

Activation of Acid Sphingomyelinase Drives Lysosomal Fusion to Membrane Lipid Raft Clusters in Coronary Endothelial Cells

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ABSTRACT

Lipid rafts (LRs) has been reported to be able to cluster NADPH oxidase subunits in endothelial cell membrane to form a LR-redox signaling platform in response to death receptor activation which was related to the increased activity of acid sphingomyelinase (ASM), a lysosomal glycoprotein and may catalyze the degradation of membrane-bound sphingomyelin into phosphocholine and ceramide. The fusion of lysosomal vesicles has also been regarded as a participator during this LR-redox platform formation. However, the relationship among ASM, lysosomal fusion and LR clustering is still far from being understood. The present study was to determine whether the ASM activation may drive lysosomes to traffic and fuse to cell membrane and facilitate lipid raft (LRs) clustering. By confocal microscope, it was found that both activators [Phosphatidylinositol (PI) and bis (monoacylglycerol) phosphate (Bis)] and inducer of ASM [Butyrate (Buty)] increased LR clustering in bovine coronary arterial endothelial cells (CAECs). Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS) analysis demonstrated that PI, Bis, Buty could induce ceramide production which could be reversed by ASM siRNA transfection. Stimulation by PI, Bis and Buty resulted in a lysosomal fusion to cell membrane from cytosol, as shown by increased fluorescence resonance energy transfer (FRET) efficiency (from 5 to 19.1%, 13.5%, 14.2% and 18.6%) between lysosome marker Lamp1 and LR. Lysosome-membrane fusion pattern of fluorescence changes in FM1-43 quenching and dequenching assays were shown in PI and Bis treated cell. In isolated bovine coronary arteries, FasL, PI and Bis were found to induce impairment of endothelium-dependent vasodilation, which could be prevented by lysosome functional inhibitor, bafilomycin A1 and lysosomal fusion inhibitor, vacuolin-1. It is concluded that ASM activation serves as a driving force leading to lysosomal trafficking and fusion to cell membrane in CAECs, which plays an important role in LR clustering and impairment of endothelial function (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 raft redox signaling platforms took 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 found that the LR-redox platform formation was related to the increased activity of acid sphingomyelinase (ASM) and lysosomal vesicles fusion to the cell membrane (Antioxid Redox Signal 2007; 9: 817-828 and Antioxid Redox Signal 2007; 9: 817-828 and Arterioscler Thromb Vasc Biol 2008; 28: 2056-2062).

However, it remains unknown how the ASM is involved in this lysosomal fusion and LR-redox platform formation. Given the fact that hydrolysis of sphingomyelin into ceramide by ASM may lead to the fusion of organelles, the present study was designed to examine whether ASM is first activated in response to stimuli and lead to ceramide production, which drives lysosomes to traffic and fuse to cell membrane and facilitate LR clustering.

METHODS

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 excitation wavelength of the acceptor (TRITC) was increased from 50 to 98 to bleach the acceptor fluorescence. After the intensity of the excitation 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: $E = (FITC_{post} - FITC_{pre}) / FITC_{post} \times 100\%$ (Arterioscler Thromb Vasc Biol 2008; 28: 2056-2062).

RNA interference. SiRNAs were purchased from INVITROEN. The DNA target sequence is: 5'-AAGGCCGTGAGTTCTACCT-3'. 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 (Antioxid Redox Signal 2007; 9: 817-828 and Arterioscler Thromb Vasc Biol 2008; 28: 485-490).

Ceramide analysis by Liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS-MS). C14, C16, C17, C18, C20, C22, C24 ceramides were analyzed with a reported method (J Chromatogr A 2002; 949: 225-233). Briefly, CAECs were first extracted with chloroform/methanol and then separated on a HPLC system. Finally, the MS detection was carried out using an Applied Bio systems 3200 Q trap with a turbo V source for TurbolonSpray (Ontario, Canada).

Lysosomal fusion detection by quench and dequench of fluorescence dye. CAECs were loaded with FM1-43 with or without bromide phenol blue (BPB), then scanned for 10 min under confocal microscope ($\lambda_{excitation}=488\text{ nm}$) after treatment to detect the change of fluorescence. If lysosomal fusion to cell membrane happens, the BPB would enter or come out of lysosome which cause decrease and increase of fluorescence and were named as quench and dequench process respectively (Arterioscler Thromb Vasc Biol 2008; 28: 2056-2062).

RESULTS

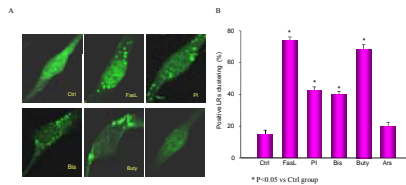


Fig.1 LR clustering induced by ASM activation. To address whether the activation of ASM lead to LR clustering, we conducted confocal microscope to compare the effects induced by FasL (as a positive control) and ASM activation. Arsenic trioxide (Ars), which can induce *de novo* synthesis of ceramide was used as negative control. It is shown that FasL, PI, Bis and Buty, but not Ars induced significant LR clustering in CAECs which means ASM activation could result in LR formation just as that did by FasL.

