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Ultrastructural observations on the hypothalamic arcuate nuclei of aged rats in the fasting/refeeding model

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The arcuate nucleus of the hypothalamus (ARH) is involved in the control of energy homeostasis. This is the first study on the ultrastructural response of ARH neurons in aged rats after short-term fasting and subsequent refeeding. Male Wistar rats (24 weeks old) were fasted for 48 or 96 hours and were then refed for 24 hours. The controls were normally fed. The rats received water ad libitum. *In both groups of fasting animals, we observed a rearrangement of the arcuate rough endoplasmic reticulum (RER) and Golgi complexes to form membranous whorls. Moreover, refeeding for 24 hours did not reverse this process. The RER was frequently found to be well organized into lamellar bodies composed of several cisternae. The membranous whorls were composed of concentric layers of endoplasmic reticulum and Golgi complexes. In addition, multiform lipofuscin granules were observed in close relationship with Golgi complexes and membranous whorls. Lipofuscin granules within the neurons of the arcuate nucleus are assumed to be a morphological manifestation of oxidative stress phenomena, which are presumably implicated in the formation of membranous whorls in both fasting and fasting/refed animals. This observation correlates with a significant increase in 8-isoprostane serum levels in the fasting and fasting/refed animals as compared to the fed control rats.* (Folia Morphol 2009; 68, 2: 79–83)

Key words: arcuate nucleus, fasting/refeeding, whorls, oxidative stress

INTRODUCTION

The arcuate nucleus of the hypothalamus (ARH) plays an important role in the control of food intake and energy homeostasis. Oxidative stress-dependent signal transduction via leptin and the leptin receptor has been reported in a number of cell systems [7, 9]. However, the significance and relevance of oxidative stress in ARH is unclear. Membranous whorls, composed of concentric layers of endoplasmic reticulum and cisternae of Golgi complex, were only found in the ARH of rats exposed to mercuric chloride, being an oxidative stress inducer [8]. Whereas, testosterone deficiency following either castration or chronic morphine treatment [3, 12] stimulated the increase in ARH membranous whorls. Interestingly, testosterone therapy inhibited the process. The purpose of the study was to investigate the effect of fasting and subsequent refeeding on ultrastructural alterations of the endoplasmic reticulum/Golgi network in the arcuate nucleus of aged rats as well as potential correlation of these changes with oxidative stress phenomena. The results may prove useful for a better understanding of obesity, including the process of losing weight, especially in

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the elderly, as well as the pathology of certain endocrine diseases.

MATERIAL AND METHODS

Animals

The study was performed on aged (24-month-old) male Wistar rats fasting for either 48 hours (Group II) or 96 hours (Group III) and then being refed for 24 hours. The control rats (Group I) were fed *ad libitum.* 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 07:00 to 19:00). The 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). 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 Gdańsk, Poland.

Dietary manipulation and sampling

The control rats (Group I) were sampled at the same time as the fasting/refed animals. The rats in Groups II and III were sacrificed at 8:00 after 24 hours of feeding. Food consumption was calculated per 2 rats housed together in a cage, and the body weight change was determined for each rat individually. The initial body weight of the rats in groups I, II, and III was 457 ± 3 g, 507 ± 3 g, and 520 ± 10 g, respectively (mean ± SD, n = 4 in each group). The body weight of the rats in groups II and III after fasting was 455 ± 5 g and 460 ± 20 g, whereas after fasting/refeeding it was 482 ± 8 g and 477 ± 18 g, respectively. The rats in group I consumed 9.3 ± 1.3 g of chow per 100 g bodyweight over 24 hours. After 24 hours of refeeding the average food consumption of the old rats in Groups II and III was 9.5 ± 0.8 g and 8.5 ± 0.3 g of chow per 100 g bodyweight, respectively. The rats were provided with water *ad libitum*.

