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Critical Role of Lipid Raft Redox Signaling Platforms in Endostatin-Induced Coronary Endothelial Dysfunction

Si Jin, Yang Zhang, Fan Yi, Pin-Lan Li

Objective—Endostatin (EST) was found to initiate a redox signaling cascade associated with activation of NADPH oxidase in endothelial cells (ECs). The present study tested whether EST stimulates clustering of ceramide-enriched lipid rafts (LRs), which assembles and activates NADPH oxidase to form redox signaling platforms.

Methods and Results—Using confocal microscopy, we first demonstrated a colocalization of LR clusters with NADPH oxidase subunits, gp91^{phox} and p47^{phox} in the ECs membrane on EST stimulation. Immunoblot analysis of floated detergent-resistant membrane fractions found that in LR fractions NADPH oxidase subunits gp91^{phox} and p47^{phox} are enriched and that the activity of this enzyme increased dramatically, as measured by electron spin resonance (ESR) spectrometry. This EST-increased LR platform formation was shown to be attenuated by inhibition or RNA interference of acid sphingomyelinase (A-SMase). Functionally, EST pretreatment significantly impaired bradykinin or A23187-induced vasodilation in isolated small coronary arteries, which could be partially reversed by LR disruptors.

Conclusions—The early injury effect of EST on the vascular endothelium is associated with the formation of redox signaling platforms via lipid raft clustering. (*Arterioscler Thromb Vasc Biol.* 2008;28:485-490)

Key Words: endostatin ■ NADPH oxidase ■ coronary ■ endothelium ■ sphingolipid

Endostatin (EST), a ≈20-kDa C-terminal fragment of collagen XVIII located in the basement membrane zones around blood vessels, is a naturally occurring peptide in the body.^{1,2} In vivo, EST has been reported as one of the most potent endothelial cell (EC)-specific inhibitors of angiogenesis and tumor growth, and in vitro, extensive studies have demonstrated that EST specifically influences many cell processes such as proliferation and migration of ECs, EC apoptosis, and so on.³ Besides these generally known actions, recent studies from our laboratory demonstrated that EST reduced the NO bioavailability through enhanced O₂⁻ production in the intact coronary endothelium, suggesting a potential role of EST in the impairment of endothelial function.⁴ This effect of EST was further demonstrated to be associated with the ceramide-mediated signaling pathway.⁴ However, it remains unknown how EST-stimulated ceramide leads to O₂⁻ production and whether this peptide indeed produces endothelial dysfunction when it acts on intact vessels for a short time.

Recently, a growing body of evidence suggests that ceramide plays essential role in lipid raft (LR) clustering in a variety of cell types. When ceramide production is increased, LRs, the lipid microdomains that consist of cholesterol, sphingolipids, and some associated proteins,^{5,6} can be clustered to form large ceramide-enriched macrodomains or platforms, mediating or amplifying transmembrane signals. There is increasing evidence that LRs clustering as a general mechanism participating in the

initiation of receptor-mediated transmembrane cell signal transduction.⁷⁻¹⁰ Along with this line, we recently introduced a new concept regarding LR redox signaling platforms which is importantly involved in the redox regulation of endothelial function.¹¹ We have demonstrated that these LR redox signaling platforms are characterized by gp91^{phox} aggregation and p47^{phox} translocation as well as by activation of acid sphingomyelinase (A-SMase) and subsequent production of ceramide.¹¹ Given that EST stimulates ceramide production through A-SMase and that increased production of ceramide in the cell membrane is able to facilitate the formation of ceramide-enriched platforms,^{5,6} the present study hypothesized that EST stimulates O₂⁻ production through the formation of the LR redox signaling platforms in coronary arterial ECs (CAECs) and thereby leads to impairment of endothelium-dependent vasodilation in coronary arteries. We used a series of molecular and physiological approaches to test this hypothesis.

Materials and Methods

Confocal Analysis of LR Clusters and Its Colocalization With NADPH Oxidase Subunits or A-SMase in CAECs

The primary cultures of bovine CAECs were obtained as we described previously.^{4,11,12} All studies were performed using CAECs of 2 to 4 passages. For microscopic detection of LR platforms, CAECs were grown on glass coverslips and then treated with 100 nmol/L EST (Upstate-Millipore) for 30 minutes to induce clustering

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of LRs. In additional groups of cells, methyl- β -cyclodextrin, MCD (Sigma, 1 mmol/L) and Nystatin (Nyst) (Sigma, 10 μ g/mL) were added to pretreat the cells for 15 minutes before EST stimulation.

