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Effects of hyperleptinemia in rat saliva composition, histology and ultrastructure of the major salivary glands

Elsa Lamy[a,*], Sandra Neves[b], Joana Ferreira[b], Lénia Rodrigues[a], Gonçalo da Costa[c], Carlos Cordeiro[c], Luísa Fialho[d], Mónica Lima[e], Ana Rodrigues Costa[b,f], Célia Miguel Antunes[b,f], Orlando Lopes[a,e], Francisco Amado[g,h], Fernando Capela e Silva[a,e]

[a] Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, 7002-554 Évora, Portugal
[b] Departamento de Química, Escola de Ciências e Tecnologia, Universidade de Évora, 7002-554 Évora, Portugal
[c] Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal
[d] Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Universidade de Évora, 7002-554 Évora, Portugal
[e] Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, 7002-554 Évora, Portugal
[f] Instituto de Ciências da Terra (ICT), Universidade de Évora, 7002-554 Évora, Portugal
[g] Departamento de Química, Universidade de Aveiro, 3810 Aveiro, Portugal
[h] Química Orgânica, Produtos Naturais e Agroalimentares (QOPNA), Universidade de Aveiro, 3810 Aveiro, Portugal

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ABSTRACT

Objective: To study the effect of the satiety hormone, leptin, in saliva proteome and salivary gland histology and ultrastructure.
Design: Increases in blood leptin levels were induced through mini-pump infusion in male Wistar rats, during a period of 7 days. Saliva was collected before and at the end of the experimental period, for proteomic analysis, and major salivary glands were collected, at the end, for biochemical, histological and ultrastructural analysis.
Results: Immunohistochemistry revealed the presence of leptin receptors in major salivary glands. Salivary amylase levels and enzymatic activity were decreased in saliva, whereas the enzymatic activity of this protein was increased in the cytosol of parotid gland cells. Transmission electron microscopy allowed the observation of high number of electron-dense granules in cytosol of parotid acinar cells, from leptin treated animals.
Conclusions: Increased levels of plasmatic leptin result in changes in saliva composition and salivary glands function. To our knowledge, this is the first study providing evidences for a potential role of leptin in salivary gland secretion and saliva composition. An understanding of how appetite/satiety factors influence saliva composition and how this composition influences food processing in mouth may be relevant in understanding ingestivebehaviour.

1. Introduction

Leptin is a hormone mainly produced by adipose tissue that influences energy balance, inducing satiety and energy expenditure, as extensively reviewed (e.g. Allison & Myers, 2014; Elmquist, Elias, & Saper, 1999; Zhou & Rui, 2013). The involvement of leptin in food intake and energetic balance results mainly from its action in the hypothalamic appetite/satiety centres, with an impact on ingestive and digestive processes. The observation that the functional form of leptin receptor (ObRb) is present in peripheral tissues including liver, heart, kidneys, lungs, small intestine, testes, ovaries, spleen, pancreas, salivary glands and adipose tissue, among others (Schulz & Widmaier, 2007), also indicates that leptin has a possible role in energy regulation and metabolism at a peripheral level.

The mouth is the first door for the entry of food in digestive tract, being the place where taste and aroma perception takes place and where digestion is initiated. The presence of the functional form of leptin receptor in taste buds has been observed (Shigemura, Miura, Kusakabe, Hino, & Ninomiya, 2003) and the effects of leptin on oral food perception have been reported: sweet taste sensitivity has been suggested to decrease with increasing levels of leptin (Meredith, Corcoran, & Roper, 2015), although this is a non-consensual issue, with studies observing no decreases in sweet responsiveness in response to increased levels of this hormone (Glendinning et al., 2015).

Saliva, a fluid resultant from the secretion of three pairs of major (parotid, submandibular and sublingual) and numerous minor salivary

* Corresponding author.
E-mail address: ecsl@uevora.pt (E. Lamy).

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glands, is involved in food digestion, perception and acceptance (Méjean et al., 2015). Although salivation being mainly under the control of the autonomic nervous system, some degree of regulation by hormones has already been reported (Gröschl, 2009). The presence of leptin receptors in salivary glands from humans (Bohlender, Rauh, Zenk, & Gröschl, 2003; De Matteis, Puxeddu, Riva, & Cinti, 2002) and animals (Dall'Aglio, Maranesi, Pascucci, Mercati, & Ceccarelli, 2012; Dall'Aglio, Bazzucchi, Mercati, & Ceccarelli, 2015) suggests that this hormone may have a direct influence on salivary gland function. One study reported decreases in the dimensions of rat salivary glands as a result of hyperleptinemia induced through infusion of a recombinant adenovirus containing rat leptin cDNA (Higa, Shimabukuro, Fukuchi, Komiya, & Takasu, 2002). Other studies reported changes in saliva composition induced by satiety (Harthoorn et al., 2007) and in obesity condition (Lamy et al., 2015; Rodrigues et al., 2015), although evaluation of leptin levels has not been performed (Harthoorn et al., 2007). Moreover, increasing levels of circulating leptin relate to the activation of the sympathetic nervous system (Harlan & Rahmouni, 2013), which, in turn, is known to influence saliva protein composition (Proctor & Carpenter, 2007).

