nature medicine

Induction of leptin resistance through direct interaction of C-reactive protein with leptin

Ke Chen[1,6], Fanghong Li[1,6], Ji Li[1], Hongbo Cai[2], Steven Strom[2], Alessandro Bisello[3], David E Kelley[3], Miriam Friedman-Einat[4], Gregory A Skibinski[5], Mark A McCrory[5], Alexander J Szalai[5] & Allan Z Zhao[1]

The mechanisms underlying leptin resistance are still being defined. We report here the presence in human blood of several serum leptin-interacting proteins (SLIPs), isolated by leptin-affinity chromatography and identified by mass spectrometry and immunochemical analysis. We confirmed that one of the major SLIPs is C-reactive protein (CRP). *In vitro*, human CRP directly inhibits the binding of leptin to its receptors and blocks its ability to signal in cultured cells. *In vivo*, infusion of human CRP into *ob*/*ob* mice blocked the effects of leptin upon satiety and weight reduction. In mice that express a transgene encoding human CRP, the actions of human leptin were completely blunted. We also found that physiological concentrations of leptin can stimulate expression of CRP in human primary hepatocytes. Recently, human CRP has been correlated with increased adiposity and plasma leptin. Thus, our results suggest a potential mechanism contributing to leptin resistance, by which circulating CRP binds to leptin and attenuates its physiological functions.

Molecular and physiological evidence accumulated in the past decade has established that leptin is a crucial adipocyte hormone involved in regulation of energy intake and expenditure[1,2]. Null mutations in genes encoding leptin and leptin receptor cause hyperphagia, severe obesity and hyperglycemia in both rodents and humans[2–4], and leptin replacement in leptin-deficient animals and humans can have profound normalizing effects on food intake and body weight. Paradoxically, the majority of obese individuals have elevated rather than depressed levels of leptin[5]. Therapeutic trials with exogenously administered leptin, which raises leptin levels, have not induced substantial weight loss[6]. The seemingly contradictory observation that leptin seems to be ineffective in preventing obesity has spawned the concept of 'leptin resistance'[7,8], and recent studies have begun to elucidate potential molecular mechanisms for this resistance. For example, elevation of the protein suppressor of cytokine signaling-3 (SOCS-3), which is induced by leptin, might diminish the actions of leptin in the central nervous system[8].

Here, we explored the hypothesis that leptin resistance might be partially attributed to interactions between leptin and plasma circulating factors. Such factor(s) might bind leptin and impede its transport or otherwise neutralize its actions. To account for clinical observations that obesity correlates with increased levels of leptin, these putative factors are postulated to rise in proportion to the severity of obesity, much like leptin itself. We report the identification of several blood-borne factors termed SLIPs, detected by elution from a leptin-conjugated affinity column. We have confirmed that SLIP-1 is CRP. CRP not only binds to plasma leptin but also impairs leptin signaling and attenuates its physiological effects *in vivo*. Furthermore, leptin directly stimulates expression of CRP in human primary hepatocytes *in vitro*. Together, these findings suggest that CRP is not only a marker for obesity-related comorbidities, but that CRP itself is involved in the regulation of adiposity through interaction with leptin and promotion of leptin resistance.

RESULTS

Purification of SLIPs and Identification of SLIP-1

To identify SLIPs, we passed human and rat sera through columns coupled with human and mouse recombinant leptin peptides, respectively. After extensive washing and elution with an acidic glycine solution (**Supplementary Fig. 1** online), we found five major protein species with apparent molecular weights on silver-stained SDS-PAGE gels of ~30, ~42, ~65, ~70 and ~85 kDa (**Fig. 1a**) retained by the human leptin column. We termed these proteins human SLIP-1, SLIP-2, SLIP-3, SLIP-4 and SLIP-5, respectively. A substantial amount of human leptin (identified by western blot assay; data not shown) was also present in these eluates (**Fig. 1a**), probably as a result of association and coelution with SLIPs. All five human SLIPs have rat counterparts, as passage of rat serum through the mouse leptin-affinity column yielded proteins of similar molecular weights (**Fig. 1b**). Human soluble leptin receptors, although present in the eluates (**Supplementary Fig. 1** online), was not represented by any of the SLIPs. The low level of human soluble leptin receptors (relative to

[1]Department of Cell Biology & Physiology, [2]Department of Pathology and [3]Division of Endocrinology & Bone Metabolism & Obesity and Nutrition Research Center, University of Pittsburgh, 3500 Terrace Street, BST/S-326, Pittsburgh, Pennsylvania 15261, USA. [4]Institute of Animal Science, ARO, Volcani Center, P.O. Box 6 Bet-Dagan 50-250, Israel. [5]Department of Medicine, Division of Clinical Immunology & Rheumatology, University of Alabama at Birmingham, 1900 University Boulevard, THT-437B, Birmingham, Alabama 35294, USA. [6]These authors contributed equally to this work. Correspondence should be addressed to A.Z.Z. (azhao@pitt.edu).

Received 9 August 2005; accepted 3 March 2006; published online 2 April 2006; doi:10.1038/nm1372

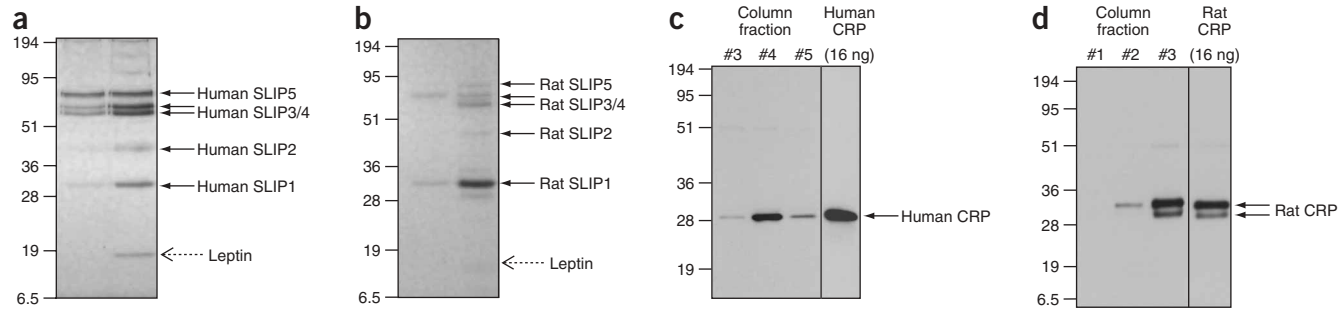

Figure 1 Purification of SLIPs and identification of SLIP-1. (**a**,**b**) Five major SLIPs were identified from the eluate of leptin-affinity columns (**Supplementary Fig. 1**) on a silver-stained SDS gel with apparent molecular weight of 30-, 42-, 65-, 70-, and 85-Kd, correspondingly named as the human (**a**) or rat (**b**) SLIP-1, 2, 3, 4, and 5. Serum leptin could also be co-eluted with SLIPs (dashed arrows), which was further confirmed by Western blot assays with antibodies specific to leptin (data not shown). (**c**,**d**) Confirmation of SLIP-1 as CRP with specific antibodies to human- or rat-CRP in Western blot assays. The column fractions (#3, #4, and #5 for human leptin affinity column; #1, #2, and #3 for mouse leptin affinity column) were run together with corresponding CRP standards (16 ng for each). The arrows indicate the specific CRP bands.

the five major SLIPs) in the column fractions results, at least in part, from their very low concentrations in human plasma.

