Regulatory Peptides 153 (2009) 77–82

Contents lists available at ScienceDirect

Regulatory Peptides

journal homepage: www.elsevier.com/locate/regpep

Evaluation of new leptin fragments on food intake and body weight of normal rats

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ARTICLE INFO

Article history:
Received 16 June 2008
Received in revised form 31 October 2008
Accepted 30 November 2008
Available online 7 December 2008

Keywords:
Synthetic peptides
Leptin fragments
Energy balance
Food intake

ABSTRACT

Leptin, a protein hormone originating from adipose tissue, circulates in the plasma and affects the energy balance by interacting with the hypothalamus. Leptin plays an important role in the regulation of a variety of physiological functions, including food intake, body temperature and body weight maintenance. Tertiary structure of the leptin molecule reveals the existence of a four-helix bundle that is characteristic of the short-helix cytokines. To identify regions of the leptin molecule responsible for its bioactivity, we have recently synthesized six peptides based on the protein three-dimensional structure. Our results indicated that the fragments Ac-hLEP$_{92-115}$-NH$_2$ (IV) and Ac-[Ser^{117}]-hLEP$_{116-140}$-NH$_2$ (V) were recognized by leptin receptor present in hp-75 cells validating that this region of the molecule contain the functional epitope of the leptin molecule. In the present study, a new series of decapeptides encompassing the region of fragments IV and V of leptin were synthesized, and their effects on body weight and food intake were assessed when administered into the lateral cerbroventricle of normal rats. Peptides were synthesized by SPPS, purified by RP-HPLC and characterized by LC/ESI-MS. We also performed a conformational study of the peptides by circular dichroism in order to correlate the biological activity and secondary structure of the leptin fragments. Among the fragments tested, we found that Ac-hLEP$_{110-119}$-NH$_2$ (VI) induce a significant reduction in both body weight and food intake. The use of synthetic leptin-derivate fragments may offer the basis for the development of compounds with potential application in human obesity or to its related metabolic dysfunctions.

1. Introduction

All over the world a growing number of people suffer from obesity. Obesity is a disorder characterized by increased adipose tissue mass as a result of systemic imbalance between energy intake and expenditure. Leptin, mainly produced by adipose tissue [1,2], plays a central role in the regulation of food intake, body weight and in a variety of physiological functions [3–10]. Mutations in the leptin pathway can be a cause of human obesity [11]. Moreover, total absence or resistance [12,13] to leptin causes morbid obesity [1], diabetes [14] and hypogonadism [15]. This 16 kDa protein hormone is produced by the *ob*-gene [2,16], that is located on chromosome 7q31.3 [17]. Leptin receptors located in the hypothalamus led to different signals that produces changes in food intake [18–20] and energy expenditure [21,22]. Leptin tertiary structure reveals the existence of a four-helix bundle that is characteristic of the short-helix cytokines [23,24].

In the past years, different research groups have tried to identify bioactive leptin fragments. Samson and coworkers found that the N-terminal leptin fragment (OBGRP 22–56) inhibited food intake after central administration in adult male rats [25]. Grasso and coworkers [4,26–31] showed that a short peptide denominated OB3 and specially its analogue [D-Leu^4]-OB3 (Ser^{116}-Cys-Ser-D-Leu-Pro^{120}-Gln-Thr) were able to reduce food and water consume in C57BL/6J *ob/ob* mice. Oliveira and coworkers based on the tridimensional structure of leptin have studied six leptin-related peptides and found that the fragment Ac-[Ser^{117}]-Lep$_{116-140}$-NH$_2$ (**V**) induced Fos-ir in hypothalamic nuclei that express leptin receptor long form [32] in normal rats. They also found that the fragments Ac-hLEP$_{92-115}$-NH$_2$ (**IV**) and Ac-[Ser^{117}]-hLEP$_{116-140}$-NH$_2$ (**V**) were recognized by leptin receptor present in hp-75 cells [33].

