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Forum Review

Lipid Raft Redox Signaling Platforms in Endothelial Dysfunction

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ABSTRACT

In response to various stimuli, membrane lipid rafts (LRs) are clustered to aggregate or recruit NADPH oxidase subunits and related proteins in vascular endothelial cells (ECs), forming redox signaling platforms. These LR signaling platforms may play important roles in the normal regulation of endothelial function and in the development of endothelial dysfunction or injury under pathological conditions. This LR-mediated mechanism now takes center stage in cell signaling for the regulation of many cellular activities or cell function such as ECs redox signaling, phagosomal activity of phagocytes, and cell apopotosis of lymphocytes. This brief review summarizes current evidence that relates to the formation of LR redox signaling platforms and their features in ECs, the functional significance of these signaling platforms in mediating death receptor activation-induced endothelial dysfunction, and the mechanisms initiating or promoting the formation of these platforms. It is expected that information provided here will help readers to understand this new signaling mechanism and perhaps extend the LR signaling platform concept to other research areas related to death receptors, redox signaling, endothelial biology, and cell/molecular biology of the cardiovascular system. *Antioxid. Redox Signal.* 9, 1457–1470.

INTRODUCTION

THERE IS INCREASING EVIDENCE THAT CLUSTERING of distinct cholesterol- and sphingolipid-rich membrane rafts [lipid rafts (LRs)] is importantly involved in transmembrane signaling in a variety of mammalian cells (47, 90). Many receptors, including tumor necrosis factor α (TNF-α) receptors, Fas, DR3, 4, 5, insulin receptors, and integrins, as well as other postreceptor signaling molecules can be aggregated within the LR clusters to form signaling platforms (34, 114). The formation of these LR signaling platforms with aggregation of different signaling molecules may represent one of important mechanisms determining the variety of transmembrane signaling; it also robustly amplifies signals from activated receptors. Among these LR signaling platforms, we recently reported that some large redox signaling molecules are also aggregated or recruited into LR clusters and subsequently produce superoxide ($O_2^{\cdot -}$) and other reactive oxygen species (ROS). This type of LR signaling platforms with ROS production has been referred to as LR redox signaling platforms (114), which now takes center stage in endothelial cells (ECs) redox signaling by death receptors, as commented by a journal editorial commentary (98). In addition, this LR signaling mechanism has also been reported to play important roles in the normal regulation of many other cell or organ functions and in the development of different pathological conditions of different cells or organs. This brief review will provide information on the basic principles and mechanisms of LR clustering or signaling platform formation and the possible relevance of LR redox signaling platforms, with a focus on cardiovascular cells or tissues.

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LRs AND TRANSMEMBRANE SIGNALING

Membrane LRs

It has been indicated that LRs are present in a variety of mammalian cells and that this special small lipid structure is considered to be an important signaling component in the cell membrane (88, 90). LRs consist of dynamic assemblies of cholesterol and lipids with saturated acyl chains that include sphingolipids and glycosphingolipids in the exoplasmic leaflet of the membrane bilayer. In addition, phospholipids with saturated fatty acids and cholesterol in the inner leaflet are important elements for LRs. By interdigitation and transmembrane proteins, the long fatty acids of sphingolipids in the outer leaflets couple the exoplasmic and cytoplasmic leaflets, which form a very stable and detergent-resistant membrane structure (65, 90). This stable structure is one of the most basic features of LRs. Different from this stable membrane structure, a large portion of cell membrane lipid (60–80%) is more fluid, as it mostly consists of phospholipids with unsaturated and kinked fatty acid chains, as well as cholesterol. Another interesting feature of these membrane LRs is their capacity of including or excluding proteins to variable extents when cells respond to different physiological or pathological stimuli. Many proteins have been demonstrated to have LR affinity such as glycosyl-phosphatidylinositol (GPI) anchored proteins, doubly acylated proteins, cholesterol-linked proteins, and some other transmembrane proteins, including ion channels, tyrosine kinases, and different membrane exchangers or transporters (90, 97).

LR clustering

Recently, the clustering of cell membrane LRs has been reported to form important signaling platforms instead of being silent building blocks (3, 90). These membrane signaling platforms play important roles in the transmembrane signaling in a variety of mammalian cells. In this regard, initiation of intracellular signaling cascades is associated with aggregation or reduction of cell surface receptors through LR clustering in the plasma membrane (34, 37). These receptors aggregated in LR clusters are many, including T-cell receptor/CD3 complex, B-cell receptors, CD2, CD40, CD44, L-selectin, insulin receptors, or integrins, which transfer the signal to these transmembrane signaling proteins or proteins in inner leaflets of the cell membrane when they aggregate within LR clusters. This completes the transmembrane signaling process (33, 89). Recent studies have indicated that several death receptors, including tumor necrosis factor receptor (TNFR), Fas, and DR 4 and 5 produce their detrimental effects through this mechanism (60, 90). During LR clustering, aggregated receptors or other signaling molecules could be either constitutively located in LRs or translocated by trafficking or recruitments upon stimulation (12, 20). This dynamic clustering of lipid microdomains may represent a critical common mechanism in transmembrane signal transduction.

It has been reported that clustered LRs contain different compositions of proteins, constituting platforms or macrodomains that form a new mixture of molecules, including different signaling molecules and crosslinkers or enzymes (88, 90). The formation of LR platforms activates, facilitates, or amplifies signal transduction. There is considerable evidence that LR clustering could be formed as a ceramide-enriched membrane platform and that ceramide production or enrichment is through sphingomyelinase (SMase)-catalyzed cleavage of choline from sphingomyelin (SM) in individual LRs (39, 42). However, ceramide-enriched membrane platforms might also be formed without existence of classically-defined LRs simply through a fusion of several ceramde molecules. These ceramide molecules could come from LRs or other membrane fractions. This topic is discussed in detail in Dr. Gulbins's review of this forum.

LR clustering in endothelial cells (ECs)

LR clustering or platform formation is implicated in the regulation of a number of biological processes in different cells, including cell growth, differentiation and apoptosis, T-cell activation, tumor metastasis, and neutrophil and monocyte infiltration (39). The clustering of receptor molecules within ceramide-enriched membrane platforms might have several important functions such as establishment of a close proximity of many receptor molecules (37), facilitation of transactivation of signaling molecules associating or interacting with a receptor, and amplification of the specific signaling of the activated receptors.

In vascular ECs, LR-mediated signaling from membrane LRs has also been demonstrated to occur in response to different stimulations. There is evidence that the LR clustering in ECs aggregates cell-associated heparan sulfate proteoglycans (HSPGs) localized in these individual LRs, which facilitates the entry of human immunodeficiency virus type 1 (HIV-1) into these cells, causing infection (50). In studies on leukocyte migration, the presence of E-selectin in LRs on ECs was found to be necessary for its action to associate with and activate PLCγ, suggesting the importance of a localization of E-selectin in LRs to leukocyte-endothelial cell interactions (50). As a signaling mechanism, LRs and their temporal–spatial organization with caveolae are also reported to be involved in signaling of vascular endothelial growth factors (VEGF), nitric oxide synthase (74), and H_2O_2 (109). Downstream effector response to LR clustering in these ECs include receptor autophosphorylation, cAMP production, caspase activation, decrease or increase in nitric oxide (NO), reorganization of the actin cytoskeleton, and Ca^{2+} mobilization (58, 106). In addition, LRs have also been implicated in promitogenic signaling in ECs. All these studies have indicated that LRs and LR clustering are present in vascular ECs and may play an important role in various activities of ECs. With respect to the functional significance of endothelial LRs, there is increasing evidence that the LR-mediated signaling mechanisms may contribute to the regulation of several important endothelial functions, such as endothelial barrier function (80), endothelium-dependent vasodilator or constrictor response, endothelial metabolic function (70, 114), and its anticoagulation and antithrombolic functions (59). Although some reports have suggested that LR regulation of endothelial function may be associated with their clustering or aggregation, dissociation or egression, and internalization or endocytosis, LR clustering or aggregation to form macrodomains or platforms in EC membrane is a major mechanism mediating transmembrane signaling. In this regard, work in our laboratory has demonstrated that it is the LR clustering that importantly participates in the redox signaling of ECs, which contributes to the regulation of endothelium-related vasomotor response in intact