Determining the identity and physiological relevance of each of the SLIPs is an ongoing process, but the following completed studies have characterized SLIP-1. We excised human SLIP-1 from SDS-PAGE gel and subjected it to a mass spectrometry assay. This analysis identified human SLIP-1 as human CRP (**Supplementary Table 1** online). Similarly, rat SLIP-1 was identified as rat CRP (**Supplementary Table 2** online). We further confirmed the identities of human SLIP-1 and rat SLIP-1 in western blot assays using species-specific antibodies to CRP (**Fig. 1c**,**d**).

Direct interaction between CRP and leptin

We confirmed interaction of CRP with leptin in an immunoprecipitation assay. We purified rat CRP from fresh rat serum to >95% purity, using a previously established protocol[9] (**Supplementary Fig. 2** online). This degree of purity was comparable to that of commercial human CRP preparation (**Supplementary Fig. 2** online). We premixed the purified human CRP and rat CRP proteins with recombinant human and mouse leptin, respectively, before addition of species-specific antibodies to leptin. The concentrations of CRP and leptin in the precipitation reactions were all within the physiological ranges observed in humans or rats[10,11]. In parallel experiments, we also

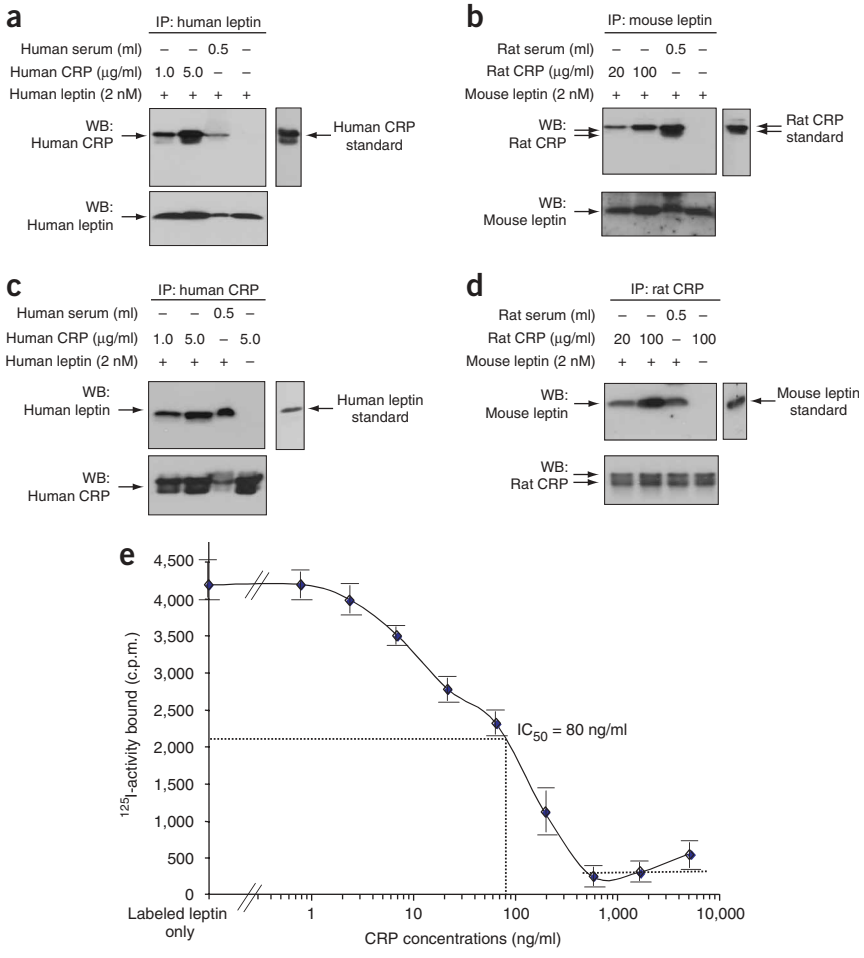

Figure 2 Determination of CRP and leptin interaction. (**a**) Immunoprecipitation of human leptin from a mixture of leptin and human CRP or a mixture of leptin and human serum. The precipitate was subjected to western assays with antibodies to human CRP. The level of precipitated leptin was assessed with human leptin-specific antibody. (**b**) The experiments were performed as in **a** except that rat CRP, mouse leptin and rat serum were used. (**c**,**d**) Western blot detection of leptin in the CRP immunoprecipitates from the mixture of CRP and leptin as well as the mixture of leptin and serum. Loading of standards: ~40 ng human CRP (**a**) or rat CRP (**b**), 2 ng human leptin (**c**) or mouse leptin (**d**). (**e**) ^{125}I-labeled human leptin (~2 ng/ml) was preincubated with varying amounts of human CRP before being added to HEK293 cells transfected with the human leptin receptor, OB-Rb. The IC_{50} value is approximately 80 ng/ml of CRP. The dashed line indicates bound ^{125}I-leptin in the presence of 2,000-fold excess of cold leptin.

