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Inhibition of ADP-ribosylation suppresses aberrant accumulation of lipidated apolipoprotein B in the endoplasmic reticulum

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ABSTRACT

ApoB-crescent, an endoplasmic reticulum (ER)-lipid droplet amalgamation structure, is a useful marker to indicate aberrant lipidated apolipoprotein B accumulation in the hepatocyte ER. Blockade of the ER-to-Golgi transport by either vesicle transport inhibitors or dominant-negative Arf1 caused a significant increase in ApoB-crescents. However, a low concentration of Brefeldin A induced the same result without affecting protein secretion, suggesting ADP-ribosylation as an additional mechanism. ADP-ribosylation inhibitors not only suppressed the increase of ApoB-crescents, but also rapidly dissolved existing ApoB-crescents. These results implicate the involvement of ADP-ribosylation in the ApoB-crescent formation and maintenance process at the ER.

1. Introduction

Apolipoprotein B-100 (ApoB) is a protein that constitutes very low-density lipoproteins (VLDL). In hepatocytes, ApoB is lipidated cotranslationally to form pre-VLDL, which thereafter acquires additional lipids to become mature VLDL. Because the VLDL concentration in the blood needs to be strictly controlled to maintain systemic lipid metabolism, it is important to understand the regulatory mechanism of VLDL secretion.

The VLDL secretion from hepatocytes is regulated mainly by ApoB protein degradation [1]. If the co-translational lipidation does not occur properly, nascent ApoB is dislocated from the Sec61 translocon to the cytoplasm, ubiquitinated, and degraded by proteasomes [2]. In addition to this well-characterized mechanism to degrade poorly-lipidated ApoB, ApoB after lipidation is also subjected to intracellular degradation [2–6]. In previous studies, we defined a degradation pathway of lipidated ApoB at the juncture of ER and lipid droplets (LD) and showed that Derlin-1 and UBXD8 play critical roles [7,8]. When this ApoB degradation pathway is suppressed by proteasome inhibition, docosahexaenoic acid (DHA), UBXD8 knockdown, or other methods, a unique ER-LD amalgamation structure called an 'ApoB-crescent' increased significantly [7,8]. These findings along with other results suggested that the ApoB-crescent formation is induced by an abnormal accumulation of lipidated ApoB in the ER lumen. Conversely, ApoB-crescents are regarded as a convenient way to monitor the lipidated ApoB degradation process.

In the present study, we explored the mechanism of ApoB-crescent formation in detail. We hypothesized that if an increase in lipidated ApoB induces ApoB-crescents, then blockade of the ER-to-Golgi transport should also increase their numbers. To test this hypothesis, we applied several different methods to suppress the secretory pathway and found that all of them indeed increased ApoB-crescents. Surprisingly, brefeldin A (BFA) at a low concentration increased ApoB-crescents without significantly affecting secretion. Because this BFA effect was repressed by ADP-ribosylation inhibitors, we concluded that an unknown target(s) of ADP-ribosylation is involved in the degradation process of lipidated ApoB. The results are discussed with regards to the mechanism of ApoB-crescent formation.

Abbreviations: ADP, adenosine diphosphate; ALLN, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; ApoB, apolipoprotein B-100; Arf1, ADP-ribosylation factor 1; BFA, brefeldin A; DAPI, 4,6-diamidino-2-phenylindole; DHA, docosahexaenoic acid; ER, endoplasmic reticulum; GDP, guanine diphosphate; GEF, guanine nucleotide exchange factor; GTP, guanine triphosphate; LD, lipid droplet; LPDS, lipoprotein deficient serum; MIBG, m-iodobenzylguanidine hemisulfate salt; MTP, microsomal triglyceride transfer protein; NAM, nicotinamide; NDGA, nordihydroguaiaretic acid; OA, oleic acid; VLDL, very low-density lipoprotein

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2. Materials and methods

Please see Supplementary Data for details.

2.1. Cells and transfection

Huh7, HepG2, and 3Y1 cells were used. When appropriate, cells were incubated in medium containing 10 μM *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN) (Sigma–Aldrich) to inhibit proteasomal functions. Oleic acid (OA) and DHA (Sigma–Aldrich) were complexed with fatty acid-free BSA (Wako) at a molar ratio of 6:1 and applied at a final fatty acid concentration of 0.4 mM.

2.2. Plasmids

pcDNA3.1(+)/HA tagged-human Arf1(WT) and Arf1(T31N) were kindly donated by Dr. Naoko Morinaga (Chiba University). Human CtBP1-S/BARS cDNA was obtained by reverse transcription and cloned to the pEGFP-C2 vector (Clontech).

2.3. Subcellular fractionation

Cells were disrupted by nitrogen cavitation and subjected to a sucrose density gradient ultracentrifugation [9]. Fractions were precipitated with 10% trichloroacetic acid, dissolved in the sample buffer, and analyzed by Western blotting.

2.4. Immunofluorescence microscopy and data analysis

Cells were fixed with 3% formaldehyde and permeabilized either with 0.01% digitonin or with 0.1% Triton X-100. LDs and nuclei were stained with BODIPY493/503 (Invitrogen) and 4,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich), respectively.

