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CALCIUM-SANDOZ®-INDUCED ERYTHROCYTE EXOVESICULATION AND INTERNALIZATION OF HEMICHROMIC MATERIAL INTO RAT BROWN ADIPOCYTES

MILICA MARKELIĆ, KSENIJA VELIČKOVIĆ, I. GOLIĆ, MIRELA UKROPINA, MAJA ČAKIĆ-MILOŠEVIĆ, VESNA KOKO and ALEKSANDRA KORAĆ *

University of Belgrade, Faculty of Biology, Institute of Zoology and Center for Electron Microscopy, 11000 Belgrade, Serbia

Abstract - An ultramicroscopic study of brown adipose tissue (BAT) of rats treated with Ca-SANDOZ® (480 mg/l) for 3 days, revealed erythrocyte exovesiculation and migratory erythrocytic complexes from the capillaries to adipocyte cytoplasm and mitochondria. Two types of erythrocytic material transfer were observed: (i) numerous exocytic vesicles with electron dense material leaving the erythrocytes; (ii) furcated complexes with microholes, embedded in amorphous material. The content of red blood cell (RBC) complexes passed through the capillaries and transferred to the brown adipocytes where it was detectable in the cytoplasm and mitochondria. Light microscopy confirmed sphenoechinocytic transformation of the RBCs in the blood smears of the Ca-SANDOZ® treated rats.

Key words: Exovesiculation, red blood cell, brown adipocyte, calcium, hemoglobin, mitochondria.

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INTRODUCTION

During their life, the integrity of erythrocytes is constantly challenged. It is well documented that they are exposed to oxidative stress in the lung, to osmotic shock in the kidney, they have to pass through capillaries which have a lumen that is smaller than their size. Their rupture and release of hemoglobin could affect pathological conditions and organ failure (Lang et al., 2005). To avoid these complications, as with any other cells, red blood cells require a mechanism allowing them to be disposed without the release of intracellular content.

Since erythrocytes are devoid of nuclei and mitochondria, they lack crucial elements of the apoptotic machinery. However, recently it was observed that erythrocytes can also die by suicidal event which is characterized by cell shrinkage, membrane blebbing and plasma membrane phospholipid scrambling, with phosphatidylserine exposure at the cell surface. Lang et al. (2005) suggested the term "eryptosis" to distinguish the death of erythrocytes from the apoptosis of nucleated cells. The main cells recognizing these phosphatidylserine-exposing erythrocytes are macrophages (Fadok et al., 2000) which engulf and degrade the affected cells (Boas et al., 1998; Eda and Sherman, 2002). Significant changes in the biological properties of RBCs can be affected by minor shifts in intracellular calcium ion concentration (Allan and Thomas, 1981; Allan et al., 1989; Friederichs et al., 1989; Hagelberg and Allan, 1990), while excessive elevations of Ca^{2+} are deleterious to cell function and survival. Namely, erythrocytes have an approximately 10^4-fold smaller Ca^{2+} concentration than the blood plasma. This is controlled by the intake of Ca^{2+} in the diffusion process through the plasma membrane and its extrusion by a highly efficient Ca^{2+} pump (Schrier et al., 1980). Increased intracellular Ca^{2+} levels are associated with various concomitant chemical and structural alterations: (i) losses of K^+ and cell water, leading to cell shrinkage

(Gardos, 1958; Bookchin et al., 1987; Brugnara et al., 1993; Franco et al., 1996); (ii) polyphospholipid breakdown to diacylglycerol whose accumulation in the inner lipid layer leads to exovesiculation (Allan and Michell, 1975); (iii) changes in membrane polypeptide and phospholipid organization; (iv) spheroechinocytic shape transformations; and (v) decrease in cell membrane deformability (Kuettner et al., 1977; O'Rear et al., 1982; Mark et al., 2000).

In our previous work, we showed that upon thermogenic stimulation, the high blood flow in BAT leads to excessive erythrocyte extravasation (Radovanovic et al., 1996). Furthermore, brown adipocytes have the ability to perform phagocytosis and degradation of erythrocytes (Radovanovic et al., 1996; Grubic et al., 2008). The general aim of the present work was to study whether the great demand of this tissue for erythrocytic catabolic material could be provided by the degradation products of erythrocytes that are transferred from the capillary lumen to the BAT. In order to contribute to the elucidation of the role of erythrocyte exovesiculation in the biology of BAT cells, an electron microscopic study of rats treated with Ca-SANDOZ was performed. Calcium treatment was used in order to reveal how the erythrocytic material is transported from the capillaries to the BAT cells and their organelles, since it is known that hypercalcemia can affect the exovesiculation of RBCs (Greenwalt, 2006).