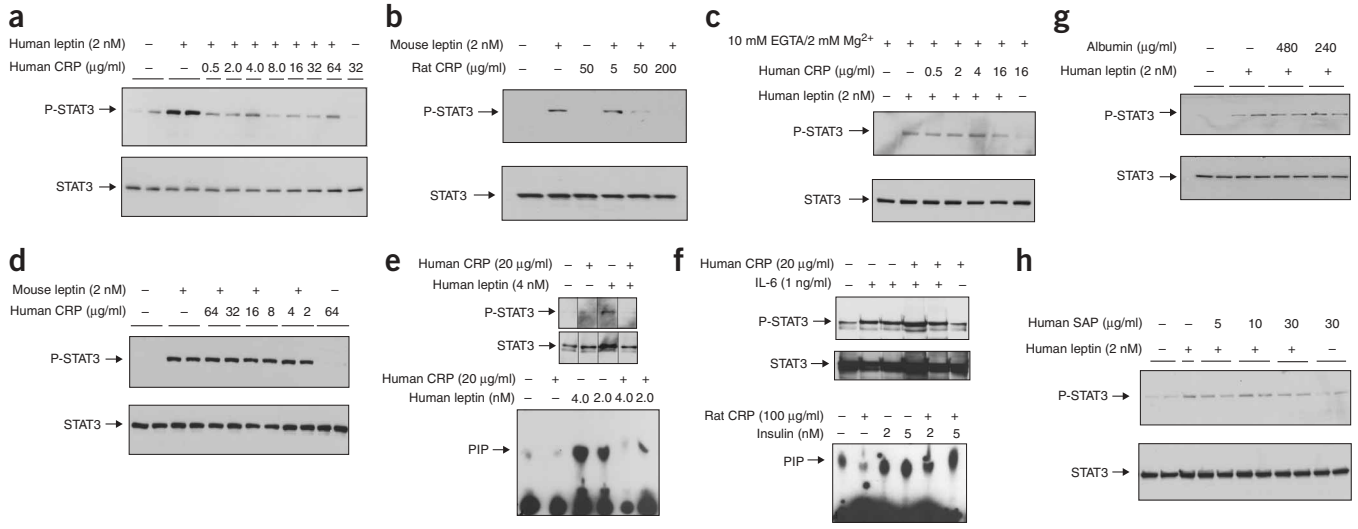

Figure 3 The effects of human or rat CRP on leptin signaling. Attenuating effects of human CRP (**a**) and rat CRP (**b**) on leptin-induced phosphorylation of STAT3 in the HEK293 cells transfected with OB-Rb. Quantitative analysis is shown in **Supplementary Figure 3** online. (**c**) Depletion of free Ca^{2+} in the medium with 10 mM EGTA (compensated with Mg^{2+}) blocked the inhibitory effect of human CRP on leptin-stimulated phosphorylation of STAT3. (**d**) Human CRP (up to 64 μg/ml) did not inhibit leptin-stimulated phosphorylation of STAT3 in a protocol similar to that in **a**. (**e**) Inhibition of human leptin signaling (phosphorylation of STAT3 (upper) and PI3 kinase activity (lower)) by human CRP in rat primary hypothalamic neurons. PIP, phosphatidyl inositol-3-phosphate. (**f**) Human CRP, when preincubated with human recombinant IL-6, did not affect IL6-stimulated phosphorylation of STAT3 in human primary hepatocytes. Similarly, rat CRP did not block insulin-stimulated PI3 kinase in 3T3-L1 adipocytes. Preincubation of human albumin (**g**) or human serum amyloid P component (SAP; **h**) with human leptin did not alter leptin-stimulated phosphorylation of STAT3.

premixed recombinant leptin with human or rat serum before immunoprecipitation. We subjected the protein precipitates that we obtained to western blot assays using specific antibodies to CRP. Immunoprecipitation with antibodies to human leptin pulled down human CRP from both leptin-CRP mixture and from human serum (**Fig. 2a**). Similarly, immunoprecipitation with antibodies to mouse leptin captured rat CRP from similar mixtures (**Fig. 2b**). Direct interaction of CRP and leptin was further shown by immunoprecipitation with antibodies to CRP, which captured leptin proteins (**Fig. 2c**,**d**).

Attenuation of leptin signaling by CRP

To examine whether CRP interferes with the ability of human leptin to bind to its receptors, we used HEK293 cells stably transfected with the long form of the human leptin receptor, OB-Rb[12]. The K_d for leptin–leptin receptor interaction in this cell line was 1.0×10^{-9} M, consistent with previous reports[12]. Preincubation of human CRP with ^{125}I-labeled human leptin reduced binding of leptin to its receptors in a dose-dependent manner (**Fig. 2e**). The 50% inhibitory concentration (IC_{50}) value was approximately 80 ng/ml of CRP in the presence of ~2 ng/ml human leptin, which yielded a molecular ratio of 5.8:1 (CRP:leptin, based on the pentameric structure of CRP). Human CRP showed lower affinity toward mouse leptin (IC_{50} > 64 μg/ml in the presence of ~2 ng/ml mouse leptin); consistent with this observation, human CRP was much less efficient in blocking the signaling capacity of mouse leptin.

To determine whether the interaction between CRP and leptin dampens the cellular actions of leptin, we assessed the ability of leptin to stimulate tyrosine phosphorylation of STAT3 and phosphatidylinositol-3 kinase (PI3K) activity in the presence of CRP *in vitro*. In the HEK293 cells overexpressing OB-Rb, both human CRP and rat CRP attenuated the tyrosine phosphorylation of STAT3 induced by human or mouse leptin, respectively (**Fig. 3a**,**b** and **Supplementary Fig. 3** online). The concentrations of human CRP and rat CRP required to block leptin-induced phosphorylation of STAT3 were within the ranges observed in human and rat plasma[10,13,14], although larger amounts of rat versus human CRP were required to achieve equivalent effects (**Fig. 3a**,**b**). Notably, attenuation of leptin signaling by human CRP required Ca^{2+}, as addition of EGTA to the medium blocked the inhibitory effects of human CRP on leptin signaling (**Fig. 3c**). Consistent with the observation of low affinity between human CRP and mouse leptin, high concentrations of human CRP were unable to block mouse leptin–induced activation of STAT3 (**Fig. 3d**).

We also tested the effects of human CRP on leptin-triggered JAK-STAT and PI3K pathways in rat primary hypothalamic neurons[15–17] (**Fig. 3e**), and found that human CRP inhibited human leptin signaling (**Fig. 3e**). It is unlikely that the inhibitory effect of CRP on leptin signaling resulted from nonspecific toxicity because high concentrations of human CRP did not suppress interleukin (IL)-6–induced activation of STAT3 in human primary hepatocytes (**Fig. 3f**). Similarly, rat CRP did not block insulin-triggered PI3K activity in 3T3-L1 adipocytes (**Fig. 3f**). This result is particularly notable, as leptin belongs to the same cytokine family as IL-6 (ref. 18), and both leptin (this study) and IL-6 bind to CRP[19]. The inability of CRP to influence IL-6 signaling is also consistent with the conclusions from a previously published study[19]. The structural basis underlying the differential effects of human CRP on the signaling of leptin and IL-6 remains to be determined. In contrast, even at concentrations of several hundred-fold higher than those of human CRP, human serum albumin had no appreciable effect on leptin-induced phosphorylation of STAT3 (**Fig. 3g**). Likewise, human serum amyloid P component, which circulates at much higher concentrations than CRP does, also did not influence leptin signaling when coincubated in cultured cells (**Fig. 3h**).