Detection of LR clusters were performed as we described previously.^{11–13} In brief, G_{M1} gangliosides enriched in LRs were stained by Alexa488-labeled cholera toxin B (Alexa488-CTX, 1 μ g/mL, 45 minutes; Molecular Probes). For dual staining detection of the colocalization of LRs and NADPH oxidase subunits, gp91^{phox}, or A-SMase, the CAECs were further incubated with mouse anti-gp91^{phox} monoclonal antibody (BD Biosciences, 1:200), or rabbit anti-A-SMase polyclonal antibodies (Santa Cruz, 1:200), separately, which was followed by Texas red-conjugated anti-mouse or anti-rabbit (Molecular Probes) secondary antibody as needed, respectively. Staining was visualized through sequentially scanning on an Olympus scanning confocal microscope (Olympus).

Isolation and Detection of LR Microdomains by Gradient Centrifugation

Because LR domains float in the membrane and are resistant to solubilization by nonionic detergents such as Triton X-100 at low temperatures, they are also termed as floated detergent resistant membrane domains (DRMD). LR microdomains or DRMD were isolated as described previously.^{11,14,15} For immunodetection of LR-associated proteins, 50 μ L of resuspended proteins were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane, and blocked as described previously.^{11,13} The membrane was probed with primary monoclonal antibodies against flotillin-1, anti-gp91^{phox}, anti-p47^{phox} (1:1000, BD Biosciences), respectively, according to corresponding protocols, overnight at 4°C followed by incubation with horseradish peroxidase-labeled anti-mouse IgG (1:5000). The immunoreactive bands were detected by chemiluminescence methods (Pierce) and visualized on Kodak Omat film.

Electromagnetic Spin Resonance (ESR) Spectrometric Detection of O₂⁻

ESR detection of O₂⁻ was also performed as we described previously.^{13,16} The ECs cell mixture loaded in glass capillaries was immediately analyzed by ESR (Noxygen Science Transfer & Diagnostics GmbH) for production of O₂⁻ at each minute for 10 minutes. The SOD-inhibitable signals were normalized by protein concentration and compared among different experimental groups. NADPH oxidase activity in both LRs fractions (mixture of fraction 3, 4) and nonraft fractions (mixture of fraction 7, 8) was measured by ESR. 50 μ L of LR fractions or nonraft fractions were mixed with 1 mmol/L spin trap CMH was substituted for ECs for reactions. The relative increase of the ESR spectrum was used to represent NADPH oxidase activity.

RNA Interference of A-SMase

Small interference RNAs (siRNAs) were purchased from QIAGEN. The DNA target sequence for A-SMase-siRNA is: 5'-AAGGCCGTGAGTTTCTACCT-3'. The scrambled small RNA (AATTCTCCGAA CGTGTCACGT) has been confirmed as nonsilencing double-stranded RNA and was used as control in the present study. Transfection of siRNA was performed using the QIAGEN TransMessenger transfection kit according to the manufacturer's instructions.

Isolated Perfused Small Coronary Artery Preparation

Fresh bovine hearts were obtained from a local abattoir. Small coronary arteries (\approx 200 μ m ID) were prepared and the vasodilator response measured as we described previously.¹¹ After measurement of control vasodilator response, EST was added in the presence or absence of MCD or Nyst in the lumen of the artery and then the vasodilator response was redetermined.

Statistics

Data are presented as means \pm SE. Significant differences between and within multiple groups were examined using ANOVA for repeated measures, followed by Duncan's multiple-range test. A