MATERIALS AND METHODS

Male Wistar rats weighing 200-250 g were used in this study. The animals were kept in individual cages, with standard food and water available *ad libitum*. The animals were cared for in accordance with the principles of the *Guide to the Care and Use of Experimental Animals*. The animals were randomly divided into two groups: 1) rats that drank Ca-SANDOZ® (Sandoz Pharmaceuticals, Germany) in an aqueous solution (480mg/l Ca^{2+} during three days); and 2) rats that drank tap water *ad libitum* (intact control).

The animals were killed by decapitation. For transmission electron microscopy the interscapular brown adipose tissue was removed and fixed immediately in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2). Specimens were postfixed in 2% aqueous osmium tetraoxide, dehydrated through graded concentrations of ethanol and embedded in Araldite (Fluca, Germany). Ultrathin sections were cut with a LKB III ultramicrotome (Broma, Sweden) and mounted on copper grids. Unstained and stained (uranyl acetate and lead citrate) sections were examined with a Philips CM12 transmission electron microscope (Philips, The Netherlands).

Routinely prepared blood smears were used for the light microscopy analysis of erythrocyte morphology.

RESULTS

Exovesiculation and dislodging of erythrocytic complexes into brown adipocytes

The intensity of delivery of the erythrocytic material was very pronounced in the Ca-SANDOZ®-drinking rats (Fig. 1). Inside of many RBCs, hemichromic material, a product of hemoglobin degradation, was visible (Fig. 1a). Also, the appearance and dislocation of numerous electron-dense, hemichromic complexes in the form of exocytic vesicles, from capillary erythrocytes to brown adipocyte mitochondria, was frequently observed. Furthermore, blood smears of the Ca-SANDOZ®-treated rats revealed sphenoechinocytic transformation of RBCs (Fig. 1c). The formation of hemichromic complexes was initiated in the capillary erythrocyte; they left the erythrocyte by budding on the plasma membrane and as dark stained vesicles, traversed the endothelium and intercellular space (Fig. 1a) entering into the cytoplasm and mitochondria of brown adipocytes (Fig. 1b). The capillary wall was narrower at the place of transfer than on the rest of the wall (Fig. 1d). In the BAT control animals, RBC exovesiculation was very rarely seen.

