VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 68, No. 4, pp. 218–223

ISSN 0015–5659
www.fm.viamedica.pl

Ultrastructural response of arcuate nucleus neurons to fasting in aged rats

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[Received 24 August 2009; Accepted 13 October 2009]

The arcuate nucleus of the hypothalamus (ARH) is involved in the control of energy homeostasis. Leptin — an adipocyte derived hormone — is known to act on the hypothalamic nuclei and thus to control body weight by food intake reduction. Oxidative stress is believed to be implicated in leptin signalling. However, its relevance for leptin-induced signal transduction within ARH remains unclear. The goal of the study was to investigate the effect of fasting on morphological alterations of the neuronal endoplasmic reticulum/Golgi network as well as on the expression of leptin receptors in the arcuate nucleus of aged rats. Male Wistar rats, aged 24 months, were fasted for 96 hours. The control animals were fed ad libitum. *Membranous whorls in the ARH neurons were visualized using the electron microscopy technique. Leptin receptors in the membranes of ARH neurons were determined immunohistochemically (IHC), and soluble leptin receptors in the plasma as well as plasma isoprostanes were quantified immunochemically (ELISA). An intense formation of membranous whorls was observed, directly associated with the cisternae of the rough endoplasmic reticulum, as well as lamellar bodies. Interestingly, the whorls were often localized near a well-developed Golgi complex. Moreover, some Golgi complexes displayed an early stage of whorl formation. Groups of residual lipofuscin granules were found in the immediate proximity of the whorls. An increased immunoreactivity with neuronal leptin receptors suggests that hypersensitive neurons may still effectively respond to the fasting serum levels of leptin, mediating ultrastructural transformation of ARH neurons during short-term fasting. Having observed a significant accumulation of lipofuscin granules and a marked increase of total 8-isoprostane serum level in the fasting rats, we hypothesize that signal transduction within the neurons of ARH is dependent on oxidative stress phenomena.* (Folia Morphol 2009; 68, 4: 218–223)

Key words: arcuate nucleus, fasting, whorls, leptin receptors

INTRODUCTION

Membranous whorls composed of concentric layers of endoplasmic reticulum and Golgi complexes were found in the arcuate nucleus of the hypothalamus (ARH) after treatment with mercuric chloride an oxidative stress inducer [14]. The whorls were also described in neurons of the arcuate nucleus of colchicine-treated male rats [4]. Oxidative stress dependent intracellular signal transduction by leptin and the leptin receptor has been reported in a number

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of cell systems [13, 15]. However, the significance and relevance of oxidative stress for *in vivo* leptin signalling in the arcuate nucleus of the hypothalamus still remains unclear. Oxidative stress induction by leptin treatment resulted in perturbed responsiveness to extracellular Ca^{2+}, and endoplasmic reticulum Ca^{2+}-ATPase was markedly modified by oxidation in the heart of the mouse [15]. The results of our study are meant to attract attention to the formation of whorls from rough endoplasmic reticulum (RER) and Golgi complexes in the arcuate neurons of aged male rats fasting for 96 hours. To date, there has been no ultrastructural information available on ARH neurons during fasting.

MATERIAL AND METHODS

Animals

Inbred male Wistar rats, aged 24 months, were used in the experimental model: control rats (n = 6) and rats fasting for 96 hours (n = 6). The animals were housed 2 per cage and they were maintained at 20 ± 1°C on a controlled 12-hour light regime (with the light on from 7:00 to 19:00). The control rats were fed with standard chow containing (w/w) 13% protein, 55.5% carbohydrate, 2.5% lipid, 1% calcium, 0.75% phosphates, and 27% indigestible compounds (Labofeed B, Kcynia, Poland). The animals were cared for and treated according to the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes". The study was approved by the Local Ethical Committee for Animal Experiments in Gdansk, Poland.

