Effects of acidosis on leptin secretion from 3T3-L1 adipocytes and on serum leptin in the uraemic rat

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ABSTRACT
Marked hyperleptinaemia and metabolic acidosis are common findings in patients with chronic renal failure. In animal models, both leptin administration and acidosis reduce food intake. However, leptin causes loss of body fat, while acidosis induces muscle wasting. Whether a low pH and leptin production are related has not been studied. Leptin secretion was measured in cultured 3T3-L1 adipocytes exposed to acid or control pH for up to 96 h. In addition, serum leptin was compared between acidotic and bicarbonate-treated uraemic Wistar rats using the remnant model. Leptin levels in the culture medium were decreased at an acid pH of 7.1 compared with a control pH of 7.5 at 96 h (562 ± 78 and 831 ± 103 pg mL⁻¹, respectively; mean ± S.E.M.; P = 0.037). Similarly, serum leptin in uraemic rats was found to be lower in the acidotic group than in the bicarbonate-treated group, although this observation fell just short of statistical significance (1273 ± 171 compared with 2059 ± 376 pg/mL; P = 0.07). In conclusion, acidosis decreases leptin secretion from cultured adipocytes. Accordingly, acidotic uraemic rats seem to exhibit lower serum leptin levels than their bicarbonate-supplemented counterparts. This study is the first report providing a link between acidosis and leptin levels.

INTRODUCTION
Protein–energy malnutrition is a common problem in patients with chronic renal failure (CRF) [1,2]. Numerous factors contribute to malnutrition, of which a reduced food intake due to anorexia and increased energy expenditure are among the most important. Since such malnutrition is associated with high morbidity and mortality in these patients [3], it is crucial to delineate the mechanisms mediating these effects.

Leptin, a 167-amino-acid protein transcribed from the ob gene (leptin gene) and produced mainly in white adipose tissue, has recently attracted attention both scientifically and in the media. After interaction with its receptors, leptin induces a complex response, including limitation of food intake and stimulation of energy expenditure. Evidence supporting this claim was provided by the dramatic decrease in body weight in laboratory rodents injected with leptin [4]. In human subjects, the plasma leptin concentration is correlated with body fat mass [5], leading to the idea that obesity may be associated with leptin insensitivity. As an exception, uraemic patients show markedly increased serum leptin levels that are much greater than would be predicted by their adiposity. Impaired renal elimination accounts only in part for this elevation, and therefore the cause of uraemic hyperleptinaemia is still awaiting a convincing explanation [6–10]. The pathophysiological significance of circulating leptin levels in patients with CRF also remains unclear. Longitudinal studies are still needed to prove a definite role for leptin in the regulation of body weight in this setting. However, recent data indicate that a high serum leptin/body fat mass ratio may be associated with weight loss in uraemic patients.

Key words: acidosis, adipocyte, leptin, rat, uraemia.
Abbreviations: CRF, chronic renal failure; GFR, glomerular filtration rate.
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undergoing haemodialysis, suggesting that hyperleptinaemia might contribute to the anorexia and malnutrition associated with CRF [11].

The impact of metabolic acidosis, a common component of uraemia, on nutrition status is well known. There is abundant evidence from both animal and human studies that uraemic metabolic acidosis leads to malnutrition by increasing protein degradation in skeletal muscle [12–14]. Acidosis stimulates the oxidation of branched-chain amino acids and the secretion of catabolic hormones (e.g. cortisol), and promotes ubiquitin-dependent proteolysis [15]. In addition, severe acidosis induces anorexia in animal models [16]. Furthermore, correction of metabolic acidosis in uraemic patients undergoing continuous ambulatory peritoneal dialysis has been shown to improve several nutrition parameters, such as mid-arm muscle circumference and body weight [17]. However, whether acidosis has any significant effect on adipose tissue, and in particular on leptin release, is unknown.

As leptin and acidosis are important players in energy and protein balance respectively, and since both reduce food intake in animal models, it was tempting to suggest a relationship between them. The present study was designed to investigate a possible effect of low pH on leptin secretion and levels. This hypothesis was tested both in vitro and in vivo, respectively by measuring leptin secretion in cultured adipocytes exposed to acid or control pH and by comparing serum leptin levels between acidic and bicarbonate-treated uraemic rats using the remnant model.

Part of this work was presented at the American Society of Nephrology 31st Annual Meeting, Philadelphia, U.S.A., 25–28 October 1998, and at the 9th International Congress on Nutrition and Metabolism in Renal Disease, Vienna, Austria, 29 August–1 September, 1998.

MATERIALS AND METHODS

The study comprised two experimental protocols.