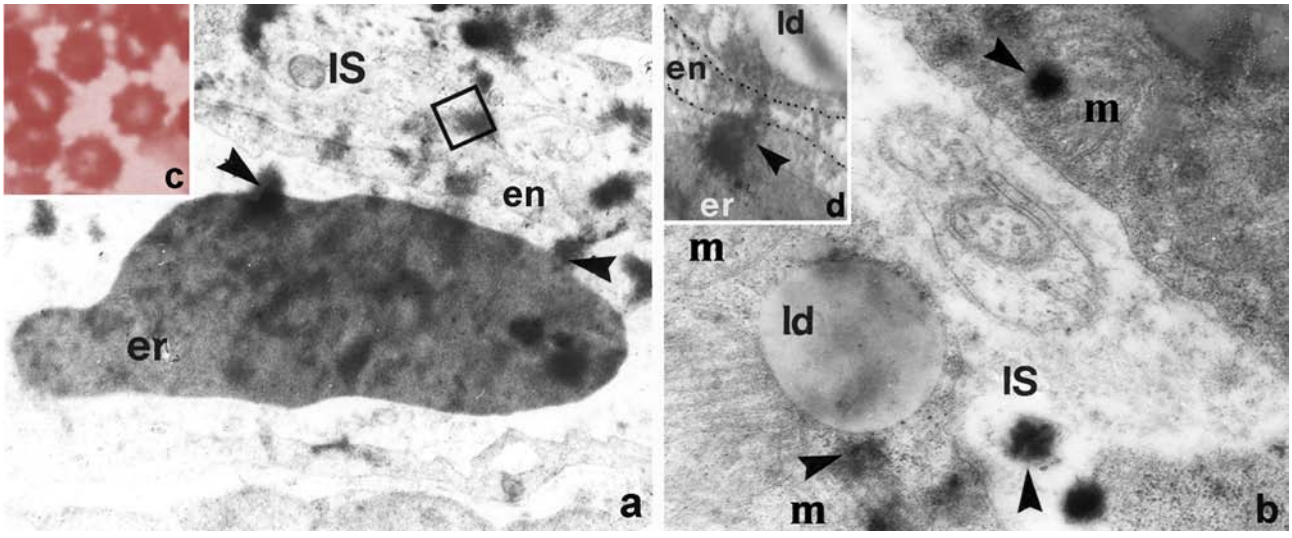

Fig. 1 (a;b;c;d). The electron micrographs reveal the process of erythrocyte exovesiculation and route of hemichromic complexes migration in BAT of Ca-SANDOZ® treated rats: (a) degradation of hemoglobin and hemichromic material formation inside of erythrocyte (**er**), budding of erythrocytic vesicles (**arrowhead**) and transfer of hemichromic material (**open square**) through endothelial cells (**en**) and intercellular space (**IS**); (b) numerous Ca-SANDOZ® - induced complexes (**arrowhead**) from the intercellular space enter brown adipocyte cytoplasm and mitochondria (**m**). At the place of erythrocytic material transfer, endothelial wall (**en**) is narrowed (d). Light microscopy of blood smears shows sphenoechinocytic transformation of erythrocytes (c). (**ld**) - lipid droplet. Magnifications: (a) 16000x; (b) 17000x; (c) 20x; (d) 35200x.

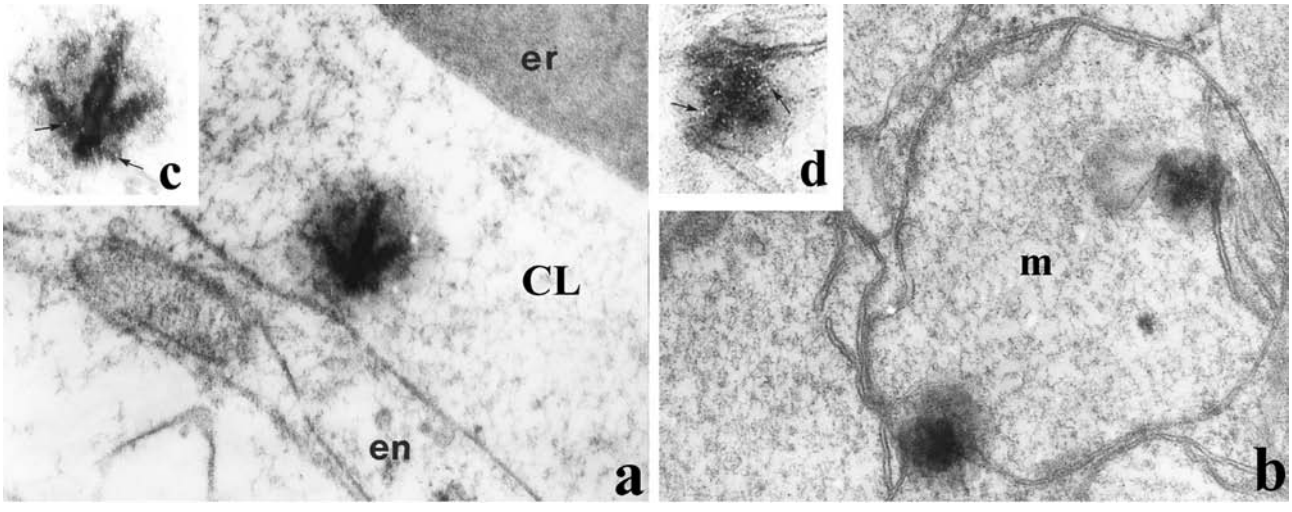

Fig. 2 (a;b;c;d). Ca-SANDOZ®-induced furcated migratory complexes: (a) in the capillary lumen (**CL**) a swallow-like shape complex with microholes (**arrow**) (c), (b) two complexes in swelling mitochondria (**m**) of brown adipocyte, one in the entering phase and the other in the mitochondrion in close contact with cristae, both with microholes (**arrows**) (d). (**er**) - erythrocyte. Magnifications: (a) 68200x, (b) 124400x, (c) 84000x, (d) 123200x.

Microholes and dark clouds were observed during the bypassing process in migratory Ca-SANDOZ®-induced complexes

Some of the Ca-SANDOZ®-induced erythrocytic complexes were furcated, surrounded by an amorphous material and with microholes (Fig. 2). These migratory formations were analyzed in more detail: (i) a swallow-like complex leaving the erythrocyte, in close contact with the capillary wall (Fig. 2a); (ii) another two formations were observed in a brown adipocyte, one of them entering a mitochondrion; the other was cross-like and in close contact with mitochondrial cristae (Fig. 2b). All three formations were embedded in a cloud-like amorphous material and had visible microholes (Fig. 2c, d).

