Lysosomal Targeting of Acid Sphingomyelinase in the Formation of Lipid Raft-Redox Signaling Platforms of Coronary Arterial Endothelial Cells

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ABSTRACT

Lipid rafts (LRs) are emerging as an important cellular signaling mechanism in the regulation of a variety of cell functions. Recent studies have demonstrated that LRs are able to cluster NADPH oxidase subunits in endothelial cell membrane to form a LR-redox signaling platform in response to death receptor activation. However, the mechanisms mediating the formation of this LR-redox signaling platforms are still poorly understood. In the present study, we examined the role of a sortilin-mediated lysosomal targeting and trafficking of acid sphingomyelinase (ASM), an essential enzyme activating LRs clustering and platform formation in bovine coronary arterial endothelial cells (CAECs). By confocal microscopy and detection of fluorescence resonance energy transfer (FRET), ASM was found to be targeted on lysosomal membrane via sortilin in response to Fas ligand(FasL) stimulation as shown by detection of ASM with sortilin or Lamp-1, a lysosomal membrane marker. The FRET efficiency between these molecules was over 20% compared to 5% in control cells, indicating a FasL-stimulated lysosomal targeting of ASM via sortilin. After CAECs were transfected by an siRNA of sortilin, this FasL stimulated targeting of ASM was abolished and therefore LRs clustering could not be formed, resulting in failure of LR-redox signaling platform formation in cell membrane, as shown by substantial reduction of LR clusters containing NADPH oxidase subunits gp91*phox* or p47*phox*. This action of sortilin siRNA was also confirmed by Western blot analysis of isolated LRs membrane fractions, showing that sortilin siRNA efficiently prevented FasL-induced translocation of gp91*phox* or p47*phox* into LR microdomains. Consequently, FasL-induced superoxide production via NADPH oxidase in LRs clusters was also abolished by sortilin siRNA, as measured by electron spin resonance (ESR) spectrometry. It is concluded that lysosomal targeting of ASM through sortilin is present in CAECs and this targeting mechanism is attributed to the formation of LR-redox signaling platforms in these cells in response to FasL stimulation (Supported by NIH Grants HL-57244, HL-75316, and DK54927).

BACKGROUND

- Lipid rafts consist of dynamic assemblies of cholesterol, lipids with saturated acyl chains such as sphingolipids and glycosphingolipids in the exoplasmic leaflet of the membrane bilayer.
- Recent studies in our laboratory have indicated that lipid rafts can mediate the formation of different signaling platforms. One of such signaling platforms was named as lipid raft redox signaling platforms, which was commented to be taking center stage in endothelial cell redox signaling for death receptors (*Hypertension 2006;57(1)74-80 and Hypertension 2006;47(1):16-18*).
- We further explored the mechanisms mediating the formation of lipid raft signaling platforms related to the production of ceramide, a critical triggering factor in this process, and indicated that lysosomal vesicles, which contain the ceramide producing enzyme, acid sphingomyelinase (ASMase), have an important impact on the formation of these signaling platforms (*Antioxid. Redox Signal 9, 1417–1426*).

However, it remains unknown how ASMase or ceramide particiaptes in the formation of these LR-redox signaling platforms and how ASMase is translocated toward cell membrane. Given the fact that sortilin is an important intracellular transporter of targeting ASMase to lysosomes, the present study was designed to examine its role in the agonist-stimulated formation of LR redox signaling platforms in bovine coronary artery endothelial cells.

METHODS

Confocal Immunofluorescence Analysis. Cells grown on glass coverslips were treated as indicated in the figures presented in this poster, washed, fixed in 4% paraformaldehyde, and blocked with 1% BSA for 30 min. Confocal visualization and detection of lipid raft clusters were performed as we described in previous studies (*Hypertension 2006;57(1)74-80 and Antioxid. Redox Signal 9, 1417–1426*).

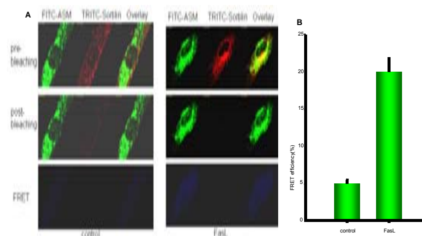
Fluorescence Resonance Energy Transfer(FRET). An acceptor bleaching protocol was employed to measure the FRET efficiency. After the pre-bleaching image was normally took, the laser intensity at the excitement wavelength of the acceptor (TRITC) was increased from 50 to 98 and continued to excite the sample for 2 minutes to bleach the acceptor fluorescence. After the intensity of the excitement laser of the acceptor was adjust back to 50 and the post-bleaching image was then took. The FRET image was obtained by the subtract of the pre-bleaching image from the post-bleaching image (in blue). After measuring the FITC fluorescence intensity in the pre-, post-, and FRET image, the FRET efficiency was calculated through the following formula: E=(FITCpost - FITCpre)/FITCpost x 100%.

RNA interference. SiRNAs were purchased from INVITROEN (CAT#HSS109429). The scrambled RNA (AATTCTCCGAACGTGTC ACGT) has been confirmed as non-silencing double stranded RNA and was used as control in the present study. Transfection was performed according to the instruction manual.

