Insulin-induced iron loading in the rat brown adipose tissue: histochemical and electron-microscopic study

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In the present study we have reported an iron-loading in the rat brown adipose tissue (BAT) after 3-day treatment with insulin (4 IU/kg). Light microscopy showed numerous iron-positive cells (Perls' stain) mainly macrophages and brown adipocytes, while electron-microscopic examination revealed lipofuscin granules and phagosomes as iron-containing components. These results clearly indicate that iron participates in damaging of brown adipocytes.

Key words: insulin, iron, brown adipose tissue, rat, histochemistry, electron microscopy.

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Insulin stimulates the uptake and accumulation of iron in isolated fat cells (Davis et al., 1986) increasing the number and distribution of transferrin receptors on the cell surface (Tanner and Lienhard, 1987). Transferrin receptors colocalize with glucose transporters within intracellular vesicles in 3T3-L1 adipocytes (Tanner and Lienhard, 1989; Ramm et al., 1994). Thus, in response to insulin, the transferrin receptors are recruited to the adipocyte plasma membrane together with glucose transporters (Tang et al., 1998). As we have recently shown, high dose of insulin (4 IU/kg) applied to rats for 3 days induces massive glucose uptake and accumulation of glycogen in brown adipocytes (Korac et al., 2000). The aim of the present study was to examine correlation between hyperinsulinemia and iron accumulation in BAT.

It is also known that iron may acts as co-factor in potentiating of the cell injury. Modest tissue concentration of iron induces lipid peroxidation and accumulation of damaged macromolecules (Powell and Yapp, 2000). But, exess iron is extremely harmful and may even promote cell death (Halliwell and Gutteridge, 1999). Our previous results have revealed that hyperinsulinemia causes either lipofuscin accumulation (Korac et al., 1998) or apoptosis of brown adipocytes (Korac et al., 1999). This was the reason to analyse the type and ultrastructural features of iron-containing cells, in the light of relation between iron accumulation in BAT and tissue damage.

Materials and Methods

Experimental animals and design

Male rats of Wistar strain (Rattus norvegicus) weighing 200-210 g were used for the experiments. The animals were born at and acclimated to 22 ± 1°C. They were maintained under intermittent 12 h periods of light and dark and were given commercial rat food (Subotica, Yugoslavia) and tap water ad

libitum.

The rats were divided into two groups, each consisting of six animals. The first group was treated intraperitoneally with insulin (ICN Galenika, Belgrade) at a dose of 4 IU/kg body mass, over 3 days (one injection daily). The second group was injected with 0.9% saline (1 mL/kg) in the same manner as the first group and served as control. Three hours after the last injections, all animals were killed by decapitation with a guillotine (Harvard Apparatus, USA). The interscapular portion of their BAT was removed and processed for light and electron microscopy.

Light microscopy

A half portion of BAT was fixed for 48 hours in neutral buffer solution containing 10% formaldehyde. After fixation, the tissue was routinely processed, embedded in paraffin and 5mm thick sections were obtained. For staining with Prussian blue (Perls' reaction), the sections were incubated in freshly prepared mixtures of 2% potassium ferrocyanide and 2% hydrochloric acid for 15 min. They were washed well in water and counterstained with 0.1% nuclear fast red for 5 min. The sections were then washed again in water, dehydrated, cleared and mounted in DPX. The sections were examined by Leica (Germany) light microscope.

Electron microscopy

The remaining half of BAT was cut into small pieces, were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and postfixed in 1% osmium tetroxide in the same buffer. The specimens were dehydrated through serial alcohol solutions of increasing concentration, and were embedded in Araldite (Fluka, Germany). The blocks were trimmed and cut with glass knives on an LKB III ultramicrotome (Broma, Sweden). The thin sections were mounted on copper grids, stained either with uranyl acetate and lead citrate or unstained and examined with a Philips MC 12 electron microscope.

Results

In BAT of insulin treated rats, staining with Prussian blue was observed in phagocytic cells (macrophages) situated in the interstitium and in brown adipocytes (Figure 1b). Staining was strong and cytoplasmatic. In some brown adipocytes fine blue granules were also observed. By contrast, none of the sections from BAT of control rats were stained with Prussian blue (Figure 1a).