Abbreviations: 2-Cl-Z, 2-chloro-benzyloxycarbonyl; AAA, amino acid analysis; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate; Bzl, benzyl; CD, circular dichroism; CH_3CN, acetonitrile; CHO, Formyl; CSF, Cerebrospinal fluid; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, N,N'-dimethylformamide; DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide; EDT, 1,2 ethanedithiol; HF, hydrogen fluoride; hLEP, human sequence leptin; HOBt, N-Hydroxybenzotriazole; ICV, intracerebroventricular injection; i-PrOH, isopropanol; LC/ESI-MS, liquid chromatography electrospray ionization mass spectrometry; MBHA, 4-methylbenzhydrylamine; MeOH, methanol; Mob, 4-methoxybenzyl; NMP, N-methylpyrrolidone; OcHex, cyclohexyl; RP-HPLC, reversed phase high performance liquid chromatography; RT, retention time; SDS, sodium dodecylsulfate; SPPS, Solid Phase Peptide Synthesis; t-Boc, Terc-butyloxycarbonyl; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol and Tos, p-toluenesulfonyl.

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doi:10.1016/j.regpep.2008.11.013

Herein, we report the design, synthesis, chemical characterization, and body weight and food intake responses induced by a new series of leptin fragments in comparison to that expected for the whole leptin when intracerebroventricularly administrated in normal adult rats.

2. Materials and methods

2.1. Peptide synthesis, purification and chemical characterization

All acetylated and amidated leptin fragments (Table 1) were synthesized manually using a common protocol for solid-phase methodology [34]. MBHA-resins with substitutions degree varying from 0.4 to 0.8 mequiv/g were used. The t-Boc amino acid derivatives Arg(Tos), Boc-Asp(OcHex), Cys(Mob), Glu(OcHex), His(Tos), Lys(2-Cl-Z), Ser(Bzl) and Trp(CHO) were obtained from Novabiochem (Darmstadt, Germany) and Advanced ChemTech (Louisville, KY, USA). All solvents were reagent grade or better and used from freshly opened container without any further purification. The N^{α}-terminal protections were removed with TFA 50% in DCM in the presence of 2% anisole for 20 min, followed by sequential washes with i-PrOH containing 2% anisole, 10% TEA in DCM, MeOH and DCM. Couplings were carried-out using 2.5 excess of DIC in DCM-DMF (1:1, v/v). Boc-Asn was coupled using 2.5-fold excess of DIC-HOBt (1:1). Coupling times were 1–2 h. Recouplings of 1 h were done when needed using 2.5 excess TBTU or BOP in the presence of excess DIEA in DMSO-NMP (2:1, v/v). The dry protected peptidyl-resins were exposed to anhydrous HF in the presence of 5% anisole or EDT and 5% DMS 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 0.1% TFA in CH_3CN/H_2O (60:40) and lyophilized. The crude lyophilized peptides were purified in two steps by preparative reversed phase high performance liquid chromatography (RP-HPLC) on a Waters (Milford, MA, USA) system (Model Delta 600), using linear gradients (slope 0.33% B/min). Briefly, they were loaded on a Jupiter C_{18} column (from Phenomenex, Torrance, CA, USA) (21,2×250 mm, 15 μm particle size, 300 Å pore size) at a flow rate of 10.0 mL/min and eluted with solvent A (TEAP, pH 2.25) and solvent B [TEAP, pH 2.25/CH_3CN (40:60)], 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 (60:40)] at a flow rate of 10.0 mL/min. Selected fractions containing the purified peptide were pooled and lyophilized. The purified peptides were characterized by LC/ESI-MS (Liquid Chromatography–Electrospray Ionization Mass Spectrometry) and by amino acid analysis (AAA). LC/ESI-MS data were obtained on a Micromass instrument, model ZMD coupled on a Waters Alliance model 2690 system and a Waters photodiode array model 996, using a Phenomenex Gemini C_{18} column (2,0×150 mm, 3,0 μm particle size, 110 Å pore size); solvent A: [0.1% TFA/H_2O] and B: [0.1% TFA in CH_3CN/H_2O (60:40)]; gradient: 5–95% B for 30 min, λ range: 190–300 nm. Mass measurements were performed in a positive mode in the following conditions: mass range between 500 and 3,930 *m/z*; nitrogen gas flow: 4.1 L/h; capillary: 2.5 kV; cone voltage: 47 V; extractor: 8 V; source heater: 105 °C; solvent heater: 400 °C; ion energy: 1.0 V and multiplier: 996 V. AAA of the peptides previously hydrolyzed in 6 N HCl at 110 °C for 72 h, were performed by ion-exchange chromatography in a Biochrom 20 Plus amino acid analyzer (Pharmacia LKB Biochrom Ltd, Cambridge, England) using the three-buffer system under standard conditions recommended by the manufacturer.