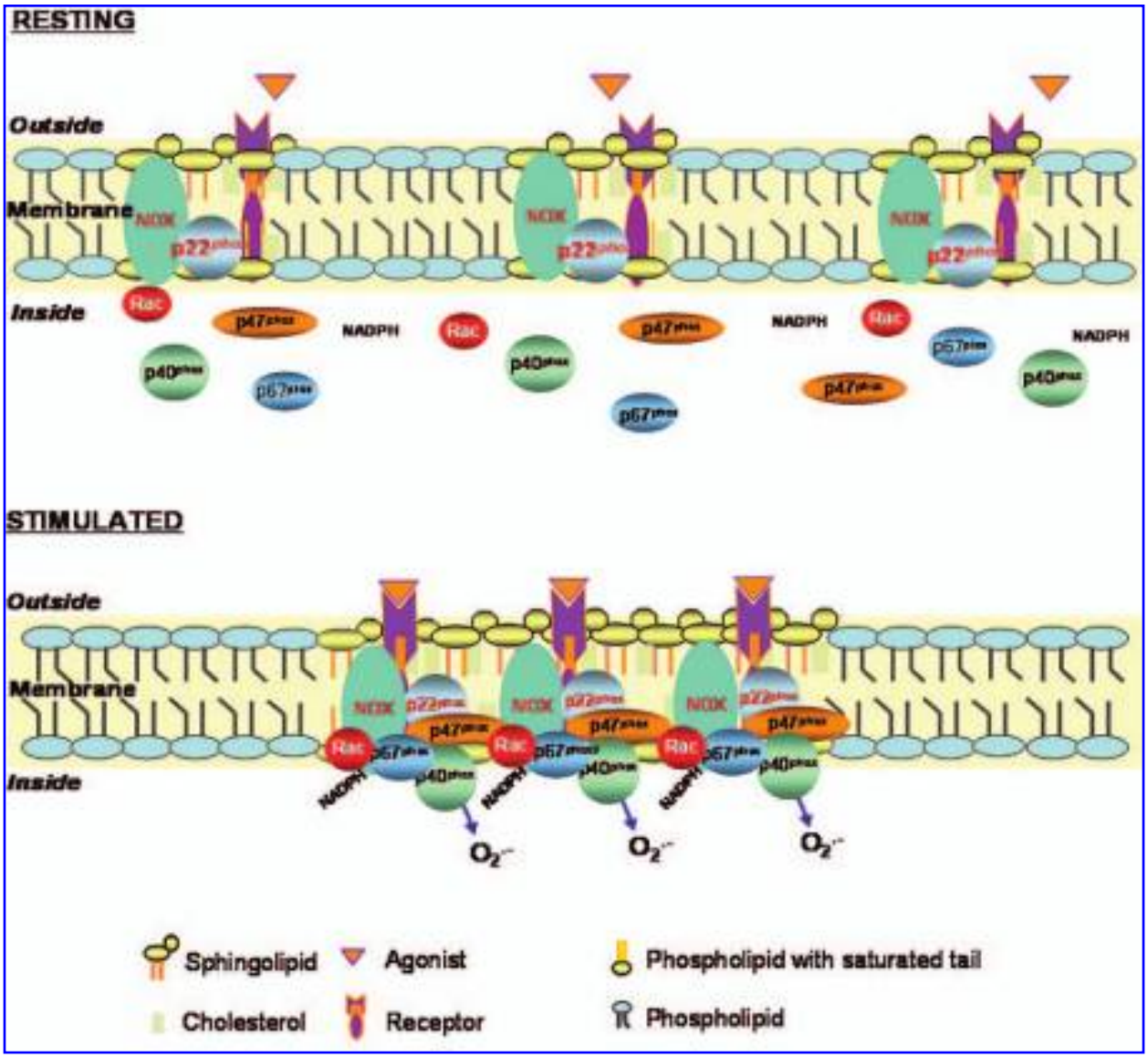

FIG. 1. A hypothetic model showing LRs and LRs clustering to form a redox signaling platform. Under resting condition, individual LRs with attached receptors are present in the membrane of ECs (*panel for resting cells*). These individual LRs are dynamic microdomains and carry several membrane-bound or attached proteins or enzymes such as G-proteins, protein kinases, or the subunits of NADPH oxidase gp91^{phox} and p47^{phox}. When ligands or agonists bind to their receptors on individual LRs, the clustering is activated to form a number of LR macrodomains or platforms with aggregation or recruitment of receptors, NADPH oxidase subunits, and other proteins such as Rac GTPase. Clustering of these proteins and enzymes leads to activation of NADPH oxidase and production of $O_2^{\cdot -}$, which results in a prominent amplification of the transmembrane signal (*panel for stimulated cells*).

coronary arteries. This LR-mediated redox signaling mechanism in the vascular endothelium will be a major topic in following sections.

LR REDOX SIGNALING PLATFORMS ASSOCIATED WITH NADPH OXIDASE

Redox signaling is emerging as an essential mechanism in the regulation of biological activity of a variety of cells. In vascular ECs, $O_2^{\cdot -}$ can be derided from activation of nonphagocytic NADPH oxidase (NOX) proteins, which is a family of gp91^{phox}-like proteins including NOX1, NOX2 (also known as gp91^{phox}), NOX3, NOX4, NOX5, and two related proteins, DUOX1 and DUOX2; xanthine/xanthine oxidase; mitochondrial NOX, or uncoupled nitric oxide synthase (NOS). However, overwhelming evidence has now accumulated showing that nonmitochondrial NOXs are a major source of $O_2^{\cdot -}$ for the redox regulation of vascular endothelial and smooth muscle function (14, 36). It has been estimated that this nonmitochondrial NOX-derived $O_2^{\cdot -}$ constitutes >95% of the production of $O_2^{\cdot -}$ in the vasculature, especially upon stimulation (68, 77). Therefore, the role of NADPH oxidase is now highlighted in the normal regulation of endothelial function and in the development

of related diseases associated with endothelial dysfunction such as atherosclerosis, hypertension, and diabetes.

Activation of NADPH oxidase in ECs

NADPH oxidase, first identified and characterized in neutrophils, catalyzes one-electron reduction of oxygen to produce $O_2^{\cdot -}$ using NADPH as the electron donor (8, 36). Vascular ECs express an NADPH oxidase similar to that identified in neutrophils. This endothelial NADPH oxidase has similar subunits to those in neutrophils or phagocytes, including gp91^{phox}, p22^{phox}, p47^{phox}, p40^{phox}, and p67^{phox}. A cytosolic GTPase, Rac, also importantly participates in activation of NADPH oxidase by assembling an NADPH oxidase complex on the cell membrane. Recent studies have shown that NADPH-derived $O_2^{\cdot -}$ production was primarily detected in 100,000 *g* membrane fraction; therefore, this enzyme is likely to localize mainly in cell membranes in these cells. Functionally, this endothelial NADPH oxidase shares some, but not all, of the characteristics of neutrophil NADPH oxidase. One major difference is that endothelial NADPH oxidase continuously generates a low level of $O_2^{\cdot -}$ even in unstimulated cells, although its activity can be further increased by several agonists. However, neutrophil NADPH oxidase primarily produces $O_2^{\cdot -}$ when the cells are stimulated. In regard to the isoform of NOXs, gp91^{phox} (NOX2) has been considered as the major isoform of NOX proteins in vascular ECs (19, 32). The functional role of this NOX isoform has been confirmed by decrease in phorbol ester-induced $O_2^{\cdot -}$ production and endothelium-dependent relaxation in gp91$^{phox-/-}$ mice (32). In addition to gp91^{phox}, NOX4 mRNA is also detectable in ECs. It appears that NOX4-dependent oxidase functionally contributes to the basal $O_2^{\cdot -}$ production in ECs (2).

Despite extensive studies, the precise mechanisms for the production of $O_2^{\cdot -}$ by endothelial NADPH oxidase are still poorly understood. Some studies have suggested that this endothelial enzyme may produce $O_2^{\cdot -}$ by transfer of electrons from a reduced substrate to molecular oxygen in a way similar to neutrophil gp91^{phox}. In the electron transfer process for neutrophil NADPH oxidase, the cytosolic NADPH binds to gp91^{phox} and releases two electrons (53). These electrons were transferred to two molecules of oxygen on the extracellular side of the membrane via FAD and heme and results in production of two molecules of $O_2^{\cdot -}$ (53). Recent studies have indicated that activation of NADPH oxidase in ECs is involved in several signaling pathways, including Rac/Ras, arachidonic acid metabolites, and ceramide, during stimulation by agonists or hemodynamic forces (107). There is evidence that different stimuli activate this oxidase by assembling or aggregating its membrane-bound and cytosolic subunits (11, 53). The assembly of the active NADPH oxidase requires translocation of cytosolic subunits p47^{phox}, p67^{phox}, and Rac to the plasma membrane, where these subunits interact with gp91^{phox} and p22^{phox} and associate with other cofactors in the membrane to form a functional enzyme complex. The p47^{phox} translocation has been considered as a key step, to some extent, a marker event, for the assembly and activation of NADPH oxidase since it is first translocated during the assembly process of these enzyme subunits. It has been reported that p47^{phox} translocation is initiated by the phosphorylation of this subunit at various phosphorylation sites by protein kinase C (PKC), protein kinase A (PKA), or mitogen-activated protein kinase (MAPK) (26, 76). Studies using either tissues from p47$^{phox-/-}$ mice or specific inhibitors have shown a crucial role for p47^{phox} in endothelial NADPH oxidase activation by several agonists such as angiotensin II, TNF-α, VEGF, and by chronic oscillatory shear (28, 100). However, for a long time it is unknown how p47^{phox} translocation and subsequent assembly of other NADPH oxidase subunits occur on the cell membrane. As discussed below, the LR clustering or formation of LR macrodomains represents an important mechanism mediating this assembly or activation process of NADPH oxidase.

Demonstration of LR redox signaling platforms

Recent studies have demonstrated that LR clustering occurs in coronary arterial ECs, as shown by the formation of large membrane LR patches or macrodomains (114). Various agonists or stimulations were tested including FasL, anti-Fas CH11, TNF-α, endostatin, H_2O_2, 7-dehydrocholesterol, platelet aggregation factor, acetylcholine, and prostaglandins (13, 85). It was demonstrated that some agonists such as FasL, TNF-α, and endostatin induced an aggregation of membrane-bound gp91^{phox} and translocation of cytosolic p47^{phox} into LR clusters, whereby NADPH oxidase activity was markedly increased. In addition, these LR clusters with recruitment or aggregation of NADPH oxidase subunits formed a number of LR–NADPH oxidase complexes, which produce ROS including $O_2^{\cdot -}$ to act on downstream effectors and induce physiological or pathological cell response. Now these LR–NADPH oxidase clusters or complexes that possess redox signaling function have been referred to as membrane LR redox signaling platforms, which could transmit or amplify the signals produced by agonists or extracellular stimuli across cell membrane (114).

As graphically described in Fig. 1, under resting conditions, individual LRs with attached receptors are present in the membrane of ECs (panel for resting cells). These individual LRs are dynamic microdomains and carry several membrane-bound or attached proteins or enzymes such as G-proteins, protein kinases, or the subunits of NADPH oxidase gp91^{phox} and p47^{phox}. When ligands or agonists bind to their receptors on individual LRs, their clustering is activated to form a number of LR macrodomains or platforms with aggregation or recruitment of receptors, NADPH oxidase subunits, and other proteins such as Rac GTPase. Clustering of these proteins and enzymes leads to activation of NADPH oxidase and production of $O_2^{\cdot -}$, which results in a prominent amplification of the transmembrane signal (panel for stimulated cells). $O_2^{\cdot -}$ will mediate redox signaling to produce various responses depending on targeted effector molecules within cells.

Fluorescent and confocal microscopy of LR redox signaling platforms. A method commonly used for detection of LR clusters, including ceramide-enriched platforms not from classical defined LRs, is to detect a colocalization of lipid components and aggregated or recruited NADPH oxidase subunits or other molecules. Although individual LRs are too small to be resolved on the cell surface by standard light microscopy, clustered LRs could be visualized by fluorescence or other staining techniques if their components are crosslinked

with antibodies or lectins (37). Therefore, fluorescent or confocal microscopy of LR patches and spots is widely used as a common method to detect LRs in membranes of living cells. One of LR markers is FITC–CTX (FITC-cholera toxin), and the reason of FITC-CTX being used is based on the fact that G_{M1} is only relatively specific for rafts, which selectively partitions into LRs (33, 44). Using this marker, our recent confocal microscopic analysis demonstrated that LRs were distributed through ECs membrane in a random punctuate staining pattern. Upon stimulation, these endothelial LRs formed multiple onpolarized patches randomly distributed throughout the cell membrane, as shown in Fig. 2, middle panels. These FITC-CTX patches have been confirmed as LR clusters by numerous studies (37). Various agonists such as FasL, endostatin, and TNF-α stimulate the formation of this LR clusters (50, 114). With regard to the shape of LR clusters under fluorescent or confocal microscope, the polarized patches usually detected in T or B lymphocytes upon stimulation could not be identified in ECs (34). Instead, rather random patching sites were observed as shown in Fig. 2, the panel with FasL stimulation. It seems that LRs are clustered to form signaling platforms at different locations depending on cell types, and therefore the shape and features of the LR clusters are much related to the function of cells.

