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Brief exposure to copper activates lysosomal exocytosis

Karina Peña, Jessica Coblenz, Kirill Kiselyov*

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, United States

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ABSTRACT

Copper (Cu) is essential mineral, but its toxicity necessitates existence of powerful machinery responsible for the extraction of excess Cu from the cell. Cu exposure was recently shown to induce the translocation of Cu pump ATP7B to the lysosomes followed by lysosomal exocytosis. Here we sought to investigate the mechanisms underlying the effect of Cu on lysosomal exocytosis. We found that brief exposure to Cu activates lysosomal exocytosis, which was measured as a release of the lysosomal digestive enzyme β-hexosaminidase (β-hex) into the extracellular medium and by the presence lysosomal protein LAMP1 at the plasma membrane. Such release depends on calcium (Ca) and on the lysosomal SNARE VAMP7. ATP7B knockdown using RNAi suppressed the basal lysosomal exocytosis, but did not affect the ability of Cu to activate it. ATP7B knockdown was associated with sustained oxidative stress. The removal of Ca from the extracellular medium suppressed the Cu-dependent component of the lysosomal exocytosis. We propose that Cu promotes lysosomal exocytosis by facilitating a Ca-dependent step of the lysosomal exocytosis.

1. Introduction

Lysosomal exocytosis has been originally described as a means of repairing plasma membrane via recruitment of the lysosomal membrane to a place of membrane damage [1]. Lysosomal fusion with the plasma membrane depends on a specific set of SNARE components [2], suggesting a regulated process. Therefore, the significance of lysosomal exocytosis likely extends beyond pathological conditions of membrane rupture, possibly including response, adaptation, or signaling involvement. The latter idea finds support in the recent series of evidence on the role of lysosomes in transition metal extraction from cells [3,4].

Transition metals such as Fe, Zn and Cu enter cells via plasma membrane transporters or via endocytosis followed by absorption through lysosomal/endosomal transporters [5–7]. While all cells require some levels of transition metals, an excessive exposure to transition metals is toxic, necessitating their tight regulation. In the cytoplasm, transition metals are bound to chelating proteins, exported via plasma membrane transporters or absorbed into organelles, which is followed by exocytosis or metal-filled organelles. Among the transporters implicated into transition metals absorption into lysosomes are Zn transporters ZnT2 and ZnT4 (SLC30A2 and SLC30A4), and a Cu transporter ATP7B. Suppression of these transporters was shown to significantly affect Zn and Cu handling [3,4].

Lysosomal transition metal importers are regulated in a variety of ways. ATP7B, the Cu transporter whose loss is responsible for Wilson's disease [8], responds to Cu exposure by moving from trans-Golgi to the lysosomes [3,9]. Cu absorption by the lysosomes is followed by its extraction from the cells via SNARE-dependent lysosomal exocytosis [3]. Thus, the main mechanism of ATP7B regulation appears to be translocation to the lysosomes or perhaps formation of the new population of ATP7B-bearing lysosomes. In addition, ATP7B interacts with p62 subunit of dynactin facilitating lysosomal transport toward the apical pole of hepatic cell where Cu is released [3]. ZnT transporters, especially ZnT2, have been shown to translocate to the lysosomes in response or in parallel to Zn exposure, and structural determinants of such translocation have been proposed [10]. At the same time, transcription of genes coding for several ZnTs is regulated by the transcription factor MTF-1, which responds to Zn and other transition metals [11]. These data show that lysosomal metal uptake capacity is regulated by the cytoplasmic transition metals. Whether or not Cu regulates the rate of the lysosomal metal extraction has not been consistently explored. It is the main question of the present studies.

Abbreviations: β-hex, β-hexosaminidase; RNAi, RNA interference; siRNA, short interfering RNA; SNARE, SNAP (Soluble NSF Attachment Protein) Receptor; TBHP, tert-Butyl hydroperoxide.

* Corresponding author at: Department of Biological Sciences, University of Pittsburgh, 4249 Fifth Avenue, Pittsburgh, PA 15260, United States. Tel.: +1 4126244317.
E-mail address: Kiselyov@pitt.edu (K. Kiselyov).

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2. Materials and methods

2.1. Cell culture and treatments

HeLa cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Lonza) supplemented with 10% FBS (Atlanta Biologicals) (growth medium) at 37 °C in the presence of 5% CO_2. For Cu treatments, cells were incubated with 100 μM $CuCl_2$ for the indicated time in growth medium or regular buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 g/L glucose), as indicated for each experiment. For TBHP treatments, cells were incubated for 1 h with 400 μM TBHP (Invitrogen, Carlsbag, CA) in regular buffer. For $LaCl_3$ experiments, cells were pre-treated for 5 min with the indicated concentration of $LaCl_3$ in regular buffer. $LaCl_3$ was removed, added back and kept in the medium for the length of the assay.

2.2. siRNA transfection

Control (universal negative control #1), VAMP7, and ATP7B siRNAs were acquired from Sigma–Aldrich (St Louis, MO). Cells were transfected using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbag, CA). Transfections were performed as described by manufacturer's protocol. Briefly, cells were seeded at subconfluency and transfected next day either with control siRNA, or siRNA for VAMP7 or ATP7B. Media was changed 16–24 h later. siRNA efficiency was measured by qPCR.

