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Serum Leptin Levels in Septic Men Correlate Well with C-Reactive Protein (CRP) and TNF-alpha but not with BMI

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Summary

Leptin, an adipocyte-derived signaling factor, is a member of the IL-6 cytokine family. However there is no direct evidence of leptin stimulation of the acute phase protein (APP) synthesis which is typical for all other IL-6-like factors. The purpose of this study was to characterize the dynamics of circulating leptin in relation to ten APPs. We used postoperative septic patients as a model of cytokine network hyperstimulation and intensive APP reaction. The prospective study was performed on 22 patients with proven postoperative intraabdominal sepsis after large abdominal surgery. Plasma levels of leptin, TNF-α, IL-1β, soluble IL-2 receptor (sIL-2R), IL-6 (ELISA analysis) and ten APPs (nephelometric analysis) were estimated. We have demonstrated a statistically significant elevation of plasma leptin concentrations in the septic group compared with healthy subjects ($p<0.001$). The correlation of plasma leptin and BMI during postoperative sepsis was diminished. The regression coefficient was the highest for leptin and CRP ($r=0.48$, $p<0.05$), and for leptin and alpha-1-antitrypsin ($r=0.46$, $p<0.05$) in the septic group. There was significant correlation between TNF-α and leptin ($r=0.47$, $p<0.05$) and between IL-6 and leptin ($r=0.45$, $p<0.05$) in septic patients. No significant correlation was found between leptin and "negative" APP and between leptin and IL-1β. Leptin has thus been shown as an acute phase reactant with a potential hematopoietic, immunomodulatory and hepatocyte stimulating activity during the infectious and non-infectious stress response. The significant correlation between leptin and CRP and leptin and alpha-1-antitrypsin indicates that leptin can participate in APP synthesis regulation during a systemic inflammatory response.

Key words
C reactive protein • Inflammation • Leptin • Tumor necrosis factor

Introduction

Acute phase proteins (APPs) are archetypal examples of plasma proteins. Higher plasma levels of APP are required during the acute phase response following an inflammatory stimulus. APPs are implicated in a wide range of physiological processes after infection or physical trauma. Main APPs include the C reactive protein (CRP) in humans, serum amyloid P component in rodents and serum amyloid A protein in all mammalians. Other groups of APPs have protease inhibiting activity (alpha-1-antitrypsin, alpha-2-macroglobulin etc.) or metal-binding and scavenger effects (ceruloplasmin, hemopexin, and haptoglobin). APPs also include some coagulation factors (e.g. fibrinogen) and complement proteins.

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Mediators of the systemic inflammatory response syndrome (SIRS) radically alter the biosynthetic profile of the liver. Among a spectrum of soluble factors that initiate the APP reaction, the IL-6 cytokine family plays a crucial role (Akira and Kishimoto 1992). The high structural homology (four-chain structure of alpha helix) and 130 kDa receptor subunit similarities define this group of cytokines. The IL-6 family cytokines (IL-6, oncostatin M, ciliary neurotrophic factor – CNTF, IL-13 etc.) are pluripotent mediators with diverse immunoregulatory, metabolic and hematopoietic activity.

Leptin (OB protein), a 16 kDa soluble polypeptide was initially described as an adipocyte derived hormone. The adipose tissue is a major source of leptin and its circulating concentrations indirectly reflect body fat stores. Leptin is an important signal in the regulation of food intake and energy balance. Its concentrations are elevated in the majority of obese individuals, and leptin levels usually correlate with adiposity and the body mass index (Haluzík *et al.* 1999).

The leptin receptor is expressed primarily in the hypothalamus (Elias *et al.* 1999). The brain is an established critical site of leptin function, yet little is known about leptin concentrations in the central nervous system relative to the plasma levels, other endocrine mediators, and psychiatric diagnoses (Dallongeville *et al.* 1998). However, it has recently been shown that leptin plays an important role in many aspects of the neurohumoral inflammatory response. Leptin modulates the hypothalamo-pituitary-adrenal axis, but the clinical importance of this effect is not completely clear. The leptin receptor is also expressed in human vasculature and in primary cultures of human endothelial cells. *In vitro* and *in vivo* assays have revealed that leptin has angiogenic activity (Sierra-Honigmann *et al.* 1998).

