Ca^{2+}-dependent and Ceramide-mediated Membrane Repair with Annexin V Recruitment and Aggregation in Mouse Endothelial Cells

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BACKGROUND AND AIMS

- It has been reported that plasma-membrane disruption of mammalian cells occurs normally under physiological conditions or upon different pathologic stimuli. However, these cells also have strong resealing or repair competence to antagonize such membrane disruption.
- Annexin V has been shown to interact with biological membranes and may participate in the instant resealing or repairing process of the cell plasma membrane during its injury upon various stimuli.
- The diabetes patients are more prone to various microvascular complications such as stroke, retinal vascuopathy and glomerular sclerosis, which are mainly associated with early injury of the vascular endothelium and consequent hyperpermeability of microvascular beds.

In the present study, we attempted to test whether annexin V is critical in the control of a Ca^{2+}-dependent, ceramide-mediated membrane repairing mechanism in coronary endothelial cells (CECs). This annexin V-associated instant plasma membrane repairing protects Ecs from plasma membrane injury and dysfunction induced by hyperglycermia or diabetes mellitus.

METHODS

Endothelial cell culture. Coronary arterial endothelial cells were isolated from mouse hearts previously described and primary cultures of these cells were used for experiments as we described previously (*J Mol Med.* 2013;91:25-36).

Measurement of EC membrane resealing during Laser holing under confocal microscope: This assay is a modification of method used by Howard AC *et al.* (*Nat Commun*. 2011; 2:597). ECs were washed three times in Hank's buffer, then incubated in a buffer with or without 1.2 mM Ca^{2+}, containing 2.5 μM FM 1–43 (Invitrogen) and maintained over ice until imaging. The laser injury was produced using a 2 photon laser scanning confocal microscope (LSM 510, Zeiss) coupled to a 10-W Argon/Ti-sapphire laser at 100% power, five laser iterations and a 15 pixels diameter circle bleach area placed over the membrane edge. Fluorescence intensity of FM1-43 was monitored over the time period of 2 min and quantitated with the subtraction from initial fluorescence.