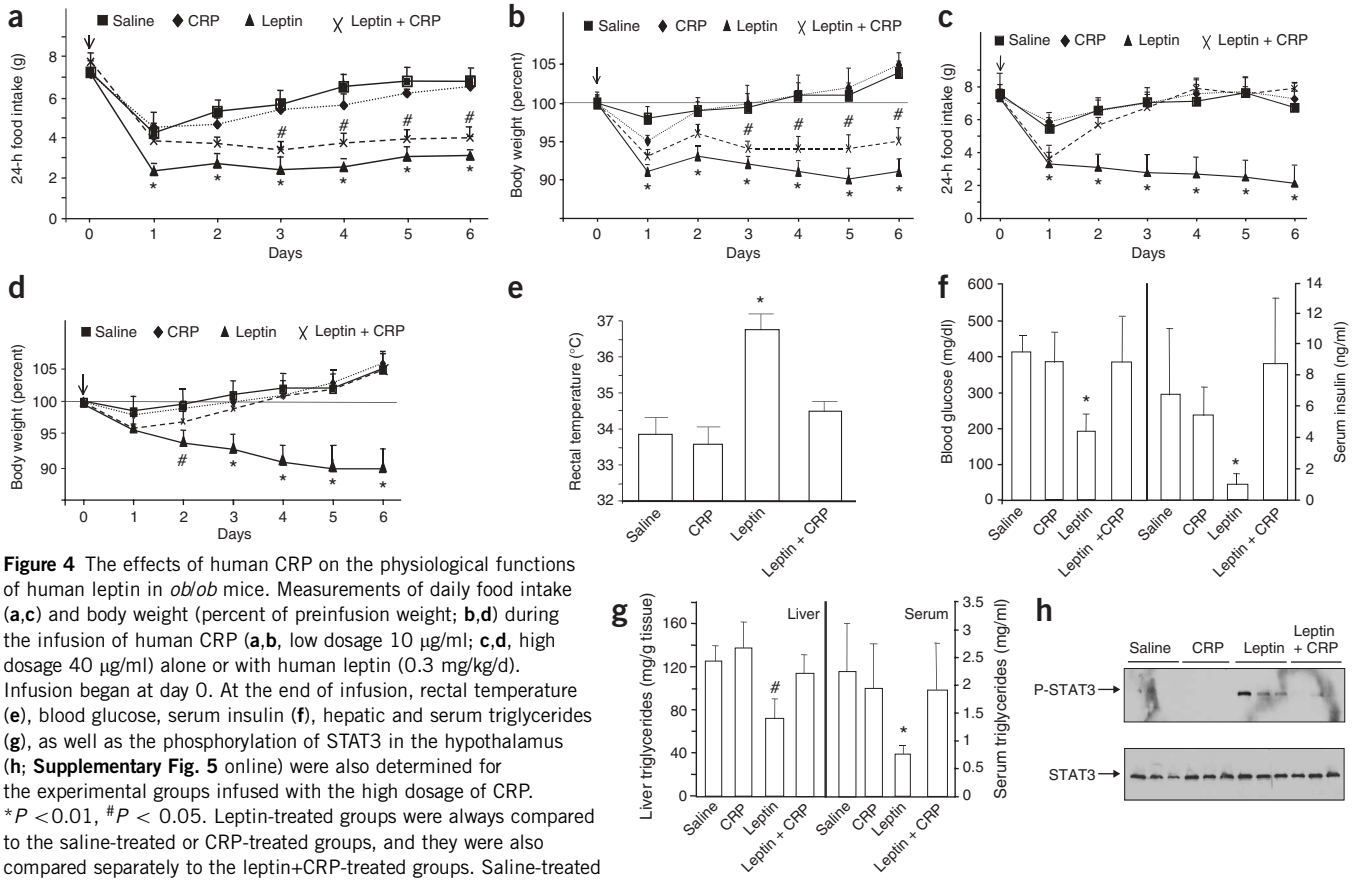

Figure 4 The effects of human CRP on the physiological functions of human leptin in *ob*/*ob* mice. Measurements of daily food intake (**a**,**c**) and body weight (percent of preinfusion weight; **b**,**d**) during the infusion of human CRP (**a**,**b**, low dosage 10 μg/ml; **c**,**d**, high dosage 40 μg/ml) alone or with human leptin (0.3 mg/kg/d). Infusion began at day 0. At the end of infusion, rectal temperature (**e**), blood glucose, serum insulin (**f**), hepatic and serum triglycerides (**g**), as well as the phosphorylation of STAT3 in the hypothalamus (**h**; **Supplementary Fig. 5** online) were also determined for the experimental groups infused with the high dosage of CRP. $^{*}P < 0.01$, $^{\#}P < 0.05$. Leptin-treated groups were always compared to the saline-treated or CRP-treated groups, and they were also compared separately to the leptin+CRP-treated groups. Saline-treated mice ($n = 5$); CRP- or leptin-treated mice ($n = 6$); leptin+CRP-treated mice ($n = 7$).

Attenuation of human leptin functions by human CRP

Because of the leptin-binding abilities of CRP, we postulated that elevation of circulating CRP through infusion would negate the satiety and weight-reduction effects caused by leptin. Because of their high basal plasma concentrations of CRP, rats are not an ideal model system in which to test this hypothesis. Mice, however, have only trace plasma levels of CRP, and in this species, CRP is not an acute-phase reactant[20]. Accordingly, we used *ob*/*ob* mice to examine the effects of exogenously administered human CRP upon leptin-mediated metabolic changes.

We administered differing amounts of human CRP, either alone or with human leptin, through microosmotic pumps implanted subcutaneously into 8-week-old *ob*/*ob* mice (**Supplementary Fig. 4** online). Using an ELISA that detects both free and bound forms of leptin, we found that serum leptin concentrations were almost identical in the mice infused with leptin only and with both leptin and CRP (**Supplementary Fig. 4** online). During the 6-d continuous infusion, human leptin produced the expected reduction in food intake and body weight in the *ob*/*ob* mice (**Fig. 4a–d**). Although coadministration of human CRP at a low infusion dosage (10 μg/d) only partially attenuated these effects of human leptin (**Fig. 4a**,**b**), at a higher concentration (40 μg/d), human CRP completely blocked the actions of leptin to restrain appetite and induce weight loss (**Fig. 4c**,**d**). At the high-infusion dosage, the serum concentration ratio of human CRP to human leptin was approximately 10 (**Supplementary Fig. 4** online), substantially higher than the ratio achieved at IC_{50} (**Fig. 2e**). Consistent with these observations, there was also attenuation of leptin-induced energy expenditure as gauged by recordings of body temperature (**Fig. 4e**). Notably, the serum concentrations of human CRP attained in these mice are similar to the values observed in ostensibly healthy human donors[10,21] (**Supplementary Fig. 4** online). Although administration of leptin to the *ob*/*ob* mice alleviated diabetes, as judged by the lowering of blood glucose, serum insulin (**Fig. 4f**) and serum and hepatic triglycerides (**Fig. 4g**), coinfusion of human CRP blocked these effects (**Fig. 4f**,**g**). Additionally, activation of STAT3 in the hypothalamic tissues of *ob*/*ob* mice was also blocked by coinfusion of human CRP (**Fig. 4h** and **Supplementary Fig. 5** online), further confirming the negative effects of CRP on the actions of leptin. Administration of human CRP alone did not affect food intake and body weight (**Fig. 4a–c**). Thus, the impact of CRP was dependent upon the presence of leptin.

These *in vivo* data were obtained from mice given infusions of a mixture of CRP and leptin stored in a single microosmotic pump. We also infused human CRP and leptin in separate osmotic pumps (**Fig. 5**). The total human leptin concentrations in the sera of all groups infused with leptin ("leptin only" or "leptin plus CRP") were comparable (15–20 ng/ml). For reasons not yet clear to us, with the separate-pump approach higher infusion dosages were required to achieve serum concentrations of human CRP in the *ob*/*ob* mice comparable to those achieved with the single-pump approach. Also, compared to infusion from a single pump, the effects of human CRP on leptin were achieved later. This probably resulted from the time required to achieve diffusion

