

Effect of Obesity on Plasma Clusterin: A Proposed Modulator of Leptin Action

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ABSTRACT: Clusterin, a protein constituent of HDL, was recently shown to bind plasma leptin *in vitro* and has been proposed to modulate leptin activity. To gain insight into a possible role for plasma clusterin in human obesity, we measured plasma clusterin, leptin, soluble leptin receptor (sObR), and lipoproteins in 70 obese adolescents (12.4 ± 1.6 y; BMI-SD score (SDS-BMI) 2.35 ± 0.47) before and after 3 wk of weight reduction in a dietary camp and in 44 normal weight controls. Binding of plasma leptin to HDL or clusterin was studied using ultracentrifugation and immunoaffinity chromatography. During weight reduction, clusterin decreased from 14.6 ± 4.1 to 10.3 ± 2.9 mg/dL, $p < 0.001$) in obese adolescents, whereas sObR increased. However, baseline plasma clusterin in obese adolescents did not differ from controls. Clusterin did not correlate with SDS-BMI, weight loss, leptin, or lipoproteins. Only ~1% of plasma leptin was associated with clusterin/apoA-I complexes or with HDL. Our results do not support a role for plasma clusterin as an important leptin-binding protein or modulator of leptin action. The decrease of plasma clusterin during weight reduction may be an effect of the hypocaloric diet rather than being directly linked to weight loss. (*Pediatr Res* 69: 237–242, 2011)

Leptin, the product of the *OB* gene, is a 16 kD plasma cytokine secreted predominantly by adipocytes, which plays a central role in the regulation of body weight (1). Leptin is transported into cerebrospinal fluid (CSF) and interacts with leptin receptors expressed in hypothalamic nuclei to regulate appetite and energy expenditure (2).

In plasma, leptin circulates in free form and in protein bound form (3). The major leptin-binding protein in plasma is a soluble form of the leptin receptor (sObR) (4). Binding to sObR modulates leptin signaling by sequestration of leptin from interaction with the signaling-competent isoform of the leptin receptor (ObRb) and prolongs the half life of leptin in the circulation (5,6). In addition to sObR, leptin was reported

to bind to $\alpha 2$ -macroglobulin (7) and several other as yet uncharacterized proteins (3). Previous data from this laboratory suggested that a variable fraction of circulating leptin may be associated with HDL (8). Recently, Bajari *et al.* (9) identified clusterin (apolipoprotein J), a minor protein component of HDL, as a leptin-binding protein *in vitro*.

Clusterin is a heterodimer of two disulfide-bonded 40 kD glycoprotein subunits (10). Clusterin occurs in many body fluids and its mRNA is found in most tissues. Clusterin exists in a secretory form and an intracellular form and has been implicated in processes as diverse as sperm maturation, complement inhibition, apoptosis, cancer promotion, and Alzheimer's disease (11–13). Clusterin may have a protective effect against atherosclerosis (14–16). In blood, most clusterin is associated with a dense subfraction of lipid-poor HDL (10,17). Data from mice suggest that leptin/clusterin complexes can circulate with HDL (9). In contrast to leptin bound to sObR, the leptin/clusterin complex can transduce the leptin signal via ObRb *in vitro* (9). The leptin/clusterin complex also binds to members of the LDL receptor family resulting in uptake and probably degradation (9). This led to the suggestion that clusterin may modulate leptin activity by binding either to signaling receptors or to receptors mediating degradation. This possibility seemed of particular interest, because clusterin occurs in plasma at a fairly high concentration (8–14 mg/dL), which constitutes a large molar excess over both, plasma leptin and sObR. Therefore, we hypothesized that the concentration of plasma clusterin and the binding of leptin to clusterin or HDL may play a role in the regulation of body weight by leptin.

The aims of this study were 1) to compare plasma clusterin levels in obese and normal weight adolescents, 2) to determine whether plasma clusterin concentrations correlate with the degree of obesity or with plasma leptin, 3) to study the effects of weight reduction on plasma clusterin levels, 4) to measure HDL bound plasma leptin, and 5) to study the interaction of leptin with clusterin in human plasma.

