Protective Role of Instant Membrane Resealing in NLRP3 Inflammmasome Activation of Mouse Endothelial Cells

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BACKGROUND AND AIMS

- 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 rafts (MRs) and an important MR sphingolipid, ceramide play a critical role in cell membrane repair during cell injury, and acid 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 the 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 Ca^{2+} and PI production in MVECs. Plasmic membrane damage and intracellular Ca^{2+} response to different stimulants were determined by using Propidium iodide (PI) as an indicator of membrane open and resealing and by fura-2, a Ca^{2+}-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;304(5):C458-66*).

Confocal microscopy, Western blot analysis, Laser-sort cytometry and measurements of cathepsin B activities were conducted as we described currently (*Biochim Biophys Acta. 2015 Feb;1853(2):396-408*).

RESULTS

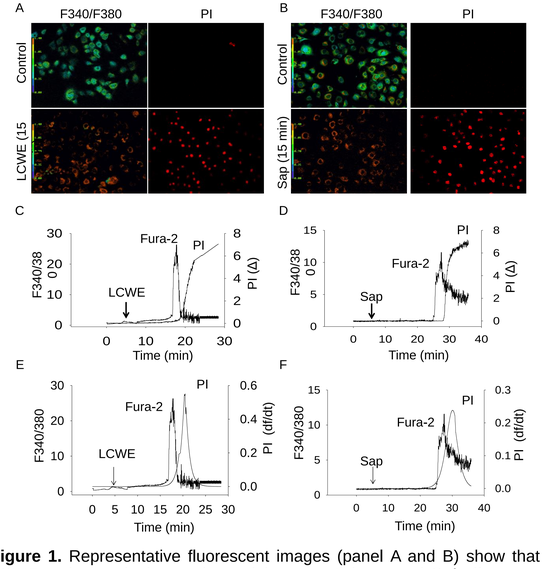

Figure 1. Representative fluorescent images (panel A and B) show that both LCWE and saponin (a membrane injury agent) increased Ca^{2+} (from green to red color) and PI entry into cells (red). These changes in Ca^{2+} and PI fluorescence were recorded, as shown by panel C and D. Given that PI cannot be dissociated from their binding to DNA for red fluorescence, the *df/dt* 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).

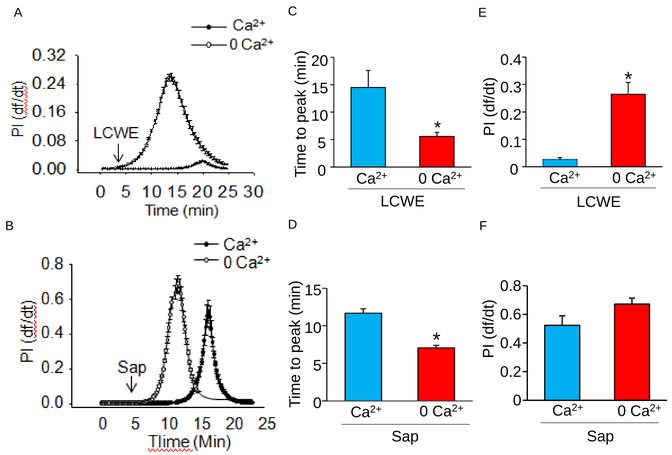

Figure 2. We demonstrated that Ca^{2+} plays an important role in the plasma membrane instant resealing, as shown by the Ca^{2+}-dependence of the PI fluorescence rising peaks and its df/dt. In the presence of Ca^{2+}, both LCWE and saponin produced much less membrane injury, as MVECs in Ca^{2+}-free Hank's solution needed much shorter time to reach maximal df/dt compared to cells in normal Hank's solution (A, B). The time to peak of PI fluorescence rising and the maximal *df/dt* were summarized in panel C-F. LCWE or saponin-induced cell membrane injury as shown by maximal *df/dt* was significantly larger in MVECs bathed in Ca^{2+}-free Hank's solution compared to the cells in Ca^{2+} Hank's solution, as shown by short time to the peak of PI fluorescence rising (C and D) and by increased dt/df (E and F).

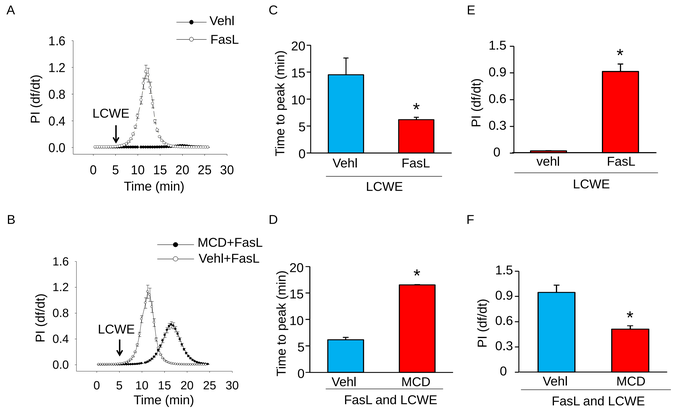

Figure 3. We further tested whether MRs clustering alters membrane resealing during LCWE-induce plasma membrane injury in MVECs by measuring the peak of the flow velocity of PI and df/dt. The peak response was more sensitive after pretreatment of FasL for 30 min. The lipid raft disruptor, MCD could attenuate 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 restored the level of vehicle treated cells (C, D). In contrast, the df/dt was increased significantly by FasL indicating weak or no resealing. However. MCD markedly reduced FasL-induced increases in df/dt (E, F). These results suggest that FasL-induced MRs clustering impair cell membrane resealing, which can be blocked by disruption of the MRs.