Ultrastructural study

The animals, both the control and fasting/refed groups, were deeply anaesthetized with 10% ketamine and then perfused transcardially with a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) containing $CaCl_2$. After perfusion, the brains were removed from the skulls and stored in the same fixative overnight at 4°C. Next, from the tissue blocks containing hypothalamus, serial coronal slices (500 μm thick) were cut on a vibratome 1000S (Leica, Germany). Small tissue specimens containing arcuate nucleus were taken bilaterally under a binocular stereomicroscope and then left in the fixative for 3 hours and post-fixed in 1% osmium tetroxide for 2 hours. After dehydration in alcohols and propylene oxide, the specimens were embedded in Epon 812. In order to precisely localize the individual arcuate nuclei, Epon semithin (1 μm) sections were cut on a Reichert OmU3 ultramicrotome, stained with 1% toluidine-blue solution and examined by light microscopy. After a suitable area was found, the specimens were trimmed and ultrathin sections were then cut. The ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEM 1200 EX II electron microscope.

Immunochemistry

Moreover, total 8-isoprostane serum levels were measured using commercially available Elisa kits (Cayman, Ann arbor, MJ, USA), as a reliable marker of oxidative stress induced lipid peroxidation *in vivo.*

RESULTS

Group I (fed control)

In general, the neurons of the arcuate nucleus were round in shape and contained a large, centrally placed nucleus (Fig. 1). The nuclear envelope exhibited deep invaginations. Usually each neuron contained a prominent nucleolus localized eccentrically. A narrow band of cytoplasm was poor in organelles: sparse cisternae of the rough endoplasmic reticulum (RER), patches of Golgi complexes in the perinuclear region, and few lipofuscin granules. Other organelles including ribosomes and mitochondria were distributed quite evenly in the cytoplasm. Occasional short lamellar bodies, composed of two parallel cisternae of the RER with electron dense material in between, were observed in the cytoplasm. The membranous whorls consisted of closely apposed concentric cisternae of the RER devoid of ribosomes were found in the control animals very rarely.

Groups II and III (having fasted for 48 h or 96 h and then having been refed for 24 h)

In fasted as well as in fasted/refed animals, the neurons of the arcuate nucleus were characterized by large, irregular nuclei with prominent nucleoli

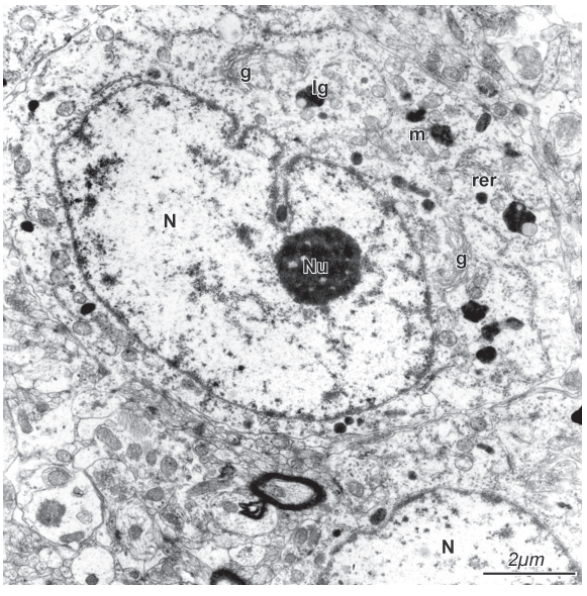

Figure 1. Control (normally fed) old rat. Two neurons in the arcuate nucleus are separated by neuropile. The large nucleus (N) with a prominent nucleolus (Nu) is localized near the invagination of the nuclear envelope. The cytoplasm contains Golgi complexes (g), cisternae of the rough endoplasmic reticulum (rer), mitochondria (m), and lipofuscin granules (lg). Bar: 2 μm.