Protocol study I: acidosis in cultured adipocytes

Cell culture

3T3-L1 cells from the European Collection of Animal Cell Cultures (ECACC; ref. 86052701) were plated at 5 × 10⁴ cells per well in six-well plates and routinely maintained in 2 ml of Dulbecco’s modified Eagle’s medium containing 10% (v/v) non-heat-inactivated foetal bovine serum, 500 units/ml penicillin, 500 μg/ml streptomycin and 10 mg/l Phenol Red at 37°C under a 5% CO₂ atmosphere. When cells were confluent, differentiation was initiated by the addition of 500 μM 3-isobutyl-1-methylxanthine, 250 nM dexamethasone and 330 nM insulin to the medium for 2 days. The cells were then further cultured in regular medium without any addition of hormones for 4 days, and finally were exposed for up to 4 days to test medium at acidic pH 7.1 or control pH 7.5. The pH was adjusted by the addition of HCl or NaHCO₃. NaCl was added to the acidic cultures to maintain a constant Na concentration. This test medium comprised minimal essential medium, 10% (v/v) non-heat-inactivated foetal bovine serum, 500 units/ml penicillin and 500 μg/ml streptomycin. The medium was changed three times per week during the propagation phase, and every other day during both the differentiation and the test phases. At the end of the experiments, the plates were frozen at −20°C until the cell content was used for analysis.

Leptin quantification

Leptin concentrations in cell culture media were determined at 2 and 4 days after exposure to the test medium using a sensitive and specific sandwich ELISA for mouse leptin (Quantikine M; R&D Systems, Minneapolis, MN, U.S.A.). Briefly, an affinity-purified polyclonal antibody specific for mouse leptin was bound as the capture antibody to microtitre plates, to which leptin samples (50 μl of sample mixed with 50 μl of assay diluent) were immobilized. Bound leptin was detected by the addition of 100 μl of polyclonal antibody specific for mouse leptin conjugated to horseradish peroxidase. After addition of a substrate solution (tetramethylbenzidine), the intensity of the colour obtained was measured at 450 nm. Recombinant mouse leptin was used as a standard.

DNA, protein and triacylglycerol quantification

After thawing, the cells were scraped into 10% (w/w) HClO₄. Triacylglycerol was extracted from the scraped cell suspension into chloroform and dried down under a stream of oxygen-free nitrogen, followed by enzymic assay using a commercially available kit (Boehringer Mannheim; no. 701882). The remaining precipitated cell suspension in HClO₄ was heated at 70°C for 20 min. Total precipitated protein was determined by the method of Lowry et al. [18], and total digested DNA in the soluble fraction was determined colorimetrically (595 nm) [19].

Protocol study II: acidosis in the remnant model

Animals

To assess the effect of pH on serum leptin concentration in vivo, archived frozen sera from acidic (n = 10) and bicarbonate-treated (n = 9) uraemic rats were assayed. Details of this study protocol have been published previously [20]. In brief, female Wistar rats (initial weight
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Approx. 160 g) underwent right nephrectomy and ligation of two left renal artery branches as a single procedure under general anaesthesia (2.7 ml/kg Hypnorm/Hypnovel/water; 1:1:2, by vol.; intraperitoneal). Postoperatively, they were fed on a 20% (w/w) casein feed mixed with water and methylcellulose for 2 weeks, ranked for severity of injury based on proteinuria, serum urea and creatinine, and allocated to one of two groups. Group 1 rats (acidotic remnants) were fed with a paste mixed with water and methylcellulose supplemented with 1.3 g of NaCl per 100 g. Group 2 rats (bicarbonate-treated remnants) were pair-fed with the same diet but supplemented with 1.9 g of NaHCO$_3$ per 100 g instead of NaCl. This procedure equalized the sodium intake between the two groups. Food consumption was recorded daily, and body weight was measured weekly. Glomerular filtration rate (GFR) was measured using the method of Nankivell et al. [21] by intraperitoneal injection of technetium-99m-labelled diethylenetriaminepenta-acetic acid at 12 weeks.

Animals were examined individually on a daily basis, and were killed by exsanguination under anaesthesia (2.7 ml/kg Hypnorm/Hypnovel/water; 1:1:2, by vol.; intraperitoneal) when they developed terminal uraemia, characterized by lethargy, anorexia and failure to gain weight. Arterial blood was collected into a heparinized, air-free syringe for blood gas determination, and serum was analysed for urea and creatinine. The remaining serum was frozen at $-70^\circ\text{C}$ until used for serum leptin analysis.