In these LR macrodomains or platforms, colocalization of receptors and NADPH oxidase with LRs should be most important evidence for the presence of LR redox signaling platforms. By a double staining of ECs with FITC-CTX and anti-Fas or anti-gp91^{phox} antibodies, our recent studies showed that Fas and gp91^{phox} were colocalized with LR elements stained by FITC-CTX on coronary arterial ECs when they were stimulated with FasL, as also shown in Fig. 2, right panel for merged images. Similar to gp91^{phox}, other NADPH oxidase subunits were also found clustered in LR platforms such as p47^{phox}, Rac GTPase, and other molecules (114). However, if ECs were pretreated with LR disruptor or cholesterol depletion reagents such as methyl-β-cyclodextrin (M-β-CD) or filipin, LR clustering and aggregation of NADPH oxidase subunits were completely abolished, suggesting that colocalization of ganglioside G_{M1} and NADPH oxidase represents LR-mediated clustering on the cell membrane of ECs.

Flotation of detergent-resistant membranes. Fluorescent or confocal microscopy described above could confirm the occurrence of LRs clustering in response to activation of different receptors in ECs and represent one of the most important approaches to confirm LR redox signaling platforms in living cells. Another important detection of LR clusters often used is the flotation of detergent-resistant membranes (DRM) to identify associated proteins or receptors in LR fractions. DRM complex or detergent-insoluble glycolipid-enriched domains (DIG) float to low density fraction during sucrose gradient centrifugation and are enriched in raft proteins and lipids, which provides a simple means of identifying possible LR components, especially LR-associated proteins (22, 88, 90). Many signaling proteins abundant in LRs have been confirmed by this DRM flotation such as many GPI-anchored proteins, some transmembrane proteins such as intestinal epithelial sucrose–isomaltase, and numerous acylated proteins such as heterotrimeric G-proteins, Ras, and Src family tyrosine kinases. Also,

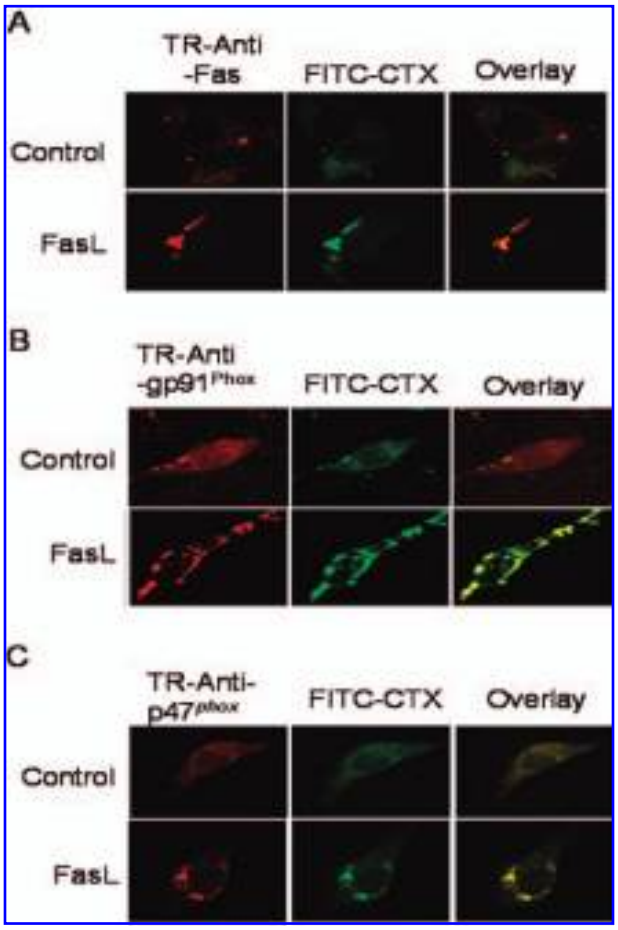

FIG. 2. Confocal images showing colocalization of LRs and Fas, gp91^{phox} and p47^{phox} in coronary arterial ECs. LRs were evenly spreaded throughout the cell membrane under control condition as indicated by weak diffused green FITC fluorescence (*control*). Upon stimulation with FasL, these endothelial LRs form multiple patches on the cell membrane as displayed by *large and intense green fluorescence patches*. Furthermore, Fas, gp91^{phox} or p47^{phox} evenly spread throughout the membrane under normal conditions; FasL aggregated Fas, gp91^{phox} or p47^{phox} as shown by *red fluorescence patches*. FITC-CTX and Fas, gp91^{phox} or p47^{phox} colocalized as indicated after merging the fluorescence stainings. CTX, cholera toxin B; TR, Texas red.

many receptors such as T-cell receptors, B-cell receptors, EGF receptors, insulin receptors, angiotensin II receptors, prostanoid receptors, Fas, and integrins have been shown in floated DRM (33, 35, 89). In combination with proteomic techniques developed recently, this membrane flotation technique can help demonstrate unidentified molecules including receptors, enzymes, and adaptors (63).

For detection of LR redox signaling platforms in ECs, DRMs prepared by gradient centrifugation and Western blot analysis were used to examine the distribution of membrane subunits of NADPH oxidase, either natively located in membrane or recruited into membrane LRs in recent studies from our laboratory and by others (84, 114, 120). It was found that gp91^{phox} was abundant in the LR fractions and that p47^{phox} was also en-

riched by recruitment when coronary arterial ECs were stimulated by FasL. However, these NADPH oxidase subunits have a very low level in LR fractions (fraction 3–5) when these cells are under resting conditions. Considering that the membrane translocation of $p47^{phox}$ is closely associated with NADPH oxidase activation, this increase in $p47^{phox}$ in the LR fraction may indicate activation of the enzyme with LR clustering (114). This membrane flotation and Western blot approach was also used in studying LR clustering with NADPH oxidase subunits in neutrophils. Similarly, under resting conditions, $gp91^{phox}$ and $p22^{phox}$ were found proportionally present in LR fractions. Some cytosolic subunits including $p67^{phox}$ and $p40^{phox}$ were also detected in LR fractions at a very low rate (3% or 5%). However, $p47^{phox}$ was not detected in the LR fractions. Upon activation of Fcγ receptors of these cells by IgG-opsonized *S. aureus* particles, NADPH oxidase subunits were significantly increased in the LR fractions. In particular, $p47^{phox}$ was translocated to membrane LR fractions and its content in these LR fraction increased by ~40-fold. In the presence of M-β-CD or filipin, however, increases in these NADPH oxidase subunits in the LR fraction were substantially blocked (121). Taken together, these results confirm an important coupling of LRs with NADPH oxidase subunits, which may lead to enhanced NADPH oxidase activity and produce the redox regulation of cell functions.

In addition to the two commonly used methods for detection of LR clusters discussed above, several other methods are also reported to be used for detection of LR clusters or single LRs, which include cross linking and fluorescent resonance energy transfer (FRET) measurement (24, 49, 78, 86), fluorescent recover after photobleaching (FRAP) (25), electron microscopy (5, 39), high-speed particle tracking (52), and atom force microscopy (62, 72). However, these methods are rarely used for detection of LRs and their clustering in ECs, and there is no report that these methods are used for detection of the promaxity of NADPH oxidase subunits to LR-associated proteins or lipid components. Much effort is needed to develop or optimize these methods for studies on these NADPH oxidase-based LR redox signaling platforms. It should be noted that, although often used in determination of LRs in living cells, FRET-based methods were reported to have yielded controversial results regarding LR clustering. An excellent review article by Silvius and Nobi discusses this aspect in detail (86).

$O_2^{\cdot-}$ production from LR redox signaling platforms. Although there are several methods that could be used to determine whether NADPH oxidase aggregated in LR clusters is activated, the most definitive measurement is to analyze $O_2^{\cdot-}$ production in isolated LR fractions of DRM by membrane flotation. Using fluorescence microplate spectrometry with the trapping dye, dihydroethidium (DHE), LR-enriched fractions were reacted with NADPH oxidase substrate, NADPH or NADH, and NADPH-dependent DHE oxidation assessed (114). FasL significantly increased NADPH oxidase activity in LR fractions by fourfold, compared to microsomes. This increased production of $O_2^{\cdot-}$ was blocked by M-β-CD pretreatment, suggesting that clustered LRs with aggregation and recruitment of NADPH oxidase subunits do activate $O_2^{\cdot-}$ production (114). This increased $O_2^{\cdot-}$ production in LR fractions from FasL or other stimuli has also been confirmed by using electron spin resonance (ESR) spectrometry (see an accompanying paper by Jin *et al.* in this forum).. In addition, LR-clustering enhanced $O_2^{\cdot-}$ production was found to be stimulated or boosted in single cell fluorescent imaging analysis or electrode probing by LR clustering agonists such as FasL, TNF-α, endostatin, and angiotensin II in ECs or Fcγ activation in neutrophils (110, 114, 120). Although these assays measured $O_2^{\cdot-}$ production within cells, rather than directly on membrane LRs, blockade of the enhanced $O_2^{\cdot-}$ production by LR disruptors or by NADPH oxidase inhibitors defined their association of $O_2^{\cdot-}$ with LRs clustering during agonist stimulation (43, 114). Taken together, these findings support the view that LR clustering contributes to activation of NADPH oxidase by aggregation and recruitment of its subunits to LR platforms, which constitutes a LR redox signaling platform, regulating downstream effector response and influencing cell function.

These results obtained by linking NADPH oxidase activity to LR clustering undoubtedly enhance our understanding of how this enzyme is functioning. In previous studies, several other mechanisms were also proposed to mediate agonist-induced activation of NADPH oxidase in different cells. For example, in neutrophils the cytosolic components of this enzyme are translocated, guided by Rac1, to the plasma membrane when they are stimulated by bacteria or other ligands such as Fcγ activators. Then, they assemble with plasma membrane-bound subunits to form a functional enzyme to produce $O_2^{\cdot-}$. In contrast to neutrophils, some reports have indicated that, in unstimulated ECs, NADPH oxidase subunits exist as preassembled complexes in a predominantly perinuclear location associated with the intracellular cytoskeleton, which may account for the low level ROS-generating activity. Upon stimulation, the activity of this NADPH oxidase is enhanced (57). It has also been reported that TNF-α induced translocation of $p47^{phox}$ to cytoskeletal elements in ECs (55), but VEGF caused translocation of $p47^{phox}$ to membrane ruffles through association with WAVE1 in this type of cells (108). In addition, angiotensin II-induced activation of NADPH oxidase and ROS production are involved in the scaffold for various signaling molecules such as G-protein-coupled receptor and tyrosine kinase in vascular smooth muscle cells. However, our recent studies have demonstrated that a translocation of cytosolic subunits such as $p47^{phox}$ happens when ECs were stimulated by TNF-α or its lipid mediator, ceramide (117). By analyzing all these results, it is interesting to find that activation of NADPH oxidase is associated with assembling or congregating of its subunits no matter whether there is translocation of cytosolic subunits to cell membrane. The LR clustering may provide a driving force for this assembling of NADPH oxidase, and therefore the formation of LR redox signaling platforms with NADPH oxidase may be an essential mechanism for redox signaling mediated by this enzyme.

