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Chronic central leptin infusion differently modulates brain and liver insulin signaling

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ABSTRACT

Recent studies reported the impact of leptin on peripheral insulin sensitivity and glucose utilization. However, little is known concerning the effect of central leptin on hypothalamic and hepatic insulin efficiency. This study aimed to determine the consequence of chronic intra-cerebroventricular (ICV) leptin or murine leptin antagonist (MLA) infusion on hypothalamic and hepatic insulin signaling pathways, in rats.

A 2-week central leptin infusion enhanced insulin-dependent Akt phosphorylation in the liver without changing PTP-1B protein expression, associated to insulin receptor (IR) upregulation and reduced IRS-1 phosphorylation on Ser302 residue. In the hypothalamus, a chronic ICV leptin infusion induced PTP-1B associated with a specific decrease in insulin-dependent Akt phosphorylation. In contrast, a chronic MLA infusion did not alter IR and PTP-1B expressions in hypothalamus and liver.

Our results underline a brain leptin-dependent increase in hepatic insulin efficiency as mirrored by IR up-regulation, increased insulin-dependent Akt phosphorylation and reduced IRS-1 phosphorylation on Ser302 residue.

1. Introduction

Growing evidence suggests a strong link between the adipocyte leptin–hypothalamus axis and the onset of insulin resistance and type 2 diabetes (Kalra, 2009). This relationship is, most likely, primary located in the hypothalamus which is considered as the central brain region involved in the control of energy homeostasis and food intake. In the hypothalamus, and particularly in the arcuate nucleus, leptin and insulin are major regulators of energy homeostasis and body weight through their respective receptors. Both hormones increase energy expenditure and inhibit food intake. Thus, the hypothalamic alteration of insulin or/and leptin signaling could contribute to the onset of insulin resistance, considered as a common feature of type 2 diabetes, and obesity characterized by a leptin resistance state (Münzberg et al., 2004).

Such evidence accumulated over the last decade concerning the possible cross-talk between insulin and leptin sensitivity at both central and peripheral levels which may possibly link the insulin and leptin resistance, and consequently lead to obesity and type-2 diabetes. Studies performed in hepatic or neuronal cells reported that both leptin and insulin share several signaling pathways such as JAK-2/STAT-3, IRS/PI3kinase/Akt and MAP kinase (Szanto and Kahn, 2000; Carvalheira et al., 2003, 2005; Benomar et al., 2005a,b, 2009). Furthermore, leptin treatment of leptin deficient ob/ob mice reduces blood insulin and glucose concentrations suggesting improved insulin sensitivity (Pelleymounter et al., 1995). This treatment also improved glucose utilization and insulin sensitivity in liver and muscle in normal rodents (Sivitz et al., 1997; Harris, 1998; Yaspelkis et al., 1999). Leptin also increases peripheral insulin sensitivity in diabetic or insulin-resistant rats (Yaspelkis et al., 2001, 2004; Lin et al., 2002; Lam et al., 2004; Morton et al., 2005). Interestingly, a chronic central leptin treatment increases insulin-stimulated muscle and brown adipose tissue glucose utilization (Cusin et al., 1998; Park et al., 2008). It has been also reported that leptin directly modulates insulin secretion by reducing insulin mRNA expression and insulin secretion in pancreatic β cells (Emilsson et al., 1997; Fehmann et al., 1997; Kieffer et al., 1997; Ishida et al., 1997; Pallett et al., 1997; Poitout et al., 1998; Ookuma et al., 1998).

Furthermore, the cross-talk between insulin and leptin signaling may also involve a negative regulator, Phosphotyrosine

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Phosphatase 1B (PTP-1B). PTP-1B is able to dephosphorylate several components of leptin and insulin signaling pathways such as: IRS1/2, JAK2 (Benomar et al., 2009). We have previously demonstrated a cross-desensitization between leptin and insulin signaling pathways involving PTP-1B in human neuroblastoma cell line (Benomar et al., 2005a,b). Indeed, previous studies have evidenced a resistance to diet-induced obesity and increased leptin signaling in PTP-1B-deficient mice (Bence et al., 2006). Likewise more recent studies reported hypersensitivity to leptin in neuron-specific PTP-1B knockout mice (Klaman et al., 2000).

