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Original Contribution

Ferritin-stimulated lipid peroxidation, lysosomal leak, and macroautophagy promote lysosomal "metastability" in primary hepatocytes determining *in vitro* cell survival

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ABSTRACT

Several pathologies are associated with elevated levels of serum ferritin, for which growth inhibitory properties have been reported; however, the underlying mechanisms are still poorly defined. Previously we have described cytotoxic properties of isoferritins released from primary hepatocytes *in vitro,* which induce apoptosis in an iron and oxidative stress-dependent mode. Here we show that this ferritin species stimulates endosome clustering and giant endosome formation in primary hepatocytes accompanied by enhanced lysosomal membrane permeability (LMP). In parallel, protein modification by lipid peroxidation-derived 4-hydroxynonenal (HNE) is strongly promoted by ferritin, the HNE-modified proteins (HNE-P) showing remarkable aggregation. Emphasizing the prooxidant context, GSH is rapidly depleted and the GSH/GSSG ratio is substantially declining in ferritin-treated cells. Furthermore, ferritin triggers a transient upregulation of macroautophagy which is abolished by iron chelation and apparently supports HNE-P clearance. Macroautophagy inhibition by 3-methyladenine strongly amplifies ferritin cytotoxicity in a time- and concentration-dependent mode, suggesting an important role of macroautophagy on cellular responses to ferritin endocytosis. Moreover, pointing at an involvement of lysosomal proteolysis, ferritin cytotoxicity and lysosome fragility are aggravated by the protease inhibitor leupeptin. In contrast, EGF which suppresses ferritin-induced cell death attenuates ferritin-mediated LMP. In conclusion, we propose that HNE-P accumulation, lysosome dysfunction, and macroautophagy stimulated by ferritin endocytosis provoke lysosomal "metastability" in primary hepatocytes which permits cell survival as long as in- and extrinsic determinants (e.g., antioxidant availability, damage repair, EGF signaling) keep the degree of lysosomal destabilization below cell death-inducing thresholds.

Introduction

In maintaining proper cell function, the indispensable need for iron is a challenging issue since ferrous iron and H_2O_2 will produce highly reactive hydroxyl radicals via the Fenton reaction [1] which will initiate further oxidative effects such as lipid peroxidation. Hence, by controlling cellular Fe^{2+} availability the iron storage protein ferritin is pivotal to cell integrity serving as cytoprotectant especially under prooxidant conditions [2]. However, growth inhibitory properties have been reported for certain ferritin isoforms belonging to the class of secreted "serum" ferritins [3] including acidic isoferritins released from primary rat hepatocytes *in vitro*, which have been shown to exert cytotoxic effects [4,5].

Secreted serum ferritins will enter the cell via receptor-mediated endocytosis (RME) which involves specific ferritin binding sites as well as the transferrin receptor (TfR) [6]. On RME,

Abbreviations: ACD, accidental (necrotic) cell death; AO, acridine orange; CM, conditioned medium; DAPI, 4′,6-diamidino-2-phenylindole; DFO, desferrioxamine mesylate; EGF, epidermal growth factor; ELC, endo-/lysosomal compartment; GFL, green AO fluorescence; GSH, glutathione (reduced form); GSSG, oxidized glutathione; ferritin-CM, ferritin-enriched conditioned medium; HNE, 4-hydroxy-2-nonenal; HNE-P, HNE-modified proteins; IF, immunofluorescence; LC3, autophagy-related microtubule–associated protein 1 light chain 3; LMP, lysosomal membrane permeability; LEU, leupeptin; 3-MA, 3-methyladenine; MEM, minimum essential medium; MOMP, mitochondrial outer membrane permeability; PCD, programmed cell death; PI3K, phosphoinositide-3-kinase; PI3K-Vps34, phosphoinositide-3-kinase vacuolar protein sorting 34; RFL, red AO fluorescence; RME, receptor-mediated endocytosis; ROS, reactive oxygen species; TfR, transferrin receptor

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ferritin is directed to the endo-/lysosomal compartment (ELC) where the ferritin-stored iron is liberated on proteolytic degradation [7], though proteolysis-independent mechanisms may also exist [8]. Iron can exit the ELC via iron transporters such as DMT-1, Zip14, and TRMPL-1 [9–11] or is directly transferred to iron consumers such as mitochondria by a "kiss and run" mechanism [12]. Thus, the ELC pool of redox-active iron is regulated by the balance between the transferrin/ferritin endocytosis and the export of iron to cellular acceptors including cytosolic ferritin. Additionally, autophagy of metalloproteins and iron-rich organelles destined for lysosomal degradation [13] will also affect the ELC iron balance. Concerning the uptake of serum ferritin, a reported iron content of 160–180 iron atoms per ferritin molecule [14] appears low with respect to a maximum storage capacity of more than 4000 iron atoms [2]. Nevertheless, 80–90 times more iron will enter the ELC when ferritin is taken up via TfR-based RME instead of iron-loaded transferrin binding two iron atoms. In addition, albeit TfR-based ferritin endocytosis will be regulated by cellular iron levels, ferritin may enter the cell also via binding to non-iron-regulated asialoglycoprotein receptors [15] which will charge the ELC iron pool in a less controlled way. Hence, ferritin endocytosis represents a considerable challenge for the ELC and a tight control of the "labile" lysosomal iron pool is mandatory to prevent lysosomes from becoming iron overloaded "suicide bags" [16]. Previously we have hypothesized that lysosomal iron overload, enhanced lipid peroxidation, and resulting lysosomal membrane permeability (LMP) could be causal to the cytotoxic properties of acidic serum ferritins released from primary hepatocytes in vitro [17]. Although this concept is supported by experimental evidence demonstrating an increased vulnerability of iron overloaded lysosomes to oxidative stress including the possible onset of cell death [13,18], the ability of serum ferritins to mediate cell death at physiologically relevant concentrations by ELC interference has not yet been demonstrated. Importantly, high amounts of hepatocyte-derived serum ferritin [$>$ 1 μg/ml, i.e., $>$ 2 nM] stimulate necrotic cell death while apoptosis is induced at concentrations [50–500 ng/ml, i.e., 0.1–1 nM] which are also found in normal human and rat serum [12–300 ng/ml which equals ~0.02–0.6 nM] [4,19,20]. Thus, together with the fact that serum ferritin levels above 300 ng/ml are considered pathologic [19] and the reported implications of serum ferritins in immunity and disease [3], ferritin-mediated cell death definitely represents an issue of medicinal interest. Moreover, it has been suggested that ferritin serves the iron transfer between cells of the brain and in the liver [21,22]—without doubt a process that demands special adaptation of the recipient cell to increased lysosomal iron levels.

