Human leptin forms complexes with α2-macroglobulin which are recognized by the α2-macroglobulin receptor/low density lipoprotein receptor-related protein

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Abstract

Objective: To identify binding proteins of leptin in human plasma.
Methods: Binding was evaluated by electrophoresis, size exclusion chromatography (SEC), Western blotting, and radiolabeling. Quantification of leptin and the different forms of α2-macroglobulin (α2-M) was performed by ELISA.
Results: Leptin interacts with the proteinase inhibitor, α2-M. 125I-labeled leptin specifically binds to the transformed inhibitor, which arises by reaction with proteinases or with reactive primary amines. No leptin binding was observed to the native α2-M, which abundantly occurs in plasma. The complex formation between leptin and α2-M was found to proceed within minutes and was stable, as it resisted separation by SEC and electrophoresis. The Kd of the complex was 2.14 ± 0.78 μmol/l. Complex formation with transformed α2-M did not interfere with the immunological determination of leptin in plasma. The leptin–α2-M complex was found to be recognized by the α2-M receptor/low density lipoprotein receptor-related protein. By computer analysis, a simple model is presented showing that the degree of transformation of α2-M may significantly influence the leptin concentration in blood.

Conclusions: The proteinase inhibitor, α2-M, may act as a leptin-binding protein in human plasma. Binding of leptin to transformed α2-M and its rapid clearance by the α2-M receptor may significantly influence the bioavailability of leptin in human plasma.

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Introduction

Leptin (16 kDa), a product of the obese gene, is secreted from adipose tissue and it is suggested that in mice it acts on the central nervous system to regulate body weight through the control of appetite and energy expenditure (1, 2). Its role in man may be more complex, and remains to be elucidated. One major effect of leptin may be to inhibit synthesis and release of the hypothalamic neuropeptide Y, which increases food intake, decreases thermogenesis, and increases levels of insulin in plasma (3).

In rodents and humans the level of leptin is highly correlated with the size of the body fat mass. Recent data suggest that in obesity it is not the absence of leptin but leptin resistance which may be the cause of this conditions (4). In blood, leptin is thought to be bound to other proteins (5–7). Such binding proteins, known for a number of growth factors and cytokines, may alter the clearance of these biomolecules, influencing their bioavailability or protecting them from degradation (8). To understand the physiology of circulating leptin, it is important to determine its interaction with these circulatory components.
α2-Macroglobulin (α2-M), a proteinase inhibitor in blood and tissue, is known to act as such a binding protein for numerous cytokines and growth factors (9). Recently, we have shown that α2-M is also involved in binding and transport of human growth hormone (10). These biologically important polypeptides mainly bind to transformed α2-M (α2-Mt), which is the receptor-recognizable form of the inhibitor. The receptor of α2-M (α2-M-R) was found to be identical to the low density lipoprotein receptor-related protein (LRP) (11, 12). α2-M-R/LRP is known to confer binding and endocytosis of multiple ligands such as α2-Mt, lipoproteins (13) and diverse proteinase–proteinase inhibitor complexes (14). The objective of the present study was to analyze the binding proteins for circulating leptin. Such plasma components capable of interacting with leptin have been suggested, but their identities have not been elucidated up to now. We identified α2-M as such a leptin-binding factor in human plasma. We provide evidence that leptin binds only to α2-Mt but not to the...
native inhibitor. The complex was found to be recognized by α2-M-R/LRP.

**Subjects and methods**

**Patients**

Fresh blood was obtained from the Clinic of Internal Medicine of the University of Leipzig from a healthy, female volunteer (age 23 years; body mass index 20.2 kg/m²; leptin concentration 6.21 ng/ml).

**Reagents**

Recombinant human leptin and rabbit anti-human leptin antibodies were purchased from PeproTech, Inc. (Rocky Hill, NY, USA). Purified, native α2-M, purified α2-M-R/LRP, and horse radish peroxidase (HRP)-conjugated immunoglobulin (Ig) of rabbit anti-human α2-M were obtained from BioMac GmbH (Leipzig, Germany).