To analyze effects on ApoB-crescents, at least 10 pictures were randomly taken, and the ratio of ApoB-crescent-positive cells was quantified. The results from three independent experiments were averaged, and the statistical difference from the control was examined using the Student's *t*-test.

2.5. Electron microscopy

Cells cultured on coverslips were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and post-fixed in a mixture of 1% osmium tetroxide and 0.1% potassium ferrocyanide in the same buffer.

2.6. Microsomal triglyceride transfer protein (MTP) activity assay

The MTP activity of mouse liver tissue homogenate (50 μg protein), HepG2 cells (50 μg protein), and 1 μg purified MTP was measured using the fluorescence assay as described [10].

3. Results

3.1. ApoB-crescents increased significantly by blockade of the ER-to-Golgi transport

The ApoB-crescent is an ER-LD amalgamation structure, which is recognized as crescent-shaped ApoB labeling around LDs by immunofluorescence microscopy [9]. To examine whether blockade of the ER-to-Golgi transport increases ApoB-crescents, we tested three vesicle transport inhibitors that work by different mechanisms. BFA down-regulates COPI vesicle formation by suppressing the GDP/GTP exchange activity of Arf1-GEF [11,12], whereas Exo1 accelerates GTP hydrolysis through the activation of Arf1-GAP [13]. NDGA has been suggested to modulate the dynein-dynactin-related process [14] and to disrupt cisternal organization of the Golgi complex [15]. When Huh7 cells were treated with these reagents, secretion of ApoB was virtually abolished, but the intracellular amount of ApoB did not decrease significantly (Fig. 1A), suggesting an increase of ApoB in the ER. Under this condition, a significant increase of ApoB-crescents was observed (Figs. 1B, C).

We also tested the effect of transient expression of Arf1(T31N), the dominant-negative form of Arf1 that down-regulates the ER-to-Golgi transport [16]. The ApoB-crescent formation was increased in cells expressing Arf1(T31N), but not in cells expressing wild-type Arf1 or EGFP alone (Fig. 1D).

The increase of ApoB-crescents by the above treatments was completely inhibited when MTP inhibitor (MTPi; BAY13-9952 at 100 nM) was given simultaneously (data not shown), confirming that lipidated ApoB is responsible for the ApoB-crescent formation. The result corroborated that blockade of the ER-to-Golgi transport caused accumulation of lipidated ApoB in the ER and thereby increased ApoB-crescents.

3.2. A low concentration of BFA increased ApoB-crescents without inhibiting secretion

In the above experiment, cells were treated with BFA for 1 h at a concentration of 5 μg/ml. This condition is generally used to block the ER-to-Golgi transport. We found, however, that ApoB-crescents increased even when BFA was applied to Huh7 cells at much lower concentrations, i.e., 5–10 ng/ml (Fig. 2A). In cells treated with 10 ng/ml BFA for 12 h, the Golgi delineated by immunofluorescence labeling of GM130 showed disintegration (Fig. 2B) and ApoB and transferrin secretion decreased (Fig. 2C), but neither of these changes were observed in cells treated with 5 ng/ml BFA for 12 h (Figs. 2B, C). It was further confirmed that ApoB-crescents induced to form by 5 ng/ml BFA take a similar ultrastructure to those caused by proteasomal inhibition or DHA [7] (Fig. 2D). This result suggested that BFA does not increase ApoB-crescents simply by blocking the ER-to-Golgi transport, but that other mechanisms may be involved.

3.3. ADP-ribosylation inhibitors suppressed ApoB-crescent formation

Besides inhibition of Arf1-GEF, BFA is known to increase ADP-ribosylation of several proteins [17,18]. To test whether ADP-ribosylation is related to the ApoB-crescent formation, we examined the effect of three ADP-ribosylation inhibitors: MIBG, which is specific to mono-ADP-ribosylation [19,20], and coumarimycin A1 and NAM, which are effective for both mono- and poly-ADP-ribosylation [21]. When Huh7 cells were treated for 6 h with 5 μg/ml BFA in the presence of one of the ADP-ribosylation inhibitors, ApoB-crescent formation was suppressed significantly (Figs. 3A, B). This result indicated that the effect of BFA on the ApoB-crescent formation was exerted mainly through facilitating ADP-ribosylation. Because MIBG is thought to be most specific to mono-ADP-ribosylation among the three reagents [21], the following experiments were mainly performed using MIBG, but the other two inhibitors were also tested in most cases.

MIBG also inhibited the increase of ApoB-crescents that was induced by other reagents. In the presence of MIBG, either proteasomal inhibition with 10 μM ALLN or treatment with 0.4 mM DHA failed to increase ApoB-crescents (Fig. 3C). The inhibitory effect of MIBG on ApoB-crescent formation was similar even when cells were treated with a mixture of ALLN and 3-methyladenine to suppress both autophagy and proteasomes (Fig. 3D).

The effect of ADP-ribosylation inhibition on ApoB was further examined by western blotting of subcellular fractions (Fig. 3E). In Huh7 cells treated with 10 μM ALLN alone for 12 h, a significant