So far issues directly associated with ferritin-induced apoptosis have been dissected showing an upregulation of p53, stimulation of Fas (CD95), Bid cleavage, and mitochondrial outer membrane permeability (MOMP) [5]; however, little is known on potential upstream mechanisms such as the involvement of lysosomes. Therefore, we investigated the ELC in ferritin-treated primary rat hepatocytes following a single-cell, live-imaging-based approach using acridine orange (AO), a fluorescent dye which accumulates in intact acidic organelles comprising endosomes, lysosomes, and auto(phago)lysosomes. In addition, macroautophagy has also been addressed by immunofluorescent (IF) staining and Western blot analysis for the autophagy marker LC3 (microtubule-associated protein 1 light chain 3) [23]. Functional aspects of a putative ferritin–ELC interference, including the analysis of cytotoxic endpoints (apoptosis, necrosis), have been examined by employing (i) the macroautophagy inhibitor 3-methyladenine (3-MA) [24], (ii) inhibition of lysosomal proteolysis by leupeptin (LEU), and (iii) proliferative stimulation via EGF/insulin (EGF/I), which counteracts ferritin cytotoxicity [5,25]. Initiated by earlier findings [17], also the involvement of protein modification by 4-hydroxynonenal and changes of the cellular GSH levels and the glutathione redox status (GSH/GSSG) have been addressed.

As will be shown, treatment of primary hepatocytes with secreted isoferritins at physiologically relevant concentrations induced an "expansion" of the ELC including the onset of macroautophagy, rapidly shifting the levels of aldehyde-modified (HNE-His adducts) protein, which was accompanied by GSH depletion and a drop of the GSH/GSSG ratio. Based on this and the observed interference with 3-MA, LEU, and EGF/I we suggest that the prooxidant challenge exerted by serum ferritin endocytosis generates a "metastable" ELC condition which triggers cell death when cellular stress responses fail to adequately counteract ferritin-based lysosome dysfunction.

Materials and methods

Animals/materials

Adult 8–12 weeks old female Fischer 344 rats (Charles River, Germany) were kept in a temperature and humidity-controlled room with a 12 h light–dark cycle. Food and water were provided ad libitum. Animals were allowed to acclimate for at least 2 weeks prior to hepatocyte isolation. MEM with Earle's salts and nonessential amino acids were obtained from Invitrogen, Vienna, Austria. Antibiotics were obtained from Lonza through Szabo-Scandic, Vienna, Austria. Collagenase and other cell culture chemicals—unless otherwise specified—were obtained from Sigma-Aldrich, Vienna, Austria. Recombinant human EGF was obtained from Sanova, Vienna, Austria. Percoll was obtained from GE Healthcare, Vienna, Austria. Plasticware was obtained from Sarstedt, and Falcon/Beckton Dickinson, Austria.

Cell culture medium

Serum-free minimum essential medium (Invitrogen No. 41500–018), supplemented with aspartate (0.2 mM), serine (0.2 mM), pyruvate (10 mM), penicillin (100 U), and streptomycin (100 μg/ml) was used, indicated as MEM in the text.

Isolation of primary parenchymal hepatocytes

Parenchymal rat hepatocytes were isolated by the two-step *in situ* collagenase liver perfusion technique described by Michalopoulos et al. [26]. After washing twice in calcium-free buffer (142 mM NaCl, 6.7 KCl, 10 mM Hepes, pH 7.4) cell numbers and viability (trypan blue exclusion assay) of the freshly isolated cells were determined. Only preparations with a viability $\geq$ 90% ($\geq$ 80% for GSH analysis) were used for experimental work. Dead and remaining nonparenchymal cells were removed from the crude cell pellet in a self-generating Percoll density gradient as described previously [4].

Preparation of ferritin-enriched, conditioned medium and ferritin purification

The number of 5×10^6 viable cells was plated on 90-mm-diameter collagen-coated petri dishes containing 10 ml MEM and incubated for 3 h under standard culture conditions (37 °C, 5% CO_2, 95% relative humidity) and the culture supernatant, i.e., the ferritin-conditioned medium (ferritin-CM), was collected. After removal of detached cells and cell debris by centrifugation (250 g, 10 min) the ferritin-CM was stored at 4 °C. Ferritin was purified from the ferritin-CM as described previously [4], yielding a fraction of H-chain-rich, acidic isoferritin referred to as "ferritin" in the text [5].