Protective Role of Instant Membrane Resealing in NLRP3 Inflammasome Activation of Mouse Endothelial Cells
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> Instant cell membrane resealing may importantly protect endothelial cells (ECs) from injury, and a rapid membrane resealing or repairing during injury is an important adaptive mechanism, which is essential for the regulation of cell survival and cell function.

> Membrane rattr (MRs) and an important MR sphingolipid, ceramide play a critical role in cell membrane repair during cell injury and a sphingomyelinase (ASM), the ceramide-producing enzyme that promotes MR clustering has been reported to participate in the control of cell membrane resealing.

The present study was designed to test whether MR- and ceramide-associated cell membrane resealing is critically implicated in the regulation of vascular function or related diseases due to endothelial dysfunction and injury.

**METHODS**

Simultaneous measurement of Ca2+ and PI production in MVECs. Plasma membrane damage and intracellular Ca2+ response to different stimuli were determined by using Propidium iodide (PI) as an indicator of membrane open and resealing and by fura-2, a Ca2+-sensitive fluorescent dye, respectively. A fluorescent microscopic imaging system with high speed wave-length switching was used as described previously (Am J Physiol Cell Physiol. 2013 Mar 1(3):C648-66).

Confocal microscopy, Western blot analysis, Laser-sort cytometry and measurement of caspase B activities were conducted as we described currently (Biochem Biophys Acta. 2015 Feb;1852(2):396-408).

**RESULTS**

Figure 1. Representative fluorescent images (panel A and B) show that both LCWE and saporin (a membrane injury agent) increased Ca2+ (green to red color) and PI entry into cells (red). These changes in Ca2+ and PI fluorescence were recorded, as shown by panel C and D. Given that PI cannot be dissociated from its binding to DNA for red fluorescence, the shift of PI was calculated to represent the speed of PI entry into cells, which is used as an indicator of cell membrane injury/repair (E and F).

Figure 2. We demonstrated that Ca2+ plays an important role in the plasma membrane instant resealing, as shown by the Ca2+-dependence of the PI fluorescence rising peaks and its shift. In the presence of Ca2+, both LCWE and saporin markedly increased less membrane injury, as MVECs in Ca2+-free Hank’s solution needed much shorter time to reach maximal dtt compared to cells in normal Hank’s solution (A, B). This time to peak of PI fluorescence rising and the maximal dtt were summarized in panel C-F. LCWE or saporin-induced cell membrane injury as shown by maximal dtt was significantly larger in MVECs bathed in Ca2+-free Hank’s solution compared to the cells in Ca2+ Hank’s solution, as shown by short time to the peak of PI fluorescence rising (C and D) and by increased dtt (E and F).

Figure 3. We further tested whether MRs clustering alters membrane resealing during LCWE-induced plasma membrane injury in MVECs by measuring the peak of the flow rate (Vmax) of FasL and its shift. The peak response was more sensitive after pretreatment of FasL for 30 min. The lipid raft disruptor, MCD could attenuate FasL-induced effects on this peak response (A, B). The time to peak response was much shorter in FasL-treated cells than its vehicle. However, after pretreatment of cells with MCD with FasL together, the time of peak response was remarkably delayed. Confocal microscopic imaging showed that the resealing of these cells was significantly impaired by FasL. It was significantly weakened by FasL, indicating weaker peak or no resealing. However, MCD markedly reduced FasL-induced increases in dtt (E, F). These results suggest that FasL-induced MRs clustering impaired cell membrane resealing, which can be blocked by disruption of the MRs.

Figure 4. Next, we demonstrated that membrane injury by LCWE enhanced Nlrp3 inflammasome inflammasome as shown by confocal co-localization of Nlrp3 with ASC or caspase-1, which was blocked by artificial plasma membrane resealing reagent (VA64) (A, B, C). By Western blot analysis, cleaved or active caspase-1 was shown increased by LCWE, which was blocked by VA64 indicating that membrane resealing can block LCWE-induced Nlrp3 inflammasome activation (D). Correspondingly, LCWE-induced caspase-1 activity increase and IL-1β production, the indicators of inflammasome activation were blocked by VA64, further confirming the protective role of membrane resealing in the Nlrp3 inflammasome activation.

Figure 5. Similarly, silencing of ASM gene, a MR key lipid-ceramide producing enzyme markedly inhibited FasL-enhancement of LCWE-induced Nlrp3 inflammasome formation and activation in MVECs, as shown by confocal microscopic co-localization of Nlrp3 with ASC or caspase-1 (A-C) for Nlrp3 inflammasome formation, cleaved or active caspase-1 level and caspase-1 activity (D, E).

SUMMARY AND CONCLUSION

> A Ca2+ dependent membrane resealing was shown in MVECs in response to LCWE or saporin, which was markedly attenuated by FasL, a death receptor ligand that stimulates membrane raft (MR) clustering in ECs.

> FasL-induced membrane resealing and consequent enhancement of plasma membrane injury were almost completely blocked by a MR disruptor, methyl β-cyclohexodrin (MCD).

> The impaired plasma membrane resealing by FasL led to the enhanced formation and activation of Nlrp3 inflammasome in MVECs.

> FasL-enhanced inflammasome activation was blocked by an artificial membrane resealer, VA64 and ASM inhibition, and this enhanced inflammasome activation is due to increased lysosomal permeability and cathepsin B release.

These results suggest that a Ca2+-dependent and MR-associated instant cell membrane resealing occurs instantly in ECs in response to injurious factors, which prevents Nlrp3 inflammasome activation by stabilization of lysosomes.