**Preparation of samples**

Citrated blood was centrifuged to remove blood cells. Unless immediately analyzed, the plasma was kept at −20°C. No serum was used for analysis. This was to guarantee that more than 99% of α2-M in the plasma was in the native form. *In vitro* transformation of isolated α2-M and α2-M in plasma was achieved by treatment of the samples with 200 mmol/l methylamine (MA) (Sigma Chemical Co., St Louis, MO, USA) for 4 h at room temperature. The resulting samples, designated as α2-M-MA and MA-plasma respectively, were extensively dialyzed against PBS (50 mmol/l sodium phosphate, 150 mmol/l NaCl, pH 7·4).

**Quantification of total and α2-Mt**

The concentrations of total and α2-Mt were measured by ELISA using the test kits MacroNat and MacroTrans respectively, obtained from BioMac GmbH.

**Binding studies by size exclusion chromatography (SEC)**

Mixtures of ¹²⁵I-leptin with plasma and MA-plasma were separated by SEC, using a Superdex 200 column (16 mm × 60 mm) from Pharmacia (Uppsala, Sweden). The column was equilibrated with PBS at a flow rate of 1 ml/min. The sample volume was 200 μl. Fractions of 1 ml were collected. As a control, the tracer was chromatographed in the presence of 2% BSA under the same conditions.

**Electrophoresis**

Resolution of native α2-M and α2-Mt was achieved by rate electrophoresis on 5% polyacrylamide slab gels as previously described (16). Polyacrylamide gel electrophoresis (PAGE) was performed in 3–15% polyacrylamide slab gel gradients using Tris–glycine buffer. Sodium dodecyl sulfate (SDS) electrophoresis was done in the same gradient gels according to Laemmli (17). The gels were stained with Coomassie Brilliant Blue R 250. In some experiments, the gels were dried and exposed at −80°C to Hyperfilm-MP (Amersham).

**Receptor binding studies**

Titer plates were coated with purified α2-M-R/LRP (2 μg/ml). After washing, the plates were incubated with increasing concentrations of ¹²⁵I-leptin–α2-M-MA complex, which had been made by reacting 100 μg of α2-M-MA with 5 ng ¹²⁵I-leptin at 37°C for 30 min. Further processing of the plates is described in the legend of Fig. 5.

**Computer modeling**

The impact of the leptin–α2-M interaction on the clearance of leptin was analyzed in terms of a steady state model. The dependence of stationary leptin concentration on the rate of transformation of α2-M was analyzed using the software tool GEPASI (18).

**Results**

When human recombinant ¹²⁵I-leptin was incubated with plasma at 37°C for 1 h and analyzed by SEC, the main peak of radioactivity (peak II) represented unbound or free leptin when compared with the elution profile of tracer alone (Fig. 1). In the case of plasma treated with MA, an additional peak of radioactivity (peak I) appeared, corresponding to the elution position of human α2-M (arrow). Approximately 30% of the total radioactivity applied was found within this peak. Treatment of plasma with the reactive MA causes transformation of α2-M (16). The transformed α2-M (α2-M-MA) can easily be separated from native inhibitor by rate electrophoresis (Fig. 2). In this
experiment, radiolabeled leptin was incubated with purified \( \alpha_2 \)-M and \( \alpha_2 \)-M-MA and subjected to electrophoresis. The radioactivity was found to be associated only with \( \alpha_2 \)-M-MA (fast-moving form). No binding of \( ^{125} \)I-leptin to native \( \alpha_2 \)-M (slow-moving form) was observed. The complex formation was found to proceed rather fast: within 30 min of incubation approximately 70 to 80% of maximum binding was reached (results not shown).

Leptin–\( \alpha_2 \)-M complexes were also detectable by Western blotting using polyclonal antibodies directed against leptin (Fig. 3). In the absence of SDS, leptin immunoreactivity was found to be associated with \( \alpha_2 \)-M-MA. Free leptin, which was not bound, appeared as a diffuse band as it tends to aggregate under this electrophoretic condition. The complex was found to dissociate by treatment with SDS under nonreducing conditions. The bound leptin was released and moved as free leptin in SDS-PAGE.