**Leptin quantification**

Leptin concentrations in rat serum were determined using the mouse leptin ELISA described above. It is known that mature mouse leptin shares approx. 96% sequence identity with the rat protein. In addition, in rat adipocyte cultures, Mueller et al. [22], using an RIA technique, found that leptin values in the culture media obtained with a mouse assay were highly correlated with those obtained using a rat-specific assay ($r = 0.97; P < 0.0001$). Therefore it was concluded that measurements of serum rat leptin with a mouse ELISA would provide a reliable measurement of leptin concentrations. The outline of the assay was similar to that used for cell supernatant assays. Briefly, 50 µl of serum sample was diluted with 50 µl of assay diluent to bind the immobilized antibody specific for leptin, and 100 µl of enzyme-linked polyclonal specific antibody was added to the well. The intra- and inter-assay coefficients of variation for this assay are 3.8% and 5% respectively.

**Statistical analysis**

Results are expressed as means ± S.E.M. The $n$ values represent the numbers of cell culture wells for protocol I and the numbers of rat serum samples in protocol II. All comparisons were made on paired data sets using a two-tailed $t$ test; $P < 0.05$ was considered statistically significant.

**RESULTS**

**Effects of acidosis on leptin secretion in vitro**

Differentiation of 3T3-L1 cells into adipocytes became evident within 2 days after removal of the hormone cocktail. The complete adipocyte phenotype, characterized by accumulation of lipid deposits into droplets and enlargement of the cells, was achieved in the majority of the cells approx. 1 week after induction. Initially, preliminary experiments revealed that leptin secretion into the medium peaked 6–10 days after differentiation was initiated. Therefore cells were exposed to test medium from day 6 onwards.

As shown in Figure 1, the mean leptin concentration in the medium was shown to be lower at the more acidic pH of 7.1 compared with at the control pH of 7.5; the

![Figure 1: Effect of pH on leptin secretion from 3T3-L1 adipocytes](image)

**Figure 2: Effect of pH on total DNA, protein and intracellular triacylglycerol (triglyceride) levels in 3T3-L1 adipocytes**

Leptin concentrations were measured by ELISA in each separate culture well 0–48 h and 48–96 h after medium at pH 7.1 (grey bars) or pH 7.5 (black bars) was added. Data are pooled from three independent experiments ($n = 27–33$ culture wells for each set of conditions); * indicates a significant difference from the value at pH 7.5 ($P = 0.037$).

![Figure 2: Effect of pH on total DNA, protein and intracellular triacylglycerol (triglyceride) levels in 3T3-L1 adipocytes](image

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Table 1  Data for uraemic rats

Group 1 contained acidotic rats, and group 2 contained bicarbonate-supplemented rats. Because of variable sensitivity to general anaesthesia, which resulted in a range of PaO2 values in arterial blood at the time the rats were killed, terminal arterial pH is expressed both as a ‘raw’ pH and as a ‘corrected’ pH adjusted using the Henderson–Hasselbach equation to represent a pH value at a standard PaCO2 of 5.6. NS, not significant.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n = 10)</th>
<th>Group 2 (n = 9)</th>
<th>P  value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (days)</td>
<td>112 ± 14</td>
<td>100 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>34.2 ± 0.7</td>
<td>33.1 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>186 ± 10</td>
<td>197 ± 17</td>
<td>NS</td>
</tr>
<tr>
<td>GFR at 12 weeks (ml/min)</td>
<td>0.60 ± 0.18</td>
<td>0.66 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine (umol/l)</td>
<td>287 ± 47</td>
<td>314 ± 52</td>
<td>NS</td>
</tr>
<tr>
<td>Arterial pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>6.94 ± 0.04</td>
<td>7.21 ± 0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>Corrected</td>
<td>6.87 ± 0.04</td>
<td>7.32 ± 0.05</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum leptin (pg/ml)</td>
<td>1233 ± 171</td>
<td>2059 ± 376</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Figure 3  Effect of bicarbonate supplementation on serum leptin levels in uraemic rats

Each data point represents the serum leptin concentration (measured by ELISA) in one of ten acidotic (○) or nine bicarbonate-treated (■) uraemic rats. *P = 0.07 compared with values in group 2.

