Cysteine cathepsin S processes leptin, inactivating its biological activity

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Abstract

Leptin is a 16 kDa hormone mainly produced by adipocytes that plays an important role in many biological events including the regulation of appetite and energy balance, atherosclerosis, osteogenesis, angiogenesis, the immune response, and inflammation. The search for proteolytic enzymes capable of processing leptin prompted us to investigate the action of cysteine cathepsins on human leptin degradation. In this study, we observed high cysteine peptidase expression and hydrolytic activity in white adipose tissue (WAT), which was capable of degrading leptin. Considering these results, we investigated whether

recombinant human cysteine cathepsins B, K, L, and S were able to degrade human leptin. Mass spectrometry analysis revealed that among the tested enzymes, cathepsin S exhibited the highest catalytic activity on leptin. Furthermore, using a Matrigel assay, we observed that the leptin fragments generated by cathepsin S digestion did not exhibit angiogenic action on endothelial cells and were unable to inhibit food intake in Wistar rats after intracerebroventricular administration. Taken together, these results suggest that cysteine cathepsins may be putative leptin activity regulators in WAT. *Journal of Endocrinology* (2012) **214**, 217–224

Introduction

Leptin is a small protein primarily produced by adipocytes, consists of 167 amino acid residues, and is encoded by the *ob* gene on chromosome 6, the murine homolog of the human leptin gene *LEP*. The role of leptin in energy homeostasis is demonstrated in the genetic obese mouse (*Ob/Ob*), which exhibits profound obesity, type II diabetes, and hyperphagia (Zhang *et al.* 1994, Tartaglia 1997, Lago *et al.* 2007). In addition to its important function in regulating food intake (Zhang *et al.* 1994, Attele *et al.* 2002, Sahu 2004), leptin is involved in several processes such as angiogenesis (Anagnostoulis *et al.* 2008), blood pressure control (Haynes 2005), osteogenesis (Bertoni *et al.* 2009), fertility (Thomas *et al.* 2004), immune response, inflammation (Lago *et al.* 2007), and atherosclerosis (Taleb *et al.* 2007).

The activities of leptin are mediated through its receptor (OB-R), which is encoded by the diabetes (*db* (*Lepr*)) gene on chromosome 4. Leptin receptor null mice (*db/db*) exhibit a phenotype similar to that of *ob/ob* mice, which is used as a model for obesity (Tartaglia 1997). The clearance of leptin by the kidneys is believed to be the end point of leptin action,

and the bioavailability of leptin is defined by the ratio of free/bound leptin (Huang *et al.* 2001, Lammert *et al.* 2001).

Recent studies have shown that the cysteine peptidases cathepsins L, S, and K are present in the adipose tissue (Naour *et al.* 2010), and these cathepsins are thought to be important molecules in adipogenesis and atherosclerosis (Lafarge *et al.* 2010, Naour *et al.* 2010). Although human cathepsins were initially associated with the protein recycling machinery, they are also implicated in a variety of events outside the lysosomes or present on cell membranes where they participate in selective and controlled processes (Turk *et al.* 2000, 2012, Vasiljeva *et al.* 2007). This feature is specifically supported by cathepsin S, which is involved in many pathophysiological processes (reviewed in Arnlow (2012)), such as atherosclerosis (Sukhova *et al.* 2003) and adipogenesis (Taleb *et al.* 2005, 2006a,b). Cathepsin S has been reported to be a marker of obesity and is highly correlated with body mass index (Taleb *et al.* 2005), overexpressed in obese human subjects, and shows decreased expression after induced weight loss (Taleb *et al.* 2005). Furthermore, the cathepsin S gene is one of the most deregulated genes in the adipose tissue of obese subjects (Naour *et al.* 2010).