RESULTS

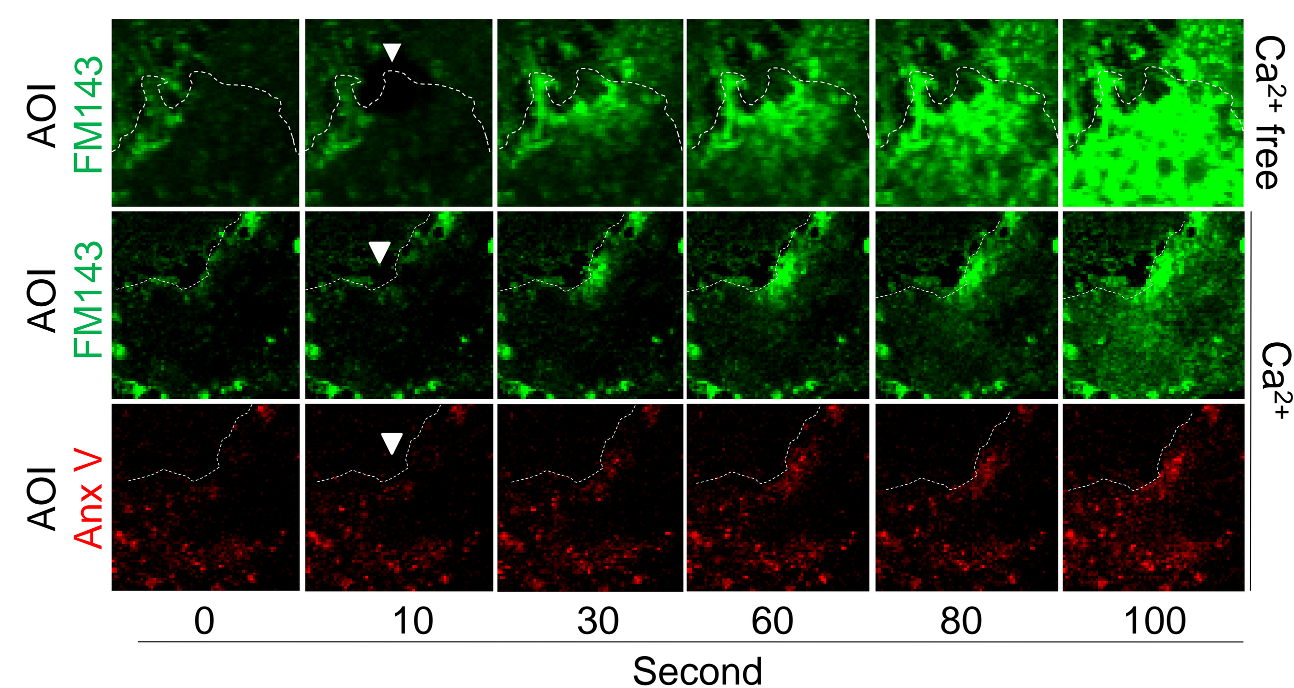

Figure 1. First, we tested whether annexin V plays an important role in the plasma membrane resealing. The images of mouse ECs subjected to laser wounding were recorded in the absence or presence of calcium after introduction of the RBITC-Annexin V protein. The entry of FM1-43 dye into cells was monitored to indicate cell membrane injury when laser wounding was performed at time = 0 sec (arrowheads marks laser disruption site). The FM1-43 dye influx over 2 minutes after laser injury was dramatically increased in a time-dependent manner in the absence of Ca^{2+}. However, the presence of Ca^{2+} led to a rapid resealing of laser wounding. At the same time, in Annexin V transfected cells the red fluorescence was observed to be aggregated at the edge of injury (red fluorescence), indicating that Annexin V is recruited at the injury site and thereby participates in calcium-dependent membrane resealing.