Thus, though the impact of leptin treatment on insulin sensitivity of peripheral tissues is now well established, little is known concerning the impact of a chronic central leptin infusion on insulin signaling pathways and its direct or indirect incidence on hypothalamic and hepatic insulin responsiveness.

In the present study, we attempted to reveal the potential link between central leptin and, hypothalamic and hepatic insulin sensitivity. To address this question, the activation of hypothalamic and hepatic insulin-dependent signaling pathways (such as Akt and STAT-3) in response to a 2-week ICV leptin infusion was investigated. We have focused on hypothalamic STAT-3, an important component of leptin/insulin signaling involved in the regulation of food intake; and on hypothalamic and hepatic Akt phosphorylation, which is a good indicator of central and peripheral insulin-sensitivity. The protein-tyrosine phosphatase PTP1B, a major negative regulator of leptin and insulin signaling (Benomar et al., 2009), and insulin receptor (IR) expression levels have been also measured in liver and hypothalamus.

2. Materials and methods

2.1. Animals

All procedures in rodents were conducted according to the guidelines of laboratory animal care and were approved by the local governmental commission for animal research. Animals were maintained on a 12 h light–dark cycle. Adult male Wistar rats weighing 200-250 g (Charles River, L'Arbresle, France) were housed individually in cages designed to allow the recording of food intake. Food (chow) and water were available *ad libitum*.

2.2. Hormones and antibodies

Recombinant rat leptin and murine leptin antagonist (MLA) were produced in *Escherichia coli* as previously described (Salomon et al., 2006). Human insulin Actrapid was provided by Novo Nordisk (Bagsværd, Denmark). Phospho-STAT-3 (Tyr705), STAT-3, phospho-Akt (Ser473), Akt, phospho-IRS-1 (Ser302) and β-tubulin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-PTP-1B (H-135) and anti-insulin Rβ (C-19) antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Secondary antibodies (from mouse and rabbit) conjugated to peroxidase were purchased from Sigma–Aldrich, Missouri, USA.

2.3. Chronic intracerebroventricular (ICV) infusion

The mini-osmotic pumps (Alzet, model 2002, constant pumping rate 0.5 μl/h), were implanted under pentobarbital anesthesia (50 mg/kg, Sanofi-Aventis, Libourne, France) in rats pre-treated with the muscle relaxant xylazine (Rompun, Bayer Puteaux, France). The brain infusion cannula was implanted to the right lateral ventricle by the aim of a stereotaxic frame using the following coordinates: 0.8 mm anterior to bregma, 1.5 mm lateral, and 3.5 mm dorso-ventral (atlas of Paxinos and Watson, 1997). The cannula was fixed to the skull with stainless steel screws and dental cement. The osmotic pumps were housed in a subcutaneous pocket in the midscapular area of the animal's back. Rats received either saline (0.9%), leptin (0.25 μg/μl) or MLA (1 μg/μl) for 14 days.

2.4. Body weight and food intake

Body weight and food intake were daily measured at 09:00 am.

2.5. Intraperitoneal (IP) insulin injection

To test the impact of a chronic central leptin infusion on the insulin sensitivity, overnight fasted rats were treated with insulin (1 U/kg body weight) by IP injection 30 min prior to euthanasia.

2.6. Determination of plasma glucose, insulin and leptin levels

At the end of the experiment, the animals were killed by decapitation and trunk blood was collected in heparinized tubes on ice. Plasma glucose levels were measured immediately with a blood monitoring system (Accu-Chek, Roche). The plasma was isolated and stored at −20 °C for insulin and leptin determinations. Plasma leptin levels were determined by a two-step radioimmunoassay (RIA), using Linco's rat leptin [^{125}I] assay system (Linco Research, St. Charles, MO, USA). For measuring plasma insulin levels, a DiaSorin sensitive [^{125}I] RIA system (Sallugia, Italy) was used.