Despite the description that both lysosomal and proteasomal pathways contribute to the degradation of leptin (Rau *et al.* 1999, Uotani *et al.* 1999, Lee & Fried 2006), the enzymes involved in the processing of this adipokine have not been described yet. In the present work, we demonstrated that leptin is processed by cysteine cathepsins and that cathepsin S renders leptin inactive, suggesting that cysteine cathepsins are key elements in leptin physiology.

Materials and Methods

Animals and experimental surgery

Fifteen male Wistar rats at 12–14 weeks of age weighing 240–260 g were submitted to i.c.v. cannula implantation as described previously (Telles *et al.* 2003). Two days later, the correct placement of the cannulae was assessed by a rapid dipsogenic response to angiotensin II (20 ng/l), and the animals that presented a positive response were selected, allowed to recover from surgery for at least 5 days, and separated into three animal groups containing five rats each. Then, the animals were submitted to i.c.v. injections of 10 µg leptin (group 1), 10 µg leptin incubated with 0.24 µM cathepsin S (group 2), and 10 µg leptin incubated with 0.24 µM heat-inactivated cathepsin S (group 3). The incubations were performed in 50 mM sodium phosphate buffer, pH 6.0, for 1 h at 37 °C. The injections were repeated for 3 days in 24 h intervals, and the weights of the animals and food intake were measured daily. Statistical analysis of food intake data was performed using two-way ANOVA followed by Bonferroni's posttests. $P < 0.05$ was considered statistically significant. All procedures were conducted in accordance with the Ethics Committee of Federal University of São Paulo (Brazil) and by the Animal Care Committee from the Institute of Biomedical Sciences, University of São Paulo (Brazil). Epididymal white adipose tissue (WAT) was obtained from C57Bl6/J mice that had free access to food and water in a 12 h light:12 h darkness cycle in a ventilated cabinet (Alesco Ind. & Com. Ltd., Monte Mor, SP, Brazil). The animals were killed by cardiac exsanguination under anesthesia. WAT samples were sonicated in buffer containing 50 mM sodium phosphate, pH 6.0, and quantified using a BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA) with BSA as a standard.

Proteins

Recombinant human leptin was purchased from R&D (Minneapolis, MN, USA). The recombinant human cathepsins S and K were expressed in *Pichia pastoris*, as described previously (Linnevers *et al.* 1997). Cathepsins L and B were purchased from Sigma Chemical Co. The molar concentrations of the studied cathepsins were determined by active site titration with E-64 according to the method previously described (Barrett *et al.* 1982).

RNA extraction and RT-quantitative PCR

Total RNA from epididymal WAT was extracted using TRIzol reagent (Invitrogen Inc.). Total RNA (2.5 mg) was reverse transcribed using the first-strand cDNA synthesis (Invitrogen) and submitted to PCR using the Quantitect Sybr green master mix (Qiagen) on a MX3005P thermocycler (Agilent Technologies, Santa Clara, CA, USA). Cycling conditions were 50 °C for 2 min followed by 95 °C for 10 min (Hot start), 50 cycles at 95 °C for 15 s; 60 °C for 25 s, and 72 °C for 30 s. Acquisition was done at the 72 °C step. Primers used were sense 5'-tccccagaatcttggact-3' and antisense 5'-tcttcagggtcttcgttc-3' for cathepsin K, sense 5'-gcctcaggtgttgaaacct-3' and antisense 5'-gttgctgattccccgttg-3' for cathepsin L, and sense 5'-ccattgggactcttggaaga-3' and antisense 5'-ttcatgccacttgtaggt-3' for cathepsin S. Cyclophilin A was used as internal control (Christoffolete *et al.* 2006).

