

REVIEW

Membrane raft redox signalosomes in endothelial cells

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(Received date: 7 February 2010; In revised form date: 5 April 2010)

Abstract

Membrane rafts (MRs) are specialized microdomains in the cell membrane with an altered lipid composition. Upon various stimulations, MRs can be clustered to aggregate or recruit NADPH oxidase sub-units and related proteins to form MR redox signalosomes in the membrane of cells like vascular endothelial cells (ECs). Multiple protein complexes, like MR redox signalosomes, are now considered to play a crucial role in the regulation of cell function and in the development of different cell dysfunctions. To form such redox signalosomes, ceramide will be generated from the hydrolysis of sphingomyelin by lysosomal acid sphingomyelinase that has been translocated via lysosome fusion to the MR area. In this brief review, current information is provided to help understand the occurrence and function of MR redox signalosomes. This may increase enthusiasm of the scientific community for further studies on the molecular mechanisms and the functional significance of forming such MR redox signalosomes.

Keywords: Lipid microdomains, ceramide, reactive oxygen species, lysosome, signal transduction, endothelium

Introduction

Recently, redox signalling is emerging as an essential mechanism in the regulation of the biological activity of a variety of cells. In vascular cells, the production of superoxide ($O_2^{\cdot-}$) can be induced by activation of NADPH oxidase (Nox), xanthine/xanthine oxidase or uncoupled nitric oxide synthase (NOS). Overwhelming evidence is now accumulating that non-mitochondrial Nox is a major source of $O_2^{\cdot-}$ in the vessel wall for the redox regulation of vascular endothelial and smooth muscle function [1–8]. It has been estimated that this non-mitochondrial Nox-derived $O_2^{\cdot-}$ constitutes greater than 95% of $O_2^{\cdot-}$ production in the vasculature, especially upon stimulation [5,9]. Despite many studies having demonstrated that the phosphorylation and translocation of Nox sub-units are of importance in activation of this enzyme [10,11], it remained unknown what physical force drives the aggregation of Nox sub-units so that they are assembled together, until we reported that lipid rafts (LRs) or membrane rafts (MRs) provide a driving force

[12–20]. In these studies, we demonstrated that MR clustering occurred in arterial endothelial cells (ECs) and that some agonists, such as Fas ligand (FasL), tumour necrosis factor- α (TNF- α), endostatin and homocysteine, induced aggregation of Nox sub-units such as gp91^{phox} and p47^{phox} into MR clusters, whereby Nox activity markedly increased. Now this MR-Nox cluster or complex that possesses redox signalling function has been referred to as MR redox signalling platforms, constituting a membrane signalosome that transmits or amplifies the signals produced by agonists or extracellular stimuli across the cell membrane [19,21]. This brief review will describe the nature of such MR signalosomes and discuss some of their functions with a focus on vascular ECs. It should be noted that the MR redox signalosomes here represent a transmembrane multiple protein signalling complex, which will not include the redoxosomes that require the endocytosis of key plasma membrane components, leading to Nox activation in the endosomal compartment. The readers who are interested

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in redoxosomes are directed to an excellent review article currently published [22].

Nature and functions of membrane rafts

Lipid raft (LR) or membrane raft (MR)

The concept has been well established that biological membranes are a mosaic of different compartments or domains that can form a number of types of sub-domains due to the interaction between membrane components. It is assumed that LRs consist of dynamic assemblies of cholesterol and lipids with saturated acyl chains, such as sphingolipids and glycosphingolipids in the exoplasmic leaflet of the membrane bilayer and phospholipids with saturated fatty acids and cholesterol in the inner leaflet [23]. Since long fatty acids of sphingolipids in the outer leaflets couples the exoplasmic and cytoplasmic leaflets by inter-digitation and transmembrane proteins stabilize this coupling, LRs are very stable and detergent resistant [24,25]. The sizes of individual LRs are hypothesized to vary in different cell types from 50–200 nm in diameter. Given its small size, a raft may contain only a sub-set of all available raft proteins. It has been estimated that the number of proteins in each raft depends on the packing density, but it probably carries no more than 10–30 proteins. Therefore, raft clustering is important for transmembrane signalling through its amplification. By comparing the ratio of the main raft and non-raft exoplasmic leaflet lipids, it was found that ~45% of the cell surface in fibroblasts and ~30% in lymphocytes are made up of sphingolipids [26].