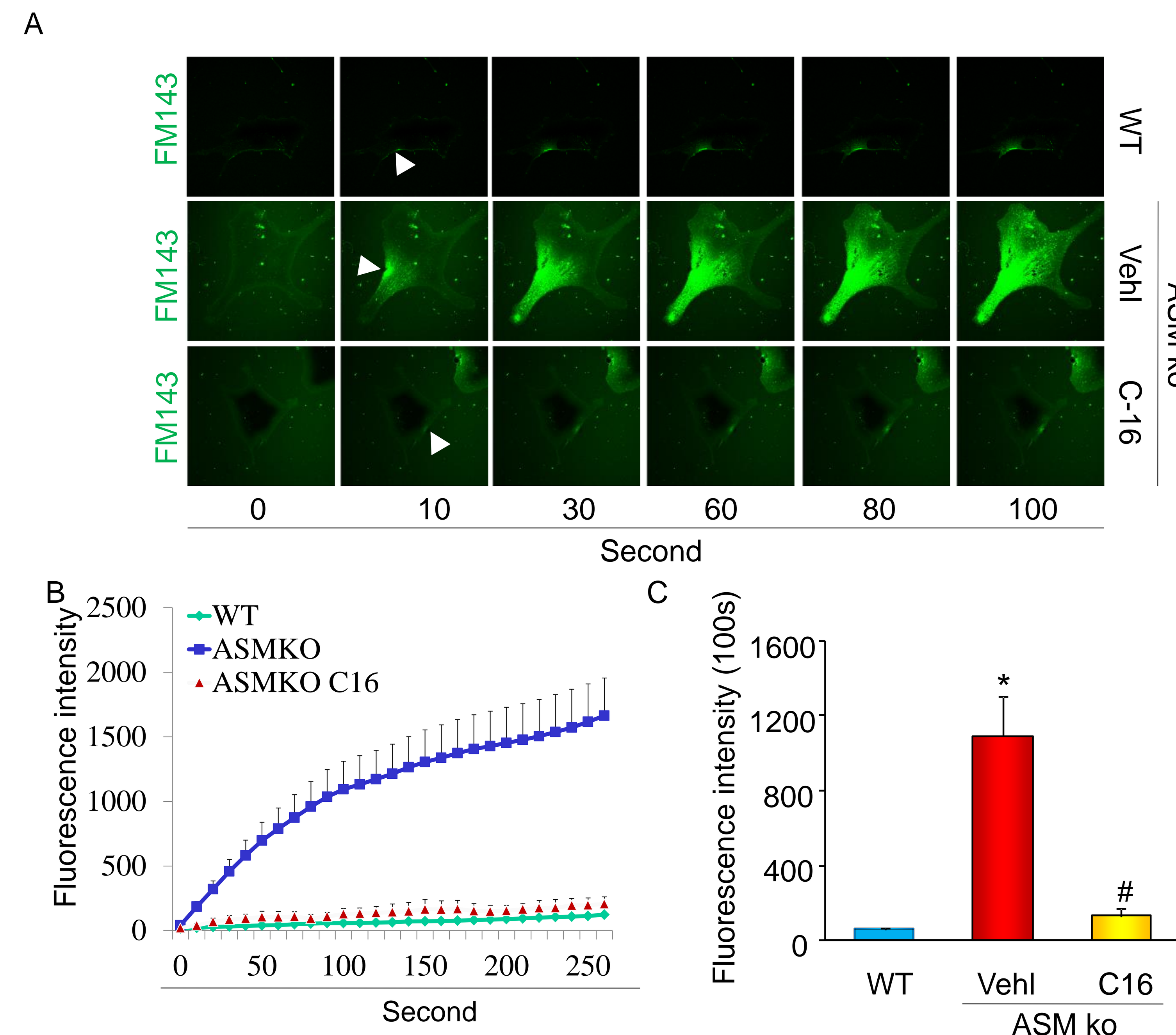

Figure 2. Role of ASM and ceramide in plasma membrane resealing. It was found that in the presence of Ca^{2+} ECs from ASM KO mice lacked resealing as shown by intensive FM1-43 entry into the cells. However, addition of ceramide-16 (C-16, 10 μM) for 30 min rescued cell resealing as shown by decreases in FM1-43 fluorescence after laser wounding (A). These data summarized in panel B and C provide clear evidence that ASM-derived ceramide is an important mediator for the ability of ECs to calcium-dependently reseal membrane wounds.

