Redox Biology 1 (2013) 498–507

Contents lists available at ScienceDirect

Redox Biology

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

Research Paper

The role of nitric oxide signaling in food intake; insights from the inner mitochondrial membrane peptidase 2 mutant mice ☆

Changjie Han [a,b], Qingguo Zhao [a], Baisong Lu [a,*]

[a] Wake Forest University Health Sciences, Institute for Regenerative Medicine, Winston-Salem, NC 27157, United States
[b] Department of Human Anatomy and Embryology, The Second Military Medical University, Shanghai 200433, China

ARTICLE INFO

Article history:
Received 7 October 2013
Received in revised form
14 October 2013
Accepted 16 October 2013

Keywords:
Immp2l
Mutant mice
Food intake
Superoxide
Nitric oxide
Energy expenditure

ABSTRACT

Reactive oxygen species have been implicated in feeding control through involvement in brain lipid sensing, and regulating NPY/AgRP and pro-opiomelanocortin (POMC) neurons, although the underlying mechanisms are unclear. Nitric oxide is a signaling molecule in neurons and it stimulates feeding in many species. Whether reactive oxygen species affect feeding through interaction with nitric oxide is unclear. We previously reported that *Immp2l* mutation in mice causes excessive mitochondrial superoxide generation, which causes infertility and early signs of aging. In our present study, reduced food intake in mutant mice resulted in significantly reduced body weight and fat composition while energy expenditure remained unchanged. Lysate from mutant brain showed a significant decrease in cGMP levels, suggesting insufficient nitric oxide signaling. Thus, our data suggests that reactive oxygen species may regulate food intake through modulating the bioavailability of nitric oxide.

Introduction

Reactive oxygen species (ROS) are implicated in feeding regulation through various mechanisms. For example, ROS are involved in brain lipid sensing, which regulates food intake [1]. Additionally, regulation of free radical generation in the mitochondria by UCP2 mediates ghrelin's action on NPY/AgRP neurons [2]; suppression of ROS promotes feeding by diminishing the activation of pro-opiomelanocortin (POMC) neurons and enhancing the activity of NPY/AgRP neurons, whereas expression of ROS activates POMC neurons and reduces feeding [3]. Whether alternate mechanisms are involved in ROS regulation of feeding requires further investigation.

Nitric oxide (NO), a signaling messenger, is involved in feeding control in multiple species, including chicken [4,5], mice [6–9] and rats [10,11]; moreover, NO is a central component in neuropeptide regulation of appetite [12]. Blockade of NO synthesis reduces adiposity in high fat-induced obese mice [13] and obese Zucker rats [14]. Three nitric oxide synthases (NOS), the constitutive neuronal NOS (nNOS), endothelial NOS (eNOS), and the inducible NOS (iNOS), are responsible for the synthesis of NO. Leptin, a hormone produced by adipocytes, inhibits brain NO synthesis and decreases food intake [15,16]. Guanylate cyclase, the principal NO receptor, synthesizes cyclic guanosine monophosphate (cGMP) upon activation by NO.

The hypothalamus is a critical part of the brain that contains nuclei, such as the arcuate nucleus (ARC), the ventromedial nucleus (VMN) and the dorsomedial nucleus (DMN), which contain multiple neurons that receive signals from insulin and leptin to regulate food intake [17–19]. In the ARC, neuropeptide Y (NPY) and Agouti-related peptide (AgRP), which are co-expressed by NPY/AgRP neurons, stimulate food intake [20–24]. Gene products from pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which are co-expressed by POMC neurons, suppress food intake [25,26]; therefore, ROS may be acting through NPY/AgRP and POMC neurons [2,3]. Pharmacological activation of AMP-activated protein kinase (AMPK) in the hypothalamus will cause increased food intake [27], NO production, and neuronal activity [28]. AMPK is activated via phosphorylation of threonine 172 of AMPKα subunit [29].

Superoxide and NO have opposing effects on blood vessel contraction [30]. The reaction of superoxide with NO yields peroxynitrite at a speed several times faster than its own dismutation by superoxide dismutases [31–33]. Recently, we generated the

Abbreviations: *Immp2l*, IMP2 inner mitochondrial membrane peptidase-like; cGMP, cyclic guanosine monophosphate; ROS, reactive oxygen species; UCP2, uncoupling protein 2; NPY, neuropeptide Y; AgRP, agouti related protein; POMC, pro-opiomelanocortin; NO, nitric oxide; NOS, nitric oxide synthase; CART, cocaine- and amphetamine-regulated transcript; CYC1, cytochrome c1; GPD2, mitochondrial glycerol phosphate dehydrogenase; ADSC, adipose-derived stromal cells; AMPK, AMP-activated protein kinase.

* Correspondence to: Wake Forest University Health Sciences, Institute for Regenerative Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, United States. Tel.: +1 336 713 7276; fax: +1 336 713 7290.
E-mail address: blu@wakehealth.edu (B. Lu).

2213-2317/$ - see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.redox.2013.10.003

Immp2l mutant mouse model through random transgenic insertional mutagenesis [34,35]. IMMP2L is a mitochondrial inner membrane peptidase, which cleaves the space-sorting signal peptide sequences from cytochrome c1 (CYC1) and mitochondrial glycerol phosphate dehydrogenase (GPD2). Accordingly, the signal peptide sequences of CYC1 and GPD2 are not cleaved in *Immp2l* mutant mice. The mitochondria from mutant mice generate excessive superoxide ions, but they show no obvious deficiencies in ATP generation and membrane potential maintenance [35]. Mutant mice exhibit erectile dysfunction [35], defective oogenesis [35] and bladder dysfunction [36], which may result from inactivation of NO by superoxide. The mutant mice also develop age-dependent spermatogenic impairment [35,37], accelerated aging [38], and increased ischemic brain damage [39] due to increased oxidative stress.