Abbreviations: C, cholesterol; CSF, cerebrospinal fluid; *d*, density; sObR, soluble leptin receptor; SDS-BMI, BMI-SD score; TG, triglycerides

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Table 1. Body weight, SDS-BMI, plasma leptin, and plasma sObR in obese adolescents before and after weight reduction and in normal weight controls (mean \pm SD)

	Obese (n = 70)			Control (n = 44)	
	Before weight reduction	After weight reduction	p before vs after weight reduction	p before weight reduction vs control	
Age (yr)	12.4 \pm 1.6	—	—	12.5 \pm 1.7	n.s.
Body weight (kg)	75.7 \pm 16.3	70.7 \pm 15.4	<0.001	45.8 \pm 10.1	<0.001
SDS-BMI	2.35 \pm 0.47	2.1 \pm 0.75	<0.001	0.2 \pm 0.8	<0.001
Plasma leptin (ng/mL)	22.9 \pm 13.0	9.0 \pm 7.8	<0.001	7.5 \pm 4.7	<0.001
Plasma sObR (U/mL; n = 27)	23.5 \pm 7.6	33.0 \pm 12.7	<0.001	n.d.	n.a.

n.s., not statistically significant; n.d., not determined; n.a., not applicable.

METHODS

Patients and controls. Seventy obese adolescents participating in a 3-wk dietary camp on an inpatient basis (31 girls and 29 boys) with a mean age of 12.4 \pm 1.6 y were studied (Table 1). Forty-four normal weight healthy controls (18 girls and 22 boys) with a mean age of 12.5 \pm 1.7 y were recruited from adolescents participating in the STYJOBS study (<http://clinicaltrials-lhc.nlm.nih.gov/ct2/show/NCT00482924>; Table 1). The BMI-SD score (SDS-BMI) was calculated using the reference values of Kromeyer-Hauschild *et al.* (18). Informed consent was obtained from the parents of all obese adolescents and controls. The study protocol was approved by the Ethics Review Board of the Medical University of Vienna (Protocol no. 2282003).

Diet. After measurement of baseline values, the obese group received a conventional hypocaloric mixed diet for 3 wk. Mean daily energy intake during the 3-wk period was 957 \pm 237 kcal, with a minimum intake of 50 g protein and 116 g carbohydrate/d. No electrolyte or vitamin supplementation was provided. After 3 wk of diet, the mean weight loss was 5.0 \pm 1.3 kg (range, 2.7–8.1 kg), resulting in a decrease of SDS-BMI from 2.35 \pm 0.74 to 2.1 \pm 0.75 (Table 1).

Laboratory methods. Blood samples were collected by venipuncture after an overnight fast on the day before starting the diet and on the last day the participants received the diet. Plasma cholesterol (C) and triglycerides (TGs) were determined by enzymatic methods on a Kodak Ektachrome autoanalyser (Johnson & Johnson, Rochester, NY). HDL(C) was determined using polyanion precipitation (19). LDL(C) was calculated using the Friedewald formula. ApoA-I and apoB were quantitated by immunoturbidimetric methods (Roche Diagnostics GmbH, Mannheim, Germany). Plasma leptin and sObR were measured in duplicates by ELISA (BioVendor, Inc., Modrice Czech Republic). The intra-assay coefficient of variation of these ELISAs was 8.3 and 13.1%, respectively.

The ELISA for plasma clusterin was performed according to Trougakos *et al.* (20) with minor modifications. Briefly, 96 well plates were coated with 60 ng/well of polyclonal goat anti-human clusterin antibody (pAb-sc6419, Santa Cruz Biotech, Inc., Santa Cruz, CA), blocked with 0.1% wt/vol BSA, 0.1% Tween 20 in PBS pH 7.4 (PBS-T) for 30 min and washed with PBS-T. Samples were diluted 1:32 with blocking solution and 100 μ L were added to the wells. Plates were incubated for 90 min, washed with PBS-T, and incubated with 25 ng/well of murine MAb to human clusterin (MAB-G7, Quidel Corporation, San Diego, CA) in 100 μ L blocking solution for 90 min. After washing, plates were incubated with 10 ng/well of horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad Laboratories, Vienna, Austria) in 100 μ L blocking solution for 90 min and washed with PBS-T, followed by a color reaction using tetramethylbenzidine (DakoCytomation, Hamburg, Germany). The intra-assay and interassay coefficients of variation were 4.8 and 10%, respectively. The assay was standardized using recombinant His-myc-Clusterin-his produced in HEK293 EBNA cells and purified with Ni-NTA-agarose (21).