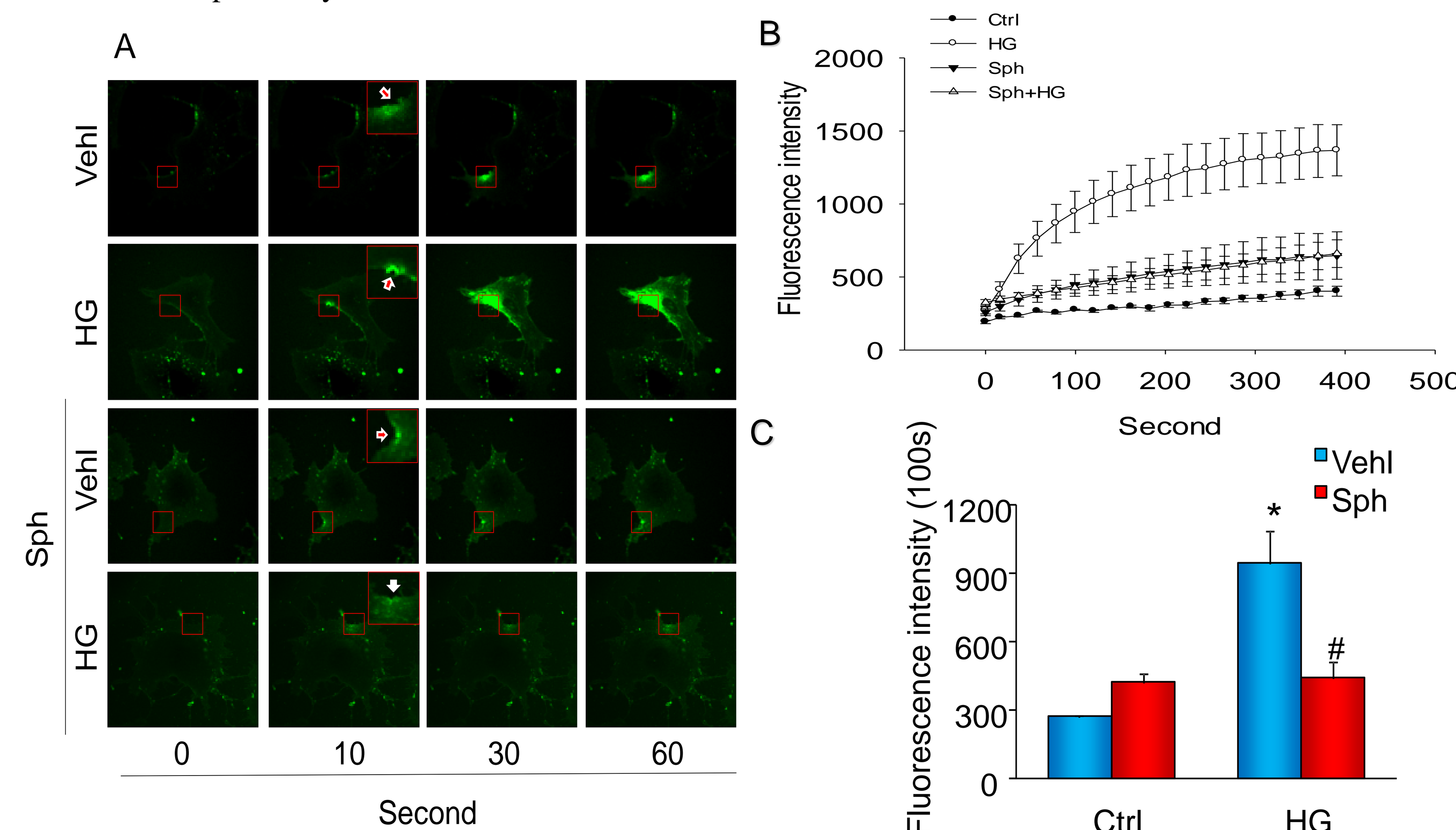

Figure 3. To determine the pathological relevance of annexin V-associated membrane resealing in ECs, we tested whether high gluocose (HG) alters the resealing function of ECs and thereby leads to endothelial dysfunction. As shown in panel A, pretreatment of mouse ECs with HG for 24 hours markedly reduced EC membrane resealing of laser-induced wounding, as shown by time-dependent increase in FM1-43 flurorescence. However, when these cells were pretreated with sphingomyeline (Sph), HG-induced impairment of membrane resealing was blocked. These results were summarized in panel B and C, showing that HG-induced impairment of membrane resealing was substantially attenuated. It is clear that HG impairs an ASM-ceramide-dependent membrane resealing resulting in membrane injury of ECs.