Leptin is secreted in a circadian fashion with a nocturnal rise in both obese patients and healthy subjects. The kidneys play a crucial role in leptin excretion in man. Therefore, renal failure is usually accompanied by a marked elevation of plasma leptin levels.

It was recently revealed that leptin is structurally similar to the granulocyte colony-stimulating factor (G-CSF), a member of the IL-6 cytokine family. The structure of the leptin receptor is not completely known, but it shows significant homology with G-CSF receptor units. G-CSF is a stimulating factor of APP synthesis *in vitro* and *in vivo.* However, there is no direct evidence of leptin stimulation of APP synthesis which is typical for all other IL-6 like factors yet.

The purpose of this study was to characterize the levels of circulating leptin in relation to ten APPs. We investigate postoperative septic patients as a model of cytokine network hyperstimulation and intensive APP reaction. To obtain a more complex picture of septic SIRS we compared plasma leptin changes with four proinflammatory cytokines and the cytokine soluble receptor.

Methods

The Ethical Committee of our institution approved this study protocol after informed consent had been obtained from the subjects.

We studied 22 surgical patients – males, (34-66 years) with proven postoperative intraabdominal sepsis after large abdominal surgery: after hemipancreatectomy (7 patients, 34-52 years), resection of colorectal carcinoma (12 patients, 52-66 years), and polytrauma (3 patients, 36-48 years), BMI = 24.8±3.4 $kg.m^{-2}$. Five patients had diabetes mellitus II and four patients had arterial hypertension treated with drug therapy. Patients were recruited for this study over a period of 12 months.

Plasma levels of leptin, tumor necrosis factor-alpha (TNF-α), IL-1, soluble IL-2 receptor (sIL-2R), and IL-6 were estimated one day after the clinical signs of sepsis (R. C. Bone's sepsis criteria) had appeared. Blood samples were collected at 08:00 h in the fasting state.

The control group consisted of 14 healthy men, aged 28-42 years, mean BMI 23.3±3.6 $kg.m^{-2}$. None of the subjects in the control group suffered from diabetes mellitus, hypertension or any other chronic disease.

For all measurements, 10 ml venous blood were collected into a chilled syringe with EDTA and immediately centrifuged at 1600x*g* for 15 min. Plasma was stored at –80°C until analysis.

Leptin was assayed by commercially available ELISA (Bio Vendor) kits for human leptin. Samples were run in duplicate. The sensitivity of the method was 0.5 ng/ml. The intra- and interassay coefficients of variation were below 5 %.

TNF-α, IL-1β, IL-6 and IL-8 were measured by ELISA for human cytokines (Immunotech/Coulter Company, Hamburg). The intra- and interassay coefficients of variation were below 5 %. The monitored APP were CRP, alpha-1-antitrypsin, alpha-2-macroglobulin (A2M), haptoglobin, transferrin,

prealbumin, albumin, alpha-1-acid glycoprotein, hemopexin and ceruloplasmin (nephelometric analysis).

Table 1. Body mass index (BMI), plasma levels of leptin, BMI, 10 APPs, TNF-α, IL-1β, IL-6 and sIL-2R in both septic and control group.

Parameters	Septic group	Control group
BMI (kg/m^2)	24.8±3.4	23.3±3.6
Leptin (μg/l)	36.8±11.2^{+++}	3.5±1.2
CRP (mg/l)	240.8±86.6^{+++}	5±5
Alpha-1-antitrypsin (g/l)	3.9±1.1^{++}	1.8±0.3
Alpha-2-macroglobulin (g/l)	1.8±0.5	1.8±0.3
Alpha-1-acid glycoprotein (g/l)	2.0±0.6^{++}	0.7±0.2
Haptoglobin (g/l)	4.2±1.2^{++}	1.7±0.4
Hemopexin (g/l)	1.4 ±0.4^{+}	0.9±0.3
Ceruloplasmin (g/l)	0.33±0.10^{+}	0.20±0.04
Albumin (g/l)	32.4±7.6^{+}	44.8±9.4
Prealbumin (g/l)	0.11±0.04^{++}	0.26±0.08
Transferrin (g/l)	1.9±0.7^{+}	2.5±0.5
TNF-α (ng/l)	422.4±214.5^{+++}	61.4±9.2
IL-1β (ng/l)	42.2±7.9	35.4±8.9
IL-6 (ng/l)	3321±1821^{+++}	118±49
sIL-2R (ng/l)	5469±1614^{+++}	581±145