WAT cysteine peptidase catalytic activity

The cysteine peptidase activity in WAT lysates (10 µg) was determined using 10 µM fluorogenic peptide benzyloxycarbonyl-L-phenylalanyl-L-arginine-4-methylcoumaryl-7-amide (Z-FR-MCA; Sigma Chemical Co.) as a substrate. The assays were performed in 50 mM sodium phosphate, pH 6.0, at 37 °C. The cysteine proteases present in the extract were preactivated with 1.0 mM dithiothreitol (DTT) for 5 min before the addition of the substrate. The hydrolysis was continuously monitored using a Hitachi F-2000 fluorimeter and the increase in fluorescence was measured at $\lambda_{em} = 380$ and $\lambda_{ex} = 460$ nm. To define the specificity for cysteine peptidases, the assays were also performed in the presence of specific cysteine inhibitor E-64 (5 µM). The measurements were performed in triplicate, and the results are expressed as mean $\pm$ S.D.

Leptin cleavage assay by cysteine proteases

Human recombinant leptin (24 µM) was incubated with WAT lysates (10 µg) in 50 mM sodium phosphate, pH 6.0, at 37 °C for 60 min in the presence or absence of E-64 (5 µM). After incubation, the samples were separated on a 12.5% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Subsequently, the membrane was washed with PBS solution and incubated with antihuman leptin monoclonal antibody (primary antibody 1:1500; Sigma Chemical Co.). The membrane was then washed again with PBS and incubated with IgG-rabbit conjugated with peroxidase (secondary antibody 1:2500; Sigma Chemical Co.). TMB (3,3', 5,5' - tetramethyl benzidine) membrane peroxidase substrate was used as a substrate.

The recombinant human cathepsins were preactivated with 1.0 mM DTT in 50 mM sodium phosphate, pH 6.0, at 37 °C for 5 min, and the catalytic activities of the enzymes were analyzed in the same buffer at 37 °C using

10 μM Z-FR-MCA as a substrate. The hydrolysis was monitored for 10 min in a Hitachi fluorimeter F-2000 at $\lambda_{\text{ex}}=380$ and $\lambda_{\text{em}}=460$ nm. Subsequently, the active cathepsins (0.24 μM) were incubated with recombinant human leptin (24 μM) for 15 min in 50 mM sodium phosphate, pH 6.0 or pH 7.0, at 37 °C. In another set of experiments, 24 μM leptin was incubated with 0.24 μM , 0.4 μM , or 2.4 μM of recombinant human cathepsin S in 50 mM sodium phosphate, pH 6.0, for 15 min at 37 °C. To confirm the specificity of the assay, 5 μM inhibitor E-64 was preincubated for 5 min with 2.4 μM of cathepsin S before the addition of leptin. Subsequently, the samples were subjected to mass spectrometric analyses or were separated on 12.5% SDS–polyacrylamide gels. An unstained protein molecular weight marker (Fermentas, Hanover, MD, USA) was used as a molecular mass standard.

Mass spectrometry analysis of leptin processing by cysteine cathepsins

The cleavage of leptin (24 μM) by cathepsins B, K, L, and S (0.24 μM of each) was analyzed using a Matrix-assisted laser desorption/ionisation–time of flight mass spectrometry (MALDI–TOF/MS) mass spectrometer (Bruker Daltonics, Bremen, Germany). The samples were incubated in 50 mM sodium phosphate buffer, pH 6.0 or pH 7.4, for 1 h at 37 °C. The assay with cathepsin S (0.24 μM) was also performed in the presence of 5 μl E-64. Subsequently, the samples were mixed (1:1) in sinapinic acid (10 mg/ml) matrix solution and 2 μl sample was spotted onto the MALDI target plate and dried at room temperature. The mass spectra were obtained operating in linear, positive ion mode previously calibrated with serum melittin and horse heart cytochrome *c*. For the analysis of leptin control, a pulsed ion extraction delay of 250 ns, ion source voltages of 20 and 18.25 kV, and an ion source lens voltage of 7.00 kV were used. The spectra were acquired by accumulating 50 laser shots at 90% laser power in the m/z range of 18–75 kDa. The analysis of leptin fragments formed by cathepsin S hydrolysis was performed using a pulsed ion extraction delay of 260 ns, ion source voltages of 20 and 18.50 kV, and an ion source lens voltage of 8.50 kV. The spectra were acquired by accumulating 50 laser shots at 32% laser power in the m/z range of 2–20 kDa.