Since the concept of LR was proposed in 1997 [27] to explain the inhomogeneity of plasma membrane microdomains, tremendous research efforts worldwide have been spent in this field. However, the majority of these studies failed to identify such individual LRs in the membrane of living cells. Therefore, at a recent 'Key Stone Symposium on Lipid Rafts and Cell Functions', which brought together leading scientists working from different angles in the raft field, the term 'lipid raft' was replaced by 'membrane raft (MR)'. The use of MR seems to be more appropriate since raft formation is not driven solely by lipids, but also involves protein interactions [28].

Caveolar and non-caveolar MRs

Generally, there are two types of MRs, namely, caveolar and non-caveolar rafts. Caveolar MRs are formed in cell types that express caveolin proteins. Polymerization of caveolins bends the membrane to form caveolae. However, the lipid components in caveolar or non-caveolar rafts are difficult to differentiate. There is considerable evidence that some cell types have only caveolar or non-caveolar membrane rafts,

but some cell types may have both in the plasma membrane [29]. It is possible that caveolar and non-caveolar MRs mediate different signalling pathways, thereby participating in the regulation of different cell function or cell responses to agonists and other stimuli [30,31].

It is well known that caveolar MRs are an important platform for the action of endothelial NOS (eNOS) to produce NO regulation of endothelial function. Among the binding partners of caveolins, their interaction with eNOS has been extensively studied [32]. It has been reported that binding of eNOS to the caveolin scaffolding domain inhibits eNOS activity [33], while loss of caveolin expression increases eNOS activity [34]. Recent data have yielded new insights regarding the regulation of eNOS by caveolins and caveolae [29]. It has been shown that endothelium-specific expression of eNOS and co-localization of eNOS with caveolins in ECs is important for NO-mediated vasodilation and maintenance in blood pressure [35]. Some data also suggests that antioxidant treatments can enhance the generation of NO, which depends on the formation of an oestrogen receptor alpha/caveolin-1/c-*Src* complex that leads to increased phosphorylation and activity of eNOS in these ECs [36]. In this regard, the caveolin-1-mediated formation of caveolae in ECs represents another form of MR clustering, which is present under resting conditions. In general, NOS in caveolae is constitutive and most activators of this enzyme may not alter the location of NOS in caveolae. This is different from non-caveolar MRs, which largely depends on clustering or de-clustering in response to various stimuli. In this review, we will mainly focus on the non-caveolar MR functions related to redox signalling.

MR clustering and functioning

The most important role of MRs on plasma membrane is their function in signal transduction. These MRs form concentrating platforms when ligand binding causes activation of individual receptors, and thereby recruit or aggregate various signalling molecules, activating different signalling pathways [37,38]. It has been demonstrated that many well-known signalling molecules such as trimeric G-protein, Ras, PIP2, sphingomyelin, K channels, tyrosine kinase and phosphatases can be recruited or aggregated in membrane raft platforms [31,39–41]. It is obvious that this dynamic clustering of lipid microdomains may represent a critical common mechanism in transmembrane signal transduction. In regard to the mechanisms for MR clustering, recent studies, including our work [14,42] and those from the laboratory of Dr Gulbins [43], have indicated that acid sphingomyelinase (ASMase) activation and translocation produce ceramide, which facilitates MR clustering and the patching or capping of Fas in different cells in response to death receptor

activation. It is believed that ceramide can modify the subtle intermolecular interactions within and among MRs and lead to their clustering and signalling.

