Brief Communication

MOLECULAR METABOLISM

Time-resolved hypothalamic open flow microperfusion reveals normal leptin transport across the blood–brain barrier in leptin resistant mice

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ABSTRACT

Objective: The inability of leptin to suppress food intake in diet-induced obesity, sometimes referred to as leptin resistance, is associated with several distinct pathological hallmarks. One prevailing theory is that impaired transport of leptin across the blood–brain barrier (BBB) represents a molecular mechanism that triggers this phenomenon. Recent evidence, however, has challenged this notion, suggesting that leptin BBB transport is acquired during leptin resistance.

Methods: To resolve this debate, we utilized a novel cerebral Open Flow Microperfusion (cOFM) method to examine leptin BBB transport in male C57BL/6J mice, fed a chow diet or high fat diet (HFD) for 20 days.

Results: Basal plasma leptin levels were 3.8-fold higher in HFD-fed mice ($p < 0.05$). Leptin administration (2.5 mg/kg) elicited similar pharmacokinetic profiles of circulating leptin. However, while leptin reduced food intake by 20% over 22 h in chow-fed mice, it did not affect food intake in HFD-fed mice. In spite of this striking functional difference, hypothalamic leptin levels, as measured by cOFM, did not differ between chow-fed mice and HFD-fed mice following leptin administration.

Conclusions: These data suggest that leptin transport across the BBB is not impaired in non-obese leptin resistant mice and thus unlikely to play a direct role in the progression of pharmacological leptin resistance.

Keywords Obesity; Hypothalamus; Leptin; Leptin resistance; Blood–brain barrier; Leptin transport

1. INTRODUCTION

Leptin is a 16 kDa hormone that is secreted from white adipocytes to signal satiety in the brain. It plays a critical role in the neuroendocrine regulation of body-weight [1–3]. Null-mutations in the leptin-encoding gene cause morbid obesity in mice and humans [3,4]. This monogenetic obesity is corrected by treatment with recombinant leptin [3,5]. In contrast, common polygenetic obesity [6] is associated with increased leptin levels and treatment with additional leptin has limited efficacy to lower body-weight under these circumstances [7–9]. One prevailing theory is that impaired transport of leptin across the blood–brain barrier (BBB) plays a central role to this blunted responsiveness to exogenous leptin – sometimes referred to as *leptin resistance.* This theory of a defect in leptin transport across the BBB is rooted in several observations. First, early studies suggested a lower ratio of leptin in the cerebral spinal fluid (CSF) to serum leptin in obese humans [10,11], possibly reflecting saturation in leptin transport. Second, rodents fed a high-fat diet develop resistance to peripherally administered leptin before they develop resistance to centrally injected leptin [12,13]. Third, using radiolabeled leptin, researchers found a reduced brain-to-serum ratio of leptin in obese relative to lean animals [14].

However, leptin resistance describes a complex phenomenon that can emerge from several distinct entry points. Beyond impairments in leptin transport, perturbations in the leptin receptor signaling cascade and disruptions in neural circuits downstream of leptin target neurons can result in leptin resistance [9,15]. The relative contribution and the sequential manifestation of these events reported to be associated with functional leptin resistance remain unclear. Given this uncertainty, we took advantage of recent methodological advances to specifically quantify leptin transport across the BBB in the early stages of diet-induced leptin resistance (i.e. the inability of exogenous leptin to suppress food intake). Cerebral Open Flow Microperfusion (cOFM) is a new *in vivo* technique that allows for continuous sampling of the interstitial fluid in brain tissue [16–18], enabling the time-resolved

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assessment of leptin BBB transport in response to peripheral administration of recombinant leptin. Using simultaneous measurements of plasma leptin levels, we generated the first combined profile of peripheral and hypothalamic leptin pharmacokinetics examining diet-induced leptin resistance *in vivo*.

2. MATERIALS AND METHODS

2.1. Animals

All animal experiments were approved either by the Danish Animal Experimental Inspectorate or the Austrian Federal Government (BMWFW-66.010/0035-WF/V/3b/2017) and were performed in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes. Mice were housed on a 12:12-h light-dark cycle at 21–22 °C.

2.2. Assessment of pharmacological leptin responsiveness

Fourteen-week-old male C57BL6/J mice were maintained on a standard rodent chow diet and were single-housed 14 days before a diet switch, for which mice were randomized to remain on chow diet or to receive high-fat diet (Research diets, D12331) for 20 days. Mice had *ad libitum* access to food and water. At 5 pm on day 20, mice were injected intraperitoneally with either a bolus of recombinant mouse leptin (2.5 mg/kg; R&D Systems, USA) or a vehicle solution. Food was weighed at 2, 6, 14, and 22 h after the injection and the difference to pre-injection food was defined as food intake.