DISCUSSION

In the BAT of Ca-SANDOZ® and, to a smaller degree, in the BAT of the untreated, control animals, signs of erythrocytic material transfer to brown adipocytes were visible. Bearing in mind our previous study in which the erythrophagocytic activity of brown adipocytes was shown (Grubic et al., 2008), the intake of RBCs or their material could be considered as one of the features of brown adipocytes, at least in the BAT of rats.

Ca-SANDOZ®-induced exovesiculation of erythrocytes

As previously described by other investigators (Gardos, 1958; Beaven and Gratzer, 1980; Friederichs et al., 1992), calcium-loaded RBCs showed progressive spheroechinocytic morphological changes, and the release of microvesicles from the spicules in the form of membrane-encapsulated red cell fragments (Allan and Thomas, 1981; Muller et al., 1981; Laczko et al., 1985; Comfurius et al., 1990; Bucki et al., 1998; Mark et al., 2000; Kelemen et al., 2001; Wolfs et al., 2003; Liu et al., 2005). Up to 20% of the total RBC membrane lipids are consumed to form microvesicles following an elevation of Ca^{2+} (Allan et al., 1976; Butikofer et al., 1989). Two types of vesicles, differing in size, have been described; these have been referred to as microvesicles (150 nm diameter) and nanovesicles (60 nm diameter) (Allan et al., 1980). The vesicles released from erythrocytes after treatment with calcium and ionophore A23187 are free of cytoskeletal components and are specifically enriched in glycosylphosphatidylinositol (GPI)-anchored proteins (Butikofer et al., 1989) which are known to be specifically located in the lipid rafts of different cell types (Simons and Toomre, 2000), including erythrocyte (Lauer et al., 2000; Salzer and Prohaska, 2001). There is increasing evidence that lipid rafts are involved in membrane budding and vesiculation during diverse biological processes (Huttner and Zimmerberg, 2001). Recent work on overhydrated hereditary stomatocytosis (OHSt) has described a rare type of hemolytic anemia where the erythrocytes assume a stomatocytic shape, exhibiting an inward bending of their membrane and major leaks of Na^+ and K^+ across the plasma membrane. Wilkinson et al. (2008) showed that the Ca^{2+}-dependent release of exovesicles is altered in stomatin-deficient OHSt erythrocytes. They suggested that actin and stomatin are linked on lipid rafts in normal erythrocytes, and that when internal Ca^{2+} is increased, this bridge is broken, possibly by competition from calcium-dependent proteins such as annexin VII, sorcin (Maki et al., 2002) or copine 1 (Creutz et al., 1998). Moreover, the phospholipid scramblase is activated and the aminophospholipid translocase is inhibited (Zwaal and Schroit, 1997; Zhou et al., 2002), thereby leading to a randomization of the phospholipid asymmetry over the two membrane leaflets and to phosphatidylserine exposure on the erythrocyte surface (Lang et al., 2003a). Phosphatidylserine on the erythrocyte surface is recognized by phagocytes, primarily macrophages (Fadok et al., 2000, and these affected cells are rapidly engulfed and degraded (Boas et al., 1998; Eda and Sherman, 2002). The elimination of these cells without the release of intracellular content prevents inflammation (Gulbins et al., 2000). Moreover, phosphatidylserine-exposing RBCs can adhere to the vascular wall (Lang et al., 2005). The calcium-activated cellular loss of K^+ contributes to the triggering of eryptosis by causing the loss of cell water and con-

comitant shrinkage of the erythrocyte (Allan and Thomas, 1981; Lang et al., 2003b). The latter factors are essential for the release of the vesicles from the echinocytic erythrocytes.

It could be assumed that in our Ca-SANDOZ® experiment, Ca^{2+} exposure led to the decrease in life span of mature erythrocytes by facilitating exovesiculation which is visible as migratory erythrocytic complexes, probably representing vesicles disassembled from affected cells, passing through endothelial cells and entering brown adipocytes. Namely, although earlier studies showed no effect of increased plasma Ca^{2+} concentration in the absence of calcium ionophore A23187 (Mark et al., 2000), our results indicate that Ca-SANDOZ®-induced hypercalcemia *per se* affects the formation of exocytic vesicles with electron dense content from the RBC plasma membrane, at least in the BAT of rats.