2.3. β-Hexosamindase activity assay

HeLa cells on 12-well plates were washed once with regular buffer and 250 μl of buffer was added to each well. For Cu treatments, cells were incubated with 250 μL of $CuCl_2$ diluted in regular buffer. Buffer was collected at the indicated times and incubated with 300 μl of 3 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide (N9376, Sigma–Aldrich, St Louis, MO) for 30 min at 37 °C in 0.1 M citrate buffer (0.1 M sodium citrate, 0.1 M citric acid, pH 4.5). Reactions were stopped by adding 650 μl borate buffer (100 mM boric acid, 75 mM NaCl, 25 mM sodium borate, pH 9.8) and the absorbance was measured in a spectrophotometer at 405 nm. To determine total cellular content of β-hexosamindase, cells were lysed with 250 μl of 1% Triton X-100 in PBS and after a 10,000 × *g* spin for 5 min at 4 °C, 25 μl of the cell extracts were used for the enzyme activity reaction. Enzyme activity was determined as the amount of 4-nitrophenol produced. Absorbance was calibrated with different amounts of 4-nitrophenol (N7660, Sigma–Aldrich, St Louis, MO) in 0.1 M citrate buffer.

2.4. Flow cytometric surface LAMP1 assay

HeLa cells plated on 6-well plates were incubated with 100 μM $CuCl_2$ in regular buffer for 1 h at 37 °C or left untreated (control). Cells were trypsinized and washed in PBS before fixing in 1% paraformaldehyde (PFA) for 30 min. To detect surface LAMP1, fixed cells were incubated with CD107a LAMP1 antibody APC conjugated (Life Technologies, Carlsbag, CA) in 5% BSA in PBS for 30 min. Cells were washed and resuspended in PBS prior to analysis in BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.5. Reverse transcriptase and quantitative PCR (qPCR)

For qPCR assays, cell were seeded in 12-well plates, transfected, and treated as indicated. Total RNA was isolated from HeLa using TRIzol (Invitrogen) according to manufacturer's protocol. cDNA was synthesized with MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA) using 2 μg of total RNA and 0.5 μg of oligo$(dT)_{18}$ (IDT, Cralville, IA) as primer. qPCR was carried out using 1:500 dilutions of cDNA, 2X SYBR Green (Fermentas, Glen Burnie, MD), and 4 μM primer mix per 10 μl reaction. For gene expression analysis, the following primers (IDT, Cralville, IA) were used: *HMOX1*, forward 5′-GAGACGGCTTCAAGCTGGTGAT-3′ and reverse 5′-CCGTACCAGAAGGCCAGGTC-3′; *ATP7B*, forward 5′-GTGGGCAATGACACCACTTT-3′ and reverse 5′-TGGGTGCCTTTGACATCTGA-3′, and *RPL32*, forward 5′-CAACATTGGTTATGGAAGCAACA-3′ and reverse 5′-TGACGTTGTGGACCAGGAACT-3′. To ensure amplification of cDNA only, all primers were designed to span exons and negative RT reactions were performed as control. The Relative Quantification method on the 7300 Real Time System (Applied Biosystems, Foster City, CA) was used to perform qPCR. Samples were amplified with the following program: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles at 95 °C for 15 s followed by 60 °C for 1 min. Samples were run in triplicates. At least 3 biological replicates were performed per condition. Relative gene expression was calculated using the $\Delta\Delta^{Ct}$ method, where Ct represents the cycle threshold. Δ^{Ct} values were calculated as the difference between the target genes and the expression of the endogenous gene RPL32 and $\Delta\Delta^{Ct}$ values were calculated relative to untreated controls. Data are presented as fold increase.

Statistical significance was calculated using a one-tailed, unpaired *t*-test with $p < 0.05$ considered significant. Data are presented as mean ± SEM.

3. Results and discussion

To analyze the dependence of lysosomal exocytosis on Cu we exposed HeLa cells to fresh buffer containing normal levels of free Ca (1 mM) and measured β-hex levels in the extracellular medium, followed by measuring β-hex content in the total cellular lysate. Fig. 1A shows that cells gradually released β-hex, and at the 1-h time point, cells released about 20% of their β-hex content, which is in line with the previously published data [4]. The addition of 100 μM $CuCl_2$ to the extracellular medium significantly increased the β-hex exocytosis rate (Fig. 1A). At the 1-h mark, the amount of β-hex released by Cu-treated cells was 41.9% higher than in control cells ($n = 3$, $p < 0.05$). The effect was concentration-dependent, as exposure to 1 and 10 μM $CuCl_2$ had no effect on β-Hex release (Fig. 1D). Flow cytometry analysis revealed that the plasma membrane levels of lysosomal protein LAMP1 were increased when cells were treated with 100 μM $CuCl_2$ for 1 h (Fig. 1B), which is in agreement with the β-Hex data. In addition, lysosomal exocytosis was increased in retinal pigment epithelium cells (RPE1) exposed to 100 μM $CuCl_2$ for 1 h (Fig. 1C, 367.7% increase, $n = 3$, $p < 0.05$). Together, these data indicate that Cu stimulate lysosomal exocytosis.

In the previously published studies we showed that in agreement with the SNARE/Ca-dependent model of lysosomal exocytosis, β-hex release is enhanced by intracellular Ca and suppressed by the removal of SNARE components [4]. Accordingly, we find that the stimulatory effect of Cu on lysosomal exocytosis is mediated by a SNARE-dependent process, since VAMP7 knockdown reduced the basal and Cu-induced lysosomal exocytosis by 13 and 30%, respectively (Fig. 2A, $n = 3$, $p < 0.05$).

The dependence of lysosomal exocytosis on Ca was analyzed by increasing intracellular Ca levels with ionomycin and by removing extracellular Ca. Fig. 2B shows that Ca ionophore ionomycin increased both basal and Cu-induced lysosomal exocytosis by 27% and 49% respectively ($n = 3$, $p < 0.05$). The fact that ionomycin was more effective in stimulating lysosomal exocytosis when Cu was present suggests that Cu facilitates a Ca-dependent step of the lysosomal exocytosis.

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