In vitro angiogenesis assay on Matrigel

BD Matrigel Matrix (B and D Biosciences, Bedford, MA, USA) was thawed on ice and then 15 μl /well were distributed into 96–well plates and allowed to polymerize for 1 h at 37 °C. Then, leptin (1 μM), cathepsin S (15 nM), and leptin in the presence of cathepsin S were incubated for 1 h at 37 °C and submitted to heating inactivation. The samples were added to 100 μl RPMI medium supplemented with 0.2% FCS containing the 5×10^3 cells/well human umbilical vein endothelial cells (HUVECs). The plates were incubated

at 37 °C for 18 h, and the number of pro-angiogenic structures (closed rings) per well was counted as described previously (Paschoalin *et al.* 2007). The assays were carried out in triplicate, and the average value was determined for each sample. As a control, HUVECs were plated on Matrigel without any addition. Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls posttest. Significance was set as $P < 0.05$.

Figure 1 Cysteine peptidase in epididymal WAT. (A) Real-time analyses of cathepsins in WAT. (B) Measurements of WAT (10 μg) proteolytic activity in 50 mM sodium phosphate buffer, pH 6.0, using Z-FR-MCA (10 μM) as a substrate in the absence (filled circle) or presence (open circle) of E-64 (5 μM). (C) Activity of WAT (10 μg) on exogenous recombinant human leptin (10 μg) followed by immunoblotting analysis using leptin monoclonal antibody.

Results

Cathepsins in WAT are able to degrade leptin

The WAT RT-qPCR analyses indicated that there are high levels of cathepsins L and K expression and a moderate expression of cathepsin S in epididymal WAT (Fig. 1A). The catalytic activity of cysteine peptidase in mouse WAT extract (10 µg) was monitored using Z-FR-MCA as a substrate (10 µM) in the presence or absence of E-64 (5 µM).

The results revealed a high hydrolytic activity completely abolished in the presence of specific cysteine protease inhibitor E-64 (Fig. 1B). To investigate this endogenous WAT activity on leptin, mouse WATs (20 µg) were incubated with human recombinant leptin (exogenous) with or without E-64 (5 µM). The proteolytic activity after 2 h of incubation at pH 6.0 was analyzed on a SDS gel electrophoresis followed by immunoblotting using human leptin monoclonal antibody. The results demonstrated that the endogenous WAT cysteine enzymes are able to degrade leptin (Fig. 1C).

Figure 2 Mass spectral analysis of leptin hydrolysis by cathepsins at pH 6.0 and 7.4. Human recombinant leptin was incubated at pH 6.0 (A) and pH 7.4 (B) with cathepsin B (cat B), cathepsin K (cat K), cathepsin L (cat L), and cathepsin S (cat S) in the absence or presence of 5 µM E-64. The fragments resulting from hydrolysis were detected by MALDI-TOF/MS. The experimental procedure details are presented in Materials and Methods section. *(Leptin mass + H)⁺ and *(Leptin mass + 2H)²⁺/2.

Leptin is efficiently degraded by cathepsin S

Initially, to verify the involvement of cysteine cathepsins in leptin degradation, human recombinant cathepsins B, L, K, and S (0.24 μ M) were incubated with human recombinant leptin (24 μ M) at pH 6.0. The proteolytic activity after 15 min of incubation was analyzed by mass spectrometry. Our results showed that at pH 6.0, leptin was slightly hydrolyzed by cathepsins B, L, and K, whereas cathepsin S was the most efficient, generating small fragments (Fig. 2A). In addition, considering the broad pH profile presented by cathepsin S (Kirschke *et al.* 1989), we extended our investigation at pH 7.4. A mass spectrometric analysis demonstrated that at neutral pH, cathepsin S was still able to degrade leptin into small peptides, whereas no hydrolysis was observed with cathepsins B, L, and K (Fig. 2B). Furthermore, analyses using different concentrations of cathepsin S confirmed this high efficiency of leptin hydrolysis (Fig. 3A). Considering the already well-described cathepsin S substrate specificity (Choe *et al.* 2006, Lutzner & Kalbacher 2008, Oliveira *et al.* 2010), we have suggested potential cleavage sites for cathepsin S in the leptin sequence (Fig. 3B).