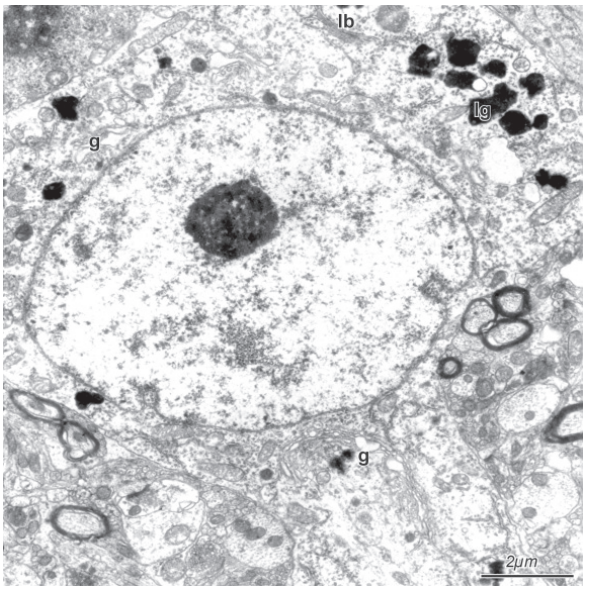

Figure 3. Old rat fasted for 96 hours and refed for 24 hours. The cytoplasm of the arcuate neuron contains extensive Golgi complexes (g), the cisternae of which are greatly dilated, while the rough endoplasmic reticulum is sparse with the exception of the upper part of the neuron where the rough endoplasmic reticulum is organized into a four-layered body (lb). Note the group of lipofuscin granules (lg) near this structure and Golgi complex. Bar: 2 μm.

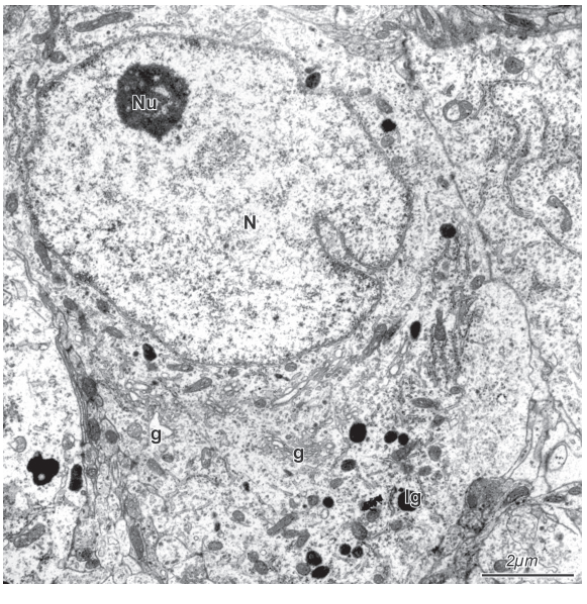

Figure 2. Old rat fasted for 48 hours and refed for 24 hours. The cytoplasm of the arcuate neuron contains extensive Golgi complexes (g) with significantly dilated cisternae, while the rough endoplasmic reticulum (rer) is sparse with the exception of the right part of the neuron. Note a group of lipofuscin granules (lg) near Golgi complexes (g); nucleus (N), nucleolus (Nu). Bar: 2 μm.

(Figs. 2, 3). The nucleoli were frequently situated in the vicinity of the nuclear envelope. In the cytoplasm, we observed rearrangement of the 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 bodies (Figs. 3, 4A, B, 5A) and membranous whorls (Figs. 4A, 5B). Lamellar bodies were formed from two to four parallel cisternae of the RER with electron dense material between them. They became longer than in the control animals and some of them were bent, probably participating in the formation of membranous whorls (Fig. 5A). Membranous whorls, however, were characterized by closely apposed concentric cisternae of the RER devoid of ribosomes. Sometimes dilatations could be seen extending from the ends of the endoplasmic reticulum (Fig. 4A). The outermost layers of the lamellar bodies and membranous whorls were irregularly covered with ribosomes and often appeared to be continuous with the RER cisternae (Figs. 4A, 5A). One to three membranous whorls were observed in a single cross section. Moreover, some very well developed Golgi complexes (Figs. 3, 4B, 5C) displayed the early stages of whorl formation by means of involution of their cisternae. All the membranous whorls usually appeared in close association with very well developed Golgi complexes. Multiform lipofuscin granules were observed in close relationship with the Golgi complexes and membranous whorls.