Dietary manipulation and sampling

The control rats (Group I) were fed *ad libitum* and sampled at the same time as the fasting animals. The fasting rats (Group II) were sacrificed at 08:00 after 96 hours of starvation. Food consumption was calculated per 2 rats housed together in a cage, and the body weight change was determined for each rat individually. The average body weight of the rats in groups I and II was 568 ± 78 g and 525 ± 38 g, respectively. The rats in Group I consumed 9.3 ± 1.3 g chow per 100 g body weight during 24 hours. The rats were provided with water *ad libitum*.

Ultrastructural study

The animals were anaesthetized with 10% ketamine and were perfused transcardially with a mixture of 4% paraformaldehyde and 1% glutaraldehyde dissolved in 0.05 M phosphate buffer (pH 7.4) supplemented with 0.3% $CaCl_2$. After perfusion, the brains were removed from the skulls and stored in the same fixative at 4°C overnight. Next, hypothalamic tissue blocks were cut down on a vibratome 1000s (Leica, Germany) into serial coronal slices (500 μm thick). Small tissue specimens containing arcuate nucleus were taken bilaterally from the rat brain under the control of a binocular stereomicroscope. The specimens were left in the fixative for 3 hours and then post-fixed in 1% osmium tetroxide for 2 hours. After dehydration with alcohols and propylene oxide, the specimens were embedded in Epon 812.

Semithin Epon sections were cut on a Reichert Om U3 ultramicrotome and then stained with 1% toluidine blue for a light microscope examination in order to identify the arcuate nucleus. After the required area had been confirmed, ultrathin sections were then cut down. The sections were stained with uranyl acetate and lead citrate and examined using a JEM 1200 EX II electron microscope (JEOL, Japan).

Immunohistochemistry

Twenty-four-month-old male rats (n = 2) fasting for 96 hours, were used to reveal expression of leptin receptors in the arcuate nucleus. Two rats were used as controls. The ABC Santa Cruz staining system was applied for paraffin sections (20 μm thick) according to Matsuda et al. [16].

Immunochemistry

Serum leptin receptor levels were determined by ELISA method using specific monoclonal antibodies from R & D Systems (USA). Serum levels of 8-isoprostanes were assessed immunochemically as described previously [12].

RESULTS

The arcuate nucleus of the rat hypothalamus is situated on both sides of the infundibular recess of the third ventricle above the median eminence. The neurons are located beneath the ependymal lining and they are usually found in groups of 3 or 4 cells surrounded by the neuropil.

Ultrastructure

Group I (fed control). The neurons of the ARH (Fig. 1A) were characterized by a large, round, and centrally placed nucleus. A nuclear envelope exhibited deep and branched invaginations. Usually each neuron contained a prominent nucleolus, often localized in the vicinity of the nuclear envelope. A narrow