Plasma lipoproteins were isolated by single-spin density gradient ultracentrifugation (22) as reported previously (8). Four milliliters of plasma were adjusted to density (d) = 1.25 g/mL with solid KBr, overlaid with KBr solutions of d = 1.063 g/mL (4 mL), d = 1.019 g/mL (4 mL), and d = 1.006 g/mL (2 mL) and centrifuged in a SW40 Ti rotor (Beckmann Coulter, CA) for 30 h at 160,000 \times g and 4°C (22). Fourteen 1 mL fractions were collected: fraction 1 and 2 contained low-density lipoproteins, fractions 3–6 LDL, fractions 8 and 9 HDL, and fraction 11–14 contained lipoprotein-deficient serum as identified by protein and C determination and SDS-PAGE. The HDL contained clusterin as demonstrated by Western blotting. Lipoproteins were dialyzed three times against 10 mM ammonium hydrogen carbonate in dialysis tubing with a cutoff of 50 kD to ensure complete removal of free leptin.

Clusterin and apoA-I containing complexes were isolated from plasma by immunoaffinity chromatography using a MAb to human clusterin (MAB-G7, Quidel Corporation) bound to CNBr-activated Sepharose 4B (Amersham, GE Healthcare, Chalfont St. Giles, United Kingdom) as described by Jenne *et al.* (23). The column was eluted with 0.2 M glycine pH 2.8. Four fractions of 1 mL were collected into 200 μ L of 1M Tris HCl pH 8, dialyzed against 10 mM ammonium hydrogen carbonate, and vacuum concentrated to a final volume of 200 μ L. Leptin in immunoaffinity and ultracentrifugation fractions was measured by RIA (SHL-81 K; Linc Research, St. Charles, MO; detection limit: 0.05 ng/mL).

Plasma or clusterin/apoA-I complexes were fractionated by 10% SDS-PAGE. The primary antibody was an affinity-purified goat polyclonal antibody against the carboxy terminus of the human clusterin- β subunit (sc-6419, Santa Cruz Biotech, Inc.). The second antibody was a horseradish peroxidase-conjugated rabbit anti-goat IgG (Sigma Chemical Co.-Aldrich, St. Louis, Mo). Fluorescence signals were analyzed in a ChemImager 4400 (Biozym Diagnostics, Oldendorf, Germany).

Statistics. Data are expressed as mean \pm SD. Means were compared using paired or nonpaired t test as indicated. The t tests and univariate linear correlation coefficients were calculated using STATISTICA 5.0 for Windows (Stat Soft Inc., Tulsa, OK); p < 0.05 were considered as significant.

RESULTS

Effect of obesity on plasma clusterin. In our study, plasma clusterin levels were not affected by obesity. Plasma clusterin measured before weight reduction did not differ between obese and normal weight adolescents (14.6 \pm 4.1 *versus* 13.2 \pm 2.4 mg/dL, n.s.; Table 2). Moreover, plasma clusterin concentrations did not correlate with body weight, SDS-BMI, or plasma leptin levels in obese adolescents or in controls (Table 3). No sex difference in plasma clusterin levels was observed (not shown).

The lipoprotein profiles of obese adolescents were characterized by elevated LDL(C) and apoB (Table 2). Plasma clusterin concentrations did not correlate with basal plasma total C, TG, HDL(C), LDL(C), apoA-I, apoB, and glucose in obese adolescents or controls (data not shown). As expected, basal plasma leptin levels were considerably higher in obese adolescents than in controls (Table 1) and higher in females than in males (33.7 \pm 13.3 *versus* 22.5 \pm 11.1 ng/mL in obese and 10.2 \pm 4.6 *versus* 5.3 \pm 3.2 ng/mL in control adolescents, respectively, p < 0.01). Plasma leptin positively correlated with SDS-BMI (r = 0.50, p < 0.01 in obese and r = 0.41, in controls, p < 0.01).

Effect of weight reduction on plasma clusterin and leptin. During weight reduction, plasma clusterin levels in obese adolescents decreased by \sim 30% (from 14.6 \pm 4.1 to 10.3 \pm 2.9 mg/dL, p < 0.001; Table 2). This decrease was confirmed by Western blot analysis (Fig. 1). The magnitude of this

Table 2. Plasma clusterin and plasma lipoproteins in 70 obese adolescents before and after weight reduction and in 44 normal weight controls (mean \pm SD)