While studying the effects of mitochondrial superoxide overgeneration on aging, we noted that mutant mice have significantly reduced body weight and fat composition compared to control mice after the age of 5 months. Data from our present study suggest that decreased food intake may account for reduced body weight and adiposity in mutant mice; furthermore, our data suggest that the mechanism of food intake decrease may be the decreased bioavailability of NO in the brain of mutant mice.

Material and methods

Animals

The generation of $Immp2l^{Tg(Tyr)979Ove}$/$Immp2l^{Tg(Tyr)979Ove}$ mutant mice has been described previously [35], and the mice have been maintained on an FVB/N background. $Lep^{ob/ob}$ mice have been described previously [40], and animals were purchased from The Jackson Laboratories. The mice were backcrossed to FVB/N background for five generations before crossing with *Immp2l* heterozygous mice. *Immp2l* and *Leptin* double mutant mice were generated by mating double heterozygous males and females. Mice were housed in the pathogen-free animal facility of Wake Forest University Health Sciences. Experiments were conducted in accordance with the National Research Council publication Guide for Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee of Wake Forest University Health Sciences. Mice were kept in microisolator cages with 12-h light/dark cycles and were fed ad libitum on a chow diet (Prolab, RMH3000). Genotypes of the mice were determined by coat color [35].

Food consumption

Food consumption was determined for mice that were individually caged in cages with lifted wire bottoms, as described previously [41]. Food spilled on the bottom of the cages was recovered and subtracted from food intake. Unless otherwise stated, the number of animals per group was equal to or greater than five. Food intake was assayed prior to noticeable differences in body weight between control and mutant mice. Food intake was normalized to body mass and analyzed by Student's *t*-test, and p values <0.05 were regarded as statistically significant.

To compare fasting induced food intake, mice were individually housed in cages with lifted wire bottoms, fasted for 15 h (from 6:00 pm to 9:00 am) or 16 h (from 10:00 pm to 2:00 pm), and then provided with food. Food intake during the first 4 h was recorded and compared by Student's *t*-test.

Blood biochemical assays

Sera were collected from mice following 6 h fasting. Free fatty acids were assayed with kits from Cayman Chemical (Ann Arbor, MI). Serum leptin, insulin, cholesterol and triglyceride were assayed as described previously [41].

Determination of cGMP and nitrate/nitrite concentrations

To avoid cGMP degradation, mice were euthanized by cervical dislocation and brain removed within 2–3 min after death. The hypothalami were dissected and snap frozen in liquid nitrogen, and stored at $-80\,^{\circ}C$ prior to measuring hypothalamic cGMP concentrations. The hypothalamic tissues were lysed in 5% trichloroacetic acid (10 ml solution per 1 g tissue) and extracted with water-saturated ether. A Monoclonal Anti-cGMP EIA Kit without Acetylation from Neweast Biosciences Inc. (Cat# 80103) was used to compare cGMP according to manufacturer's protocol. Total nitrate and nitrite contents in the trichloroacetic acid extracts were assayed with a kit from Cayman Chemical.

Magnetic resonance imaging (MRI) analysis of fat composition

Fat composition was determined by MRI using the Bruker Biospin 7 T microMRI scanner housed in the Center for Biomolecular Imaging, Wake Forest University Health Sciences as described previously [41].

Metabolic studies

Indirect calorimetry and locomotor activity were measured using the Oxymax CLAMS system (Columbus Instruments). Animals were tested at 4 months of age and prior to noticeable differences in body weight and fat composition. Oxygen consumption rate (VO_2) and CO_2 production rate (VCO_2) in individual mice were measured using metabolic chambers, and the respiratory exchange ratio (RER) was calculated to reflect energy expenditure. A photobeam-based activity monitoring system was used to detect and record ambulatory movements. The temperature of the cabinet was set at 26 °C. Energy expenditure (kJ/h) was calculated using the formula: $VO_2 \times [3.815 + (1.232 \times \mathrm{RER})] \times 4.1868$ [42] and was normalized to body weight since control and mutant mice showed similar body weight at the time of assay. All parameters were measured continuously and simultaneously for 72 h after 36 h of adaptation in singly housed mice. Three-day averages for each mouse were used for analysis.

Quantitative RT-PCR analysis of gene expression

Total RNA was extracted from hypothalamic tissues with Turbo DNase (Ambion) to eliminate DNA contamination, and reverse transcribed as described previously [43]. Primers for *Npy*, *Agrp* and *Pomc* have been described in our recent publication [41]. Other primers used include: pmchF (agcggtttcatgaacgatg) and pmchR (tcagacttgccaacatggtc) for *Pro-melanin-concentrating hormone*, OrexinF (gggtatttggaccactgcac) and OrexinR (ttcgtagagacggcaggaac) for *Orexin* gene, sGCF (ctgctggtgatccgcaattat) and sGCR (gatggtatcatagccagactcct) for *Soluble guanylyl cyclase*, eNOSF (ccttccgctaccagccaga) and eNOSR (cagagatcttcactgcattggcta) for *eNos*, nNOSF (tcctaaatccagccgatcga) and nNOSR (tcatggttgccagggaagac) for *nNos*, and iNOSF (agagagatccgatttagagtcttggt) and iNOSR (tgacccgtgaagccatgac) for *iNos*. Equal amounts of RNA from five mice per group were mixed for RT-PCR analysis. Mean ± SEM was obtained from three to four independent PCR analyses, with each PCR analysis including triplicate wells.