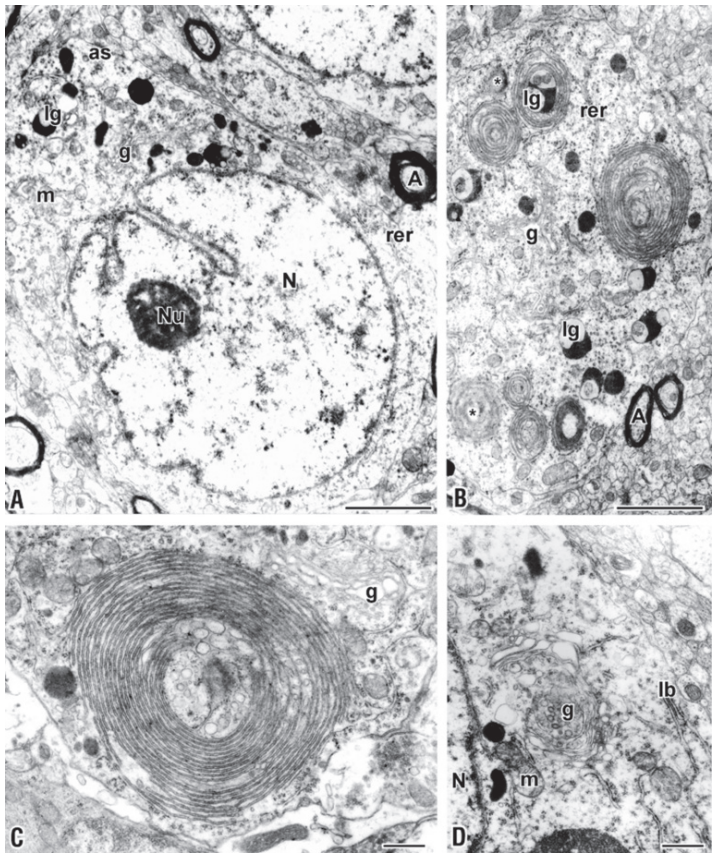

Figure 1. A. Fed control aged rat. The large nucleus (N) with a prominent nucleolus (Nu) localized near the deep invaginations of the nuclear envelope. Golgi complex (g), cisternae of rough endoplasmic reticulum (rer), mitochondria (m), and lipofuscin granules (lg). Axo-somatic synapse (as) identify a cell as the neuron. Myelinated axon (A); **B.** Aged rat having fasted for 96 hours. Eight small whorled bodies localized near the Golgi complex (g). Two of them are blurred because of oblique sectioning (asterisks). A lipofuscin granule (lg) is trapped in the centre of one of the whorls. There is a continuation of the outermost layers, which usually contain ribosomes with the rough endoplasmic reticulum cisternae (rer). Numerous multiformed lipofuscin granules (lg), myelinated axon (A). Bar: 2 μm; **C.** Aged rat having fasted for 96 hours. A large whorled body consisting of approximately 23 concentric layers of smooth endoplasmic reticulum with varying portions of cytoplasm trapped in between. Irregular vesicles apparently budding off from the smooth endoplasmic reticulum cisternae. Extensive Golgi complex (g) closely associated with the whorl can be observed; **D.** Aged rat having fasted for 96 hours. The Golgi complex (g) with lamellar and vesicular elements has taken a circular shape. Nucleus (N), lamellar body (lb), mitochondrium (m). Bar: 500 nm.

band of cytoplasm surrounding the nucleus was poor in organelles: RER, ribosomes, mitochondria, and few lipofuscin granules, as compared to the fasting group. The cisternae of the RER were distributed as single, discrete sacs in the cytoplasm. Golgi complex was generally localized in the perinuclear area. In some neurons, occasional short lamellar bodies composed of two cisternae of the RER were observed. Membranous whorls made up of closely apposed concentric cisternae of the smooth endoplasmic reticulum (ER) were observed accidentally within the neuronal perikarya.

Group II (having fasted for 96 hours). The ARH neurons of fasted animals were characterized by large invaginated nuclei with prominent nucleoli. The nucleoli were often located near the nuclear invaginations, as in the control rats. In response to fasting for 96 hours, the ARH neurons developed RER in the form of short independent fragments randomly dispersed throughout the cytoplasm, or longer ones anastomosing one another with a tendency to form lamellar and membranous whorls. The lamellar bodies were usually formed by two parallel RER membranes with electron dense material in between. At both ends they were directly associated with the RER cisternae. The membranous whorls, however, were characterized by closely apposed concentric cisternae of smooth ER. Their outermost layers, which usually contained ribosomes, were directly associated with the cisternae of the RER or the lamellar bodies. Two types of membranous whorls could be distinguished in the arcuate neurons. Small whorls

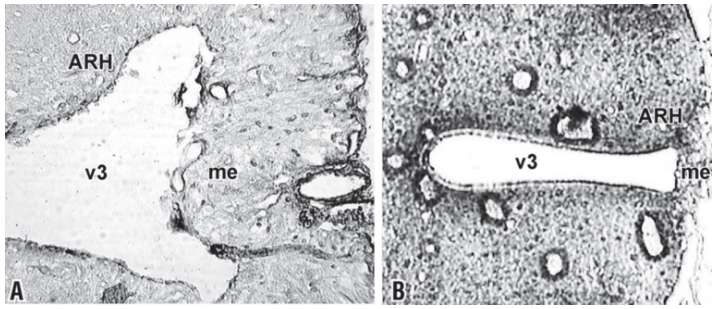

Figure 2. A. Fed control aged rat. Immunopositive reaction for leptin receptors in the arcuate nucleus of the hypothalamus (ARH). IHC test; 1 × 100; **B**. Aged rat having fasted for 96 hours. An increased immunopositive reaction for leptin receptors in the ARH (arrow). IHC test; 1 × 400; third ventricle (v3), median eminence (me).