Caveolar and noncaveolar clustering of NADPH oxidase subunits

Generally, there are two types of LRs, namely, caveolar and noncaveolar rafts. Caveolae is a subset of plasma membrane rafts in cell surface invaginations (91), and they exist in cell types that express caveolins, which are polymerized to bend the membrane to form caveolae (91). Therefore, caveolin is considered as a signature protein of caveolae. According to current

understanding, caveolae and noncaveolar LRs and their clustering may mediate different signaling pathways, participating in the temporal–spatial regulation of different signaling pathways or molecules and consequent cell responses in the same type of cells. In this regard, many studies have been done to clarify the association of NOS with caveoli. It has been indicated that the formation of caveolae may be associated with NO production and endocytosis in ECs (31, 92). However, endothelial NOS is also found in noncaveolar LRs (74), and the formation of caveolae promotes interfacing or juxtaposing of NOS with other signaling partners such as cavolin-1, dynamin-2, calmodulin, heat shock protein 90, and akt (74). There is evidence that, although cavolin-1 is important to the formation of caveolae, this protein exerts inhibitory action on NOS activity. In fact, the formation of caveolae does play a role to cluster or juxtapose various signaling components for NOS production. In this regard, the caveolin-1-mediated formation of caveoli in ECs indeed represents another form of LRs clustering, which is constitutive and present under rest condition. The noncaveolar LRs are clustered in response to agonists or stimuli. Therefore, it is not surprising that NOS could be detected in caveolar and noncaveolar LRs.

With respect to NADPH oxidase, its subunits have been indeed identified in caveoli and noncaveolar LRs (110, 120). Similar to NOS, this distribution of NOS in both LRs and caveoli may mediate different signaling pathways, participating in the temporal–spatial redox regulation of cell function in the different or even same type of cells in response to various agonists or stimuli. For example, in vascular smooth muscle cells there is strong evidence that NADPH oxidase subunits are colocalized with caveolin-1, indicating the association of this enzyme with caveoli (41, 100). Angiotensin II stimulates this caveoli-associated NADPH oxidase to produce $O_2^{\cdot -}$, which represents a redox signaling mechanism mediating the action of angiotensin II in the regulation of the cellular activity of vascular smooth muscle cells such as protein synthesis, hypertrophy, and proliferation (120). Since death factors such as TNF-α have been shown to alter endothelial function independent of caveolae-related mechanism and in other type of cells noncaveolar LR clustering are implicated in the actions of these factors (33), the formation of noncaveolar LR signaling platforms may primarily contribute to aggregation or recruitment of NADPH oxidase components in ECs. This in turn activates NADPH oxidase to produce $O_2^{\cdot -}$, mediating redox signaling in these cells. Different from caveoli LRs clustering of these noncaveolar LRs is not constitutively present, but occurs upon stimulation.

FUNCTIONAL RELEVANCE OF LR REDOX SIGNALING PLATFORMS

The functional significance of LR redox signaling platforms is still far from clear. Based on the findings discussed above, it is possible that the formation of these redox signaling platforms importantly contributes to the normal regulation of endothelial function and, in particular, to endothelial dysfunction associated with various death receptor agonists. Given that various death receptor agonists such as FasL or TNF-α are importantly involved in the pathogenesis of different vascular diseases such as atherosclerosis, hypertension, diabetic vasculopathy, and ischemia/reperfusion injury, the formation of these redox signaling platforms on the membrane of ECs in response to activation of death receptors may also be implicated in the development of these diseases. This section will address some evidence related to the possible role of the LR redox signaling platforms with NADPH oxidase in death factor-induced endothelial damage or dysfunction.

LRs and death receptor signaling

Various death factors exert their actions through a group of receptors called death receptors. These death receptors are a superfamily member of tumor necrosis factor (TNF) receptors, which are characterized by an extracellular cysteine-rich domain and a cytoplasmic death domain. These death receptors include Fas (or CD95/Apo1), TNFR1 (TNF receptor-1), DR3, DR4, DR5 (death receptor 3, 4 and 5), NGFR (nerve growth factor receptor), TRAIL (TNF-related apoptosis-inducing ligand receptors), and others such as angiostatin and endostatin receptors (46, 90). There is evidence that these receptors can be activated by their ligands under physiological or pathological conditions. Induction of apoptosis is initiated when death receptors bind with their ligands. The common apoptotic factors or ligands among an array of cytokines or bioactive factors include TNF-α, FasL, Apo3L (DR3 ligand), Apo2L (DR4, DR5 ligand), angiostatin, and endostatin (6, 102). Among of them, TRAIL is unusual as compared to the other cytokines of this family, as it interacts with a complex system of receptors consisting of two pro-apoptotic death receptors (TRAIL-R1 and TRAIL-R2) and three decoy receptors (TRAIL-R3, TRAIL-R4, and osteoprotegerin). Moreover, as a stable soluble trimer, TRAIL selectively induces apoptosis in many transformed cells but not in normal cells (81).

Formation of receptor–ligand complexes may promote conformational alteration of the receptors such as dimerization or trimerization and result in intracellular clustering of the receptor components called death domains (DD), which recruit other signaling or effector molecules activating apoptotic processes and resulting in cell death (6, 18, 102). Although it remains poorly understood how these receptors aggregate and thereby recruit intracellular signaling components to initiate cell dysfunction or apoptosis, three major pathways are proposed to mediate the postreceptor processes after activation of various death receptors including the actions of caspase-mediated execution, SMase activation, and mitochondrial uncoupling (17, 46). Recent studies have highlighted the crucial role of sphingolipid- and cholesterol-enriched membrane LRs for clustering or aggregation of these receptors and signaling molecules. Clustering of death receptors in LR platform may importantly participate in the initiation and mediation of apoptotic signaling. For example, in leukemic Jurkat cells, various receptors such as Fas, TNFR1, and TRAIL 2/DR 5 could be clustered into LR platforms when these cells were treated with the antitumor drug Aplidin (29). In the LR platforms, many downstream signaling molecules including the Fas-associated death domain-containing proteins, procaspase-8, procaspase-10, c-Jun amino-terminal kinase were also detected, which may be related to their translocation or recruitment. In addition to these classic cytosolic apoptotic factors, other signaling molecules such as the

actin-linking proteins ezrin, moesin, RhoA, and RhoGDI were also found to aggregate into Fas-enriched rafts in these antitumor drug-treated leukemic cells (30, 93). In other studies, redistribution of TNFR1 in LR and nonraft regions of the plasma membrane was observed in the cerebral cortex after traumatic brain injury in adult male Sprague–Dawley rats, suggesting that TNFR1 translocation into or out of LR clusters may be of importance in the initiation or development of brain injury (60). Some new anti-inflammatory compounds have been reported to induce T-cell apoptosis and enhance inflammation resolution through LRs clustering (4). Furthermore, endostatin induced Src-dependent activation of p190RhoGAP with concomitant decrease in RhoA activity and disassembly of actin stress fibers through LR-mediated interactions in human dermal microvascular ECs (106). These results support the view that LR-mediated signaling plays an important role in the mediation or regulation of the death receptor conformation and translocation or recruitment of downstream signaling molecules, which activate or launch the apoptotic processes.

Death receptor activation-induced endothelial dysfunction

There is considerable evidence that many different death receptors are present in vascular ECs such as Fas, TNFRI, DR3, 4 and 5, and NGFR1 (38, 106). Activation of these death factors has been reported to induce ECs apoptosis. Of these death receptors, Fas is well characterized, and both Fas and its ligand are detected in ECs from different vascular beds. However, ECs are normally resistant to FasL-induced apoptosis (93). This resistance of ECs to FasL-stimulated apoptosis may be due to the presence or induction of endogenous FasL on their cell surface since inhibition of the synthesis of this endogenous FasL with cycloheximide or activation of p53 enhanced the sensitivity of ECs to FasL-induced apoptosis. In addition, previous studies have indicated that the resistance of ECs to FasL-induced apoptosis may be due to the expression of cellular FLICE-inhibitory protein (cFLIP) that functions as a dominant-negative inhibitor of caspase-8 function. It seems clear that the Fas/FasL apoptotic pathway may primarily result in endothelial dysfunction, rather than apoptosis when Fas levels increase to certain extent in blood or vascular tissues in response to different physiological or pathological stimuli. In contrast to Fas, stimulation of ECs with cytokine TNF-α directly results in strong pro- and anti-apoptotic signals (64, 83). Both TNF-receptors, TNFR1 and TNFR2, are expressed in ECs, but TNFR1 is the major one to induce endothelial apoptosis (21, 64). However, TNFR2 activation produces a weak death signal, but strong cell survival signals in these vascular cells (21, 64). Therefore, TNF-α-induced cell apoptosis is dependent on a balance of activation of both TNF-α receptors. There is a general agreement that some protective mechanisms are present in ECs, which protect these cells from apoptosis. However, if large amounts of death factors are produced and act on ECs under certain pathological conditions, a typical apoptosis could also be produced (21). Again, endothelial dysfunction may be an important early event for the action of death factors.

Again, it is general accepted that ECs are relatively resistant to cell apoptosis due to some endogenous protective mechanisms. During stimulation of some death factors including TNF-α, FasL, and endostatin, if any, endothelial apoptosis is much dependent on the duration of their action (21, 87). Even subtle apoptotic changes could be detectable only when ECs were incubated with these factors for 6–10 h (9, 16). This suggests that some functional disturbances or signaling changes must precede cell death in ECs when exposed to death factors. Endothelium-dependent vasodilation is one of the important endothelial functions, which particularly represents the functional integrity of the endothelium in many vascular beds. Therefore, impairment of endothelium-dependent vasodilation is often considered as an endothelial dysfunction (23). Recent studies have indicated that this endothelial dysfunction may be an early response to death receptor activation (111, 116). In this regard, work in our laboratory and by others have shown that death factors such as TNF-α attenuated endothelium-dependent vasodilation in a variety of vascular beds (54, 116). Although several mechanisms are proposed to contribute to this death receptor-mediated endothelial dysfunction including inhibition of eNOS activation (66, 118) and decrease in antioxidant enzymes such as superoxide dismutase (SOD) (105), enhancement of NADPH oxidase activity to produce $O_2^{\cdot -}$ is now considered as a major contributor to the decrease in NO bioavailability, and thereby to the impairment of endothelium-dependent vasodilation (56, 61).

LR redox signaling platforms in death receptor-induced endothelial dysfunction

In recent studies, we systematically determined the role of LR redox signaling platforms with NADPH oxidase in endothelial dysfunction induced by various death factors including FasL, TNF-α, endostatin, and TNFRI, DR3, 4, and 5. It has been shown that these death factors can rapidly alter endothelial function prior to apoptosis. Given a rapid formation of LR clusters in response to death receptor activation, it is plausible that endothelial dysfunction induced by these death factors is dependent on LR redox signaling platforms-dependent activation of NADPH oxidase and $O_2^{\cdot -}$ production. Indeed, the results from several series of experiments support this view.