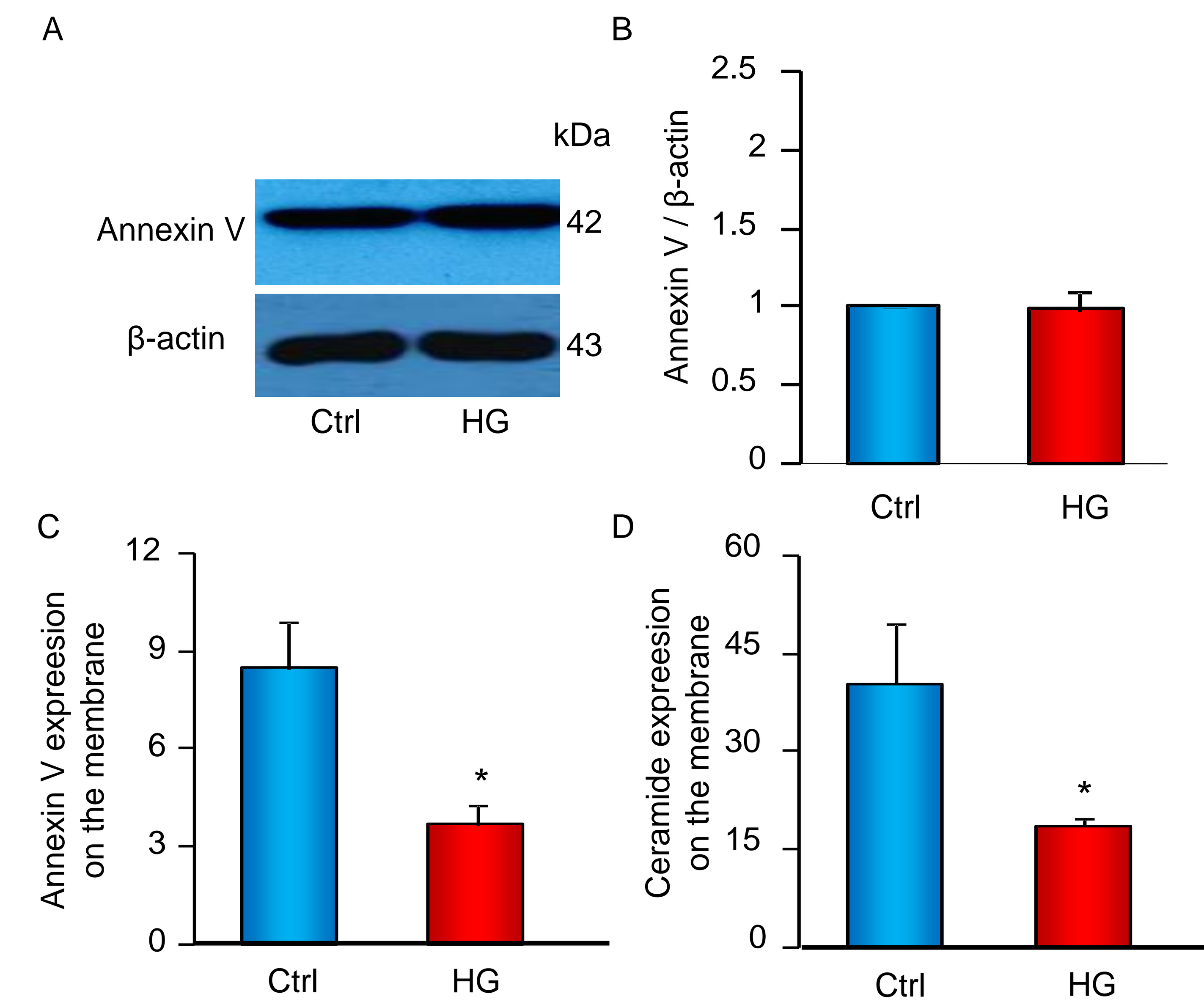

Figure 4. Western blot and flow cytometry showing the effects of HG on the levels of Annexin V and ceramide. By Western blot analysis, we did detect any changes in Annexin V expression upon HG stimulation (A, B). However, by cytometry of fluorescent ECs fluorescently labeled on the cell membrane, it was found that HG decreased the expression of Annexin V on the cell membrane ECs, accompanied by decreased ceramide (C, D). This suggests a possibility that HG may impair membrane resealing function in ECs due to reduction of Annexin V and ASM reduction on the cell membrane.