	Obese (n = 70)			Control (n = 44)	
	Before weight reduction	After weight reduction	p before vs after weight reduction		p obese before weight reduction vs control
Clusterin (mg/dL)	14.6 \pm 4.1	10.3 \pm 2.9	<0.001	13.2 \pm 2.4	n.s.
Total C (mmol/L)	4.57 \pm 0.87	3.25 \pm 0.57	<0.001	4.3 \pm 0.82	n.s.
Total TG (mmol/L)	1.07 \pm 0.58	0.73 \pm 0.25	<0.001	1.23 \pm 0.66	n.s.
HDL(C) (mmol/L)	1.34 \pm 0.33	1.22 \pm 0.26	<0.001	1.26 \pm 0.32	n.s.
LDL(C) (mmol/L)	2.82 \pm 0.69	1.66 \pm 0.42	<0.001	2.31 \pm 0.99	<0.05
ApoA-I (g/L)	147.9 \pm 29.2	121.3 \pm 18.4	<0.001	156.2 \pm 33.6	n.s.
ApoB (g/L)	76.6 \pm 17.3	54.8 \pm 10.5	<0.001	52.4 \pm 14.9	<0.001

n.s., not statistically significant.

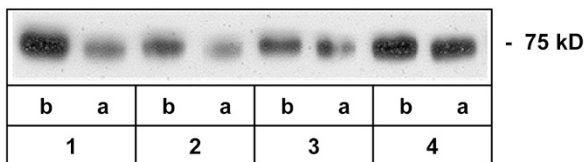
Table 3. Univariate linear correlation coefficients of plasma clusterin with SDS-BMI and plasma leptin in obese adolescents before and after weight reduction and in controls

	Obese			Control Clusterin
	Clusterin before weight reduction	Clusterin after weight reduction	Δ Clusterin	
SDS-BMI obese before weight reduction	-0.09	-0.16	0.10	—
SDS-BMI obese after weight reduction	-0.16	-0.15	0.07	—
Δ SDS-BMI	0.13	-0.06	0.08	—
Leptin obese before weight reduction	-0.08	-0.06	-0.02	—
Leptin obese after weight reduction	-0.08	-0.06	-0.15	—
Δ Leptin	0.05	0.04	-0.10	—
Clusterin after weight reduction	0.68*	—	-0.45†	—
SDS-BMI control	—	—	—	-0.04
Leptin control	—	—	—	-0.02

Plasma clusterin and leptin were measured in 70 obese adolescents before and after 3 wk of weight reduction and in 44 controls. The relation of plasma clusterin before and after weight reduction and of its changes during weight loss (Δ clusterin) with plasma leptin and SDS-BMI and their changes (Δ leptin and Δ SDS-BMI) was studied by univariate linear correlation analysis.

* $p < 0.001$.

† $p < 0.01$.

**Figure 1.** Immunoblotting of clusterin in representative plasma samples from obese adolescents before and after weight reduction (SDS-PAGE, nonreducing conditions). 1, 2, 3, and 4: patients one to four; b: before weight reduction, and a: after weight reduction.

decrease did not correlate with the changes of SDS-BMI and plasma leptin (Table 3) or with the changes of total C, TG, HDL(C), LDL(C), apoA-I, and apoB during weight loss (data not shown). Plasma clusterin before weight reduction was closely correlated with clusterin levels after weight reduction and with the decrease of clusterin levels. As in previous studies, weight loss was associated with a significant decrease in total plasma C, TG, apoB, and apoA-I (Table 2). The decrease of plasma C was mainly due to not only a reduction of LDL(C) (Table 2) but also a slight decrease of HDL(C). As in our earlier studies (8,24), plasma leptin decreased much more than SDS-BMI (Table 1), confirming reports that plasma leptin is lower during active weight loss than during maintenance of reduced body weight (25).

To compare the effect of weight reduction on plasma clusterin with that on the major leptin-binding protein, sObR, we

measured plasma sObR in a subset of 27 obese adolescents (17 boys and 10 girls; Table 1). In contrast to the decrease of plasma clusterin, sObR increased markedly during weight reduction, confirming the results of earlier studies (26,27). There was no significant correlation between clusterin and sObR ($r = 0.21$ before and 0.13 after weight reduction, respectively). Unlike plasma clusterin, sObR levels correlated negatively with SDS-BMI ($r = -0.38$, $p < 0.05$) and with plasma leptin ($r = -0.65$, $p < 0.001$). The opposite changes of plasma clusterin and sObR indicate a differential regulation of these two proteins during weight loss.