DISCUSSION

In the in vitro study it was shown that, at an acid pH of 7.1, leptin levels in the culture medium were significantly decreased compared with those at the control pH of 7.5. Unlike with cultured skeletal muscle cell models [23], a 4-day acid exposure did not lead to a protein-wasting effect in 3T3-L1 adipocytes, since total protein levels were similar at both pH values. Furthermore, total DNA and triacylglycerol levels were not significantly affected by acid, suggesting that decreased leptin secretion is not due to reduced adipose cell mass or differentiation. The demonstration at low pH of a trend towards an increase in the intracellular triacylglycerol level, an indicator of adipose cell mass, is consistent with earlier observations reporting a direct anti-lipolytic effect of acidosis in isolated rat adipocytes [24]. As adipose mass is correlated with leptin levels, down-regulation by acid of leptin secretion per unit of adipose cell mass could be even more marked than that reported in Figure 1. It could be argued that acidosis may produce a general decrease in secretory processes, and that the observation of a decrease in leptin secretion might be unspecific. However, among a number of examples, increases in aldosterone serum levels in men and in tumour necrosis factor-α protein secretion from macrophage cell lines have been demonstrated under acid conditions [25,26]. In the experiments in protocol I, it was not possible to detect any measurable level of tumour necrosis factor-α in the cell supernates, despite con-
centration of the samples by up to 10-fold. Therefore specificity could not be addressed. A toxic effect of a low pH on 3T3-L1 adipocytes is unlikely, since these cells have been exposed to a pH as low as 6.9 for up to 72 h without any apparent toxic effects [27].

Given the results with protocol I, it was important to determine whether this effect was relevant in vivo. Consistent with the in vitro findings, uraemic acidic remnant rats had near-significant lower levels of circulating leptin than their bicarbonate-supplemented counterparts. The apparent difference is not explained by confounding factors. First, the two groups of rats had similar renal function during the whole course of the study, as corroborated by assessment of GFR during the study and measurement of serum creatinine when the rats were killed. Secondly, the body weights were very similar in the two groups and, despite the lack of fat mass evaluations in these animals, it is reasonable to assume that, under uraemic conditions, the fat mass was small and quite comparable between the two groups when the animals were killed.

Acidosis caused a decrease of approx. 30% in leptin secretion in 3T3-L1 adipocytes, which was similar in magnitude to the apparent fall in the serum leptin concentration in the remnant rats. This response is comparable with that reported after short-term fasting in animals or humans [28,29]. Therefore it is postulated that acidosis may be perceived as a starvation state by the adipose tissue. Interestingly, Kolaczynski et al. [29] observed that the profiles of serum leptin and β-hydroxybutyrate during fasting and refeeding represented almost mirror images of each other in healthy humans. In subsequent experiments, the authors failed to demonstrate that ketones were leptin inhibitors, and concluded that some other factors triggered by fasting, conceivably acidosis, were responsible for the fall in serum leptin levels. More recently, Wang et al. [30] reported that the flux of glucose in the hexosamine biosynthetic pathway regulates ob gene expression in the adipose tissue and skeletal muscle of the rat. Glucose transport is a pH-dependent process, and it has been demonstrated that alkaline pH stimulates glucose transport in rat adipocytes and in skeletal muscle [31,32]. Thus it may be anticipated that metabolic acidosis produces a starvation signal to the adipocyte, by decreasing glucose transport and flux into the hexosamine biosynthetic pathway, ultimately leading to down-regulation of the leptin gene.

In patients with CRF, plasma leptin concentrations were found to be elevated compared with those in matched healthy individuals. Plasma leptin is partly cleared by the kidney [7]. However, a number of mechanisms other than impaired renal elimination or body fat mass are responsible for or contributory to the observed levels. Increased synthesis, perhaps due to stimulation by hormones accumulated in renal failure and systemic pro-inflammatory cytokine activation, has been postulated [6–10]. The impact of metabolic acidosis on leptin secretion and levels is addressed in the present paper. Since severe acidosis and leptin injection both reduce food intake in animals, the potential of a low pH to stimulate leptin secretion was initially suggested. The results clearly refute this hypothesis, and suggest the opposite. Thus acidosis associated with CRF does not contribute to uraemic hyperleptinaemia. In the light of these findings, it is interesting to note that nearly one-third of patients with end-stage renal disease maintain normal plasma leptin levels [10]. Furthermore, Nordfors et al. [33] demonstrated decreased leptin gene expression in patients with advanced CRF compared with healthy subjects. The authors postulated that chronically high leptin levels could down-regulate the ob gene. In the present paper, metabolic acidosis is suggested as an alternative mechanism for decreased leptin gene expression in uraemia.

In conclusion, we report the first evidence for a link between acidosis and leptin secretion. 3T3-L1 adipocytes exposed to acid showed a marked decrease in leptin secretion in vivo. In a retrospective cross-sectional study, remnant acidic Wistar rats had near-significant lower serum leptin levels than bicarbonate-treated animals, suggesting that, in vivo, a low pH is also likely to decrease leptin production. The mechanism(s) responsible for this effect is still unknown, but may involve glucose availability within the adipose cell. Prospective in vivo studies are required to confirm these results, since correction of acidosis, largely advocated in dialysis patients to minimize protein wasting, could potentially exacerbate uraemic hyperleptinaemia. Finally, the results suggest that metabolic acidosis is not a contributor to hyperleptinaemia in patients with CRF.

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