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SCIENCE DIRECT

Regulatory Peptides 127 (2005) 123–132

REGULATORY PEPTIDES

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Leptin fragments induce Fos immunoreactivity in rat hypothalamus

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Received 1 July 2004; accepted 10 November 2004

Abstract

Leptin presents an important role in energy balance and neuroendocrine control in mammals. In an attempt to identify regions of the leptin molecule responsible for its bioactivity, we have synthesized six peptides based on the protein three-dimensional structure. Fragments were synthesized by the solid-phase methodology, purified by reverse-phase high-performance liquid chromatography (RP-HPLC), and characterized by liquid chromatography–electrospray ionization mass spectrometry (LC/ESI-MS). They were injected intravenously and their ability to induce Fos immunoreactivity (Fos-ir) in rat hypothalamus was compared with that of the recombinant human leptin and saline. Fragment Ac-[Ser^{117}]$Lep_{116–140}$–NH_2 (V) induced Fos-ir in hypothalamic nuclei that express leptin receptor long form. No similar ability was observed for the other five fragments. To investigate whether Fos-ir was induced in the same neuronal group activated by leptin, we proceeded with a dual-label immunohistochemistry for cocaine- and amphetamine-regulated transcript (CART), a neuropeptide related to leptin action in rat hypothalamus. We found that Ac-[Ser^{117}]$Lep_{116–140}$–NH_2 (V) differentially activates CART neurons through the rostrocaudal extension of the arcuate nucleus. These results suggest that this fragment acts in the same group of neurons that mediate leptin response. This approach may offer the basis for the development of leptin-related compounds, having potential application in human or veterinary medicine.

Keywords: Synthetic peptides; Obesity; CART; Energy balance

1. Introduction

Obesity is a disorder characterized by increased adipose tissue mass as a result of systemic imbalance between energy intake and expenditure. Energy balance in mammals ought to be controlled by a feedback loop in which the amount of stored energy is sensed by the hypothalamus, which in turn adjusts food intake [1] and energy expenditure to maintain a constant body weight [2,3]. The protein hormone produced by the *ob*-gene [4,5] and denominated leptin [6], a product originating from adipose tissues [7,8], circulates in the plasma and affects energy balance by interacting with the hypothalamus [9]. Leptin plays an important role in the regulation of a variety of physiological functions [10], including food intake [11,12], body temperature, and body weight maintenance [13–15]. Total absence or resistance to leptin causes morbid obesity [8], diabetes, and hypogonadism [16].

The tertiary structure of the leptin molecule [17,18] reveals the existence of a four-helix bundle that is characteristic of short-helix cytokines. In fact, leptin receptors belong to the class I cytokine receptor family, densely expressed in the hypothalamus, particularly in the arcuate, dorsomedial, and ventromedial nuclei [19]. Currently, a variety of studies have shown that the weight-reducing effects of leptin are likely mediated by its interaction with specific receptors located mainly in these areas.

In the last years, different groups reported their attempts to identify bioactive leptin fragments. While Samson et al. [20] found that the N-terminal leptin fragment (OBGRP 22–

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0167-0115/$ - see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.regpep.2004.11.001

56) inhibited food intake after central administration in adult male rats, Rozhavskaya-Arena et al. [21] showed that a short peptide denominated OB3 (Ser^{116}–Cys–Ser–Leu–Pro–Gln^{121}–Thr) and some of its analogues reduced food and water consumption in C57BL/6J *ob*/*ob* mice.

Based on these results and on the tridimensional structure of leptin [17,18], we have recently studied the following six leptin-related peptides: Ac-Lep$_{23–47}$–NH_2 (I), Ac-Lep$_{48–71}$–NH_2 (II), Ac-Lep$_{72–88}$–NH_2 (III), Ac-Lep$_{92—115}$—NH_2 (IV), Ac-[Ser^{117}]Lep$_{116–140}$–NH_2 (V), and Ac-Lep$_{141–164}$–NH_2 (VI). I, III, IV, and VI encompass fragments of the native protein that present an α-helical structure. II and V cover the loops of the molecule. Herein, we report the design, synthesis, chemical characterization, and neuronal responses found for these fragments in comparison to that expected for the whole protein. This comparison was made by using the expression of Fos immunoreactivity (Fos-ir), a marker for cellular activation following intravenous administration of leptin [22,23]. Therefore, we attempted to investigate fragments' ability to activate hypothalamic neurons mediating leptin functions in the rat brain.