2.2. Circular dichroism (CD) studies

CD spectra were acquired on a Jasco J-810 spectropolarimeter (Tokyo, Japan) thermostated at 20 °C and continually flushed with ultra-pure nitrogen. Peptide samples were prepared by diluting the 1 mM stock solutions in water, TFE (10–70%) or in the presence of SDS (1–50 mM) to obtain a final concentration of 0.1 mM. CD spectra were recorded using a 1 mm path length rectangular quartz cell, with four accumulations at 50 nm/min scan speed, 8 s response time, 0.5 nm spectral bandwidth and wavelength range of 190 to 260 nm. Results were expressed in molar ellipticity [θ] (deg cm^2 $dmol^{-1}$).

2.3. Bioassays

Normal adult male albino rats (Wistar — weighing 240–300 g) were anesthetized by intraperitoneal injection of a mixture of Ketamine/Xylazine (66.6 and 13.3 mg/kg, respectively) and a 23-gauge cannula was stereotaxicaly implanted into the right lateral cerebral ventricle (from the bregma: anterior −1.3 mm, lateral −1.6 mm and ventral −2.5 mm). The cannula was secured to the skull with screws and dental cement. The animals were individually caged and maintained with food and water ad libitum for 1 week after surgery [35]. After this recovery period, they were submitted to an Angiotensin II drinking tests. Rats that drank water in response to an intracerebroventricular injection of 100 ng/10 μl of angiotensin II were considered to have the cannula implanted in the correct place [36]. Five days before initiation of experimentation, body weight and food and fluid intake were recorded daily. On day one of experimentation peptides or CSF were administered in the conscious unrestrained rats with free access to food and water in their home cage. Similar injections were administered for three additional days. All injections were conducted at 1700 h, in the setting of lights off at 1800 h, and on at 0600 h, in a temperature-controlled environment (24±2 °C). Daily records of body weight and food consumption were monitored. Measurements of food intake were corrected for spillage and were expressed in grams per 100 g of the rat body weight. Groups of 6 rats were used for each compound tested. All experiments

Table 1
The primary structures, molecular masses and purity of the leptin fragments tested in this study

No.	Peptide	Sequence	LC/ESI-MS [a]			
			RT (min)	Purity (%)	Calculated ($M+H^+$)	Obtained ($M+H^+$)
I	Ac-hLEP$_{98-122}$-NH_2	Ac-S-N-D-L-E-N-L-R-D-L-L-H-V-L-A-F-S-K-S-C-H-L-P-W-A-NH_2	21,6	98	2920,35	2922,3
II	Ac-hLEP$_{98-107}$-NH_2	Ac-S-N-D-L-E-N-L-R-D-L-NH_2	11,7	99	1229,31	1230,3
III	Ac-hLEP$_{101-110}$-NH_2	Ac-L-E-N-L-R-D-L-L-H-V-NH_2	18,4	97	1262,48	1262,5
IV	Ac-hLEP$_{104-113}$-NH_2	Ac-L-R-D-L-L-H-V-L-A-F-NH_2	25,1	99	1237,51	1238,5
V	Ac-hLEP$_{107-116}$-NH_2	Ac-L-L-H-V-L-A-F-S-K-S-NH_2	20,3	99	1155,41	1156,4
VI	Ac-hLEP$_{110-119}$-NH_2	Ac-V-L-A-F-S-K-S-C-H-L-NH_2	17,2	98	1145,39	1147,4
VII	Ac-hLEP$_{113-122}$-NH_2	Ac-F-S-K-S-C-H-L-P-W-A-NH_2	16,4	98	1216,43	1216,4
VIII	Ac-[Ser^{117}]-hLEP$_{113-122}$-NH_2	Ac-F-S-K-S-S-H-L-P-W-A-NH_2	13,6	98	1200,36	1201,4

[a]Purity percentages determined by RP-HPLC using a linear gradient of 5–95% B for 30 min, at flow rate of 0.4 mL/min; solvent systems: A=0.1%TFA/H_2O and B=0.1%TFA-60% CH_3CN/H_2O and a Phenomenex Gemini C_{18} column (2.0×150 mm, 3.0 μm particle size, 110 Å pore size). Determination at λ range=190–300 nm; injection volume: 20 μL, sample concentration: 1.0 mg/mL. Mass spectrometry analysis was performed on a Micromass model ZMD coupled on a Waters system Alliance model 2690, in a mass range of 500 to 3930 Da. RT: retention time.