Cathepsin S inactivates leptin biological activity

The biological activity of leptin after cathepsin S cleavage was evaluated *in vitro* and *in vivo*. We first explored the well-known leptin effect on angiogenesis stimulation (Anagnostoulis *et al.* 2008) and examined whether the leptin fragments generated by cathepsin S hydrolysis were still able to evoke a biological response. As expected, leptin presents a pro-angiogenic effect by generating closed intercellular compartments arising from endothelial cell sprouting (pro-angiogenic structures; Fig. 4). This response was completely abolished in cells treated with the products of leptin incubation with cathepsin S, whereas cathepsin S alone had no effect on angiogenesis when compared with the control (Fig. 4).

Figure 3 Effect of cathepsin S on leptin and potential cleavage sites. (A) SDS gel electrophoresis (12.5%) followed by Coomassie blue staining. Recombinant human leptin (24 μ M) was incubated with 0.24, 0.4, and 2.4 μ M cathepsin S for 15 min in sodium phosphate buffer, pH 6.0. At the same conditions, 2.4 μ M cathepsin S was incubated in the presence of 5 μ M E-64. The experimental details are described in Materials and Methods section. (B) Proposed cleavage sites (arrows) for cathepsin S in leptin based on the substrate specificity of the enzyme.

Figure 4 Influence of leptin fragments generated by cathepsin S on angiogenesis. HUVECs (control) were plated on Matrigel in the presence of recombinant human leptin, recombinant human cathepsin S (cat S), and the leptin fragments generated by cathepsin S cleavage. The number of pro-angiogenic structures was counted after 18 h. (A) A representative picture of the different treatments is shown with the respective counts. (B) The results are expressed as the number of pro-angiogenic structures. Data were analyzed using one-way ANOVA followed by Newman-Keuls posttest. $P < 0.05$. Error bars, s.d. of triplicate samples. *, denotes statistical significance.

The influence of leptin fragments generated by cathepsin S catalytic activity on food intake was also investigated by performing *in vivo* assays in Wistar rats (Fig. 5). In these experiments, the leptin fragments were injected into the lateral ventricle (i.c.v.) following the methodology previously described (Telles *et al.* 2003). The i.c.v. injections of 10 μ g leptin (group 1), 10 μ g leptin incubated with 0.24 μ M cathepsin S (group 2), and 10 μ g leptin incubated with 0.24 μ M heat-inactivated cathepsin S (group 3) showed that on the second and third day, the control group (group 1) exhibited a significant decrease in food intake compared with the first day. However, this effect was not observed when leptin was exposed to the hydrolytic action of cathepsin S (group 2). Notably, active cathepsin S is required to inactivate leptin once when heat-inactivated cathepsin S was incubated with leptin (group 3), the food intake was comparable with that of the leptin control group (Fig. 5). The animal weights were measured daily after i.c.v. and no significant variation was observed.

Figure 5 Effect of leptin fragments on rat food intake. Male Wistar rats were submitted to i.c.v. cannula implantation. One week after the surgery, the animals were divided into three groups that received leptin (group 1, black bar), leptin incubated with cathepsin S (group 2, white bar), or leptin incubated with heat-inactivated cathepsin S (group 3, gray bar). The injections were repeated for 3 days in 24 h intervals, and the food intake was measured. The results are shown as means of the food intake. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's posttests. * $P < 0.05$.