Using freshly isolated perfused small coronary arteries, FasL as a death factor added in the arterial lumen, was found to impair the endothelium-dependent vasodilator response to bradykinin (BK). This effect of FasL on BK-induced endothelium-dependent vasodilation was attenuated by the disruption of LRs using nystatin and M-β-CD, suggesting that LRs or its clustering are of importance in mediating the action of FasL. Similarly, when the arteries were pretreated by siRNA of AS-Mase or apocynin, FasL-induced impairment of endothelium-dependent vasodilation was also significantly attenuated. All these results are summarized in Fig. 3. Taken together, these findings suggest that ceramide-related LR clustering that associate with aggregation and activation of NADPH oxidase may be a contributing mechanism for FasL-mediated endothelial injury and dysfunction. This is supported by the findings obtained from detection of LR clusters with aggregation of NADPH oxidase subunits in cell preparations, as discussed above.

Another death factor, endostatin, was also reported to induce the formation of LR redox signaling platforms in endothelial

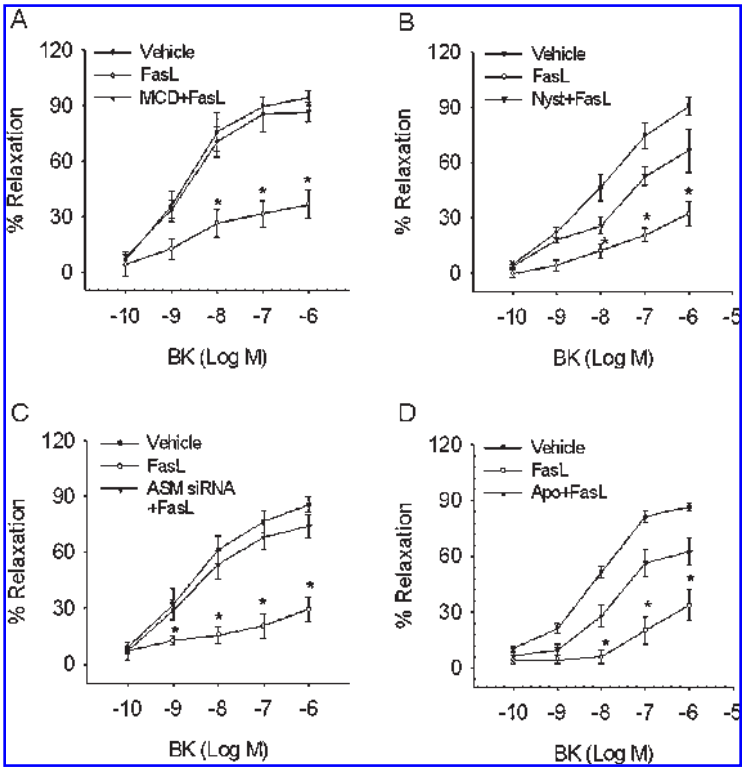

FIG. 3. Effects of FasL on endothelium-dependent coronary vasodilation before and after inhibition of the formation of LR redox signaling platforms and associated components. In response to bradykinin (BK), isolated and perfused small coronary arteries dilated in a concentration-dependent manner under control conditions (vehicle), and FasL significantly blunted this BK-induced endothelium-dependent vasodilation. When the arteries were pretreated with LR disruptors, methyl-β-cyclodextrin (MCD) (**A**) or nystatin (Nyst) (**B**), FasL-induced inhibitory action was markedly attenuated. Similarly, siRNA acid sphingomyelinase (ASM, an enzyme producing ceramide and promoting LP clustering) (**C**) or NADPH oxidase inhibitor apocynin (Apo) (**D**) could also restore FasL-impaired vasodilator response of coronary arteries to BK.

cell membranes. In these LR redox signaling platforms formed in response to endostatin, NADPH oxidase-mediated $O_2^{\cdot -}$ production was enhanced (114). We also tested whether the endostatin-induced impairment of endothelium-dependent vasodilation is attributed to the formation of LR signaling platforms in coronary arteries. It was found that the inhibitory effect of endostatin on endothelium-dependent vasodilation was also significantly attenuated when these arteries were pretreated with nystatin and M-β-CD, which disrupted the formation of LR redox signaling platforms. These results provide evidence that the formation of LR redox signaling platforms on endothelial cell membrane plays an important role in the mediation of endostatin-induced endothelial injury and dysfunction. In experiments testing the action of other death factors such as TNF-α, Apo3L, or Apo2L, similar results were obtained (unpublished data), indicating that LR signaling platform formation and consequent activation of NADPH oxidase importantly contribute to the impairment of endothelium-dependent vasodilator responses in coronary arteries induced by various death factors. Therefore, these LR redox signaling platforms seem to represent a common mechanism mediating death receptor activation-associated endothelial dysfunction or injury in coronary circulation.

The formation of LR redox signaling platforms results in increased $O_2^{\cdot -}$ production within ECs. It is known that $O_2^{\cdot -}$ and endothelium-derived vasodilator, NO are free radicals containing an unpaired electron in their outer orbit. The reaction of $O_2^{\cdot -}$ and NO may occur at an extremely rapid rate of 6.7×10^9 mol/L$^{-1} \cdot$ s^{-1}, which is three times faster than the reaction rate of NO with SOD (95, 103). It is proposed that $O_2^{\cdot -}$ is an important target for the NO action in different vessels. Therefore, this interaction of $O_2^{\cdot -}$ and NO, which reduces NO bioavailability, represents an important mechanism for the action of $O_2^{\cdot -}$ in the control of vascular tone (36, 53), in particular, for endothelium-dependent vasodilation. This decreased NO bioavailability has been suggested to be a general mechanism to mediate the vascular effects of many injury stimulations including activation of death receptors. In addition, $O_2^{\cdot -}$ interact with NO resulting in production of another reactive oxygen free radical, $ONOO^{\cdot -}$ (61, 104). $ONOO^{\cdot -}$ is a most highly reactive oxidant, which could lead to severe oxidative injury of ECs and vascular smooth muscle, resulting in endothelial or vascular dysfunction.

In addition, a new mechanism by which $O_2^{\cdot -}$ or ROS affect endothelial NO has been demonstrated (104, 119), which relates to the uncoupling of NOS and consequent reduction of NO production but increase in $O_2^{\cdot -}$ production by NOS. This uncoupling of NOS is due to increased $ONOO^-$ within cells. It is known now that once formed, $ONOO^{\cdot -}$ will oxidize BH_4 to dihydrobiopterin (BH_2) resulting in NOS uncoupling (101, 119). $O_2^{\cdot -}$ production from uncoupled NOS activity will amplify oxidative stress in ECs. Exaggerated oxidative stress in these cells will further reduce the availability of NO, resulting in endothelial dysfunction, and abolish endothelium-dependent vasodilation (48, 96, 119). In recent studies from our laboratory and others, this interaction of $O_2^{\cdot -}$ and NO had been attributed to NADPH oxidase-derived $O_2^{\cdot -}$. It is proposed that NADPH oxidase-derived $O_2^{\cdot -}$ may serve as a trigger to induce NOS uncoupling though $ONOO^{\cdot -}$, resulting in a dramatic increase in production of $O_2^{\cdot -}$ and ultimately leading to endothelial dysfunction, damage or apoptosis (117).

MECHANISMS MEDIATING THE FORMATION OF LR SIGNALING PLATFORMS IN ECs

Although there are several different models that interpret the formation of individual LRs and their clustering, it is well documented that ceramide-enriched macrodomains or platforms can be detected in a variety of cells. Ceramide produced from SMase may be an important mechanism triggering the formation of larger lipid signaling platforms by transformation of LRs in the membrane of different cell types (60, 89, 99). In ECs, we did demonstrate that in response to some agonists such as FasL or TNF-α ceramide is accumulated to form membrane signaling platforms (39, 40). In addition, it has been shown that SMases are enriched in vascular ECs, which may importantly mediate ceramide production, thereby leading to the formation of ceramide-enriched membrane platforms with aggregation or recruitment of NADPH oxidase subunits. Recent evidence is accumulating that supports this hypothesis.

Contribution of SMase and ceramide

Five types of SMase have been described to catalyze the production of ceramide in different cells in response to various agonists or stimuli (37–39). These enzymes are (a) lysosomal acid SMase (ASMase), (b) cytosolic Zn^{2+}-dependent ASMase, (c) membrane-bound magnesium-dependent neutral SMase, (d) cytosolic magnesium-independent neutral SMase, and (e) alkaline SMase. With respect to cell signaling through LRs, ASMase has been extensively studied. It is assumed that activation of the ASMase by agonists or stimuli correlates with a translocation of the enzyme from intracellular stores onto the extracellular leaflet of the cell membrane, where it promotes ceramide production, forming ceramide-enriched membrane platforms (38). During the formation of these ceramide-enriched membrane platforms, different membrane proteins, enzymes, or signaling molecules can be aggregated, and some molecules in cytosol recruited to the membrane, which may result in a bulk amplification of the signals from related receptors.

Several lines of evidence support this action of ASMase in the formation of LR signaling platforms in ECs. First, confocal microscopic analysis revealed that ASMase was accumulated in membrane LR clusters when ECs were stimulated by FasL or endostatin. Disruption or blockade of LR platform formation markedly attenuated agonists-induced activation of ASMase in these cells. Second, ASMase inhibitor blocked LR clustering and thereby restored endothelium-dependent coronary vasodilation that inhibited by death factors such as FasL, TNF-α, or endostatin. Corresponding to these results, increases in NADPH oxidase activity and $O_2^{\cdot -}$ production induced by these death factors were substantially attenuated. This further suggests that ceramide production through activation of ASMase in coronary ECs plays an important role in the formation of LR redox signaling platforms, increasing $O_2^{\cdot -}$ production and thereby contributing to endothelial dysfunction induced by these death factors. Third, small interfering RNA (siRNA) was used to knockdown ASMase genes in ECs, and FasL-induced increases in ASMase activity and ceramide levels were abolished by this RNA interference. Correspondingly, the formation of LR platforms in coronary ECs in response to death factors and subsequent production of $O_2^{\cdot -}$ were abolished in ASMase gene-silenced cells (112, 114, 115). This again provides strong evidence that endothelial ASMase is importantly attributed to the formation of LR redox signaling platforms.