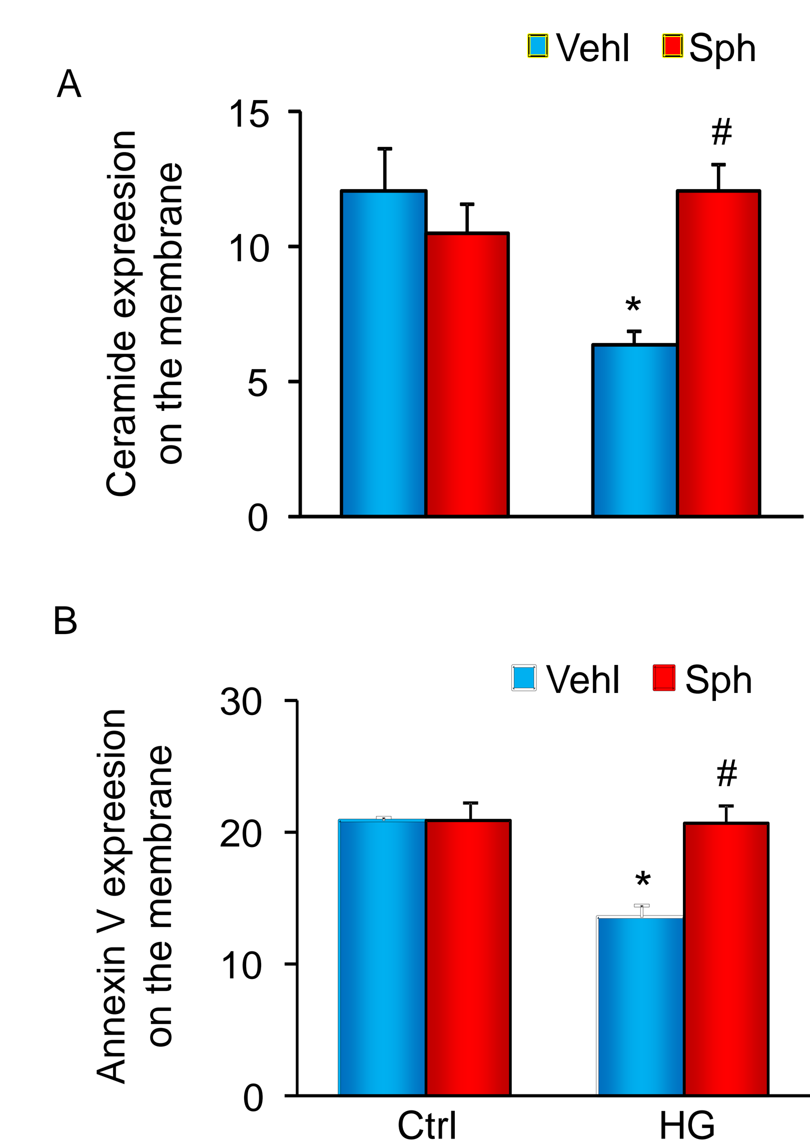

Figure 5. We also tested whether this HG-induced decreases in Annexin V and ceramide levels on the cell membrane can be restored by addition of sphingomyelinase (Sph). It was found that HG-induced decreases in membrane Annexin V and ceramide of ECs were markedly reversed after treatment of these cells with Sph.