2. Materials and methods

2.1. Peptide synthesis, purification and chemical characterization

All leptin-derived peptide fragments (Table 1) were synthesized manually using common protocols for solid-phase methodology and t-Boc strategy [24]. MBHA resins with substitutions with degrees varying from 0.4 to 0.8 mEq/g were used [25]. The following side chain-protected Boc amino acids were obtained from Bachem (Torrance, CA): Arg(Tos), Asp(OcHex), Glu(OcHex), His(Tos), Lys(2-Cl-Z), Ser(Bzl), Thr(Bzl), Thr(For), and Tyr(2-Cl-Bzl). All reagents and solvents were of analytical grade and used from freshly opened container without any further purification. The N$^{\alpha}$-terminal protections were removed with TFA 50% in DCM in the presence of 2% anisole for 20 min. Couplings were carried out using 2.5 excess of DIC in DCM–DMF (1:1, vol/vol). Both steps were monitored by Kaiser ninhydrin test [26]. Boc-Asn and Boc-Gln were coupled using 1.5-fold excess of DIC-HOBt [27] (1:1). Boc-His (Tos). DCHA incorporation employed a 1.5-fold excess of BOP [28] in the presence of excess of diisopropylethylamine (DIEA). Coupling times were 1–2 h. Recouplings of 1 h were done when needed using 2.5 excess TBTU in the presence of excess DIEA in DCM–NMP (1:1, vol/vol). When required, acetylations were performed with acetic anhydride 50% in DCM for 15 min. The dry protected peptidyl resins were exposed to anhydrous HF in the presence of 10% anisole or EDT at 0 °C for 1.5 h. Excess of HF and scavenger were eliminated under high vacuum. The crude peptides were precipitated with anhydrous diethyl ether, separated by filtration, extracted from the resin with 50% acetic acid in H_2O, and lyophilized. The crude lyophilized peptides were purified in two steps by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on a Waters Associates system (Model Prep 4000), using linear gradients (slope 0.33% B/min). Briefly, they were loaded on a Vydac C_{18} preparative RP-HPLC column (25×250 mm, 15 μm particle size, 300 Å pore size) at a flow rate of 7.0 ml/min and eluted with solvent A (TEAP, pH 2.25) and solvent B (25%/TEAP, pH 2.25/CH_3CN), detection at 220 nm. Selected fractions were collected and converted to the TFA salt by loading on a preparative column as mentioned above and eluted using solvents A (0.1% TFA/H_2O) and B [0.1% TFA in CH_3CN/H_2O (75:25)] at a flow rate of 7.0 ml/min. Selected fractions containing the purified peptide were pooled and lyophilized. The purified peptides were characterized, as shown in Table 2, by RP-HPLC and liquid chromatography–electrospray ionization mass spectrometry (LC/ESI-MS). RP-HPLC was performed on a Waters Associated system using a linear gradient of 5–95% B for 30 min of CH_3CN in two aqueous buffers: TEAP, pH 2.25, and 0.1% TFA, at 1.5 ml/min, a Vydac C_{18} column (4.6×150 mm, 5 μm particle size, 300 Å pore size) at 215 nm. The LC/ESI-MS data were obtained on a Micromass instrument, model ZMD coupled on a Waters Alliance model 2690 system using a Waters Nova-Pak C_{18} column (2.2×150 mm, 3.5 μm particle size, 60 Å pore size); solvents A (0.1% TFA/H_2O) and B [0.1% TFA in CH_3CN/H_2O (75:25)]; gradient: 5–95% B for 30 min; λ range: 190–300 nm; and mass range: 500–3930 m/z.

Table 1
Peptide sequence of the synthetic leptin fragments studied

Number	Name	Sequence
I	Ac-Lep$_{23–47}$–NH_2	Ac-P-I-Q-K-V-Q-D-D-T-K-T-L-I-K-T-I-V-T-R-I-N-D-I-S-H-NH_2
II	Ac-Lep$_{48–71}$–NH_2	Ac-T-Q-S-V-S-S-K-Q-K-V-T-G-L-D-F-I-P-G-L-H-P-I-L-T-NH_2
III	Ac-Lep$_{72–88}$–NH_2	Ac-L-S-K-M-D-Q-T-L-A-V-Y-Q-Q-I-L-T-S-NH_2
IV	Ac-Lep$_{92–115}$–NH_2	Ac-R-N-V-I-Q-I-S-N-D-L-E-N-L-R-D-L-L-H-V-L-A-F-S-K-NH_2
V	Ac-[Ser^{117}]Lep$_{116–140}$–NH_2	Ac-S-S-H-L-P-W-A-S-G-L-E-T-L-D-S-L-G-G-V-L-E-A-S-G-Y-NH_2
VI	Ac-Lep$_{141–164}$–NH_2	Ac-S-T-E-V-V-A-L-S-R-L-Q-G-S-L-Q-D-M-L-W-Q-L-D-L-S-NH_2

2.2. Animals

Sixty-two normal adult male albino rats (Wistar) weighing 240–280 g were housed one per cage in our institutional animal care facility and allowed to adapt at least 7 days prior to the onset of experiments. Animals were maintained on a 12-h light/dark cycle, in a temperature-controlled environment (21±2 °C), with free access to water and food. All experiments were carried out in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and the Institutional