Significant differences between controls and patients: $^{+}p<0.05$. $^{++}p<0.01$. $^{+++}p<0.001$

For statistical evaluation, we used the software package ANOVA© for Windows 95. As the data were normally distributed, statistical analysis was performed using the Mann-Whitney U test when comparing controls vs. septic values.

Student´s multiple comparison test was used for determining the significance among multiple time points in septic patients. Spearman´s rank correlation and correlation analysis for repeated observations were employed for evaluating the correlation between different parameters.

Results

The levels of leptin, TNF-α, IL-1β, sIL-2R, IL-6 and 10 APPs are shown in Table 1. Student´s multiple comparison test confirmed that there is a significant difference between the control group and septic group for 13 tested parameters. Significant differences between the tested groups are shown when $p<0.05$ was proved.

The regression coefficient was the highest for leptin and CRP in the septic group ($r=0.48$, $p<0.05$), for leptin and alpha-1-antitrypsin ($r=0.46$, $p<0.05$) and for leptin and alpha-1-acid glycoprotein ($r=0.45$, $p<0.05$). Figure 1 shows the correlation between leptin and CRP in the septic group ($r=0.48$, $p<0.05$).

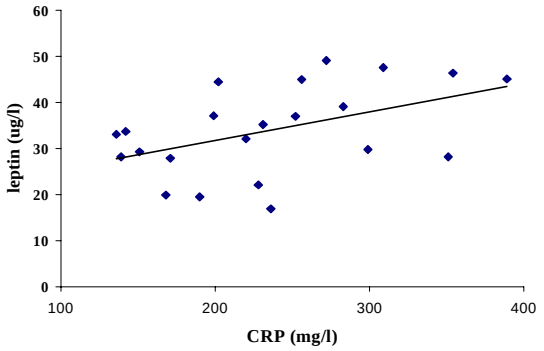

Fig. 1. *The correlation of leptin and CRP in 22 septic patients.*

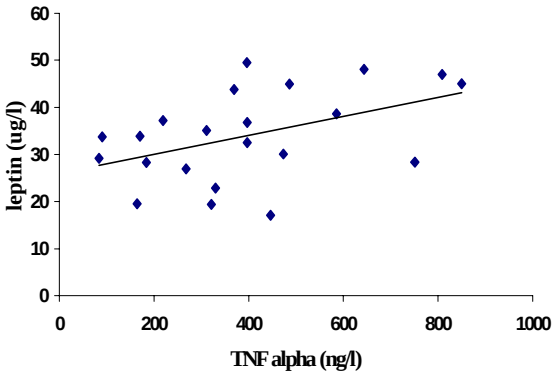

Fig. 2. *The correlation of leptin and TNF-α in septic patients.*

Among the cytokines, there was significant correlation between TNF-α and leptin ($r=0.49$, $p<0.05$) (Fig. 2) and between IL-6 and leptin ($r=0.46$, $p<0.05$) in septic patients. There was no significant correlation between leptin and "negative" APPs (albumin, prealbumin and transferrin) and between leptin and IL-1β. We demonstrated the absence of a significant correlation between plasma leptin and BMI in septic patients in

contrast to a highly significant relationship found in healthy subjects ($r=0.84$, $p<0.0001$).