In addition, clustering of various receptors in LR signaling platforms upon stimulation is another common mechanism for the initiation of transmembrane signaling (18, 71). For example, clustering of Fas has been found in lymphocytes, liver cells, and ECs, which is the prerequisite for signal transduction of death receptors and apoptosis (30, 71). An ASMase-dependent clustering of receptors was also observed for other receptors such as CD20, CD40, TNFR, and epidermal growth factor receptor (EGFR) (79, 99). In ECs, this ASMase-activated death receptor aggregation is also present since they were found redistributed into LR-enriched fractions (unpublished data). However, when the ASMase gene was silenced by its siRNA, this redistribution of death receptors such as Fas protein into LR-enriched fractions was substantially blocked. Functionally, this ASMase-mediated formation of ceramide-enriched redox signaling platforms in vascular ECs was also established. In arteries treated with ASMase inhibitors or siRNA of ASMase gene, FasL-induced impairment of endothelium-dependent vasodilation in isolated and pressurized small coronary arteries disappeared, which simulated the action of LR disruptors. This confirms that ASMase-mediated LR platform formation is importantly involved in FasL-induced endothelial dysfunction in coronary arteries (115). Taken together, it is concluded that endothelial ASMase importantly participates in redox signaling in ECs through LR redox signaling platforms.

Feed-forward action of LR redox signaling platforms

There is increasing evidence that the redox feed-forward mechanism is of importance in the regulation of cellular activity. Since $O_2^{\cdot -}$ or other ROS are very active, they may react with many molecules such as DNA, RNA, proteins, and lipids to produce corresponding effects in different cells (1, 15, 67, 94, 109). In addition to their injury actions, recent studies have indicated that ROS may modify these molecules to regulate their signaling functions (1, 15). Modifications of protein structure, lipid peroxidation, and activation of some enzymes have been reported to contribute to the actions of ROS in regulating various signaling pathways (69, 113). Since NADPH oxidase is activated in LR clusters, production of $O_2^{\cdot -}$ or other ROS may also act on lipids or enzymes in the platforms, thereby enhancing or dampening the signal transduction.

Although membrane lipids were previously considered as a major target of ROS, and lipid peroxidation is an important mechanism resulting in cellular damage induced by ROS (113), this lipid peroxidation does not mediate ROS-induced alterations of the enzyme activity within cells. Many studies demonstrated that changes in protein structure or activity is a much more sensitive indicator of cellular exposure to ROS than lipid peroxidation (51, 82). It has been reported that ROS activate many enzymes such as heme oxygenase, aconitase, tyrosine phosphatase, alkaline phosphatase, 5'-nucleotidase, and ADP-ribosylcyclase (10, 82). The dimerization of enzyme molecules induced by ROS is now considered as one of the important

mechanisms mediating the effect of ROS on cellular enzyme activity (10, 27). It has been demonstrated that the dimer formation of many enzymes is due to oxidation of the cysteine residue in the enzyme molecule, which leads to one or several disulfide bonds in enzyme molecule, resulting in the enhancement of enzymes.

Recently, Qiu *et al*. (75) demonstrated that the formation of ASMase dimer by modification of the free C-terminal cysteine significantly enhanced the activity of this enzyme. A "cysteine switch" activation mechanism is proposed by this research group to interpret the enhanced activation of ASMase, by which the free C-terminal cysteine involved zinc coordination of the active size was destroyed due to loss of free cysteine. In this way, ASMase activation is increased and ceramide produced more and more, enhancing LRs clustering and leading to feed-forward amplification. More recently, we did find that xanthine/xanthine oxidase, a $O_2^{\cdot -}$ generating system dramatically increased LRs clustering in the membrane of ECs, as shown in confocal microscopic detection (45).

In addition to the action of this protein modification, there is evidence that SM peroxidation could also promote the formation of large rafts in bilayer vesicle experiments (7). Therefore, if ROS cause SM peroxidation in LR platforms, large rafts or macrodomains may be formed. However, this SM peroxidation mechanism in promoting LR clustering has not yet been confirmed in living cells. Moreover, ROS-induced activation of certain regulatory enzymes may also contribute to this feed-forwarding amplification of LR platform formation. For example, Src kinases have been reported to be activated by ROS and then move into LR clusters. Activated Src may cause transphosphorylation, resulting in activation of related enzymes in LR clusters and thereby producing a feed-forward regulation in the formation of LR signaling platforms (73).

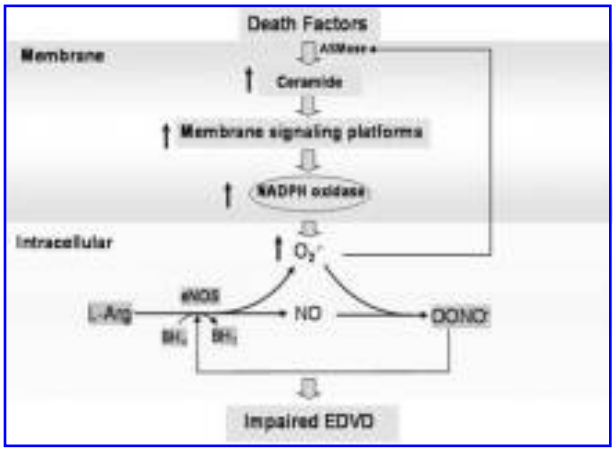

FIG. 4. Formation and action of LR redox signaling platform in response to death factors in endothelial cells. When various death factors bind to their receptors on individual LRs, ASMase is activated to produce ceramide from sphingomyelin (SM) to form a number of membrane singaling platforms, in which ASMase, NADPH oxidase subunits such as gp91^{phox} and p47^{phox}, and other proteins are aggregated and activated, producing $O_2^{\cdot -}$. $O_2^{\cdot -}$ reacts with NO to decrease NO bioavailability and to produce peroxynitrite ($ONOO^-$). Increased $ONOO^-$ uncouples NOS to produce more $O_2^{\cdot -}$ but less NO. $O_2^{\cdot -}$ or ROS may feed-forward enhance LRs clustering, forming positive amplifications. All these together constitute a redox-signaling network, resulting in endothelial dysfunction and impairment of endothelium-dependent vasodilation.

CONCLUSIONS

In this brief review, we present evidence that LR redox signaling platforms may be formed in response to death receptor activation in vascular ECs, which represents one of important mechanisms mediating the actions of various death factors on endothelial function. As depicted in Fig. 4, when various death factors bind to their receptors on individual LRs, ASMase located *in situ* or translocated from lysosomes or lysosome-like vesicles are activated to produce ceramide from SM, resulting in the formation of a number of ceramide-enriched membrane signaling platforms. In these platforms, ASMase, NADPH oxidase subunits such as gp91^{phox} and p47^{phox}, and other proteins are aggregated and activated, producing $O_2^{\cdot -} \cdot O_2^{\cdot -}$ reacts with NO to decrease NO bioavailability and to produce peroxynitrite ($ONOO^-$). Increased $ONOO^-$ uncouples NOS to produce more $O_2^{\cdot -}$ but less NO. $O_2^{\cdot -}$ or ROS may feed-forward enhance LR clustering, forming positive amplifications. All these together constitute a redox signaling network resulting in endothelial dysfunction and impairment of endothelium-dependent vasodilation. In perspective, the concept that LR redox signaling platforms participate in the regulation of normal endothelial function and contribute to the initiation or development of endothelial dysfunction will help advance the field of redox and death receptor signaling. Given that many death factors such as TNF-α, FasL, and endostatin are importantly involved in the pathogenesis of different vascular diseases such as atherosclerosis, hypertension, and diabetic vasculopathy, clarification of the pathological role of LR redox signaling platforms may direct toward the development of new therapeutic strategy for prevention or treatment of these diseases.

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ABBREVIATIONS

ASMase, acid sphingomyelinase; BK, bradykinin; cFLIP, cellular FLICE-inhibitory protein; DD, death domains; DHE, dihydroethdium; DRM, detergent resistant membranes; ECs, endothelial cells; EDVD, endothelium-dependent vasodilation; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; ESR, electron spin resonance; FasL, Fas ligand; FITC-CTX, FITC-cholera toxin; FRAP, fluorescent recover after photobleaching; FRET, fluorescent resonance energy transfer; GPI, glycosyl-phosphatidylinositol; HIV-1, human immunodeficiency virus type 1; HSPGs, heparan sul-

fate proteoglycans; L-arginine, L-Arg; LR, lipid raft; M-β-CD, methyl-β-cyclodextrin; NGFR, nerve growth factor receptor; NO, nitric oxide; $O_2^{-\cdot}$, superoxide; $OONO^-$, peroxynitrite; ROS, reactive oxygen species; siRNA, small interfering RNA; TNFR, tumor necrosis factor receptor; TNFR1, TNF receptor-1; TRAIL, TNF-related apoptosis inducing ligand receptors; VEGF, vascular endothelial growth factors.