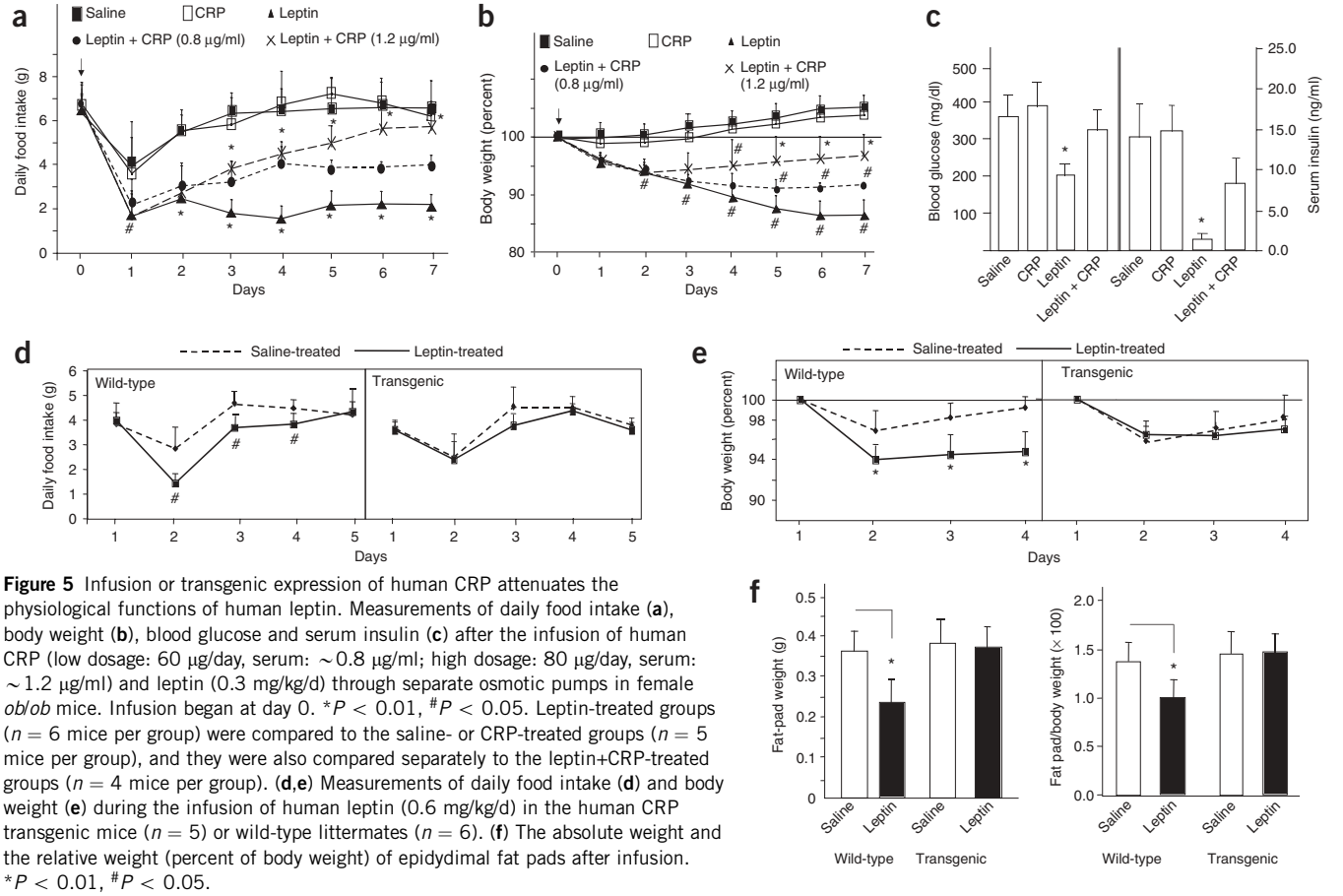

Figure 5 Infusion or transgenic expression of human CRP attenuates the physiological functions of human leptin. Measurements of daily food intake (**a**), body weight (**b**), blood glucose and serum insulin (**c**) after the infusion of human CRP (low dosage: 60 μg/day, serum: ~0.8 μg/ml; high dosage: 80 μg/day, serum: ~1.2 μg/ml) and leptin (0.3 mg/kg/d) through separate osmotic pumps in female *ob*/*ob* mice. Infusion began at day 0. $^{*}P < 0.01$, $^{\#}P < 0.05$. Leptin-treated groups ($n = 6$ mice per group) were compared to the saline- or CRP-treated groups ($n = 5$ mice per group), and they were also compared separately to the leptin+CRP-treated groups ($n = 4$ mice per group). (**d**,**e**) Measurements of daily food intake (**d**) and body weight (**e**) during the infusion of human leptin (0.6 mg/kg/d) in the human CRP transgenic mice ($n = 5$) or wild-type littermates ($n = 6$). (**f**) The absolute weight and the relative weight (percent of body weight) of epididymal fat pads after infusion. $^{*}P < 0.01$, $^{\#}P < 0.05$.

and binding equilibrium. Nevertheless, at serum concentrations that matched those achieved with a single osmotic pump, human CRP was again able to attenuate the physiological actions of human leptin (**Fig. 5a**,**c**).

To further our *in vivo* observations, we evaluated the satiety and weight-reducing functions of human leptin in mice that expressed transgenic human CRP[22]. The average baseline concentration of human CRP in these mice was approximately 15 μg/ml. Despite such high basal levels, human CRP alone did not considerably affect the food intake and body weight of the transgenic mice, consistent with the low affinity of human CRP to mouse leptin[22] (**Fig. 3d**). To evaluate the effects of human leptin in the transgenic mice, we infused a dose of human leptin (0.6 mg/kg/d) that has been known to maximally (but only temporarily) affect energy balance in wild-type mice[23]. Infusion into wild-type littermates of CRP transgenic mice produced the expected reductions in food intake (**Fig. 5d**), body weight (**Fig. 5e**) and epididymal fat-pad weight (**Fig. 5f**), similar to previously reported results[23]. In contrast, these physiological effects of human leptin were completely blunted in the CRP transgenic mice (**Fig. 5d–f**). At the end of infusion, the total human leptin concentrations in the sera of transgenic mice were higher than those in the wild-type littermates (average, 7.6 ng/ml (transgenic mice) versus 3.2 ng/ml (wild-type mice)). Although it remains to be determined whether the biochemical properties of human CRP circulating in human CRP transgenic mice are identical to those seen in humans, these results indicate a negative effect of human CRP on the physiological actions of leptin.

Stimulation of hepatic expression of CRP by human leptin

Plasma CRP concentration correlates positively with adiposity as well as plasma leptin concentrations[11,24]. Therefore, we tested whether leptin itself might stimulate expression of CRP in hepatocytes. Although a short treatment (6–8 h) did not influence expression of *CRP*, the gene encoding CRP (data not shown), incubation of human primary hepatocytes with human leptin at physiological concentrations for 24 h produced a dose-dependent accumulation of secreted CRP in the culture medium (**Fig. 6a**,**b**). Parallel examination of *CRP* expression using real-time PCR also showed a dose-dependent effect on expression of human *CRP* mRNA (**Fig. 6c**). Preincubation of human primary hepatocytes with a specific PI3K inhibitor, LY294002, completely blocked the effect of leptin on expression of CRP (**Fig. 6d**), suggesting that leptin-induced hepatic production of CRP is a PI3K-dependent process. The rapid stimulation of CRP expression during acute infection requires the activity of IL-6 (ref. 25); thus, we wondered whether the mildly elevated IL-6 in obesity (from <3 pg/ml in lean individuals to 5–10 pg/ml in obese individuals[26]) would stimulate expression of CRP in human primary hepatocytes. Although high concentrations of IL-6 produced sharp activation of CRP expression (**Fig. 6e**,**f**) similar to those reported previously[27], low concentrations of IL-6 comparable to (or 10–20-fold higher than) the range observed in obese individuals[26] did not have any effect on expression of CRP at the protein or mRNA levels (**Fig. 6e**,**f**). Consistent with these results, low concentrations of IL-6 had no discernable effect on phosphorylation of STAT3 (**Fig. 6g**), a signaling step essential for IL-6 to increase expression of CRP[28]. These