2.3. Surgery and implantation

Body-weight matched 13 -16-week-old male C57BL6/J mice were anesthetized with fentanyl (50 μg/kg, Hameln Pharma, Germany), midazolam (5 mg/kg, Erwo Pharma, Austria) and medetomidin (5 mg/kg, Orion Pharma, Austria). During stereotactic surgeries and probe implantation, anesthesia was maintained by constant inhalation of oxygen (1.5 L/min) and isoflurane (0.5% - 1%, Forane®, Abbott, Germany). The cOFM probe was implanted into the hypothalamus using the following coordinates: AP 0.7 mm, ML -1.5 mm, DV -6 mm from bregma. For post-surgical pain management, animals received an antibiotic (Baytril, Bayer, Germany) and rimadyl (Carprofen; Zoetis, Austria). After surgery, animals recovered for 14 days; then animals were either kept on chow diet or switched to high-fat diet (Research diets, D12331) for 20 days.

2.4. Interstitial fluid sampling

Animals were anaesthetized by isoflurane (0.5%, 1.5 L/h O_2) and placed on a homeothermic blanket. Body temperature was maintained at 38 °C. The healing dummy was replaced by the sampling inlet, which was connected to syringe pumps. The cOFM probe was perfused with artificial and sterile cerebrospinal fluid (aCSF) in push–pull mode. Prior to sampling, the system was equilibrated by a flush sequence using a flow rate of 2 μl/min for 5 min and a run-in sequence with 0.5 μl/min for 30 min. During the sampling procedure flow rate was constant at 0.5 μl/min. After baseline sampling of 30 min, mice received a bolus of 2.5 mg/kg recombinant mouse leptin (R&D Systems, USA) intraperitoneally. Thereafter, cOFM samples were collected over 6 h and stored at −80 °C. Corresponding blood samples were withdrawn through the tail vein into lithium-heparin coated tubes and centrifuged for 5 min at 2000 g. Plasma was stored at −80 °C. After the last sampling time point, mice were perfused with 10% neutrally buffered formalin, and brains were carefully dissected out and post-fixed overnight. Afterward, brains were kept in 1× PBS. Prior to sectioning, brains were bathed in 30% sucrose for at least 24 h. 30 μm thick sections from the entire hypothalamus were made and stained using a standard hematoxylin and eosin (H&E) protocol to verify proper probe placement.

2.5. Leptin analysis

Leptin was analyzed with a Leptin ELISA kit (Cat # 22-LEPMS-E01 from ALPCO, Salem, USA) according to the manufacturer's instructions.

2.6. Statistical analysis

Data are reported as means ± SEM. Graphs were prepared in Prism version 7.0 (GraphPad Software, San Diego, CA, USA). Data were analyzed with SigmaPlot version 13.0 (SYSTAT Software, San Jose, CA, USA). The type of statistical analysis performed is stated in the figure legends. $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Twenty days of HFD-feeding induces functional leptin resistance

Compared to chow-fed mice, HFD-fed mice did gain 1.7 g more body-weight ($p < 0.05$) but did not reach an obese state by any definition (Figure 1A). At 5 pm on day 20, mice were injected with recombinant leptin (2.5 mg/kg) or vehicle solution. In chow-fed mice, leptin decreased food intake, measured over 22 h, by ∼20% ($p < 0.05$) compared to vehicle-injected mice (Figure 1B). Leptin decreased body-weight by 2% compared to the vehicle-injected mice ($p < 0.05$) (Figure 1D). In contrast to its effect in chow-fed animals, leptin injection in HFD-fed mice had no effect on food intake or body-weight (Figure 1C). These results demonstrate that 20 days of HFD feeding induces functional leptin resistance.

3.2. Hypothalamic availability of exogenous leptin is unaltered by leptin resistance

Before assessing leptin BBB transport (as described in the methods; also see schematics in Figure 2A and Suppl. Figure 2), baseline plasma levels of endogenous leptin were 3.8-fold higher in HFD-fed mice ($p < 0.05$; Figure 2B). Following peripheral injection of recombinant leptin (2.5 mg/kg), circulating leptin levels increased in a similar manner in chow-fed and HFD-fed mice, reaching a peak of ∼1200 ng/ml at 60 min after the injection (Figure 2C) (2.5 mg/kg leptin dose, was based on a dose-titration study (Suppl. Figure 1A–B). The pharmacokinetic profiles of leptin were similar between chow- and HFD-fed mice, which is also reflected by AUCs of 247 ± 42 and 234 ± 39 $\mu g \cdot 6\text{-hours} \cdot ml^{-1}$. These data indicate that during the 6-hour period after injection, the peripheral leptin bioavailability was comparable in chow- and HFD-fed mice. In parallel, we analyzed leptin levels over time in interstitial fluid obtained from the hypothalamus by cOFM sampling (Figure 2A). The rate of leptin appearance in the hypothalamus was similar between chow- and HFD-fed mice, with peak concentrations of 4.0–5.5 $ng \cdot ml^{-1}$ at 60–120 min post-injection. Similar to the peripheral leptin levels, the overall pharmacokinetic profile of hypothalamic leptin showed no difference between the two diet groups with AUCs of 949 ± 249 and 1121 ± 204 $ng \cdot 6\text{-hours} \cdot ml^{-1}$ for chow- and HFD-fed mice, respectively ($p = 0.60$, Figure 2F). Collectively, these data indicate that leptin transport across the BBB remains normal in HFD-fed mice with reduced leptin responsiveness, eliminating that mechanism as a potential cause for leptin resistance.