Isolation of LR microdomains by gradient centrifugation. Lipid raft microdomains were isolated as we described previously (*Hypertension 2006;57(1)74-80)*. Briefly, CAECs were lysed, and cell extracts were then homogenized by 5 passages through a 25-gauge needle. Homogenates were adjusted with 60% OptiPrep Density Gradient medium to 40% and overlaid with discontinuous 30%/5% OptiPrep Density Gradient medium. Samples were centrifuged at 32,000 rpm for 30 hours at 4°C using a SW32.1 rotor. Ten fractions were collected from the top to bottom.

RESULTS

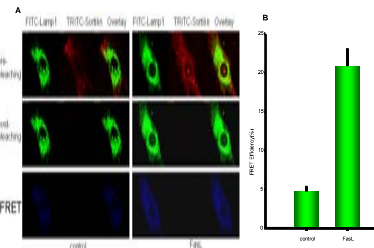
Sortilin and ASMase Trafficking Together



To confirm the close relationship between these two molecules in BCAECs, fluorescence resonance energy transfer (FRET) experiments was conducted. As shown in panel A, BCAECs were stained with FITC-labeled anti-ASMase antibody and TRITC-conjugated anti-sortilin antibody. An acceptor (TRITC) bleaching protocol was applied to calculate the FRET efficiency. Left group of images show a control cell underwent an acceptor bleaching protocol and both the pre- and post-bleaching images were taken. FRET image (in blue) was generated through the subtraction of the pre-bleaching image from the post-bleaching image. Right group of images show a FasL–stimulated cell underwent the same FRET protocol.

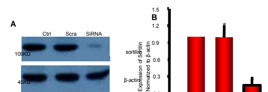
Panel B is the summarized data. FasL induced an increase in the FRET efficiency between ASMase-conjugated FITC and sortilin-conjugated TRITC, which demonstrated that both components, ASMase and sortilin are closely trafficking together.

FasL Increased Sortilin Targeting to Lysosomes



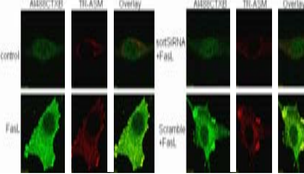
To determine the existence of FasL-stimulated lysosome trafficking with sortilin, FRET were also performed between FITC-labeled anti-lamp1 and TRITC-conjugated anti-Sortilin. It was found that FasL significantly increased the FRET efficiency. A. representative images; Panel summarized data. These results indicate that FasL induces sortilin targeting to lysosomes.

Sortilin Gene was Effectively Silenced



We used RNA interference strategy to knock down sortilin expression. By Western blot analysis, the silencing of this gene in BCAECs was effective, as shown in typical gel document (A) and summaried data (B).

Sortilin Gene Silencing Blocked LRs Clustering

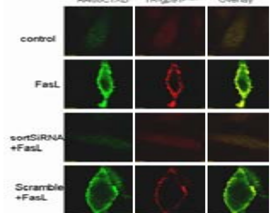


Compared to control condition, FasL stimulated LRs clustering (green patches with Al488CTXB) and translocation of ASMase into these LR clusters. When sortilin gene was silenced, FasL-induced LR clustering and ASMase translocation was blocked (sortSiRNA+FasL). But Scrambled sRNA had no effect (Scramble+FasL). It seems that sortilin-mediated targeting of ASMase importantly contributes to the formation of LR signaling platforms.

NOX Subunits Aggregation Blocked by Sortilin Gene Silencing

By detergent-resistant membrane flotation, we detected LR fractions which was positive by probing with anti-flotilin antibody. In these fractions, both NADPH oxidase (NOX) subunits, gp91*phox* and p47*phox* were increased when BCAECs were stimulated by FasL as shown in gel document above with label of FasL on the left. However, when sortilin gene was silenced in these cells, this translocation or aggregation of both NOX subunits was abolished. These results tell us that LR clusters with NOX subunits, so called LR-redox signaling platforms, are produced in associated with sortilin-mediated protein targeting process.

Sortilin Gene Silencing Abolished LR-gp91 Cluster Formation



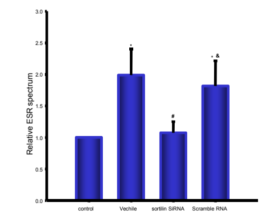
The formation of LRs with NOX subunits is an important feature of LR-redox signaling platforms. Under confocal microscope, we did demonstrate that in FasL-stimulated cells, there were fluorescent patches identified by LR markers and anti-gp91*phox* antibody shown in images with FasL on the left. Yellow patches or dot on the right indicate localization of gp91*phox* in LR clusters.

In sortilin siRNA transfected BCAECs, FasL-induced LR clustering and colocalization of gp91*phox* changes were blocked (labeled with sortSiRNA+FalL). In scrambled sRNA transfected cells, FasL was still able to stomulate LR clustering and translocation of gp91*phox*.

We also found siRNA of sortilin had similar effects to block the aggregation or translocation of p47*phox* into the LR clusters (not shown here).

These results from confocal microscopy further support the view that LR redox signaling platform formation depends on sortilin-mediated protein targeting which is mainly to lysosomes.

NOX Activity Inhibited by Sortilin siRNA



Using ESR, we found that FasL activated NOX in LRs fractions of BCAECs. However, when these cells were transfected with sortilin siRNA, FasL failed to activate NOX in these membrane fractions. This effect of sortilin siRNA was not observed in scrambled sRNA transfected cells.

CONCLUSION

It is concluded that lysosomal targeting of ASMase through sortilin is present in BCAECs and that this protein targeting mechanism is attributed to the formation of LR-redox signaling platforms in response to FasL stimulation in these cells.