REFERENCES

1. Aggeli IK, Gaitanaki C, and Beis I. Involvement of JNKs and p38-MAPK/MSK1 pathways in H2O2-induced upregulation of heme oxygenase-1 mRNA in H9c2 cells. *Cell Signal* 18: 1801–1812, 2006.
2. Ago T, Kitazono T, Ooboshi H, Iyama T, Han YH, Takada J, Wakisaka M, Ibayashi S, Utsumi H, and Iida M. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation* 109: 227–233, 2004.
3. Alonso MA and Millan J. The role of lipid rafts in signalling and membrane trafficking in T lymphocytes. *J Cell Sci* 114: 3957-3-965, 2001.
4. Ariel A, Li PL, Wang W, Tang WX, Fredman G, Hong S, Gotlinger KH, and Serhan CN. The docosatriene protectin D1 is produced by TH2 skewing and promotes human T cell apoptosis via lipid raft clustering. *J Biol Chem* 280: 43079–43086, 2005.
5. Arvanitis DN, Min W, Gong Y, Heng YM, and Boggs JM. Two types of detergent-insoluble, glycosphingolipid/cholesterol-rich membrane domains from isolated myelin. *J Neurochem* 94: 1696–1710, 2005.
6. Ashkenazi A and Dixit VM. Death receptors: signaling and modulation. *Science* 281: 1305–1308, 1998.
7. Ayuyan AG and Cohen FS. Lipid peroxides promote large rafts: effects of excitation of probes in fluorescence microscopy and electrochemical reactions during vesicle formation. *Biophys J* 91: 2172–2183, 2006.
8. Bayraktutan U, Blayney L, and Shah AM. Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. *Arterioscler Thromb Vasc Biol* 20: 1903–1911, 2000.
9. Berk BC, Abe JI, Min W, Surapisitchat J, and Yan C. Endothelial atheroprotective and anti-inflammatory mechanisms. *Ann NY Acad Sci* 947: 93–109; discussion 109–111, 2001.
10. Berruet L, Muller–Steffner H, and Schuber F. Occurrence of bovine spleen CD38/NAD + glycohydrolase disulfide-linked dimers. *Biochem Mol Biol Int* 46: 847–855, 1998.
11. Brandes RP and Kreuzer J. Vascular NADPH oxidases: molecular mechanisms of activation. *Cardiovasc Res* 65: 16–27, 2005.
12. Brown DA and London E. Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol* 14: 111–136, 1998.
13. Cahuzac N, Baum W, Kirkin V, Conchonaud F, Wawrezinieck L, Marguet D, Janssen O, Zornig M, and Hueber AO. Fas ligand is localized to membrane rafts, where it displays increased cell death-inducing activity. *Blood* 107: 2384–2391, 2006.
14. Cai H. NAD(P)H oxidase-dependent self-propagation of hydrogen peroxide and vascular disease. *Circ Res* 96: 818–822, 2005.
15. Chakraborti T, Ghosh SK, Michael JR, Batabyal SK, and Chakraborti S. Targets of oxidative stress in cardiovascular system. *Mol Cell Biochem* 187: 1–10, 1998.
16. Chang Q and Tepperman BL. Effect of selective PKC isoform activation and inhibition on TNF-alpha-induced injury and apoptosis in human intestinal epithelial cells. *Br J Pharmacol* 140: 41–52, 2003.
17. Chatterjee S. Sphingolipids in atherosclerosis and vascular biology. *Arterioscler Thromb Vasc Biol* 18: 1523–1533, 1998.
18. Chavakis E and Dimmeler S. Regulation of endothelial cell survival and apoptosis during angiogenesis. *Arterioscler Thromb Vasc Biol* 22: 887–893, 2002.
19. Cheng G, Cao Z, Xu X, van Meir EG, and Lambeth JD. Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. *Gene* 269: 131–140, 2001.
20. Cheng PC, Dykstra ML, Mitchell RN, and Pierce SK. A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J Exp Med* 190: 1549–1560, 1999.
21. Choy JC, Granville DJ, Hunt DW, and McManus BM. Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. *J Mol Cell Cardiol* 33: 1673–1690, 2001.
22. Cremesti AE, Goni FM, and Kolesnick R. Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outcome? *FEBS Lett* 531: 47–53, 2002.
23. Davignon J and Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation* 109: 11127–11132, 2004.
24. Edidin M. The state of lipid rafts: from model membranes to cells. *Annu Rev Biophys Biomol Struct* 32: 257–283, 2003.
25. Eisenberg S, Shvartsman DE, Ehrlich M, and Henis YI. Clustering of raft-associated proteins in the external membrane leaflet modulates internal leaflet H-ras diffusion and signaling. *Mol Cell Biol* 26: 7190–7200, 2006.
26. El Benna J, Faust RP, Johnson JL, and Babior BM. Phosphorylation of the respiratory burst oxidase subunit p47phox as determined by two-dimensional phosphopeptide mapping. Phosphorylation by protein kinase C, protein kinase A, and a mitogen-activated protein kinase. *J Biol Chem* 271: 6374–6378, 1996.
27. Finkel T. Signal transduction by reactive oxygen species in nonphagocytic cells. *J Leukoc Biol* 65: 337–340, 1999.
28. Frey RS, Rahman A, Kefer JC, Minshall RD, and Malik AB. PKCzeta regulates TNF-alpha-induced activation of NADPH oxidase in endothelial cells. *Circ Res* 90: 1012–1019, 2002.
29. Gajate C, An F, and Mollinedo F. Rapid and selective apoptosis in human leukemic cells induced by Aplidine through a Fas/CD95- and mitochondrial-mediated mechanism. *Clin Cancer Res* 9: 1535–1545, 2003.
30. Gajate C and Mollinedo F. Cytoskeleton-mediated death receptor and ligand concentration in lipid rafts forms apoptosis-promoting clusters in cancer chemotherapy. *J Biol Chem* 280: 11641–11647, 2005.
31. Goligorsky MS, Li H, Brodsky S, and Chen J. Relationships between caveolae and eNOS: everything in proximity and the proximity of everything. *Am J Physiol Renal Physiol* 283: F1–10, 2002.
32. Gorlach A, Brandes RP, Nguyen K, Amidi M, Dehghani F, and Busse R. A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circ Res* 87: 26–32, 2000.
33. Grassme H, Jekle A, Riehle A, Schwarz H, Berger J, Sandhoff K, Kolesnick R, and Gulbins E. CD95 signaling via ceramide-rich membrane rafts. *J Biol Chem* 276: 20589–20596, 2001.
34. Grassme H, Jendrossek V, Bock J, Riehle A, and Gulbins E. Ceramide-rich membrane rafts mediate CD40 clustering. *J Immunol* 168: 298–307, 2002.
35. Grassme H, Schwarz H, and Gulbins E. Molecular mechanisms of ceramide-mediated CD95 clustering. *Biochem Biophys Res Commun* 284: 1016–1030, 2001.
36. Griendling KK, Sorescu D, and Ushio–Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86: 494–501, 2000.
37. Gulbins E and Grassme H. Ceramide and cell death receptor clustering. *Biochim Biophys Acta* 1585: 139–145, 2002.
38. Gulbins E and Kolesnick R. Acid sphingomyelinase-derived ceramide signaling in apoptosis. *Subcell Biochem* 36: 229–244, 2002.
39. Gulbins E and Kolesnick R. Raft ceramide in molecular medicine. *Oncogene* 22: 7070–7077, 2003.
40. Gulbins E and Li PL. Physiological and pathophysiological aspects of ceramide. *Am J Physiol Regul Integr Comp Physiol* 290: R11–26, 2006.
41. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, and Griendling KK. Distinct subcellular localizations of Nox1 and

Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 24: 677–683, 2004.
42. Hoekstra D, Maier O, van der Wouden JM, Slimane TA, and van ISC. Membrane dynamics and cell polarity: the role of sphingolipids. *J Lipid Res* 44: 869–877, 2003.
43. Jackson SH, Devadas S, Kwon J, Pinto LA, and Williams MS. T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat Immunol* 5: 818–827, 2004.
44. Janes PW, Ley SC, and Magee AI. Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. *J Cell Biol* 147: 447–461, 1999.
45. Jin S, Yi F, Chen Q, Xia M, and Li PL. Contribution of lysosome-like vesicles in the formation of lipid raft redox signaling platform. *FASEB J* 21, 2007.
46. Jin Z and El–Deiry WS. Overview of cell death signaling pathways. *Cancer Biol Ther* 4: 139–163, 2005.
47. Kabouridis PS. Lipid rafts in T cell receptor signalling. *Mol Membr Biol* 23: 49–57, 2006.
48. Katusic ZS. Vascular endothelial dysfunction: does tetrahydrobiopterin play a role? *Am J Physiol Heart Circ Physiol* 281: H981–986, 2001.
49. Kenworthy AK, Petranova N, and Edidin M. High-resolution FRET microscopy of cholera toxin B-subunit and GPI-anchored proteins in cell plasma membranes. *Mol Biol Cell* 11: 1645–1655, 2000.
50. Kiely JM, Hu Y, Garcia–Cardena G, and Gimbrone MA, Jr. Lipid raft localization of cell surface E-selectin is required for ligation-induced activation of phospholipase C gamma. *J Immunol* 171: 3216–3224, 2003.
51. Kocic G, Vlahovic P, Pavlovic D, Kocic R, Cvetkovic T, and Stojanovic I. The possible importance of the cation-binding site for the oxidative modification of liver 5'-nucleotidase. *Arch Physiol Biochem* 106: 91–99, 1998.
52. Kusumi A, Nakada C, Ritchie K, Murase K, Suzuki K, Murakoshi H, Kasai RS, Kondo J, and Fujiwara T. Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu Rev Biophys Biomol Struct* 34: 351–378, 2005.
53. Lassegue B and Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* 285: R277–297, 2003.
54. Lefer AM and Ma XL. Cytokines and growth factors in endothelial dysfunction. *Crit Care Med* 21: S9–14, 1993.
55. Li JM, Mullen AM, Yun S, Wientjes F, Brouns GY, Thrasher AJ, and Shah AM. Essential role of the NADPH oxidase subunit p47(phox) in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor-alpha. *Circ Res* 90: 143–150, 2002.
56. Li JM and Shah AM. Differential NADPH- versus NADH-dependent superoxide production by phagocyte-type endothelial cell NADPH oxidase. *Cardiovasc Res* 52: 477–486, 2001.
57. Li JM and Shah AM. Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. *J Biol Chem* 277: 19952–19960, 2002.
58. Li YC, Park MJ, Ye SK, Kim CW, and Kim YN. Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents. *Am J Pathol* 168: 1107–1118; quiz 1404–1105, 2006.
59. Lopez JA, del Conde I, and Shrimpton CN. Receptors, rafts, and microvesicles in thrombosis and inflammation. *J Thromb Haemost* 3: 1737–1744, 2005.
60. Lotocki G, Alonso OF, Dietrich WD, and Keane RW. Tumor necrosis factor receptor 1 and its signaling intermediates are recruited to lipid rafts in the traumatized brain. *J Neurosci* 24: 11010–11016, 2004.
61. Lum H and Roebuck KA. Oxidant stress and endothelial cell dysfunction. *Am J Physiol Cell Physiol* 280: C719–741, 2001.
62. Lydataki S, Lesniewska E, Tsilimbaris MK, Le Grimellec C, Rochette L, Goudonnet JP, and Pallikaris IG. Observation of the posterior endothelial surface of the rabbit cornea using atomic force microscopy. *Cornea* 22: 651–664, 2003.
63. Macdonald JL and Pike LJ. A simplified method for the preparation of detergent-free lipid rafts. *J Lipid Res* 46: 1061–1067, 2005.
64. Madge LA and Pober JS. TNF signaling in vascular endothelial cells. *Exp Mol Pathol* 70: 317–325, 2001.
65. Magee AI and Parmryd I. Detergent-resistant membranes and the protein composition of lipid rafts. *Genome Biol* 4: 234, 2003.
66. Makino N, Maeda T, Sugano M, Satoh S, Watanabe R, and Abe N. High serum TNF-alpha level in Type 2 diabetic patients with microangiopathy is associated with eNOS down-regulation and apoptosis in endothelial cells. *J Diabetes Complications* 19: 347–355, 2005.
67. Milczarek R, Sokolowska E, Hallmann A, and Klimek J. The NADPH- and iron-dependent lipid peroxidation in human placental microsomes. *Mol Cell Biochem* 295: 105–111, 2007.
68. Mohazzab KM, Kaminski PM, and Wolin MS. NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am J Physiol* 266: H2568–2572, 1994.
69. Olivares–Corichi IM, Ceballos G, Medina–Santillan R, Medina–Navarro R, Guzman–Grenfell AM, and Hicks JJ. Oxidation by reactive oxygen species (ROS) alters the structure of human insulin and decreases the insulin-dependent D-glucose-C14 utilization by human adipose tissue. *Front Biosci* 10: 3127–3131, 2005.
70. Patschan S, Li H, Brodsky S, Sullivan D, De Angelis DA, Patschan D, and Goligorsky MS. Probing lipid rafts with proximity imaging: actions of proatherogenic stimuli. *Am J Physiol Heart Circ Physiol* 290: H2210–2219, 2006.
71. Pike LJ. Lipid rafts: bringing order to chaos. *J Lipid Res* 44: 655–667, 2003.
72. Poole K, Meder D, Simons K, and Muller D. The effect of raft lipid depletion on microvilli formation in MDCK cells, visualized by atomic force microscopy. *FEBS Lett* 565: 53–58, 2004.
73. Powers KA, Szaszi K, Khadaroo RG, Tawadros PS, Marshall JC, Kapus A, and Rotstein OD. Oxidative stress generated by hemorrhagic shock recruits Toll-like receptor 4 to the plasma membrane in macrophages. *J Exp Med* 203: 1951–1961, 2006.
74. Pritchard KA, Ackerman AW, Ou J, Curtis M, Smalley DM, Fontana JT, Stemerman MB, and Sessa WC. Native low-density lipoprotein induces endothelial nitric oxide synthase dysfunction: role of heat shock protein 90 and caveolin-1. *Free Radic Biol Med* 33: 52–62, 2002.
75. Qiu H, Edmunds T, Baker-Malcolm J, Karey KP, Estes S, Schwarz C, Hughes H, and Van Patten SM. Activation of human acid sphingomyelinase through modification or deletion of C-terminal cysteine. *J Biol Chem* 278: 32744–32752, 2003.
76. Rahman A, Anwar KN, and Malik AB. Protein kinase C-zeta mediates TNF-alpha-induced ICAM-1 gene transcription in endothelial cells. *Am J Physiol Cell Physiol* 279: C906–914, 2000.
77. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, and Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 97: 1916–1923, 1996.
78. Rao M and Mayor S. Use of Forster's resonance energy transfer microscopy to study lipid rafts. *Biochim Biophys Acta* 1746: 221–233, 2005.
79. Rodighiero S, De Simoni A, and Formenti A. The voltage-dependent nonselective cation current in human red blood cells studied by means of whole-cell and nystatin-perforated patch-clamp techniques. *Biochim Biophys Acta* 1660: 164–170, 2004.
80. Sanchez FA, Savalia NB, Duran RG, Lal BK, Boric MP, and Duran WN. Functional significance of differential eNOS translocation. *Am J Physiol Heart Circ Physiol* 291: H1058–1064, 2006.
81. Secchiero P, Vaccarezza M, Gonelli A, and Zauli G. TNF-related apoptosis-inducing ligand (TRAIL): a potential candidate for combined treatment of hematological malignancies. *Curr Pharm Des* 10: 3673–3681, 2004.
82. Sen CK. Redox signaling and the emerging therapeutic potential of thiol antioxidants. *Biochem Pharmacol* 55: 1747–1758, 1998.
83. Shakibaei M, Schulze–Tanzil G, Takada Y, and Aggarwal BB. Redox regulation of apoptosis by members of the TNF superfamily. *Antioxid Redox Signal* 7: 482–496, 2005.