Binding of plasma leptin to HDL or clusterin. To determine the amount of leptin bound to plasma HDL, lipoproteins were isolated by single-spin gradient ultracentrifugation in 14 patients (seven male and seven female) before and after weight reduction. Leptin was measured by RIA in HDL (fractions 8 and 9) and LDL (fractions 3–6). The concentration of leptin in HDL was 0.045 ± 0.015 ng/mL before versus 0.040 ± 0.010 ng/mL after weight reduction and thus was below the lower detection limit of the sensitive RIA (0.05 ng/mL) and even lower in LDL.

To assess the amount of plasma leptin bound to HDL by a second independent method, we isolated clusterin/apoA-I complexes from pooled plasma of two healthy young adult donors by immunoaffinity chromatography using immobilized anti-human clusterin antibody. The first fraction eluted after

the void volume (fraction 2) contained clusterin as the predominant protein. Fraction 2 contained apoA-I (M_w 28.5 kD), as shown by SDS-PAGE and Western blotting (Fig. 2). Thus, clusterin and apoA-I occurred together on the particles in this fraction as described by Jenne *et al.* (23). Approximately 55% of the protein in fraction 2 was clusterin, which was enriched ~500-fold compared with plasma, where it represents ~0.1% of total protein. Besides clusterin and apoA-I, minor amounts of other proteins like albumin could be detected. We recovered 97 μ g clusterin and 0.0164 ng leptin per milliliter of plasma in fraction 2. With a clusterin concentration of 0.17 mg/mL and a leptin level of 5.1 ng/mL in the plasma sample applied to the column, this corresponds to a recovery of 57% for plasma clusterin but of only 0.3% for leptin. This suggests that 0.5% of plasma leptin were bound to clusterin. The experiment was repeated with plasma from two other healthy donors with similar results: 29.2% of plasma clusterin and 0.4% of plasma leptin were recovered, corresponding to 1.3% of leptin bound to clusterin. In a third experiment, a control column without coupled antibody was used to rule out non-specific binding of leptin or clusterin to the column matrix. The amount of clusterin eluted from the control column was 1.1% of that eluted from an anti-human clusterin column (0.6 versus 55.3 μ g/mL plasma). Leptin levels in the eluate were below the detection limit of the RIA. Taken together, these data suggest that ~1% of plasma leptin circulates bound to clusterin. This confirms our finding that only traces of leptin were associated with HDL isolated by ultracentrifugation.

DISCUSSION

On the basis of the observation that clusterin is capable of binding to leptin *in vitro* and on the hypothesis that clusterin may be involved in the modulation of leptin activity, we asked whether plasma clusterin plays a role in human obesity. Our data collected in obese adolescents and controls do not provide evidence for such a role of clusterin. Plasma clusterin in obese adolescents did not differ from control values, and correlation analysis did not reveal any significant association

of clusterin levels with leptin concentrations or SDS-BMI in obese or normal weight probands. Similarly, Kujiraoka *et al.* (28) found no correlation of serum clusterin with BMI in a study on Japanese adults with coronary artery disease and type 2 diabetes. In rats, diet-induced obesity had no effect on plasma clusterin levels (29). Our results obtained in plasma samples do not necessarily indicate that the binding of leptin to clusterin is irrelevant for the regulation of leptin activity and body weight. It seems conceivable that clusterin binds to leptin in CSF. In CSF, clusterin is one of the major apolipoproteins (30), but sObR is not present (31). Moreover, several members of the LDL-receptor family are expressed in brain and choroid plexus (32) and could modulate leptin action by mediating the degradation of leptin/clusterin complexes (33).

Although, in our study, obesity had no effect on circulating clusterin, weight reduction resulted in a marked decrease of plasma clusterin. After 3 wk of hypocaloric diet plasma clusterin in obese adolescents had declined by 30% to below the levels in normal weight controls. A decrease during weight reduction has also been reported for apoA-I, apoA-IV, and apoB (24,34–36) but not for apoA-II or apoE (36). It remains to be determined whether the reduction of plasma clusterin levels would persist during a longer period of weight loss or during maintenance of a lower body weight, or whether clusterin would return to prediet levels, as has been described for apoA-I (36). It is unclear whether the decrease of plasma clusterin is a direct consequence of weight change, as the decrease did not correlate with the reduction of SDS-BMI or plasma leptin. Moreover, we recently observed that in adolescents with anorexia nervosa a weight gain of 4.5 kg within 6 wk did not alter plasma clusterin levels (20.3 ± 9.4 mg/dL before versus 21.5 ± 10.2 mg/dL after weight gain, $n = 10$; Huemer J and Strobl W, unpublished). It seems possible that the reduction of plasma clusterin observed in our study is an effect of the hypocaloric diet rather than of the reduction of body mass. Although data from human studies are not available, a decrease of plasma clusterin during caloric restriction has been described in nonobese rats (37). Clusterin expression is enhanced by oxidative stress, which has been proposed to be a link between the various pathologic conditions in which clusterin has been implicated (38). During weight loss, a decrease in oxidative stress occurs in obese patients (39). This may have contributed to the decrease in plasma clusterin levels observed in our study. Several studies reported an association of clusterin with plasma glucose or type II diabetes (20,28). Moreover, clusterin is regulated by IGF-1 signaling (40) and may itself modulate this pathway (41). However, in our study, no correlation of plasma clusterin with plasma glucose was observed.