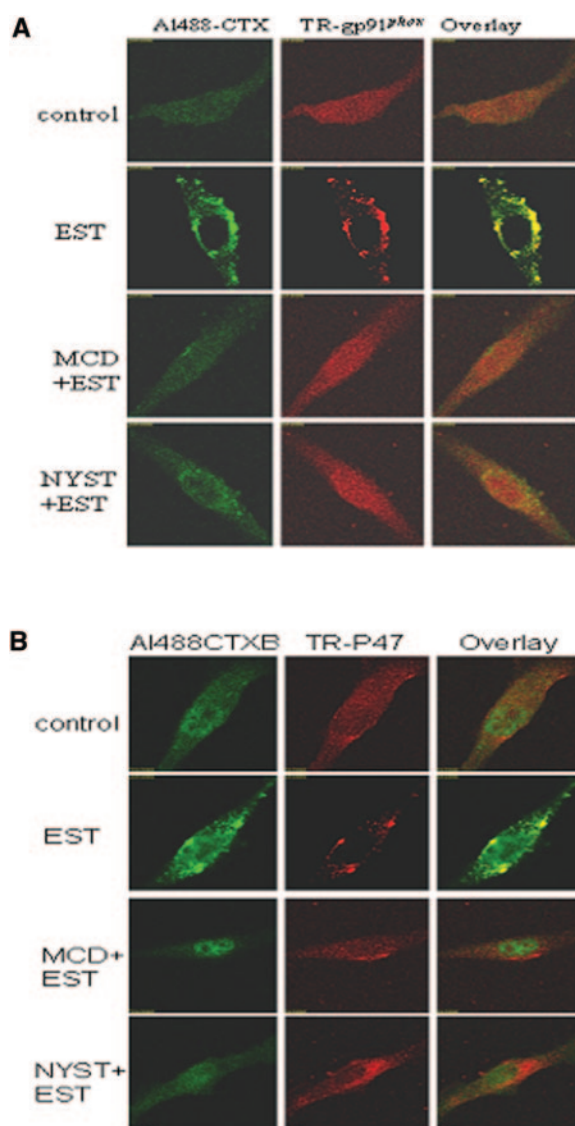


Figure 1. Representative confocal images of LR clusters (Alexa488-CTX) and gp91^{phox} (A), P47^{phox} (B) labeling (Texas red-conjugated). The overlay images exhibited yellow spots or patches (right), which represented colocalization of gp91^{phox} or P47^{phox} and LR component, ganglioside G_{M1}.2. The figure presents representative images from experiments done using 6 batches of ECs cultures.

Students *t* test was used to detect significant differences between 2 groups. *P*<0.05 was considered statistically significant.

Results

LR Clustering and Aggregation or Recruitment of Signaling Molecules in the Membrane of CAECs

As shown in Figure 1, typical LR patches in a CAECs under resting condition and during endostatin stimulation were detected by confocal microscopy (left images). Under resting condition (control), there was only a diffuse fluorescent staining on the cell membrane by Alexa-CTX (Alexa488-CTX), indicating possible distribution of single LRs without clusters for fluorescent spots or patches. However, when these cells were incubated with EST, large fluorescent dots or patches were detected on the cell membrane. These fluorescent

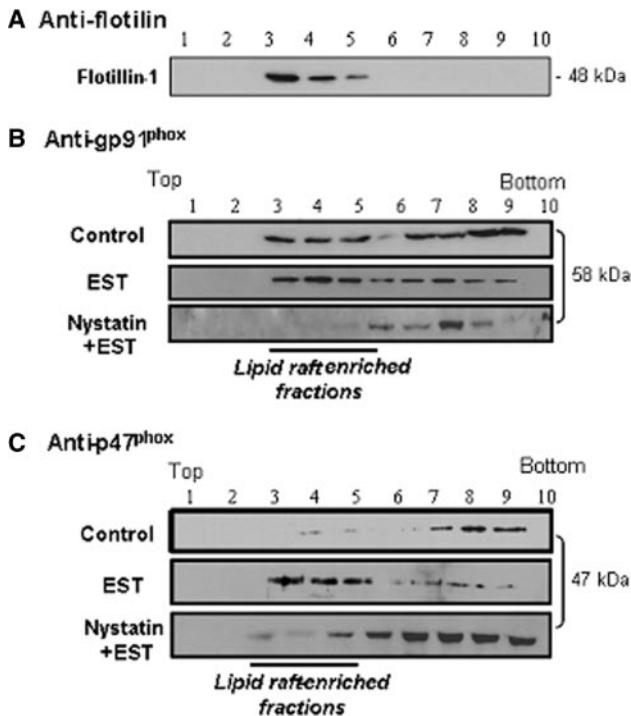


Figure 2. Distribution and localization of gp91^{phox} and p47^{phox} in floated membrane fractions from ECs. A: using LR marker protein flotillin-1, B: using anti-gp91^{phox}, and C: using anti-p47^{phox} (all were representative gels from 4 separated experiments).

patches indicate the formation of LR clusters or macrodomains. In the presence of LR disruptors, either MCD or Nyst, EST-induced formation of LR clusters was substantially attenuated and in many cells there were no LR patches detectable (panel MCD+EST or NYST+EST).