Based on what was stated above, and in the context of a deeper understanding of the mechanisms involved in oral food processing and/or perception, an evaluation of the effects of increased levels of circulating leptin (hyperleptinemia) in salivary gland histology/ultrastructure and the resultant saliva composition is of interest and is the aim of this study. For that, in this study blood leptin levels were increased through exogenous leptin administration, without inducing obesity and/or changing diet composition, in order to isolate hyperleptinemia from many other metabolic or neuroendocrine changes that do occur in these situations. For this, the effects of 7-day continuous subcutaneous infusion of leptin, in opposition to saline solution, in male rat salivary protein composition, major salivary gland histology and parotid gland ultrastructure were examined.

2. Methods

2.1. Ethical approval

All animal procedures were supervised by a scientist trained by the Federation of European Laboratory Animal Science Associations (FELASA), in compliance with Portuguese law (DL nº 113/2013, 7 August), which transposed Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The protocol was approved by the ORBEA (committee responsible for animal welfare) of the University of Evora (GD/34817/2017/P1).

2.2. Animals

24 male Wistar rats with 11–12 weeks were housed individually in plastic standard rat cages with wood chip bedding and paper tubes for enrichment, maintained at a controlled temperature of 24 °C and with a 12-h dark-light cycle. Only male rats were used to avoid the interference of sex on the observed results. Cages were cleaned every week, with changes in bedding material. Animals had *ad libitum* access to food (SAFE A04) throughout the pre-experimental week and the experimental phase. Access to water was also *ad libitum* for most of the time, with exception of 3 nights during the experimental phase, when animals were water-restricted for 12 h for the purpose of taste acceptance experiments, which are not presented in this manuscript.

2.3. Pre-experimental phase

During the week preceding the implantation of mini-pumps, growth was monitored by weighing the animals daily. Daily food consumption was assessed by weighing the food distributed and the food refused every day: the weight of the food distributed in one cage minus the food refused in that cage, the following day, represented the consumption. The animals were handled every day for a period of 10–15 min to avoid stress during the procedures in the experimental phase.

2.4. Experimental phase

Sixteen rats were implanted with mini-pumps (Alzet model 2ML1, delivery rate 10.0 µL/h), 8 of which contained leptin (recombinant rat leptin protein, CF, R&D Systems, #598-LP-01M) in phosphate buffer saline (PBS) and the other 8 only PBS (Control group). The rats in which mini-pumps containing leptin were implanted (Leptin group) received a dose of 0.2 µg/g live weight per day. A third group (un-manipulated control group) comprised 8 rats which were not submitted to any surgical procedure nor to any hormone or PBS administration, but which underwent the same daily handling procedures.

All animals, including the ones from un-manipulated control group, were prevented from eating or drinking anything the night before surgeries. Animals were anaesthetized with inhaled isoflurane in oxygen (4% isoflurane for induction and 1.5–3% isoflurane for maintenance), and an area of skin on their backs was shaved and prepped with betadine and alcohol washes. A 2–3 cm incision was made and the mini-pump positioned with the orifice facing the neck. The wound was closed with intradermal suture using "nylon" 4–0 and an injection of antibiotic (enrofloxacin) was administered.

At the end of the experiment (day 8), the animals were euthanized by exsanguination under anaesthesia and blood was collected in tubes containing EDTA and kept on ice. A small piece of the parotid glands was removed for transmission electron microscopy (TEM), as further detailed. The remaining parotid, submandibular and sublingual glands from one side were fixed in buffered neutral formaldehyde 10% (pH 7.4) for one day and processed using routine histological techniques. The contralateral major salivary glands were frozen in liquid nitrogen and stored at −70 °C for further biochemical analysis.

2.5. Plasma leptin quantification

The blood collected in EDTA-coated tubes was centrifuged at 3000 rpm, room temperature, for 15 min. Plasma concentration of leptin was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit for rats, according to the manufacturer's instructions (EZRL-83K Millipore). The test is specific for rat leptin and in terms of sensitivity, the lowest level that can be detected by this assay is 0.04 ng/mL using a 10 µL sample size.

2.6. Saliva collection

On the two days preceding the surgeries and on days 5 and 7 of the experimental phase, mixed saliva samples were collected. Each animal from each group (Control or Leptin) received a subcutaneous injection of pilocarpine [5 mg/kg live weight in phosphate buffer saline (PBS), Sigma Aldrich, # P6503]. Animals were held against the experimenter's chest with the palm of the hand while the head and mouth were stabilized by the first two fingers and the thumb. Saliva was aspirated, using a micropipette, directly from the animal's mouth over 10 min, starting from the time when saliva started to be visible in the mouth, and collected to a polyethylene tube kept on ice. Samples were centrifuged at 14,000*g* for 15 min, at 4 °C, to remove cells and food debris, and supernatants further stored at −20 °C until laboratorial analysis. On the first day of laboratorial analysis, and before analysis began, saliva samples were thawed on ice and, for each individual, the collections from the two days before surgeries, as well as the collections from days 5 and 7 of the experimental phase, were pooled. This procedure was aimed at collecting enough saliva for each animal from the period without leptin stimulation and from the period of hyperleptinemia induction.