MRs-related signalling events in ECs

In vascular ECs, MR-mediated signalling has also been demonstrated to occur in response to different stimulations. There is evidence that the MR clustering in ECs locally aggregates cell-associated heparan sulphate proteoglycans (HSPGs), which facilitates the entry of human immunodeficiency virus type 1 (HIV-1) into these cells, causing infection [44,45]. In studies on leukocyte migration, the presence of E-selectin in MRs on ECs was found to be necessary for its association and activation of phospholipase C γ (PLC γ), suggesting the importance of a localization of E-selectin in MRs to leukocyte-EC interactions [45]. As a signalling mechanism, MRs and their temporal-spatial organization with caveolae are also reported to be involved in signalling of vascular endothelial growth factors (VEGF) [46], NOS [47], H₂O₂ [48] and prostanoid receptor activation [49]. Downstream effector response to MR clustering in these ECs include receptor autophosphorylation, cAMP production, caspase activation, decrease or increase in nitric oxide (NO), reorganization of the actin cytoskeleton and Ca²⁺ mobilization [50]. In addition, MRs have also been implicated in promitogenic signalling in ECs. All these studies have indicated that MRs and MR 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 MRs, there is increasing evidence that the MR-mediated signalling mechanisms contribute to the regulation of several important endothelial functions such as endothelial barrier function [51], endothelium-dependent vasodilator or constrictor response, endothelial metabolic function [19,52] and its anti-coagulation and anti-thrombotic functions [53]. Although some reports have suggested that MR regulation of endothelial function may be associated with their dissociation or egression and internalization or endocytosis, MR clustering to form signalosomes in EC membrane is now considered to be a major mechanism mediating transmembrane signalling. In this regard, work in our laboratory has demonstrated that it is MR clustering that importantly participates in redox signalling of ECs, which contributes to the regulation of endothelium-related vasomotor response in intact coronary arteries [16].

Formation of MR redox signalosome in ECs

Nox-mediated redox signalling

Nox is a multi-sub-unit enzyme complex which was originally identified in phagocytic leukocytes. Nox

catalyses 1-electron reduction of oxygen to produce O₂⁻ using NADPH as the electron donor [54]. This enzyme is comprised of the membrane-bound cytochrome b558 (formed by the large sub-unit gp91^{phox}, also known as NOX2, and the smaller p22^{phox} sub-unit) and the cytosolic proteins p40^{phox}, p47^{phox} and p67^{phox}. In addition, the cytosolic GTPase, a small G-protein Rac, also participates in activation of Nox by assembling a Nox complex on the cell membrane. The catalytic sub-units of this enzyme are termed NOX proteins, which include several known members, namely, NOX1, NOX2 (gp91^{phox}), NOX3, NOX4 and NOX5, DUOX1 and DUOX2. Gp91^{phox} binds NADPH, contains a heme group and a flavin adenine dinucleotide and, together with p22^{phox}, supports the flow of electrons from NADPH to the oxygen molecule [55]. Similar to that identified in neutrophils, Nox expressed in vascular ECs also has several sub-units, including gp91^{phox}, p22^{phox}, p47^{phox}, p40^{phox} and p67^{phox}. Functionally, this endothelial Nox shares some, but not all, of the characteristics of neutrophil Nox.

Recent studies have indicated that activation of Nox in ECs is involved in several signalling pathways, including Rac/Ras and arachidonic acid metabolites, during stimulation by agonists or haemodynamic forces [56]. There is evidence that different stimuli may activate this oxidase by assembling or aggregating its membrane-bound and cytosolic sub-units [57]. The assembly of Nox sub-units requires translocation of cytosolic sub-units p47^{phox}, p67^{phox} and Rac to the plasma membrane, where these sub-units interact with gp91^{phox} and p22^{phox} and associate with other co-factors in the membrane to form a functional enzyme complex. The p47^{phox} translocation has been considered to be a key step and to some extent a marker event for the assembly and activation of Nox. It has been reported that p47^{phox} translocation is initiated by the phosphorylation of this sub-unit at various phosphorylation sites by PKC, PKA or MAPK [58,59]. However, it is unknown how p47^{phox} translocation and subsequent assembly of other Nox sub-units occur on the cell membrane. Emerging evidence shows that MR clustering to form signalosomes represents an important mechanism mediating the assembly or activation of Nox in ECs.

MR redox signalosomes in ECs

MR redox signalosome refers to a multiple protein complex that uses MRs as a platform to conduct redox signalling. Although individual MRs are too small to be observed on the cell surface by standard light microscopy, clustered MRs could form relatively larger microdomains or macrodomains on the cell membrane, which can be visualized by fluorescence or other staining techniques. Therefore, fluorescent or confocal microscopic detection of MR patches or

spots on the cell membrane is used as a common method in these studies. One of the common MR markers is fluorescent labelled-cholera toxin (CTX), which is used based on its capacity of binding to the raft constituent ganglioside GM₁, a glycosphingolipid that consists of a ceramide backbone [31]. Using this marker, our recent confocal microscopic analysis demonstrated that MRs were distributed through ECs membrane in a random diffuse or small punctuate staining pattern. Upon stimulation, these endothelial MRs formed multiple 'non-polarized' patches, randomly distributed throughout the cell membrane [18,19]. These fluorescent CTX patches have also been confirmed as MR clusters by other groups [60]. Various agonists such as FasL, endostatin and TNF- α were found to stimulate the formation of these MR clusters [19,45,61].

