObRb expression, and this is a direct effect of leptin in the central nervous system.

We previously demonstrated a reduction in both leptin-induced hypothalamic STAT3 phosphorylation capacity and ObRb protein in aged-obese F344 × BN male rats (Scarpace *et al.* 2001). Fernandez-Galaz *et al.* (2002) reported both reduced ObRb expression and receptor number in the hypothalami of aged-obese Wistar rats, as well as blunted physiological leptin responsiveness. These deficits in aged-obese Wistar rats were completely reversed by 3 months of moderate (~80% ad lib) CR (Fernandez-Galaz *et al.* 2002). Our present data suggest that similar defects in ObRb expression are present in young adult DIO rats and, moreover, that ObRb expression and STAT3 phosphorylation capacity can be dramatically increased in both DIO animals and their CHOW lean counterparts by 30 days of CR. It seems plausible that a similar mechanism may be involved in the changes in ObRb expression and leptin sensitivity observed in both aged-obese and DIO animals. Namely, the reductions in ObRb expression and signaling capacity in both aged and DIO animals may be due to chronic

hyperleptinemia, and, similarly, the restoration of ObRb expression and leptin sensitivity following CR in aged and DIO animals may be secondary to reduced leptin levels.

It seems unlikely that the modest 30% decrease in ObRb protein and slight attenuation in signaling capacity through the JAK–STAT cascade in DIO animals are the sole contributors to the complete physiological non-responsiveness to central leptin overexpression we previously reported in these animals (Wilsey *et al.* 2003). Rather, these data are suggestive of either downstream or parallel contributors to leptin resistance. As an example of a possible downstream contributor, the ability of leptin to stimulate the anorexic/thermogenic melanocortin system may be impaired in obesity. Consistent with this, Hansen *et al.* (2001) demonstrated that DIO Sprague–Dawley rats have reduced endogenous  $\alpha$ -melanocyte-stimulating hormone levels in the paraventricular nucleus and are hyperresponsive to the acute anorectic effects of i.c.v. injections of the peptide. It is also possible that signal transduction through parallel cascades is impaired in DIO animals. It has been recently demonstrated that the ObRb can signal through an insulin receptor substrate–phosphatidylinositol 3-kinase–phosphatidyl inositol trisphosphate cascade (Kim *et al.* 2000, Zhao *et al.* 2002, Niswender *et al.* 2003).

Regardless of the part played by parallel or downstream pathways, signaling through STAT3 appears to be an absolute requirement for leptin's effects on energy balance (Bates *et al.* 2003). As such, the potential role of even small changes in ObRb–STAT3 signaling capacity in the etiology of leptin resistance should not be ignored. Moreover, it remains a possibility that changes in ObRb expression and signaling capacity in specific nuclei of the hypothalamus most critical to the regulation of energy balance were greater than we observed by analyzing the whole hypothalamus. Additional studies are necessary to determine the effects of obesity and CR on leptin signal transduction in the arcuate and other hypothalamic nuclei.

In conclusion, diminished ObRb expression in DIO rats is associated with attenuated maximal leptin-induced STAT3 phosphorylation capacity. CR reverses these deficits, increasing both ObRb expression and signaling capacity in DIO animals to levels at or above those observed in the highly leptin-responsive CHOW animals. Furthermore, hypothalamic overexpression of leptin causes a reversible reduction in ObRb mRNA. This demonstrates that local leptin levels negatively regulate ObRb expression in the hypothalamus, and suggests that this may be one of the mechanisms behind the observed changes in ObRb expression and signaling with obesity and following food restriction. Thus, short-term CR may be a viable strategy to restore ObRb number, signaling capacity, and, ergo, leptin sensitivity in previously leptin-resistant models of acquired obesity.

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