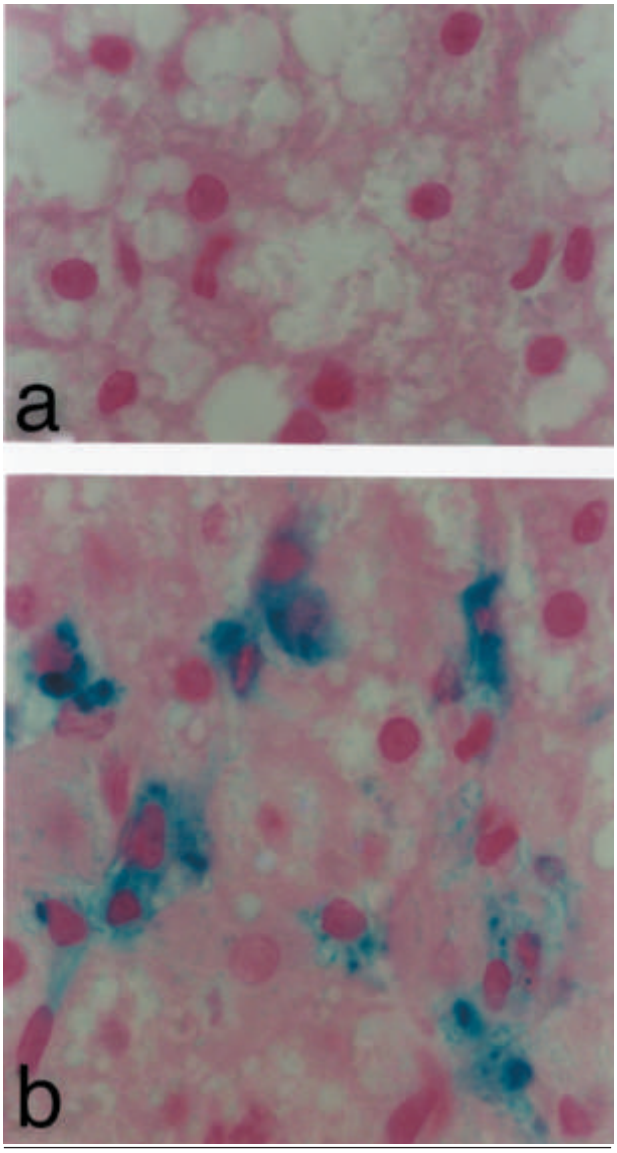

Figure 1. Staining with Prussian blue of brown adipose tissue (BAT) in control (a) and insulin treated rats (b). Iron-positive (blue) deposits are present in the cytoplasm of the majority of macrophages and brown adipocytes after insulin treatment (b). No staining was observed in BAT of control rats (a).

Electron microscopy confirmed deposition of iron in BAT of insulin treated rats (Figures 2a, b). In the macrophages electron-dense, iron-positive granules were observed mainly in the lysosomal compartment. This was especially notable in the lipofuscin granules (Figure 2a) which corresponded to the blue iron-positive granules on the light microscope