of regular structure composed of several concentric cisternae of the smooth ER were grouped together forming a cluster of 4 to 8 structures per neuronal cell (Fig. 1B). In the centre of the whorls and between their layers, varying amounts of cytoplasm were often to be found. Large multilayered whorls of more complicated structure were also observed (Fig. 1C). A number of layers exceeded 10 membranous circles per section, as compared with the small whorls. The membranes were frequently fragmented and partly replaced by irregular vacuoles, or by a row of clear vesicles. Such structures grouped from 1 to 3 were observed in a single neuron. All the whorls usually appeared in close association with a very well developed Golgi complex. We also observed an early stage of whorl formation by means of involution of the cisternae of Golgi complex. Some neurons displayed Golgi complexes composed of several long dilated cisternae, often with a tendency to circle at one end. In other neurons, the cisternae of the Golgi complex were inclined to form nearly concentric circles (Fig. 1D). Near the membranous whorls and Golgi complexes, numerous multiform lipofuscin granules were observed.

Immunohistochemical studies (IHC) revealed more intensely marked positive immunoreactivity for leptin receptor in the ARH of the aged rats having fasted for 96 hours (Fig. 2B), as compared with the control aged rats (Fig. 2A). As expected, this observation confirmed that fasting for 96 hours significantly upregulated leptin receptors in the ARH nucleus of the aged rats.

Immunochemical studies (ELISA) showed an increase in soluble leptin receptor levels in the serum of the fasting animals as compared to the controls, although the difference was statistically insignificant (Fig. 3). A significant increase in 8-isoprostane serum

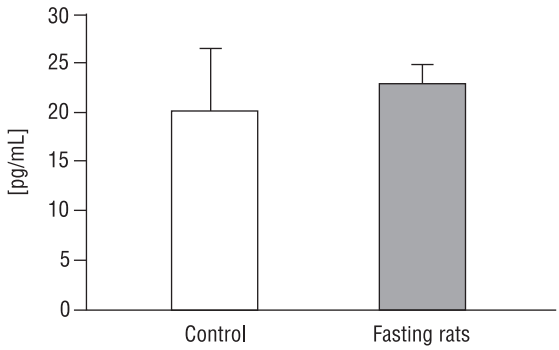

Figure 3. Serum concentration of soluble leptin receptor in fasting and control rats.

levels in the fasting animals was observed relating to the fed control rats, as described earlier [12].

DISCUSSION

In the present study, in the arcuate neurons of the aged control rats, we noted only occasional whorls. Van Houten and Brawer [22] observed these bodies in normal male rats in the ventrolateral part of the hypothalamic ventro-medial nucleus (VMN). Our studies [11] did not indicate these bodies because the specimens were taken from the centre of the middle part of the VMN. Brawer [1] and Price et al. [21] also observed the appearance of whorls in the arcuate neurons, after castration or chronic morphine treatment, which had reduced testosterone level in the blood of male rats. Interestingly, testosterone therapy inhibited the process. It is possible, as some authors have suggested [1, 3, 8], that whorls in the arcuate nucleus may mark the sites of luteinizing hormone-releasing hormone (LH-RH) synthesis. This phenomenon was confirmed by immuno-electron microscopy [18]. Whorls were stated in

ovariectomised rats [8] and in a mercury-treated female hamster [14] that had low levels of ovarian steroids. Thus, it is possible that the whorls are a sign of increased activity of the arcuate neurons due to the loss of feedback control from the gonads [1]. Since whorls are associated with RER and Golgi complex, they may contain carrier proteins and enzymes associated with the release of LH-RH [8], or those involved in the early stages of the synthesis of LH--RH [17]. In our studies, as well as those of other authors [18], there was an increase of lipofuscin/ /lysosome granules near the whorls and in the Golgi region. Some of them may capture LH-RH granules [18]. The results of our studies, in terms of the response of arcuate neurons to fasting, are consistent with the studies of Kiss [9], who also observed a wide variety of endoplasmic reticulum formations after repeated immobilization stress in the ventromedial nucleus of the hypothalamus of male rats.