To evaluate the binding affinity between \( ^{125} \)I-leptin and \( \alpha_2 \)-M-MA, the extent of complex formation under increasing concentrations of unlabeled leptin was assessed, with separation of free and bound leptin by rate electrophoresis (Fig. 4). The radioactivity associated with the fraction of \( \alpha_2 \)-M-MA was measured in the gel. The \( K_d \) was calculated to be \( 2.14 \pm 0.78 \text{mM} \).

When the binding studies were done with MA-plasma under similar conditions, a \( K_d \) of \( 3.92 \pm 1.4 \text{mM} \) was obtained, which is of the same order of magnitude compared with the purified inhibitor. As an example, the competing effect of unlabeled leptin on complex formation at a concentration of \( 4.16 \text{mM} \) is shown in the inset of Fig. 4. The ability to displace the radiolabeled leptin by unlabeled leptin indicates that the hormone is bound to specific binding sites in the inhibitor molecule.

To analyze whether leptin, when bound to \( \alpha_2 \)-M-MA, is still immunologically detectable, the leptin concentration was determined in fresh plasma and in the same plasma after reacting with MA. As seen in Table 1, no significant differences in leptin concentration could be observed in plasma before and after treatment with MA. The results indicate that leptin which is bound to \( \alpha_2 \)-M-MA is not excluded from immunological reaction with antibodies used in our assay.

The complex between leptin and \( \alpha_2 \)-M-MA was found to be recognized by the receptor for \( \alpha_2 \)-M (Fig. 5). As seen, \( \alpha_2 \)-M-MA increasingly binds to immobilized \( \alpha_2 \)-M-R/LRP in a saturable fashion. When analyzed for leptin, the binding curve parallels that of \( \alpha_2 \)-M-MA, indicating that leptin does not dissociate from the inhibitor during receptor binding. In the presence of EDTA neither \( \alpha_2 \)-M-MA nor leptin was found to be bound to the receptor. It was confirmed by control experiments that leptin itself did not bind to \( \alpha_2 \)-M-R/LRP and had no effect on the interaction between \( \alpha_2 \)-M-MA and the receptor.

The effect of increasing concentrations of \( \alpha_2 \)-M on leptin level in blood was qualitatively analyzed in terms of a skeleton model comprising both the transformation of \( \alpha_2 \)-M and the receptor-mediated endocytosis of the
leptin–α2-M complex (Fig. 6). Using quite plausible assumptions about the endocytosis rate constant of the α2-M and the K_d of the leptin–α2-M complex, we could demonstrate that the stationary leptin level drops significantly if the blood concentration of α2-M increases. For calculation, the variation of α2-M was achieved by changing the rate of transformation. However, similar results could be obtained by varying the rate constant for the endocytosis of α2-M and/or of the leptin–α2-M complex.

Discussion
In the present study we present evidence that human leptin specifically binds to the α2-M. No significant binding of leptin to native α2-M was found. The K_d value of 2.14 μmol/l obtained for the leptin binding to the purified inhibitor was found to be similar to that obtained for the interaction in whole plasma, indicating that other serum proteins did not significantly interfere with this binding.

Leptin binding to α2-M was found to be stable during chromatographic and electrophoretic separation but did not resist treatment by SDS. This gives rise to the assumption that the binding is probably conferred by hydrophobic interactions. The presence of hydrophobic binding sites in α2-M has been described earlier (19). It becomes evident, from our experiments, that the binding to α2-M of leptin is reversible, involves very specific sites and is different from that known for binding of proteinases to the inhibitor (20, 21).
indications that a putative soluble leptin receptor candidates have been described with molecular masses fulfilling this function in animal and human plasma.