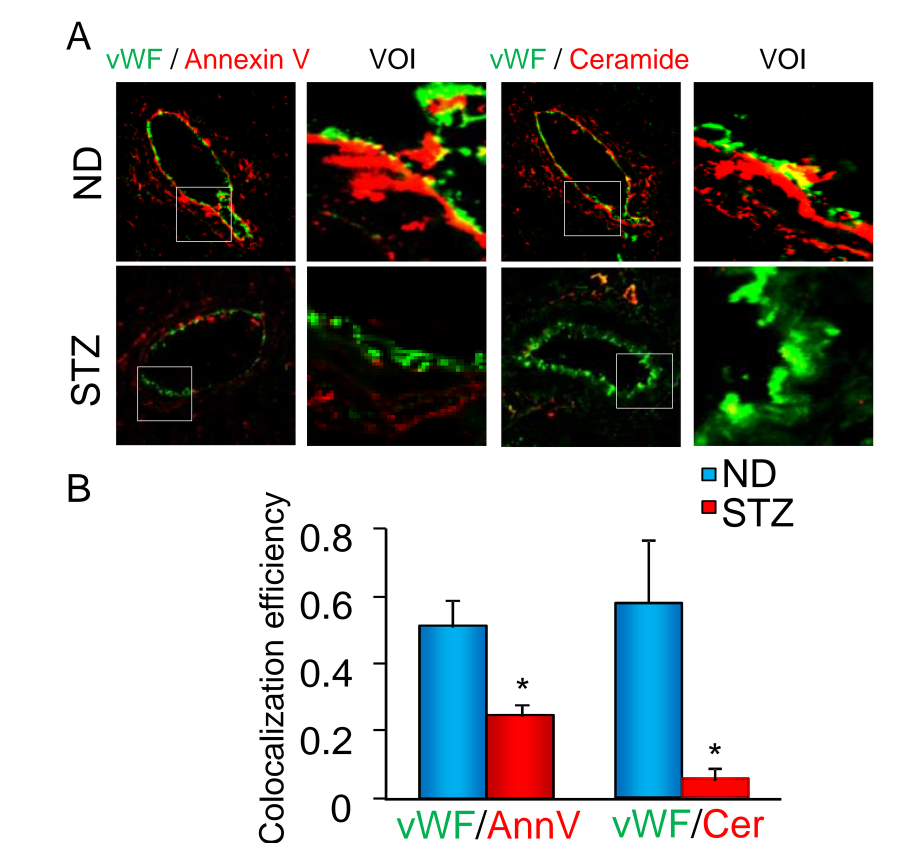

Figure 6. Confocal analysis of endothelia annexin V expression. In mice with STZ-induced diabetes, the expression of Annexin V and ceramide in the endothelial layer of coronary arteries was markedly reduced, as shown by decreased colocalization of annexin V or ceramide with an endothelial cell marker, vWF (yellow patches in panel A). These colocalization of vWF with Annexin V (AnnV) or ceramide were summarized in panel B.

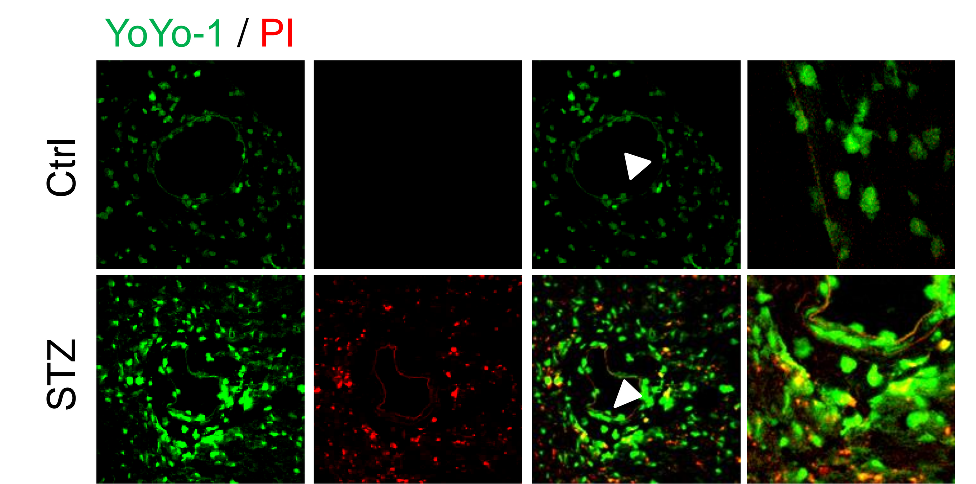

Figure 7. Using YOYO-1 and PI co-staining with a time lag i.v. injection, confocal microscopic analysis of endothelial cell plasmalemma permeability and resealing was performed. In coronary arterial wall of diabetic mice (STZ injected mice), we demonstrated that more YOYO-1 and PI staining occurred. In particular, the PI staining of the same cells after YOYO-1 staining (yellow spots) indicates the failure of resealing the cells.

SUMMARY and CONCLUSION

- Annexin V and ceramide of the plasma membrane are critical for Ca^{2+}-dependent plasma membrane resealing in coronary ECs.
- High glucose induced the plasma membrane disruption due to its effects to decrease ceramide and impair membrane resealing.
- Annexin V-associated cell membrane repair was found to protect coronary ECs from high glucose-induced dysfunction and injury.

It is concluded that annexin V participates in a Ca^{2+}-dependent and ceramide-mediated membrane resealing process in coronary ECs and that this membrane resealing may be impaired during diabetes leading to endothelial dysfunction and injury and ultimate diabetic vasculopathy.