In the middle panel of Figure 1, the cell stimulated by EST was stained by a primary anti-gp91^{phox} antibody and then by Texas red-conjugated secondary antibody. In addition to some diffuse signals under resting condition, red spots and patches were detected on the cell membrane when the cells were stimulated by EST (panel EST). In MCD or Nyst pretreatment groups, the red patches were not observed (panel MCD+EST or NYST+EST).

When two sequentially scanned images from the same cell with different wavelengths were merged together, there were a number of yellow areas either as dots or as patches resulting from green CTX and Texas red-antibody (right images). These yellow patches were considered as a colocalization of LR clusters and NADPH oxidase subunit gp91^{phox}. When the cells were pretreated by either MCD or Nyst, both CTX clusters and aggregated Texas red fluorescence were no longer observed. We also did similar experiments using p47^{phox} antibody and found very similar distribution pattern to gp91^{phox}. As shown in Figure 1B, LR clusters and colocalization of p47^{phox} were confirmed in these ECs when they are stimulated by EST, whereas p47^{phox} are diffusely distributed under control condition.

Enrichment of Both gp91^{phox} and p47^{phox} in LR Fractions of EC Membrane

To further demonstrate an involvement of LRs in aggregations or translocation of NADPH subunits, we isolated

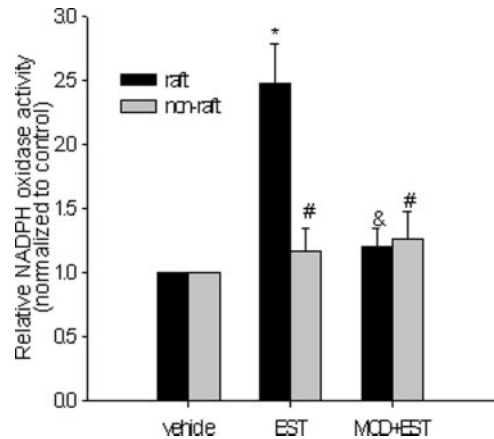


Figure 3. Effects of LRs disruption on EST-induced increase of NADPH oxidase activity in LR-enriched fractions and non-LR fractions. **P*<0.05 vs vehicle; & *P*<0.05 vs EST; # *P*<0.05 vs vehicle; n=6.

membrane fractions by detergent treatments and gradient centrifugations. As shown in Figure 2A, Western blot analysis detected positive expression of flotillin-1 in fractions 3 to 5, which were referred to as LR fractions previously.²³ The ratio of gp91^{phox} or p47^{phox} between LRs fractions and other fractions was used to analyze their aggregation or translocation, because EST induces their clustering in LRs fractions of cell membrane, rather than increases expression. Figure 2B shows that gp91^{phox} could be detected in most of the membrane fractions from CAECs; however, there was a marked increase in gp91^{phox} protein in LR microdomains when CAECs were stimulated by EST. This increase was significantly inhibited by pretreatment of cells with Nyst. Similarly, as shown in Figure 2C, p47^{phox}, a cytosolic NADPH oxidase subunit, was also found increased in LR fractions on EST stimulation and this increase also inhibited by Nyst. Similarly, another LR disruptor, MCD, blocked the enrichment of these 2 NADPH oxidase subunits in LR fractions.

NADPH Oxidase Activity in LR Microdomains of ECs

We also analyzed the NADPH oxidase activity present in both LR and nonraft fractions from control and EST-treated CAECs. We found that EST significantly increased NADPH oxidase activity by 147% in LR fractions, but only by 20% in nonraft fractions (Figure 3), which were both prevented by MCD pretreatment.

ESR Assay of EST-Induced O₂⁻ Production in CAECs

The direct consequence of activated NADPH oxidase is increased production of O₂⁻ in CAECs. Figure 4 presented summarized data obtained by ESR assay using CAECs, showing the fold changes in O₂⁻ production. After CAECs were treated with EST, O₂⁻ levels detected by ESR increased significantly. When the cells were pretreated with MCD, this EST-induced increase in O₂⁻ production was significantly reduced. MCD alone had no significant effects on the O₂⁻ level.