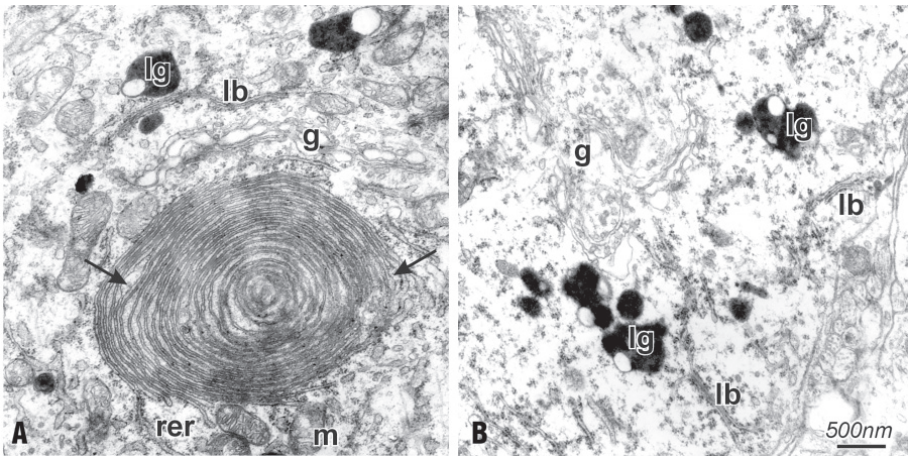

Figure 4. Old rat fasted for 48 hours and refed for 24 hours. **A.** An example of the whorled body composed of approximately 23 concentric layers of the endoplasmic reticulum in the arcuate neuron. The outermost layers are continuous with the cisternae of rough endoplasmic reticulum (rer). Dilatations can be observed extending from the ends of the endoplasmic reticulum cisternae (arrows). Simple lamellar body (lb) composed of two cisternae of the rough endoplasmic reticulum can be seen in the upper part of the neuron picture. Extensive Golgi complex (g) in close association with the membranous whorl can be observed. Multiformed lipofuscin granules (lg), mitochondria (m); **B.** Very well developed Golgi complex (g) with a tendency to wind up; numerous multiform lipofuscin granules (lg); lamellar bodies (lb). Bar: 500 nm.

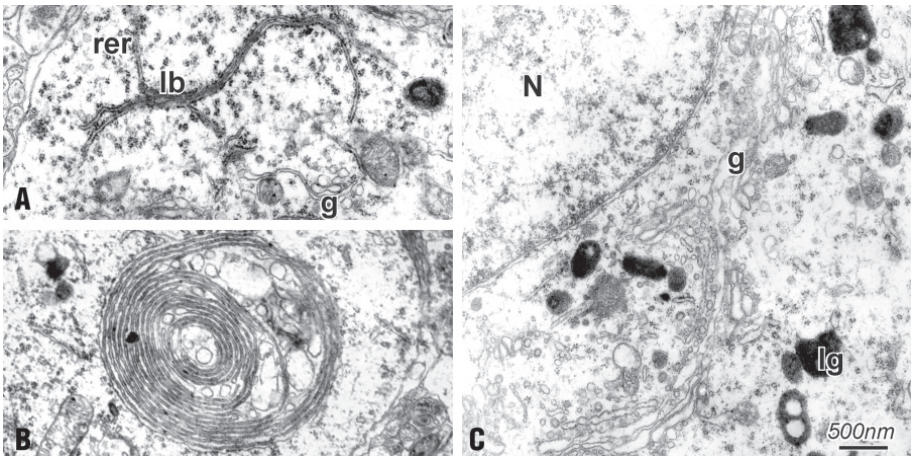

Figure 5. Old rat fasted for 96 hours and refed for 24 hours. **A.** Simple lamellar body (lb) composed of two cisternae of the rough endoplasmic reticulum (rer) which are joined by the third cistern. Part of an extensive Golgi complex (g) in the perinuclear area; **B.** The whorled body in the arcuate neuron. The lamellae are arranged concentrically around a central cytoplasmic core. Irregular vacuoles between several layers are seen; **C.** A large Golgi complex (g) composed of stacked and dilated cisternae and a series of clear vesicles. At one end of the structure, it circles slightly, enclosing part of cytoplasm containing cytoplasmic organelles. Numerous lipofuscin granules (lg) appear near the Golgi complex; nucleus (N). Bar: 500 nm