During weight reduction, plasma clusterin decreased more than apoA-I and HDL(C). This indicates a subtle change in HDL composition during weight loss. A reduction of HDL₃, but not HDL₂, was observed earlier in obese adolescents under almost identical dietary conditions (42). Our data suggest that the change in HDL composition may also have affected the HDL subfraction containing clusterin. In our study, plasma clusterin levels did not correlate with plasma C, TG, HDL(C),

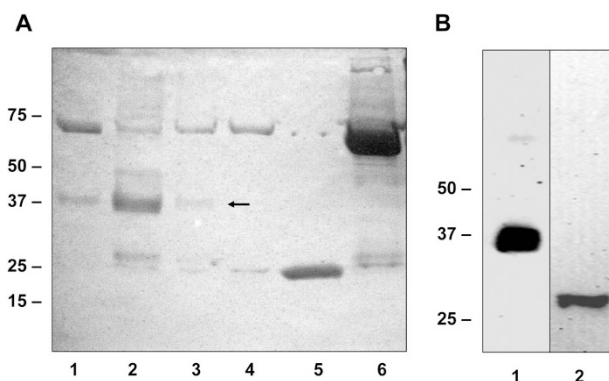


Figure 2. Isolation of plasma clusterin from plasma by immunoaffinity chromatography. (A) SDS-PAGE, reducing conditions, Ponceau stain after transfer to supported nitrocellulose. Lanes 1–4: elution fractions 1–4 and lane 5: HDL. Lane 6: plasma. Arrow, band corresponding to clusterin. (B) Immunoblotting of fraction 2. Lane 1: anti-human clusterin antibody and lane 2: anti-human apoA-I antibody.

LDL(C), apoA-I, apoB, or with their changes during weight loss. Data on the relation of plasma clusterin with lipoprotein concentrations are conflicting: one study reported a correlation of clusterin with total C and HDL(C) in females but no association with apoA, apoB, lipoprotein(a), or TG (20), whereas others found no correlation of plasma clusterin with C levels (43).

In the circulation sObR, which occurs in two different glycosylated isoforms forming di- and oligomers, represents the predominant leptin-binding activity (4). In addition to sObR, plasma leptin interacts with a number of other protein components (3,7,9,44,45). α -2 macroglobulin associates with leptin because of hydrophobic interactions with much lower affinity than sObR (7). The OB-BP1/Siglec-6 protein, expressed on B-cells, binds leptin with high affinity and may act as a sink for circulating leptin (45). Moreover, in obese spontaneously hypertensive Koletsky rats, which lack all leptin-receptor isoforms, a large portion of exogenous ^{125}I labeled leptin was reported to bind to high molecular weight plasma proteins (46). Our data on the binding of leptin to plasma clusterin or HDL, collected by density gradient centrifugation and immunoaffinity chromatography, indicate that in human plasma only little leptin circulates bound to clusterin. These data are supported by the finding of a recent HDL proteomics study, which showed the presence of other cytokines such as TGF- β or CSF-1 in HDL but did not detect leptin (47). However, *in vitro*, a direct interaction of recombinant murine clusterin with recombinant leptin has been demonstrated unequivocally (9). This direct interaction does not depend on other serum proteins such as HDL or apoA-I. Several other potential leptin-binding proteins detected by affinity chromatography (3,44) or pull-down assays (9) remain to be identified.

Taken together, our data suggest that the interaction of leptin with clusterin, which was clearly demonstrated *in vitro*, may affect only a minor portion if any of circulating leptin in human plasma. In summary, our results do not support a role for plasma clusterin as an important binding protein for plasma leptin and modulator of its action in humans.

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