Association of A-SMase Translocation and LR Clustering

As shown in Figure 5, CAECs were double-stained with Alexa488 labeled CTX and anti-A-SMase primary antibody

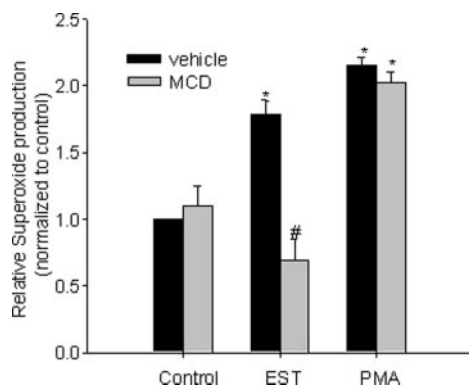


Figure 4. Effects of LR disruption on EST-induced $O_2^{\cdot-}$ production in CAECs. Summarized data depicting changes in $O_2^{\cdot-}$ production in CAECs with different treatments. * $P < 0.05$ vs control; # $P < 0.05$ vs EST group; $n = 5$.

followed by Texas red-conjugated secondary antibody. Similar to that described in Figure 1, left and middle images represent fluorescence signals from Alexa488 and Texas Red, separately. Compared with control CAECs, EST induced significant LR patches on the cell membrane, accompanied by the aggregation of A-SMase. This was shown clearly in overlaid images, where many yellow dots or patches exhibited, indicating a colocalization of CTX-stained ganglioside M_1 and A-SMase. When the CAECs were pretreated by a putative A-SMase inhibitor, desipramine, the EST-induced changes in LR clustering were blocked. In addition, when the CAECs were transfected with synthesized A-SMase siRNA (75% knocking down as shown in our preliminary experiments and previous studies¹³), EST no longer induced LR clustering and colocalization with A-SMase with LR marker (siRNA+EST). However, CAECs transfected with scramble RNA still reserved LRs clustering responses to EST (scrambleRNA+EST).

LR Disruptors Reversed EST-Induced Impairment of Endothelium-Dependent Vasodilation to BK or A23187

Incubation of small coronary arteries with EST (100 nmol/L, perfused into the lumen) had no significant effect on the basal arterial diameters, but markedly attenuated the BK-induced increase in arterial diameters. In the presence of Nyst (for 15 minutes), however, EST-induced inhibition of vasodilator response to BK was reversed. Treatment of the arteries with Nyst alone had no significant effect on basal arterial diameters or BK-induced vasodilation at the same doses used (Figure 6A). As shown in Figure 6D, the effects of Nyst to reverse the action of EST were also observed in arterial preparations that use A23187 as endothelium-dependent vasodilator. Similar results were obtained when we used another LR disruptor, MCD. EST-induced impairment of endothelium-dependent vasodilator response to BK and A23187 were also reversed by MCD treatment (Figure 6B and 6E).

In Figure 6C and 6F, 2 NADPH oxidase inhibitors, DPI and apocynin were used to preincubate the vessels and the vasodilation response to BK and A23187 were determined.

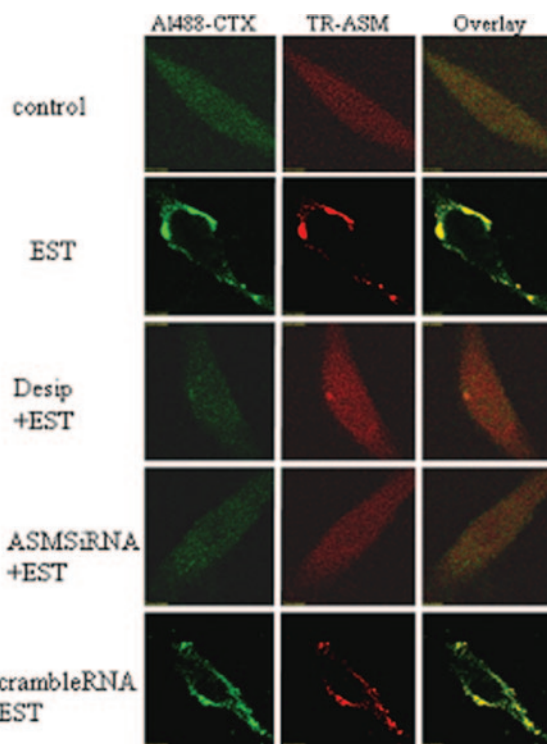


Figure 5. Confocal microscopic analysis of A-SMase in LR clusters in ECs stimulated with EST. Texas-red conjugated anti-A-SMase in the left and LR markers (A488-CTX in the middle) were used to stain cells. Desip indicates desipramine; A-SMase siRNA, A-SMase siRNA transfection. The images are representatives from 6 separated experiments.