The serum 8-isoprostane levels in the fasting/ /refed rats was 12.57 ng/mL, whereas in the fasting only animals it averaged 10.84 ng/mL — in both cases being significantly higher compared to the control level of 5.47 ng/mL ($p < 0.05$) (Fig. 6). There was no statistically significant difference between the fasting only and fasting/refed groups of animals.

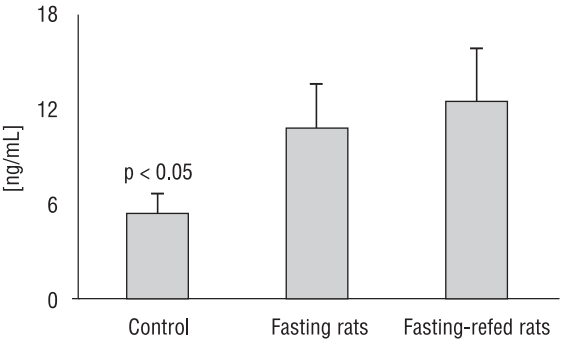

Figure 6. Serum 8-isoprostane levels in the fasting only rats as well as the fasting/refed animals, as compared to the fed control.

DISCUSSION

In our study, in the arcuate neurons of aged control rats, we noted only occasional membra-

nous whorls. Under normal conditions, Brawer [2] described them in the ependymal tanycytes of the arcuate region in male rats, whereas van Houten and Brawer [13] observed these structures in normal male rats in the ventrolateral part of the hypothalamic ventro-medial nucleus (VMN). Our previous studies [6] did not indicate these bodies because the specimens were taken from the central part of the VMN. The results of our studies, in terms of the response of arcuate neurons to fasting and subsequent refeeding, are consistent with the studies of Kiss [5], who also observed a wide variety of endoplasmic reticulum formations after repeated immobilization stress in the ventromedial nucleus of the hypothalamus of male rats. In addition, we observed the early stages of membranous whorl formation from the Golgi complexes. It was probably too short for expression of typical morphology of whorls. Our studies indicated that refeeding for 24 hours did not inhibit the process of membranous whorl formation. Brawer [1] and Price et al. [12] also observed the appearance of whorls in the arcuate neurons after castration or chronic morphine treatment, which had reduced the testosterone level in the blood of male rats. Interestingly, testosterone replacement therapy inhibited the process. It is possible, as some authors have suggested [1, 3, 4] 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 [11]. Whorls were observed in ovariectomised rats [4] and in a mercury-treated female hamster [8] 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 [4] or may be involved in the early stages of the synthesis of LH-RH [10]. In our studies, as well as those of other authors [11], there was an increase of lipofuscin/lysosome granules in the vicinity of the whorls and Golgi region. Some of them may capture LH-RH granules [11]. Lipofuscin granules have been demonstrated to be secondary lysosomes of heterogenous content that derive from autophagic vacuoles. Interestingly, formation of lipofuscin granules is assumed to be a result of intracellular oxidative stress phenomena, and a failure of their removing mechanism during ageing is probably the cause of lipofuscin accumulation in senescent neurons. Lipofuscin itself may in turn be another reason for increased oxidative stress responsible for the formation of whorls. In our experimental model, significant accumulation of lipofuscin in the close proximity of the Golgi complex and membranous whorls strongly suggests that oxidative stress may trigger topological remodelling of those membranes. Moreover, we observed a significant increase (over 2-fold) of the serum level of 8-isoprostane, being a widely accepted marker of *in vivo* lipid peroxidation, in the groups of fasting and fasting/refed rats as compared to the fed controls. Further research is needed to elucidate the intracellular mechanism of the whorl formation in neurons of ARH during short-term fasting.

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