Discussion

Since its discovery, leptin physiology and pathology has been extensively studied (reviewed in Lago *et al.* (2007), Fernandez-Riejos *et al.* (2010) and Sweeney (2010)). However, leptin-inactivating enzymes have not been described yet, and leptin action is thought to be modulated by biological availability, dictated by the free/bound ratio in plasma and its clearance by the kidneys (Huang *et al.* 2001, Lammert *et al.* 2001). In this report, we show that leptin is hydrolyzed by cysteine cathepsins and that cathepsin S is the most efficient cathepsin in this process. Furthermore, the products of leptin degradation that are generated by cathepsin S catalytic activity are biologically inactive, which places this enzyme at the end point of leptin action.

Cysteine cathepsins were classically known as enzymes involved in protein turnover; however, emerging roles of cathepsins are widely recognized in specific and controlled processes (Turk *et al.* 2001, Honey & Rudensky 2003, Turk & Guncar 2003, Mohamed & Sloane 2006). Taleb *et al.* (2006a,b) observed that cathepsin S improved human preadipocyte differentiation and attributed this effect to fibronectin degradation. Although leptin exists in both free and bound fractions, the bound form of leptin would be protected from degradation. Our hypothesis is supported by the impaired adipocyte differentiation and reduced weight gain observed in a diet-induced obese model in cathepsins L and K knockout mice and both display a lean phenotype (Funicello *et al.* 2007, Yang *et al.* 2007, 2008).

Among the tested enzymes, cathepsin S demonstrated the most significant catalytic activity toward leptin at different pH values, resulting in very small fragments (Figs 2 and 3). In order to verify whether the generated peptide fragments

can still retain biological activity, we performed experiments *in vitro* and *in vivo* to analyze the effect of the leptin products generated by cathepsin S (Figs 4 and 5). Our results demonstrated a high efficiency of this enzyme in inactivating the functional activity of leptin.

Enzymatic activities of cathepsins have not been characterized in obesity. However, the cathepsin S gene expression is described to be more influenced by changes in energy balance than cathepsins L and K (Naour *et al.* 2010). In addition, cathepsin S is highly expressed in macrophages (Shi *et al.* 1992) and increased by pro-inflammatory mediators (Cancello *et al.* 2005). Because obesity is associated with the infiltration of WAT by macrophages and an increased production of pro-inflammatory adipocytokines (Jobs *et al.* 2010, Suganami & Ogawa 2010), it is also reasonable to consider that the low levels of cathepsin S expression observed in epididymal WATs could be improved by cathepsin S from macrophages. Leptin levels are also associated with infection and inflammation (reviewed in Fernandez-Riejos *et al.* (2010)) and were found to stimulate immune cells, such as macrophages and pro-inflammatory cytokines (Santos-Alvarez *et al.* 1999, Gruen *et al.* 2007).

Studies on synthetic peptides demonstrated that the entire leptin molecule is not required for biological activity (Samson *et al.* 1996, Grasso *et al.* 1997, de Oliveira *et al.* 2008, Barrett *et al.* 2009, 2011, Martins *et al.* 2009) and leptin fragments have been detected in human serum (Stamatiadis *et al.* 2004). However, leptin proteolytic processing and identification of its derived active sequences in circulation have not been extensively studied. It has been described that both lysosomal and proteasomal pathways contribute to the degradation of leptin (Rau *et al.* 1999, Uotani *et al.* 1999, Lee & Fried 2006); however, the enzymes involved in the processing have not been identified. This is the first report that leptin action can specifically be halted via hydrolysis by cysteine cathepsins, remarkable by cathepsin S. The results presented here may contribute to the understanding of leptin action and renew the interest in cathepsins as therapeutic targets in obesity treatment. Nevertheless, additional studies are necessary to completely elucidate the biological role of cathepsins in leptin modulation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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