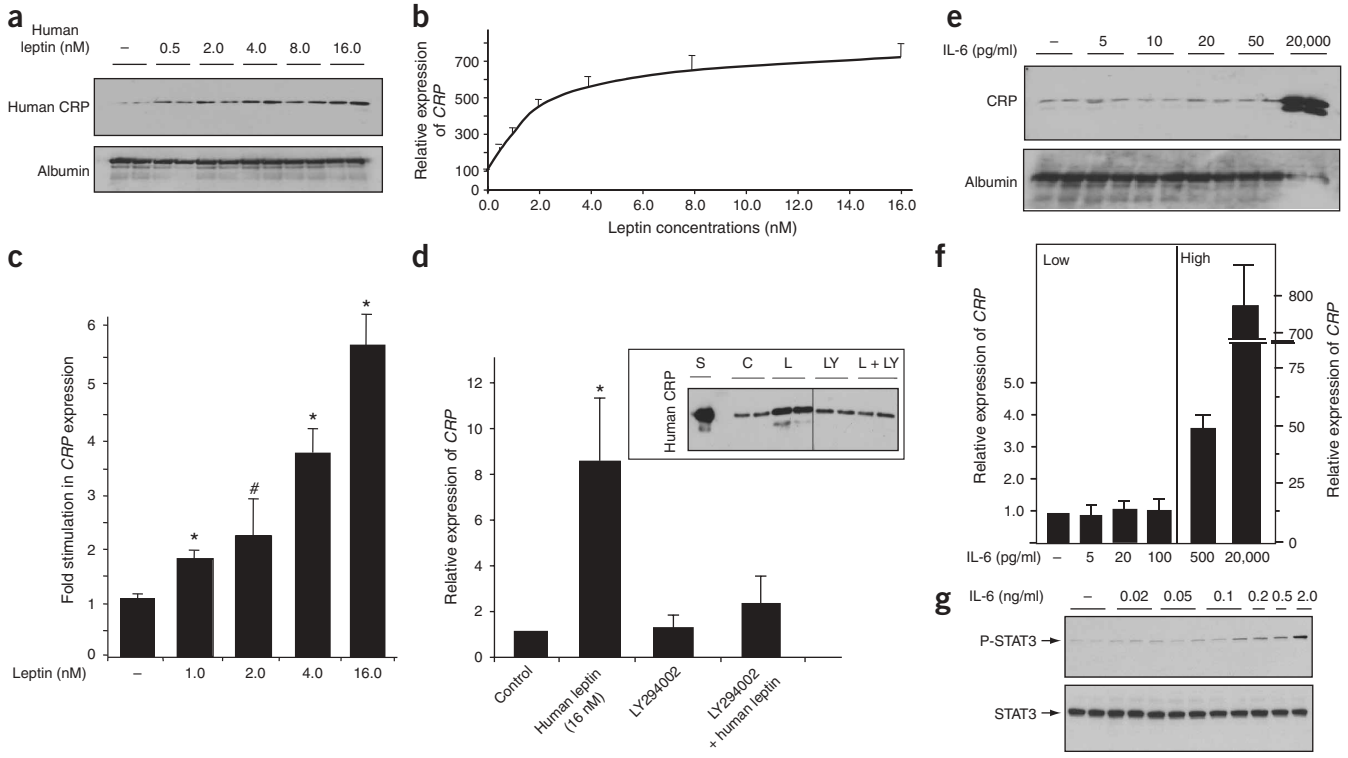

Figure 6 The effects of human leptin and IL-6 on expression of CRP. (**a**–**f**) Human primary hepatocytes were treated with human leptin or IL-6 for 24 h before western assay of secreted CRP in the medium and the real-time PCR assay of gene expression in the hepatocytes. (**a**–**c**) The stimulatory effects of leptin on the secreted CRP (**a**) and expression of *CRP* (**b**,**c**). The secreted albumin was used as a control in **a**. (**d**) A PI3 kinase inhibitor, LY294002, negated the effects of leptin on expression of *CRP* and secreted CRP (insert). $^{*}P < 0.001$, $^{\#}P < 0.01$ when compared to the control. (**e**,**f**) The effects of varying IL-6 concentrations on secreted CRP (**e**) and expression of *CRP* (**f**). (**g**) Activation of STAT3 in the hepatocytes treated with high concentrations of IL-6 (0.2, 0.5 or 2 ng/ml), but not with low concentrations of IL-6 (<100 pg/ml).

studies do not negate the presumed role of IL-6 in obesity *in vivo*; rather, they suggest that leptin might have a prominent role in elevating CRP concentrations in obese people who have no signs of overt inflammation.

DISCUSSION

Using a leptin-affinity column, we detected five major SLIPs. Of these, SLIP-1 was identified as CRP. Both human and rat CRP seemed to bind directly to leptin, and both thus inhibited the ability of leptin to activate STAT3 and PI3K. Furthermore, administration of human CRP attenuated the effects of human leptin on food intake, body weight, blood glucose and lipid metabolism in the *ob*/*ob* mice, and endogenous expression of human CRP completely negated the satiety and weight-reducing effects of human leptin in human CRP transgenic mice. The predicted physiological roles of CRP in energy balance thus probably are dependent on its interaction with leptin.

The identification of CRP as one of the major SLIPs is notable in several aspects. First, inflammation is one of the metabolic complications of obesity. CRP is an inflammatory marker and generally increases in obesity[11,21]. Secondly, elevated CRP, in obesity and insulin resistance, is predictive of high risk for cardiovascular disease[10,29]. Our findings that CRP acts to abrogate the actions of leptin by binding leptin and interfering with leptin-mediated signaling suggest a mechanism by which CRP could directly contribute to the pathogenesis of obesity and its metabolic complications. Indeed, the effects of CRP on the metabolic functions of leptin are probably confined to the context of chronic inflammation, as in conditions such as obesity. In the cases of acute infection or cancer, often accompanied by anorexia, many cytokines, such as IL-6 and tumor necrosis factor (TNF)-α, are sharply elevated and can have potent anorectic effects via the hypothalamus[30,31], which would be expected to counteract the effects of high concentrations of circulating CRP on leptin.

We also report here a stimulatory effect of physiological concentrations of leptin on the hepatic expression of human CRP. This finding is consistent with recent reports that human plasma CRP concentration is independently correlated with leptin concentration[11,24]. Controversy still exists, however, especially with respect to whether injection of recombinant leptin evokes elevation of plasma CRP[32–35]. In this regard, opposing findings have been reported in obese[34,35] and nonobese individuals[32,33]. The dose-response curve (shown in **Fig. 6b**) suggests that addition of exogenous leptin in the nonobese concentration range should be much more effective in stimulating hepatic CRP expression than addition of leptin in the concentrations found in obesity, which further implies that injection of recombinant leptin in the obese participants of leptin clinical trials should not elicit strong increases in concentrations of CRP in the blood. Potentially, our *in vitro* results obtained in human primary hepatocytes, if corroborated by future clinical studies, may help reveal an adipo-hepato-regulatory loop that involves stimulation of CRP expression by leptin and the feedback inhibition of leptin functions by CRP.

Because human CRP forms a doughnut-shaped pentameric structure with an overall molecular weight of ~120 kDa[36], an attractive hypothesis to test is that binding of leptin by CRP impedes the flow of

leptin into the central nervous system. This would explain one of the features of leptin resistance in obesity: reduced leptin transport into the medial hypothalamus[37]. Alternatively, CRP may attenuate the actions of leptin within the central nervous system, as evidence indicates that CRP can cross the blood-brain barrier[38]. In addition to its role in maintaining the pentameric structure of CRP[36], Ca^{2+} is required for the interaction between leptin and CRP. Thus, biochemical evaluation of CRP-bound leptin in human serum should use Ca^{2+}-containing buffer as opposed to the non-Ca^{2+} buffers used in previous studies[39,40]. Additionally, the substantially lower affinity of human CRP to mouse leptin relative to human leptin might help us define the interaction sites of CRP with leptin.