Leptin plasma levels correlate well with plasma concentrations of an initial inflammatory cytokine TNF-α and with plasma levels of IL-6. On the other hand, there was no significant correlation between leptin and IL-1 or leptin and sIL-2R. Nevertheless, a significant elevation of IL-1β level was not found. All other inflammatory parameters except A2M were elevated in the septic group. It was not possible to distinguish whether relatively low levels of A2M result from diminished synthesis of this protein or from its accelerated degradation.

Discussion

The human response to surgical and infectious stress is characterized by a series of inflammatory, hormonal and metabolic changes that together constitute the global stress response of an acute reaction (Kain *et al*. 1999). The stress induced release of neuroendocrine hormones (e.g. ACTH, cortisol, antidiuretic hormone) and cytokines (e.g. TNF-α, IL-1, IL-6) provoke APP synthesis, a loss of muscle proteins, thermogenesis, hyperglycemia and many other metabolic, endocrine, psychological and immune responses. The initiation of a systemic inflammatory response, its course and prognosis depend on the complex interaction of proinflammatory and anti-inflammatory cytokines, and cooperation of many other soluble and membrane bound mediators in balance with the activation of the hypothalamo-pituitary-adrenal axis.

Of the many soluble factors that initiate and maintain an inflammatory response, several hormones and cytokines specifically regulate the transcription of human APPs. These include the IL-6 cytokine family, the IL-1 cytokine family (IL-1α, IL-1β, IL-1 receptor antagonist), TNF cytokine family (TNF-α, TNF-β, lymphotoxin-β and many membrane-bound TNF-like factors), interferon-γ and the transforming growth factor-β.

Leptin was initially described as an adipocyte-derived signaling factor that, after interaction with its receptor, induced complex behavioral, endocrine and metabolic responses, including the control of body weight and energy expenditure. More recently it was reported that leptin seems, in addition to its role in metabolic control, to play an important role in the acute phase response (Barbier *et al*. 1998). During the inflammatory reaction plasma leptin is usually enhanced and may contribute to the anorexia and cachexia of infection. Leptin may also play an important role in regulating the hypothalamo-pituitary-adrenocortical axis, in angiogenesis and in regulation of the immune response.

It has been reported that leptin can induce proliferation, differentiation and functional activation of hematopoietic cells and can enhance the proliferation and phagocytic activity of macrophages (Loffreda *et al*. 1998, Santos-Alvarez *et al*. 1999). These results have identified an important and novel function for leptin, namely the up-regulation of inflammatory immune responses. Leptin receptor gene knockout mice revealed an inherent deficit in lymphopoiesis. These findings have shown that leptin itself is involved in the cytokine network of acute inflammation and stress response.

Leptin is structurally similar to the granulocyte colony-stimulating factor (G-CSF). The structure of its receptor is not completely known, but it seems to be closely homologous to G-CSF receptor units. G-GSF is a member of IL-6 cytokine family (included IL-6, IL-13, oncostatin M, CNTF and leukemia inhibitory factor – LIF).

IL-6 and related cytokines are the main regulators of the APP reaction. However, there is considerable heterogeneity in the response *in vivo* and *in vitro* of individual APP genes to the cytokines listed above. CRP mRNA transcription is induced dramatically by IL-6. The promoter of the human CRP gene contains NF-IL6, recently identified as the transcription factor, induced by the IL-6 cytokine family. Posttranslational control of CRP has also been demonstrated: the dramatic transcriptional induction of CRP by IL-6 or IL-6 related peptides is further enhanced by novel translational and posttranslational mechanisms.

The significant correlation of leptin and CRP or alpha-1-antitrypsin can reflect cytokine induction of both leptin and the main APPs during sepsis *via* similar activating mechanisms. On the other hand, it can also indicate a possible leptin co-stimulation of hepatic APP synthesis.