84. Shao D, Segal AW, and Dekker LV. Lipid rafts determine efficiency of NADPH oxidase activation in neutrophils. *FEBS Lett* 550: 101–106, 2003.
85. Shen HM, Lin Y, Choksi S, Tran J, Jin T, Chang L, Karin M, Zhang J, and Liu ZG. Essential roles of receptor-interacting protein and TRAF2 in oxidative stress-induced cell death. *Mol Cell Biol* 24: 5914–5922, 2004.
86. Silvius JR and Nabi IR. Fluorescence-quenching and resonance energy transfer studies of lipid microdomains in model and biological membranes. *Mol Membr Biol* 23: 5–16, 2006.
87. Sim BK. Angiostatin and endostatin: endothelial cell-specific endogenous inhibitors of angiogenesis and tumor growth. *Angiogenesis* 2: 37–48, 1998.
88. Simons K and Ikonen E. Functional rafts in cell membranes. *Nature* 387: 569–572, 1997.
89. Simons K and Ikonen E. How cells handle cholesterol. *Science* 290: 1721–1726, 2000.
90. Simons K and Toomre D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 1: 31–39, 2000.
91. Sowa G, Pypaert M, and Sessa WC. Distinction between signaling mechanisms in lipid rafts vs. caveolae. *Proc Natl Acad Sci USA* 98: 14072–14077, 2001.
92. Stan RV. Structure and function of endothelial caveolae. *Microsc Res Tech* 57: 350–364, 2002.
93. Stickney JT, Bacon WC, Rojas M, Ratner N, and Ip W. Activation of the tumor suppressor merlin modulates its interaction with lipid rafts. *Cancer Res* 64: 2717–2724, 2004.
94. Svegliati S, Cancello R, Sambo P, Luchetti M, Paroncini P, Orlandini G, Discepoli G, Paterno R, Santillo M, Cuozzo C, Cassano S, Avvedimento EV, and Gabrielli A. Platelet-derived growth factor and reactive oxygen species (ROS) regulate Ras protein levels in primary human fibroblasts via ERK1/2. Amplification of ROS and Ras in systemic sclerosis fibroblasts. *J Biol Chem* 280: 36474–36482, 2005.
95. Thomson L, Trujillo M, Telleri R, and Radi R. Kinetics of cytochrome c2+ oxidation by peroxynitrite: implications for superoxide measurements in nitric oxide-producing biological systems. *Arch Biochem Biophys* 319: 491–497, 1995.
96. Tiefenbacher CP. Tetrahydrobiopterin: a critical cofactor for eNOS and a strategy in the treatment of endothelial dysfunction? *Am J Physiol Heart Circ Physiol* 280: H2484–2488, 2001.
97. Tillman TS and Cascio M. Effects of membrane lipids on ion channel structure and function. *Cell Biochem Biophys* 38: 161–190, 2003.
98. Touyz RM. Lipid rafts take center stage in endothelial cell redox signaling by death receptors. *Hypertension* 47: 16–18, 2006.
99. Tsunoda S, Mazda O, Oda Y, Iida Y, Akabame S, Kishida T, Shin–Ya M, Asada H, Gojo S, Imanishi J, Matsubara H, and Yoshikawa T. Sonoporation using microbubble BR14 promotes pDNA/siRNA transduction to murine heart. *Biochem Biophys Res Commun* 336: 118–127, 2005.
100. Ushio–Fukai M and Alexander RW. Reactive oxygen species as mediators of angiogenesis signaling: role of NAD(P)H oxidase. *Mol Cell Biochem* 264: 85–97, 2004.
101. Vasquez–Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, and Pritchard KA, Jr. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci USA* 95: 9220–9225, 1998.
102. Wajant H. The Fas signaling pathway: more than a paradigm. *Science* 296: 1635–1636, 2002.
103. Wang HD, Pagano PJ, Du Y, Cayatte AJ, Quinn MT, Brecher P, and Cohen RA. Superoxide anion from the adventitia of the rat thoracic aorta inactivates nitric oxide. *Circ Res* 82: 810–818, 1998.
104. Wever RM, van Dam T, van Rijn HJ, de Groot F, and Rabelink TJ. Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. *Biochem Biophys Res Commun* 237: 340–344, 1997.
105. Whitsett JA, Clark JC, Wispe JR, and Pryhuber GS. Effects of TNF-alpha and phorbol ester on human surfactant protein and MnSOD gene transcription in vitro. *Am J Physiol* 262: L688–693, 1992.
106. Wickstrom SA, Alitalo K, and Keski–Oja J. Endostatin associates with lipid rafts and induces reorganization of the actin cytoskeleton via down-regulation of RhoA activity. *J Biol Chem* 278: 37895–37901, 2003.
107. Wingler K, Wunsch S, Kreutz R, Rothermund L, Paul M, and Schmidt HH. Upregulation of the vascular NAD(P)H-oxidase isoforms Nox1 and Nox4 by the renin-angiotensin system in vitro and in vivo. *Free Radic Biol Med* 31: 1456–1464, 2001.
108. Wu RF, Gu Y, Xu YC, Nwariaku FE, and Terada LS. Vascular endothelial growth factor causes translocation of p47phox to membrane ruffles through WAVE1. *J Biol Chem* 278: 36830–36840, 2003.
109. Yang B, Oo TN, and Rizzo V. Lipid rafts mediate H2O2 prosurvival effects in cultured endothelial cells. *FASEB J* 20: 1501–1503, 2006.
110. Yang B and Rizzo V. TNFα potentiates protein-tyrosine nitration through activation of NADPH oxidase and eNOS localized in membrane rafts and caveolae of bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol* 292: H954–962, 2007.
111. Zhang AY, Teggatz EG, Zou AP, Campbell WB, and Li PL. Endostatin uncouples NO and Ca2+ response to bradykinin through enhanced O2*− production in the intact coronary endothelium. *Am J Physiol Heart Circ Physiol* 288: H686–694, 2005.
112. Zhang AY, Yi F, Li V, Gulbins E, and Li PL. Cell membrane lipid raft-redox signaling platform mediates the actions of endostatin in coronary arterial endothelium. *FASEB J* 20: A1292, 2006.
113. Zhang AY, Yi F, Teggatz EG, Zou AP, and Li PL. Enhanced production and action of cyclic ADP-ribose during oxidative stress in small bovine coronary arterial smooth muscle. *Microvasc Res* 67: 159–167, 2004.
114. Zhang AY, Yi F, Zhang G, Gulbins E, and Li PL. Lipid raft clustering and redox signaling platform formation in coronary arterial endothelial cells. *Hypertension* 47: 74–80, 2006.
115. Zhang AY, Yi F, Zhang G, Gulbins E, and Li PL. RNA interference of acidic sphingomyelinase gene restores endothleial function through inhibition of NAD(P)H oxidase activity. *FASEB J* 19: A521, 2005.
116. Zhang DX, Yi FX, Zou AP, and Li PL. Role of ceramide in TNF-alpha-induced impairment of endothelium-dependent vasorelaxation in coronary arteries. *Am J Physiol Heart Circ Physiol* 283: H1785–1794, 2002.
117. Zhang DX, Zou AP, and Li PL. Ceramide-induced activation of NADPH oxidase and endothelial dysfunction in small coronary arteries. *Am J Physiol Heart Circ Physiol* 284: H605–612, 2003.
118. Zhang J, Patel JM, Li YD, and Block ER. Proinflammatory cytokines downregulate gene expression and activity of constitutive nitric oxide synthase in porcine pulmonary artery endothelial cells. *Res Commun Mol Pathol Pharmacol* 96: 71–87, 1997.
119. Zou MH, Shi C, and Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* 109: 817–826, 2002.
120. Zuo L, Ushio–Fukai M, Ikeda S, Hilenski L, Patrushev N, and Alexander RW. Caveolin-1 is essential for activation of Rac1 and NAD(P)H oxidase after angiotensin II type 1 receptor stimulation in vascular smooth muscle cells: role in redox signaling and vascular hypertrophy. *Arterioscler Thromb Vasc Biol* 25: 1824–1830, 2005.

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