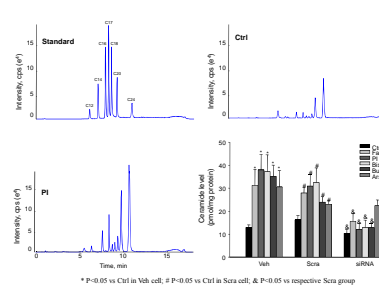


Fig.2 ceramide production induced by ASM activation. We performed the LS-ESI-MS-MS to identify and quantify different ceramide species. FasL, PI, Bis and Buty resulted in significant ceramide production which could be inhibited by ASM siRNA transfection further testifying the effect of FasL, PI, Bis and Buty were due to hydrolysis of sphingomyelin by ASM and ceramide production.

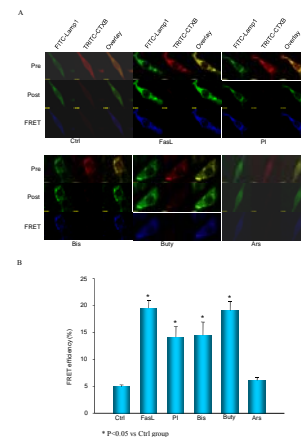


Fig.3 Lysosome targeting to LR induced by ASM activation. As shown in Panel A, there was very low FRET in control cell. After stimulation by PI, Bis, Buty but not Ars, in addition to colocalization of both molecules seen in the overlaid images (top panel), a more intense FRET image (blue on the bottom) was detected, demonstrating an energy transfer between lysosomal marker-lamp1, and LR component-GM1 ganglioside. Panel B was the summarized data. It showed that ASM activation resulted from PI, Bis and Buty stimulation increase the FRET efficiency significantly as compared to control level. The close relationship between Lamp-1 and membrane components indicated a fusion of lysosome to the cell membrane.

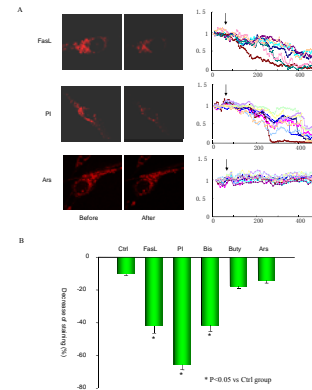


Fig.4 Quench of fluorescence induced by ASM activation. FasL, PI and Bis could cause much more decrease in FM1-43 fluorescence as compared with control group. The decrease of fluorescence induced by Buty and Ars did not show significant difference for they may need longer time to take effects.

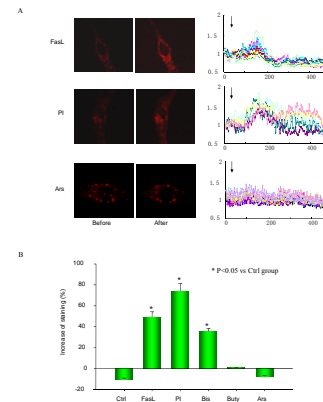


Fig.5 Dequench of fluorescence induced by ASM activation. FasL, PI and Bis induced significant increase of fluorescence which were the results of lysosomal fusion to cell membrane. The quench and dequench experiments may serve as the direct evidence for lysosomal fusion induced by ASM activation.

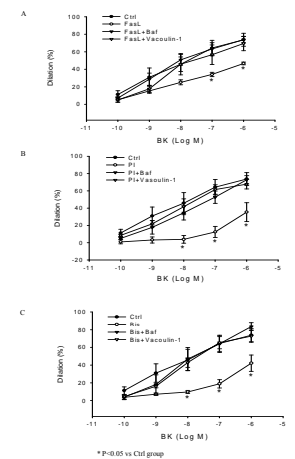


Fig.6 Impaired endothelium dependent vasodilation induced by ASM activation. Incubation of the small bovine coronary arteries with FasL, PI, and markedly attenuated the vasodilator response to bradykinin (BK), which were reversed by Baf, a specific inhibitor of vacuolar proton ATPases which can increase the pH value in lysosome and vacuolin-1, an inhibitor of calcium dependent lysosomal fusion to cell membrane. The results indicate inhibition of lysosome function or lysosomal fusion to cell membrane may block the formation of LR-redox platforms and keep the nitric oxide (NO) from lowering by superoxide, thereby protect the arterial endothelium from detrimental effects induced by FasL or ASM activation.

CONCLUSION