There is still no direct evidence that leptin regulates APP synthesis in hepatocytes. However, structural and functional similarities of leptin and other mediators of the IL-6 family speak in favor of this influence. Hematopoietic activity (mainly expressed in IL-6, G-CSF and IL-13), the alpha-helix structure of its molecule and the high homology of cytokine receptor subunits are typical for the IL-6 cytokine family. There is direct evidence that G-CSF stimulates the APP genes in hepatocytes *in vitro* in a similar manner as other IL-6 family cytokines (Sawaki *et al*. 1993, Ziegler *et al*. 1993). G-CSF receptor-transfected hepatoma cells respond to G-CSF by increasing the production of the same set of

plasma proteins as are stimulated by IL-6 or LIF, suggesting that this group of cytokines shares a common signal transduction pathway.

Recent findings have shown that leptin is involved in cytokine-APP negative feedback regulation. Leptin interacts with APP and the proteinase inhibitor A2M. Leptin specifically binds to the transformed A2M, which arises by reaction with proteinases or with reactive primary amines. The leptin-A2M complex was found to be recognized by the A2M receptor/low density lipoprotein receptor-related protein. Binding of leptin to the transformed A2M and its rapid clearance by the A2M receptor may significantly influence the bioavailability of leptin in human plasma (Birkenmeier *et al*. 1998).

As far as we know, our study concerning leptin and APP relations is the first one performed in septic subjects during the early postoperative period after large abdominal surgery. Our data have shown a dissociation in the postoperative course of leptin and BMI and a significant correlation of plasma leptin levels and the main acute phase reactant CRP. There was a less marked correlation between leptin and alpha-1-antitrypsin or leptin and alpha-1-acid glycoprotein.

It should be noted that the plasma creatinine levels of our patients did not suggest any impairment of the kidney excretory function, the major site for leptin removal. Thus the increased leptin levels cannot be explained by kidney dysfunction.

The changes of leptin concentrations observed in our study could also be affected by a reduced food intake in our patients. However, it has recently been shown that systemic inflammation is associated with an increase of serum leptin concentrations (Hernandez *et al*. 2000). The mechanism of elevated leptin levels and the blunted rhythmicity after surgery is not yet clear. The administration of endotoxin (lipopolysaccharide, LPS), a model for gram-negative infections, induces profound anorexia and weight loss in animal models. TNF and IL-1, mediators of the host response to LPS, had significant effects on energy metabolism and appetite, and also induced anorexia (Plata-Salaman 1998). These cytokines increased the levels of leptin mRNA in the adipose tissue. In mice, the administration of TNF, IL-1 and, to a lesser extent of LIF, produced a prompt and dose-dependent increase in serum leptin levels and leptin mRNA expression in the fat. IL-10, IL-4, CNTF and IL-2, cytokines not known to induce anorexia or decrease food intake, had no effect on leptin levels.

During both infectious and non-infectious stress responses (such as abdominal surgery or postoperative sepsis), leptin has been shown itself as an acute phase reactant. Significant correlation between leptin and TNF-α (also demonstrated by other authors in models of bacterial inflammation) (Yamaguchi *et al*. 1998) indicates that TNF-α can be a crucial regulator of leptin production during the early postoperative period. It is possible that proinflammatory cytokines induce OB gene transcription *in vivo via* secondary mediators such as the transforming growth factor-β.

Leptin is not only an important signal in the regulation of food intake and energy balance, but also stress related factor, structurally, functionally and perhaps evolutionary related to proinflammatory and hematopoietic cytokines. Proinflammatory cytokines such as TNF-α or IL-1 can be the main regulatory factors of leptin during this period. Leptin which is a member of the inflammatory network of cytokines and acute phase reactants, might be an essential factor for the adequate reaction during the acute phase.

In conclusion, there is no direct evidence so far for leptin regulation of APP synthesis in hepatocytes. It has recently been shown that leptin is a member of IL-6 cytokine family with many structural and functional similarities with IL-6, G-CSF and other cytokines of this group. We revealed that leptin plasma levels well correlated not only with inflammatory cytokine levels (TNF-α or IL-6) but also with main APP members (CRP, alpha-1-antitrypsin). Our study couldn't distinguish whether the relations of leptin and APP reflect cytokine induction of both leptin and main APP *via* similar activating mechanisms or not. It indirectly supports a possibility that leptin co-stimulates hepatic APP synthesis during sepsis.

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