The hemichromic content of electron-dense Ca^{2+}-induced complexes and their assumed function in brown adipocytes

The question arises about the content of calcium-induced electron-dense complexes and also, the requirement of brown adipocytes for these complexes. It has been established thaty hemichromes (hemoglobin degradation products), the precursors of Heinz bodies, can be induced by certain experimental conditions (Waugh and Low, 1985) and in normal conditions in aged cells (Sears et al., 1975). It is known that in the plasma membranes of abnormal and old RBCs, band 3 (major erythrocyte membrane protein) is found in the Heinz body-protein aggregates (Low et al., 1985). Band 3 isolated from older red cells contains higher oligomeric forms than the band 3 from younger cells, and its origin appears to be linked to red cell aging (Lutz and Stringaro-Wipf, 1983). Namely, band 3 undergoes tyrosine phosphorylation after decrease in cell volume, as occurs when erythrocytes lose K^+ and release microvesicles after treatment with Ca^{2+}/Ca^{2+} ionophore (A23187) (Minetti and Low, 1997). The oxidative transformation of oxyhemoglobin to hemichrome enhances its interaction with erythrocyte membrane. Association between denatured globin and band 3 contributed to the formation of insoluble copolymer of macromolecular dimension. Hemichrome-induced clustering of band 3 promotes the generation of epitopes on the erythrocyte cell surface. This provides a signal favoring immunologic recognition of redistributed band 3 by autologous IgG followed by erythrophagocytosis (Saha Roy et al., 2009). Moreover, the highest oligomeric forms may be directly involved in red cell senescence (Casey and Reithmeier, 1991).

Heinz bodies can also be removed by proteolytic cleavage. Microholes are found in some of our Ca-SANDOZ®-induced migratory complexes (Fig. 3). The microholes could result from proteolytic activity. High amounts of proteasomes are found in the erythrocytes under membrane complexes (Tanaka and Ichihara, 1990). Also, it was suggested that monoubiquitinated α-globin, but not free α-globin, can be degraded directly by purified 26S proteasomes (Shaeffer and Kania, 1995). The dark staining clouds surrounding migratory complexes and microholes may indicate that iron is liberated by proteolytic cleavage, as was shown in the process of erythrocytic aging (Signorini et al., 1995).

Spleen-like function of BAT and the possible role of erythrocyte exovesiculation and erythrocytic material intake in brown adipocyte

The experimental approach with Ca-SANDOZ® allowed us to have a more complete picture concerning the process of the formation of numerous dark-staining complexes and their transport to the mitochondria, suggesting a spleen-like, pitting function for brown adipocytes. Namely, Heinz bodies as well as other erythrocytic inclusions, are removed (pitted) by the spleen with little damage to the red cells during the maturation of normal RBC thanks to its deformability, allowing their passage through tight splenic sinuses by simply amputating their portions containing not-so-deformable inclusions (Crosby, 1977). Lux and John (1977) also described a high molecular weight red cell membrane protein complex that is normally removed by the spleen. It is

also possible that calcium-induced RBCexovesicles are removed in a similar way in BAT. Namely, BAT is highly vascularized and the removing of erythrocyte inclusions could be an important factor in blood flow maintenance.

In one of our previous studies, the iron loading of brown adipocytes was shown (Korac et al., 2003). Also, Gear (1965a, b) showed that radioactive iron (^{59}Fe) was most actively taken up by well-washed mitochondria during early liver regeneration. The need of brown adipocytes for hemoglobin catabolic materials is mitochondriogenic and thermogenic in the direction of energy dissipation. In isolated brown adipose mitochondria, hemin uptake caused a significant inhibition of the ATP-stimulated degradation of mitochondrial translation products without a significant effect on basal protein degradation (without ATP) (Desautels and Dulos, 1994). The inhibitory effect of hemin was specific as an immediate precursor of heme. It was suggested that heme plays a role in BAT mitochondriogenesis by its action on protein synthesis and degradation within this organelle (Atamna et al., 2002). It is well known that BAT is an important site of increased heat production and dissipation. Glucose utilization (Vallerand et al., 1990; Shimizu et al., 1991) and fatty acid oxidation as well as blood flow (Foster and Frydman, 1978) are also known to be enhanced in parallel with heat production. Also, the hemoglobin molecule displays several parallel biological functions within an organism besides the basic function of oxygen transport. For our work, it seems to be important to underline its function as a molecular heat transducer (Giardina et al., 1995).

Hence, iron loading (Korac et al., 2003), internalization of entire RBCs (Radovanovic et al., 1996; Grubic et al., 2008) and the herein presented intake of hemichromic material by brown adipocytes, indicate an important role of iron in brown adipocyte physiology. Depending on the brown adipocytes' demand for iron, the intake of complete erythrocyte or its hemichromic material, respectively, could occur.

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