Our previous studies [10], as well as those by Weigle et al. [23], showed that short-term fasting caused ~60% drop of serum leptin level. A decreased serum level of the hormone, however, seems significant enough to interact effectively with the target neurons, specifically those hypersensitive ARH neurons with markedly upregulated leptin receptors. Indeed, Otukonyong et al. [19] proved that leptin could effectively act in the pituitary-ovarian axis during fasting to improve reproductive function by partly stimulating oestrogen secretion. Moreover, we observed a tendency of soluble leptin receptor levels in the serum to increase slightly in the fasting rats, which is in accordance with Popruk et al. [20], who correlated a minor increase in the soluble leptin receptor levels with fat mass decrease. It is believed that significant levels of soluble leptin receptor in the plasma might enhance the transport of leptin across the blood-brain barrier and increase the physiological response of the target arcuate nucleus neurons to leptin [7]. Lipofuscin granules have been demonstrated to be secondary lysosomes of heterogenous content that derive from autophagic vacuoles. Interestingly, the formation of lipofuscin granules is assumed to be the result of intracellular oxidative stress phenomena, and the failure of their removal mechanism during ageing is probably the cause of lipofuscin accumulation in senescent neurons. Lipofuscin itself might, in turn, be another reason for increased oxidative stress responsible for the formation of whorls. Exogenous leptin has been found to increase lipid peroxidation in the mouse brain [13]. In our experimental conditions, significant accumulation of lipofuscin in close proxi-

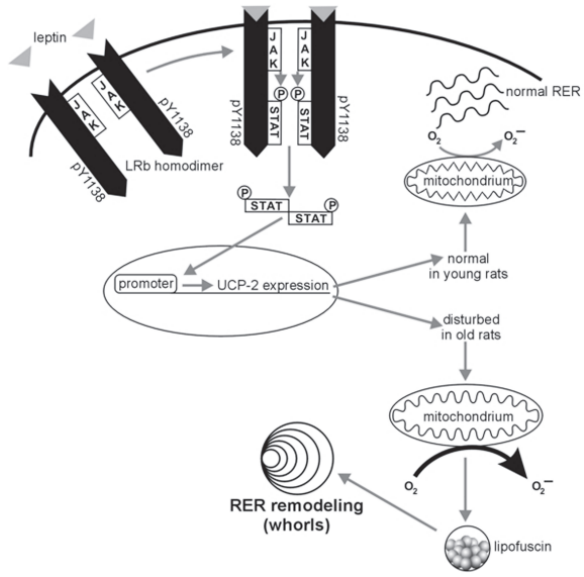

Figure 4. Presumable perturbation of UCP-2 and leptin signalling in aged rats, leading to oxidative stress dependent remodelling of endoplasmic reticulum (ER whorls).

mity to the membranous whorls and Golgi complexes strongly suggests that oxidative stress may trigger topological remodelling of those membranes. Moreover, we observed a significant increase of the serum level of 8-isoprostanes (~2.7-fold), being a widely accepted marker of *in vivo* lipid peroxidation, in the group of fasting rats as compared to the fed controls [12]. In young animals, leptin has been found to induce uncoupling protein (UCP)-2 [24], being an important neuromodulator and neuroprotectant in the central nervous system [2]. Surprisingly, the ageing process seems to reduce markedly expression of both UCP-2 [5] and leptin [6]. Taking into account the fact that UCP-2 down regulates mitochondrial generation of reactive oxygen species, one may assume that ageing-dependent disturbance of UCP-2 expression triggers oxidative stress signalling, resulting in the rearrangement of the physiological sheet-like topology of ER into the stress-like topological variant of concentric membranes (Fig. 4). Further research is needed to elucidate the intracellular mechanism of whorl formation in neurons of ARH during short-term fasting.

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