If corroborated by future clinical studies in humans, our findings point to a potential regulatory feedback loop wherein adipose production of leptin induces hepatic expression of CRP, which in turn binds leptin and dampens its action in the central nervous system and in the periphery. In the context of contemporary concerns about the pathogenesis of obesity, at first glance, this adipo-hepato axis involving leptin and CRP would seem to be detrimental to health. Yet, in periods of limited availability of food, the induction of CRP by leptin and the consequent interference with leptin action might serve to sustain positive energy balance during brief intervals of feasting. Disruption of leptin-CRP interaction may thus become a new therapeutic objective for the treatment of obesity.

METHODS

Use of animals and human samples. The use of animals and human serum samples in this study was approved by the Institutional Animal Care and Use Committee and the Institutional Review Board, respectively, at the University of Pittsburgh. Informed consent was obtained from human subjects.

Purification and identification of SLIPs. We covalently linked mouse and human recombinant leptin (from A.F. Parlow of the National Hormone and Peptide Program) to Sepharose beads with an AminoLink kit (Pierce Biotechnology), and loaded rat or human serum (1.5 ml) onto an affinity column. After allowing sera to pass through the resin, we washed the column with 15 volumes of PBS–0.5% Tween-20 (for rat samples), a Ca^{2+}-containing buffer (0.1 M Tris Cl, 0.1 M NaCl, 2 mM $CaCl_2$; for human serum samples), and eluted retained material with an acidic glycine solution. The eluate was immediately neutralized in a Tris buffer (50 mM, pH 9.5). The Proteomic Core Facility of the University of Massachusetts at Worchester performed the matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) analysis. CTL BIO Services performed the Nano-liquid chromatography–mass spectroscopy/mass spectroscopy (LC-MS/MS) in-gel protein identification.

Signaling studies. We serum-starved a HEK293 cell line stably expressing the human long-form receptor OB-Rb[41] for 2 h before addition of leptin and CRP (Chemicon or EMD Biosciences). We preincubated leptin with or without CRP in α-Minimum Essential Medium (with additional 1 mM Ca^{2+}) for 30 min at 37 °C before applying the mixtures to the cells. After a 30-min incubation with leptin, we harvested the cells with lysis buffer[42], and then subjected the resulting protein extract to western blot assays using an antibody specific for phosphorylated STAT3 (Cell Signaling). For studies involving primary hypothalamic neurons, we surgically sliced rat hypothalamus immediately after killing, and placed the tissues in DMEM for incubation with leptin in the presence or absence of CRP.

***In vivo* assessment of CRP functions.** We subcutaneously implanted micro-osmotic pumps (Durect) into *ob*/*ob* or wild-type mice according to the manufacturer's instructions. The osmotic pumps were prefilled with saline, CRP, leptin or leptin plus CRP. We monitored food intake (24 h), body weight and body temperature on a daily basis, and measured blood glucose concentrations in tail-vein blood samples using a Precision Plus glucose meter (Medisense).

Assessment of leptin, insulin, CRP and triglycerides. We measured concentrations of human and mouse leptin using ELISA kits from Chemicon and R&D Systems, respectively. We determined concentrations of mouse insulin and human CRP with ELISA kits from Linco Research and Helica, respectively. We determined concentrations of serum and tissue triglycerides with previously described protocols[43].

Measurement of IC_{50} for human CRP against human leptin. We iodinated human leptin using the Iodogen method. Briefly, we incubated 15 μg of recombinant human leptin in 100 mM phosphate buffer (pH 7.5) with 1 mCi of carrier-free $Na^{125}I$ (2,200 Ci/mmol) in a glass tube containing 50 μg Iodogen. After a 10-min incubation at room temperature (22 °C), we stopped the reaction with 100 μl 0.1% trifluoroacetic acid (TFA), and immediately purified the reaction mixture using reverse-phase high-performance liquid chromatography. The separation used a 5-min isocratic step at 20% eluant B in A, followed by two consecutive 30-min linear gradients from 20 to 50%, then from 50 to 60% eluant B in A (where eluant A is water containing 0.1% TFA and eluant B is acetonitrile containing 0.1% TFA) at a flow rate of 1.5 ml/min. We preincubated the ^{125}I-labeled leptin (~2 ng/ml) with various concentrations of human CRP in 1 ml of α-MEM for 1 h before adding the mixtures to the OBR-expressing HEK293 cells. The incubation lasted 1 h at 37 °C before we dissolved the cells in 0.1 N NaOH for scintillation counting.

Isolation of hepatocytes and hepatocytes treatment. We isolated human hepatocytes from donor livers according to a previously established protocol[44]. Approximately 24 h after seeding hepatocytes, we washed cells with serum-free Hepatocyte Maintenance Medium (glucose concentration, 11.1 mM) supplemented with 0.1 μM dexamethasone and 0.1 μM insulin, and then cultured them in the same medium for another 48 h. Subsequently, we switched cells to serum-free HMM (supplemented with 0.1 μM dexamethasone) before the stimulation with leptin or recombinant human IL-6 (R&D Systems). For experiments involving PI3K inhibitors, we pretreated hepatocytes with LY294002 (EMD Biosciences) for 1 h before hormonal stimulation.

Analysis of CRP secretion. We centrifuged culture medium to remove detached cells before we subjected it to a standard western blot assay. We used antibodies specific to human CRP (Abcam) or rat CRP (Alpha Diagnostic Intl.).

Analysis of *CRP* expression. We measured expression of *CRP* mRNA using quantitative real-time RT-PCR. The real-time PCR reaction was performed with a Taqman machine (ABI7700) as follows: 50 °C for 2 min, 1cycle; 95 °C for 10 min, 1 cycle; 95 °C for 15 sec, 60 °C for 1 min, 40 cycles. The sequences of probes and primers are listed in the **Supplementary Note** online.

Statistical analysis. All paired comparisons were subject to a two-tailed Student *t*-test with $P \leq 0.05$ considered statistically significant.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We wish to thank A. Stewart for his critical evaluation of this manuscript. This work is supported in part by a US National Institutes of Health grant (1RO1DK064383-01) and a Career & Development award from the American Diabetes Association (to A.Z.Z.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at http://www.nature.com/naturemedicine/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Friedman, J.M. & Halaas, J.L. Leptin and the regulation of body weight in mammals. *Nature* **395**, 763–770 (1998).
2. Ahima, R.S. & Flier, J.S. Leptin. *Annu. Rev. Physiol.* **62**, 413–437 (2000).
3. Zhang, Y. *et al.* Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425–432 (1994) [published erratum appears in *Nature* **374**, 479 (1995)].
4. Lee, G.H. *et al.* Abnormal splicing of the leptin receptor in diabetic mice. *Nature* **379**, 632–635 (1996).