2.7. Tissue sampling and Western blot analyses

After euthanasia, hypothalami (size of 1.2 mm on 2.4 mm) and livers were removed and immediately frozen in liquid nitrogen and stored at −80 °C. Frozen tissues were homogenized in the solubilisation buffer containing 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% nonidet-P40, 10% glycerol, proteases inhibitors (0.35 mg/ml PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin) and phosphatases inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, 20 mM sodium β-glycerophosphate and 10 mM benzamidine). After lysis under agitation for 90 min at 4 °C, insoluble material was removed by centrifugation at 14,000 rpm, for 60 min at 4 °C. Protein concentrations of the resulting supernatant were determined using a protein assay kit (Pierce, Perbio Science, France). Seventy micrograms of proteins were resolved by SDS–PAGE electrophoresis and electrotransfered to a nitrocellulose membrane. Immunoblots were blocked with 5% non-fat dry milk and then incubated with the appropriate primary and secondary antibodies. The targeted proteins were visualized by enhanced chemiluminescence reagents (ECL detection kit, Amersham Biosciences) followed by autoradiography.

2.8. Quantification of IR and PTP1B mRNA expression by quantitative RT-PCR

Total RNA from liver was extracted using Trizol® LS Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. One microgram of total denatured RNA was reverse transcribed and the cDNAs were submitted to quantitative PCR analysis. The PCR primer sequences used were as follows: PTP1B forward 5′-GCACAGCATGAGCAGTATGAGTC-3′, PTP1B reverse 5′-TCCACCCACCATCCGTTTCC-3′; IR forward 5′-TGCCACCAATCCTTCCGTTCC-3′, IR reverse 5′-TCCTCCGCCTGCCTCTCC-3′; β-actin forward 5′-CTATCGGCAATGAGCGGTTCC-3′, β-actin reverse 5′-TGTGTTGGCATAGAGGTCTTTACG-3′.

Quantitative PCR was carried out using StepOne and Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed in duplicate using the following conditions: initial activation of the hot start DNA polymerase for 15 min at 94 °C followed by denaturation for 10 s at 94 °C, annealing for 10 s at 60 °C for the three genes and extension for 10 s at 72 °C. Forty cycles of PCR were programmed to ensure that the threshold crossing point (cycle number) was attained. Fluorescence emission was monitored continuously during cycling. At the completion of cycling, melting curve analysis was carried out to establish the specificity of the amplified product. The expression level of each mRNA and their estimated crossing point in each sample were determined relative to the standard preparation using the StepOne computer software (Applied Biosystems).

A ratio of specific mRNA to β-actin amplification was then calculated to correct for any difference in efficiency at reverse-transcription.

2.9. Data analysis and statistics

Statistical analyses were performed using ANOVA (Statview software program, version 5) to detect significant inter-group differences. Values are expressed as means ± SEM. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Hypothalamic chronic leptin action blockade profoundly affects metabolic and endocrine parameters

To explore the role of hypothalamic endogenous leptin on metabolic and endocrine parameters, rats received an ICV infusion of leptin or MLA for 14 days. As expected, an ICV leptin infusion significantly reduced both body weight gain and food intake as compared to control treatment (Fig. 1A and B). In contrast, ICV MLA infusion clearly increased body weight gain and food intake as compared to controls (Fig. 1A and B). MLA action is maintained during the entire period of infusion and the leptin effect on the weight was stabilized after 8 days of treatment (Fig. 1A). ICV MLA infusion has no impact on blood glucose and plasma insulin concentration, whereas leptin infusion clearly reduced both fasting glucose and insulin levels (Fig. 1C and D). Interestingly, ICV MLA infusion sig-