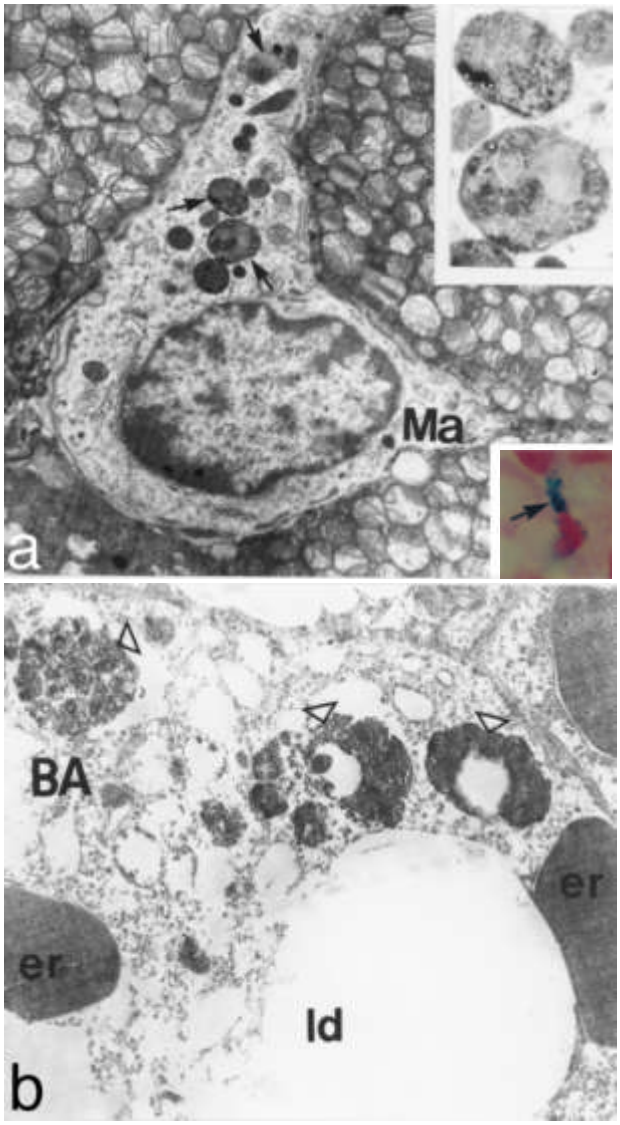

Figure 2. Electron micrographs of macrophage (a, Ma) and brown adipocyte (b, BA) from insulin treated rats. Note the numerous lipofuscin (→) in the macrophage cytoplasm which corresponds to blue, iron-positive granules (→, a-lower right insert). On unstained sections, electron-dense and iron-containing particles are visible (a-upper right insert). In the brown adipocyte (b, BA) osmiophilic aggregates (open triangles) are located near phagocytozed erythrocytes (er). Some of the aggregates partly enclose small lipid droplets (ld). The aggregates have no limiting membrane and appear embedded in the cytoplasm. There are several other abnormalities alterations, namely, the cytoplasm of brown adipocytes appears disrupted and there is loss and distortion of mitochondria. Magnification: Figure 1. (a, b)-×100, orig.; Figure 2. (a)-×7600; (a-upper right insert)-×22400; (a-lower right insert)-×100, orig.; (b)-×10850.

(Figure 2a -insert, lower right). On unstained section lipofuscin granules had electron-dense particles (Figure 2a-insert, upper right).

Some of the iron-positive brown adipocytes also showed lipofuscin which corresponded to the fine cytoplasmatic blue granules (not shown here). The majority of iron-positive brown adipocytes showed signs of the erythrophagocytosis and serious damage of organelles (Figure 2b). Namely, the cytoplasm of brown adipocytes appeared disrupted, and loss and distortion of mitochondria were obvious. Large aggregates with electron opaque cores were visible and they appeared free in the cytoplasm in the vicinity of phagocytosed erythrocytes. Two of three observed aggregates were associated with a small lipid droplet, partly enclosing it.

Discussion

BAT dysfunction is a commonly observed feature in several animal models for obesity and diabetes (Himms-Hagen, 1990; Lowell et al., 1993). Although hyperinsulinemia is also part of those metabolic syndrome diseases (Rothwell and Stock, 1988; Cusin et al., 1992), the exact molecular events linking insulin and BAT dysfunction are poorly understood.

The observation presented herein provides information on iron-accumulation in rat BAT after 3-day treatment with high dose of insulin. Using Prussian blue, the specific histochemical staining for iron (Perls' reaction), we have observed iron deposition in macrophages and brown adipocytes, suggest that hyperinsulinemia induces accumulation of iron in BAT.

Electron microscopic examination of unstained thin sections is more sensitive method than Perl's reaction for identifying storage iron. Therefore we were able to analyze through both techniques for identification of storage cells and the type of iron-aggregation. The electron microscopic examination of the former disclosed iron-containing particles mainly in the lipofuscin of brown adipocytes and macrophages.

The origin of lipofuscin is still controversial. Several investigators have regarded lipofuscin as an end product of molecular damage of cell organelles by oxygen free radicals (Tappel, 1975; Sohal et al., 1989), a process enhanced by iron. Namely, increased iron level could predispose the cell to oxidative stress. In addition, lipid peroxidation is stimulated by a wide range of iron complexes and certain membrane-associated structures can be considered as targets of iron-induced peroxidative damage: mitochondria, cytoplasmatic membranes and lysosomes (Peters et al., 1977; Britton et al.,

1987). Since brown adipocytes are extremely rich in mitochondria the observed appearance of lipofuscin in these cells could be ascribed to iron-mediated mitochondrial damage. Knowing the fact that mitochondria have a central role in cell death regulation (Kroemer et al., 1998), it could be predicted that hyperinsulinemia causes cumulative mitochondrial damage leading to apoptosis. By phagocyting apoptotic bodies, interstitial macrophages accumulate iron-riched lipofuscin, as we have observed.

Nakoe *et al.* (2001) reported intriguing evidence that most of apoptotic cells in the diaphragm muscle of dystrophic mice contain accumulated lipofuscin. We found that hyperinsulinemia induces brown adipocytes apoptosis (Korac et al., 1999), but we have not established yet a direct relationship between apoptosis and lipofuscin accumulation. Besides, no literature data, to the best of our knowledge, indicate this relationship in BAT.

The finding that erythrophagocytotic brown adipocytes are iron-positive and contain large osmiophilic aggregates is of special interest. Since brown adipocytes are capable for erythrophagocytosis (Radovanovivc et al., 1996) iron-positive aggregates seem to be, at least partly, products of erythrophagosomal degradative pathway. Collectively, our previous results and those presented here suggest that iron appears to play an important role in BAT damaging caused by hyperinsulinemia, which leading to BAT dysfunction.

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