5. Schwartz, M.W. *et al.* Evidence that plasma leptin and insulin levels are associated with body adiposity via different mechanisms. *Diabetes Care* **20**, 1476–1481 (1997).
6. Heymsfield, S.B. *et al.* Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *J. Am. Med. Assoc.* **282**, 1568–1575 (1999).
7. Gura, T. Obesity research. Leptin not impressive in clinical trial. *Science* **286**, 881–882 (1999).
8. Bjorbaek, C., El-Haschimi, K., Frantz, J.D. & Flier, J.S. The role of SOCS-3 in leptin signaling and leptin resistance. *J. Biol. Chem.* **274**, 30059–30065 (1999).
9. Tseng, J. & Mortensen, R.F. Binding of human C-reactive protein (CRP) to plasma fibronectin occurs via the phosphorylcholine-binding site. *Mol. Immunol.* **25**, 679–686 (1988).
10. Schulze, M.B. *et al.* C-reactive protein and incident cardiovascular events among men with diabetes. *Diabetes Care* **27**, 889–894 (2004).
11. Kazumi, T., Kawaguchi, A., Hirano, T. & Yoshino, G. C-reactive protein in young, apparently healthy men: associations with serum leptin, QTc interval, and high-density lipoprotein-cholesterol. *Metabolism* **52**, 1113–1116 (2003).
12. Mistrik, P., Moreau, F. & Allen, J.M. BiaCore analysis of leptin-leptin receptor interaction: evidence for 1:1 stoichiometry. *Anal. Biochem.* **327**, 271–277 (2004).
13. Aronson, D. *et al.* Obesity is the major determinant of elevated C-reactive protein in subjects with the metabolic syndrome. *Int. J. Obes. Relat. Metab. Disord.* **28**, 674–679 (2004).
14. de Beer, F.C. *et al.* Isolation and characterization of C-reactive protein and serum amyloid P component in the rat. *Immunology* **45**, 55–70 (1982).
15. Vaisse, C. *et al.* Leptin activation of Stat3 in the hypothalamus of wild-type and *ob/ob* mice but not *db/db* mice. *Nat. Genet.* **14**, 95–97 (1996).
16. Zhao, A.Z., Huan, J.N., Gupta, S., Pal, R. & Sahu, A. A phosphatidylinositol 3-kinase phosphodiesterase 3B-cyclic AMP pathway in hypothalamic action of leptin on feeding. *Nat. Neurosci.* **5**, 727–728 (2002).
17. Niswender, K.D. *et al.* Intracellular signalling. Key enzyme in leptin-induced anorexia. *Nature* **413**, 794–795 (2001).
18. Tartaglia, L.A. *et al.* Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83**, 1263–1271 (1995).
19. Pignatti, P. *et al.* High circulating levels of biologically inactive IL-6/SIL-6 receptor complexes in systemic juvenile idiopathic arthritis: evidence for serum factors interfering with the binding to gp130. *Clin. Exp. Immunol.* **131**, 355–363 (2003).
20. Zahedi, K. *et al.* Major acute-phase reactant synthesis during chronic inflammation in amyloid-susceptible and -resistant mouse strains. *Inflammation* **15**, 1–14 (1991).
21. Maachi, M. *et al.* Systemic low-grade inflammation is related to both circulating and adipose tissue TNFalpha, leptin and IL-6 levels in obese women. *Int. J. Obes. Relat. Metab. Disord.* **28**, 993–997 (2004).
22. Szalai, A.J. & McCrory, M.A. Varied biologic functions of C-reactive protein: lessons learned from transgenic mice. *Immunol. Res.* **26**, 279–287 (2002).
23. Harris, R.B. *et al.* A leptin dose-response study in obese (*ob/ob*) and lean (+/?) mice. *Endocrinology* **139**, 8–19 (1998).
24. Shamsuzzaman, A.S. *et al.* Independent association between plasma leptin and C-reactive protein in healthy humans. *Circulation* **109**, 2181–2185 (2004).
25. Mortensen, R.F. C-reactive protein, inflammation, and innate immunity. *Immunol. Res.* **24**, 163–176 (2001).
26. Yudkin, J.S., Kumari, M., Humphries, S.E. & Mohamed-Ali, V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* **148**, 209–214 (2000).
27. Castell, J.V. *et al.* Acute-phase response of human hepatocytes: regulation of acute-phase protein synthesis by interleukin-6. *Hepatology* **12**, 1179–1186 (1990).
28. Zhang, D., Sun, M., Samols, D. & Kushner, I. STAT3 participates in transcriptional activation of the C-reactive protein gene by interleukin-6. *J. Biol. Chem.* **271**, 9503–9509 (1996).
29. Ridker, P.M. High-sensitivity C-reactive protein, inflammation, and cardiovascular risk: from concept to clinical practice to clinical benefit. *Am. Heart J.* **148**, S19–S26 (2004).
30. Lennie, T.A., McCarthy, D.O. & Keesey, R.E. Body energy status and the metabolic response to acute inflammation. *Am. J. Physiol.* **269**, R1024–R1031 (1995).
31. McCarthy, D.O. Tumor necrosis factor alpha and interleukin-6 have differential effects on food intake and gastric emptying in fasted rats. *Res. Nurs. Health* **23**, 222–228 (2000).
32. Mackintosh, R.M. & Hirsch, J. The effects of leptin administration in non-obese human subjects. *Obes. Res.* **9**, 462–469 (2001).
33. Hukshorn, C.J. *et al.* Leptin and the proinflammatory state associated with human obesity. *J. Clin. Endocrinol. Metab.* **89**, 1773–1778 (2004).
34. Chan, J.L. *et al.* Recombinant methionyl human leptin administration to achieve high physiologic or pharmacologic leptin levels does not alter circulating inflammatory marker levels in humans with leptin sufficiency or excess. *J. Clin. Endocrinol. Metab.* **90**, 1618–1624 (2005).
35. Hukshorn, C.J. *et al.* The effect of pegylated recombinant human leptin (PEG-OB) on weight loss and inflammatory status in obese subjects. *Int. J. Obes. Relat. Metab. Disord.* **26**, 504–509 (2002).
36. Shrive, A.K. *et al.* Three dimensional structure of human C-reactive protein. *Nat. Struct. Biol.* **3**, 346–354 (1996).
37. Schwartz, M.W., Peskind, E., Raskind, M., Boyko, E.J. & Porte, D., Jr. Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. *Nat. Med.* **2**, 589–593 (1996).
38. Rajs, G., Finzi-Yeheskel, Z., Rajs, A. & Mayer, M. C-reactive protein concentrations in cerebral spinal fluid in gram-positive and gram-negative bacterial meningitis. *Clin. Chem.* **48**, 591–592 (2002).
39. Sinha, M.K. *et al.* 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 (1996).
40. McConway, M.G. *et al.* Differences in circulating concentrations of total, free and bound leptin relate to gender and body composition in adult humans. *Ann. Clin. Biochem.* **37**, 717–723 (2000).
41. Friedman-Einat, M. *et al.* Serum leptin activity in obese and lean patients. *Regul. Pept.* **111**, 77–82 (2003).
42. Zhao, A.Z., Zhao, H., Teague, J., Fujimoto, W. & Beavo, J.A. Attenuation of insulin secretion by insulin-like growth factor 1 is mediated through activation of phosphodiesterase 3B. *Proc. Natl. Acad. Sci. USA* **94**, 3223–3228 (1997).
43. Huan, J.N. *et al.* Adipocyte-selective reduction of the leptin receptors induced by antisense RNA leads to increased adiposity, dyslipidemia, and insulin resistance. *J. Biol. Chem.* **278**, 45638–45650 (2003).
44. Kostrubsky, V.E. *et al.* The role of conjugation in hepatotoxicity of troglitazone in human and porcine hepatocyte cultures. *Drug Metab. Dispos.* **28**, 1192–1197 (2000).