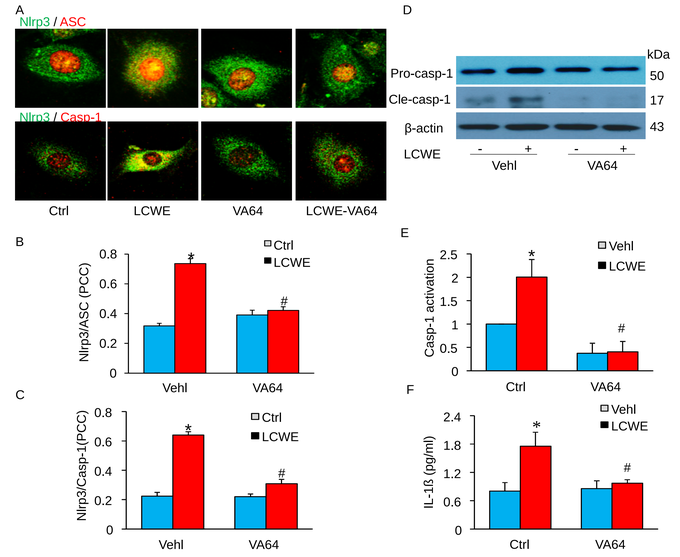

Figure 4. Next, we demonstrated that membrane injury by LCWE enhanced Nlrp3 inflammasome inflammasome as shown by confocal co-localization of Nlrp3 with ASC or caspase-1, which was blocked by artificial plasma membrane resealing reagent (VA64) (A, B, C). By Western blot analysis, cleaved or active caspase-1 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.

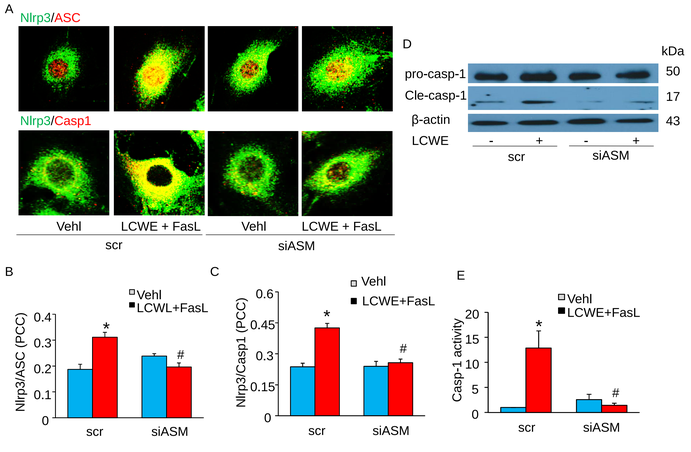

Figure 5. Similarly, silencing of ASM gene, a MR key lipid-ceramide producing enzyme markedly inhibited FasL enhancement of LCWE-induced Nlrp3 inflammasome formation and activation in MVECs, as shown by confocal microscopic co-localization of Nlrp3/ with ASC or caspase-1 (A-C) for Nlrp3 inflammasome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

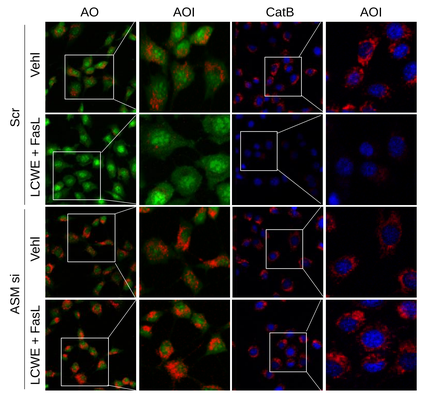

Figure 6 shows the effects of ASM siRNA transfection on FasL/LCWE-induced cathepsin B activation. It was shown that FasL/LCWE increased lysosome membrane permeability, as shown by decreased orange fluorescence intensity for acridine orange staining of MVECs. IN addition, cathepsin B activation as shown by increased red fluorescene intensity of CatB staining. These findings confirm that cathepsin B is involved in FasL/LCWE-induced Nlrp3 inflammasome activation in MVECs, which is associated with impaired membrane resealing.

SUMMARY AND CONCLUSION

- A Ca^{2+}-dependent membrane resealing was shown in MVECs in response to LCWE or sapanin, which was markedly attenuated by FasL, a death receptor ligand that stimulates membrane raft (MR) clustering in ECs.
- FasL-induced impairment of membrane resealing and consequent enhancement of plasma membrane injury were almost completely blocked by a MR disruptor, methyl-β-cyclodextrin (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 Ca^{2+}-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.