Both inhibitors significantly blocked the EST-induced impairment of vasodilation response.

Discussion

In previous studies from our laboratory and by others, EST was demonstrated to produce $O_2^{\cdot-}$ via enhanced NADPH oxidase activity and thereby regulate cell function or activities.^{4,28} However, the mechanism how this peptide activates NADPH oxidase is still poorly understood. The present study tested a hypothesis that EST induces or enhances NADPH oxidase activation through the formation of LR signaling platforms. To test this hypothesis, we first determined whether EST induces the formation of this LR redox signaling platform associated with NADPH oxidase. By using confocal colocalization analysis of LR marker, Alexa488-labeled CTXB and Texas red conjugated gp91^{phox} and p47^{phox}, 2 typical NADPH oxidase subunits, we demonstrated that EST stimulated LR clustering and enhanced enrichment of NADPH oxidase subunits in LR fractions. These results suggest that gp91^{phox} could be aggregated in response to EST stimulation. This aggregation of NADPH oxidase subunits may be of importance in the response of this enzyme to agonists in vascular ECs. Although there are many studies demonstrating an assembling or aggregation of NADPH oxidase subunits to form an enzyme complex and to produce $O_2^{\cdot-}$.²⁹ This assembling or aggregation is usually centered by its membrane subunits such as gp91^{phox} or other Nox. However, little is known whether these subunits themselves are able to aggregate or be

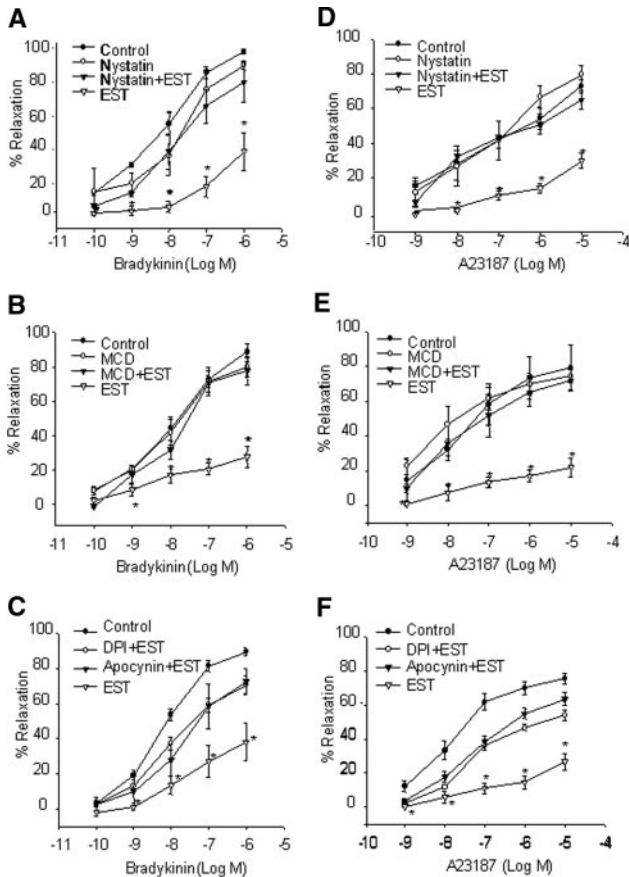


Figure 6. Effects of LR disruptors on EST-induced impairment of the vasodilator responses. A, Nyst, responses to BK; D, Nyst, responses to A23187. B, MCD, responses to BK; E, MCD, responses to A23187. C, DPI, apocynin, responses to BK; F, DPI, apocynin, responses to A23187. n=7 or 6, *P<0.05 vs control.

clustered. The present studies provide evidence that in response to EST the NADPH oxidase subunits are able to be aggregated and that this aggregation may form an NADPH oxidase-mediated redox platform, which may be a prerequisite or driving force to initiate assembling of other subunits and consequent activating cascade of this enzyme.¹¹