Furthermore, when serum instead of plasma is used, about 20 to 30% of the inhibitor is already transformed. All that may have a significant impact on the determination of leptin levels in blood. Our results demonstrate that even in the case when all α2-M in plasma is transformed, no loss in leptin concentration was observed. This indicates that the leptin-binding site(s) may be located at the surface of the inhibitor molecule accessible to anti-leptin antibodies. However, it cannot be excluded that, due to the low affinity of leptin for α2-M, leptin may dissociate from the complex in the presence of the high affinity antibody, which may be the reason why α2-M does not interfere with the leptin assay.

We have shown that the leptin–α2-M complex is recognized by the α2-M-R/LRP. Recognition is conveyed via the receptor-recognition site located on the C-terminal part of the polypeptide chain of α2-M. The fact that leptin binding did not interfere with receptor recognition indicates the existence of separate binding sites in the inhibitor. The clearance of α2-M, from the circulation by α2-M-R/LRP occurs very fast, with a half-life of approximately 5 min. Under normal conditions, the concentration of α2-M, capable of leptin binding, in plasma is approximately 7 nmol/l, which is, however, about 20 times higher than that of leptin in lean patients. Additionally, that fraction can very quickly increase by proteolytic transformation from the large pool of native α2-M (3.5 μmol/l) present in the circulation. This would suggest that α2-M and its plasma is used, about 20 to 30% of the inhibitor is already transformed. All that may have a significant impact on the distribution of leptin in blood specimens.

At present, it is commonly assumed that leptin circulates in plasma in a free form and bound to proteins. However, no proteins could be identified so far fulfilling this function in animal and human plasma. Candidates have been described with molecular masses ranging from 85 to 240 kDa (5). There are also indications that a putative soluble leptin receptor (~90 kDa) may form such complexes with leptin in plasma (6). Diamonds et al. (7) described a high molecular mass component (>450 kDa) of unknown identity which competitively bound leptin in human serum.

From our results it can be concluded that it is very important to know about the concentrations of total and α2-M in the samples when comparing results of leptin binding studies by different authors. This is because the fraction of transformed inhibitor in blood increases with sample storage and frequent freezing and thawing (22). Furthermore, when serum instead of plasma is used, about 20 to 30% of the inhibitor is already transformed. All that may have a significant impact on the distribution of leptin in blood specimens.

It is known that some proteins, when bound to leptin, become caged and escape immunological detection (21). Such an interference would have a significant impact on the determination of leptin levels in blood. Our results demonstrate that even in the case when all α2-M in plasma is transformed, no loss in leptin concentration was observed. This indicates that the leptin-binding site(s) may be located at the surface of the inhibitor molecule accessible to anti-leptin antibodies. However, it cannot be excluded that, due to the low affinity of leptin for α2-M, leptin may dissociate from the complex in the presence of the high affinity antibody, which may be the reason why α2-M does not interfere with the leptin assay.

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receptor may effectively modulate the concentration of leptin in blood, and thus in brain, as leptin is known to cross the blood–brain barrier. It is well known that α2-M binds and carries a number of cytokines and growth factors. Thus, by triggering the transformation as induced by inflammation, tissue remodeling, cell invasion etc., α2-M may play an unique role in mediating metabolic effects in different directions. At present, it is generally assumed that the hallmark of obesity in humans is leptin resistance, but not the absence of leptin. Leptin resistance could result from alterations in leptin receptor expression or receptor binding, from a disabled transport mechanism via the blood–brain barrier, or from alterations in the bioavailability or bioactivity of circulating leptin. Thus, complex formation with binding proteins may possibly restrict the availability of free leptin for its inhibitory effect on food intake. On the other hand, a decreased clearance of leptin may lead to an increased concentration of circulating leptin. Recently, a mutation in the human leptin receptor gene was detected which causes a significant shift in the distribution of leptin in the plasma of homozygous patients (23). Compared with controls, approximately 80% of leptin was found to circulate as a high molecular mass complex (<440 kDa) in plasma. Binding to a soluble dimeric leptin receptor was discussed in that paper, but the large mass of the complex found may not fit that assumption. Our preliminary results indicate that both α2-M and α2-M-R/ LR P are likely to be involved in binding and clearance of leptin. To analyze this may be a potential topic of future research.

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