In addition to aggregation of gp91^{phox}, we also found that a cytosolic subunit, p47^{phox} was enriched in membrane LR fractions on EST stimulation. This p47^{phox} enrichment in LR fractions may represent its translocation into the cell membrane. In this regard, p47^{phox} has been reported to be translocated and then interacts with membrane-bound NADPH oxidase subunits in response to agonists such as Ang II, tumor necrosis factor (TNF)- α , growth factors, and thrombin.³⁰⁻³⁴ It is now known that this translocation of p47^{phox} is governed by serine phosphorylation and that p47^{phox} protein contains a number of phosphorylation sites for PKC, PKA, and MAPK.^{32,33} The findings of the present study further demonstrate that this p47^{phox} translocation occurs in LR microdomains, in particular, in LR platforms during agonist stimulations. LR clustering may provide a driving force to promote this p47^{phox} translocation in response to agonist stimulations such as EST used in the present study.

To provide direct evidence that aggregation or assembling of NADPH oxidase components via LR clustering is

involved in the activation of this enzyme, we isolated the whole membrane fractions as well as its LR fractions from CAECs and determined NADPH oxidase-derived O₂⁻ production. It was found that EST indeed enhanced NADPH oxidase activity in the whole membrane fraction, which was inhibited by MCD, a lipid raft disruptor that could extract cholesterol from the cell membrane.⁵ In isolated LR fractions, EST more dramatically increased NADPH oxidase activity, which was also blocked by treatment of cells with MCD. We also demonstrated that EST significantly increased O₂⁻ production in intact CAECs, which was blocked by MCD. These results confirm that aggregated NADPH oxidase subunits in LR platforms are functioning as an active enzyme complex.

Next, we explored the mechanism by which EST stimulates LR clustering and thereby form LR redox signaling platforms. Given a major contributing role of ceramide to the formation of ceramide-enriched signaling platforms, it is very possible that A-SMase translocation and activation are importantly implicated in EST-induced LR clustering and redox signaling platforms formation. To test this hypothesis, we double-stained the CAECs with AI488-labeled CTXB and Texas red-conjugated anti-A-SMase and found that EST significantly increased the enrichment of A-SMase in the LR platforms. In the presence of desipramine, an inhibitor of A-SMase, this EST-stimulated enrichment of A-SMase in the LR platforms was almost completely blocked. However, although desipramine is often used as ASMase inhibitor, its action on ceramidase may be involved and thus interpretation of the results obtained by using this compound should be cautious. For this reason, we also used A-SMase specific siRNA to knock down this gene and found that clustering of LRs with A-SMase was blocked. These results together confirm that aggregation in LRs and consequent activation of A-SMase is essential to the process of LR clustering. This role of A-SMase in mediating LR clustering in ECs has been also found in response to other agonists such as FasL and TNF- α .^{13,36} It seems that the A-SMase activation is a common mechanism mediating LR clustering and corresponding signaling platforms formation.³⁷

The other important findings of the present study are that EST impaired the endothelium-dependent vasodilator responses and that this action of EST is associated with enhanced formation of LR clusters. This EST-induced endothelial dysfunction occurred no matter whether a receptor-mediated endothelium-dependent vasodilator, BK, or a non-receptor-mediated endothelial NO stimulatory agonist, A23187, was used. Although A23187 bypasses the receptor step and activates eNOS through calcium, endostatin-induced O₂⁻ production may interact with NO to generate ONOO⁻ impairing the A23187-induced vasodilator responses. Our results provide direct evidence that EST produces an early action to induce endothelial dysfunction before detectable apoptotic effect. In this regard, EST has been reported to induce a detectable apoptosis only when it was incubated with ECs at least for 6 hours.^{38,39} Given a relative resistance of ECs to apoptosis,⁴⁰ the functional impairment may represent one of the most important pathological actions of this apoptotic

peptide in ECs. Because EST-impaired vasodilator responses could be restored in the presence of LR disruptors or NADPH oxidase inhibitors or silencers, it seems that EST-induced endothelial dysfunction is attributable to increased O_2^- production from LR platforms with aggregated NADPH oxidase subunits. Increased O_2^- could reduce the bioavailability of NO, resulting in impairment of endothelium-dependent vasodilation, as shown in our previous studies and by others.^{4,41–44}

In summary, the present study demonstrated that EST is able to induce endothelial dysfunction as an early-stage acute action, which is associated with assembling and activation of NADPH oxidase in LR clusters. The clustered LRs together with activated NADPH oxidase constitute a redox signaling platform mediating the pathological actions of EST on the vascular endothelium.

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Disclosures

None.

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