Endothelial redox signalosome is centred on Nox sub-units clustering and its activation to work as an enzyme complex. Nox sub-units are the major components of such endothelial redox signalosomes. By double-staining of ECs with FITC-labelled CTX and anti-gp91^{phox} or p47^{phox} antibodies, both gp91^{phox} and p47^{phox} were found to co-localize with FITC-CTX in coronary arterial EC membrane when they were stimulated with FasL. Similarly, other Nox sub-units such as Rac GTPase were also found to be clustered in MRs [19]. However, in ECs pre-treated with MR disruptor or cholesterol depletion reagents such as methyl- β -cyclodextrin (M- β -CD) or filipin, this MR clustering and aggregation of Nox sub-units were completely abolished. By detection of fluorescence resonance energy transfer (FRET) using FITC-anti-gp91^{phox} or p47^{phox} antibody and rhodamine or TRITC-labelled CTX as a fluorescence resonance pair, we found that FasL caused a significant increase in the FRET efficiency between Rac1 and GM1, indicating the tight association between them upon death receptor activation [13]. In addition, FasL was also able to increase FRET efficiency between Fas and MR marker, GM1, which supports the close relationship or interaction between multiple molecules in MR clusters to form redox signalosomes.

By floatation of detergent-resistant membranes (DRMs) as membrane raft fractions [19,62], it was found that membrane sub-unit gp91^{phox}, although detected at a very low level in MR fractions when ECs are under resting conditions, is much more abundant in MR fractions by recruitment when cells were stimulated by FasL or other stimuli. Interestingly, although p47^{phox} is located in the cytoplasm and cannot be detected in the MR fractions under normal conditions, it was translocated to MR fractions when cells were challenged by FasL or other stimuli, such as endostatin, TNF- α or visfatin [16,19,63]. In the presence of M- β -CD or filipin, however, increases in these Nox sub-units in MR fractions were substantially blocked. Considering that the membrane

translocation of p47^{phox} is closely associated with Nox activation, this increase in p47^{phox} in the MR fraction may strongly indicate activation of this enzyme within MRs. These results confirm an important coupling of MRs with Nox sub-units, which may lead to enhanced Nox activity and produce the redox regulation of cell functions. Our recent data showed that this enrichment of Nox sub-units in MR fractions was also found in glomerular epithelial cells and glomerular ECs when they were stimulated with L-homocysteine [20,64]. In addition, MR clustering with Nox sub-units was demonstrated in neutrophils [65], which was similar to that observed in ECs. Taken together, all these findings provide evidence that the formation of Nox sub-units centred MR-redox signalosomes occurs under different physiological and pathological conditions in ECs and other cells.

Other components in MR redox signalosomes

In addition to MR components and Nox sub-units, some other molecules were also demonstrated to be present in MR redox signalosomes. These molecules include various receptors which may bind to agonists to stimulate the formation of signalosomes and molecules promoting MR platform formation and functions. In this regard, ASMase has been found to be an important enzyme in MR redox signalosomes, which activates or facilitates the formation of these signalosomes. However, ASMase is mainly present in lysosomes, but its substrate sphingomyelin is one of the major components in the outer leaflet of the plasma membrane. An important question to be answered is how the lysosomal ASMase is able to approach its substrate, sphingomyelin, in the outer leaflets of the cell membrane to produce ceramide leading to MR clustering. Recent work in our laboratory [14,16,66] and by others [67,68] demonstrated that lysosomes may rapidly fuse into the cell membrane, leading to local ASMase secretion or attachment to the surface of the cell membrane. It has been suggested that this lysosomal ASMase hydrolyses sphingomyelin to generate ceramide, leading to the formation of ceramide-enriched platforms via MR clustering. Therefore, in MR redox signalosomes, several lysosome-specific molecules such as Lamp-1 and sortilin-1 could be detected in the MR redox platforms, as shown by confocal microscopy, FRET detection and flow cytometric analysis [69,70].

In addition, SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, a core machinery for membrane fusion of organelles, have also been detected in the MR clusters associated with redox signalosomes. We found that this SNARE-centred exocytic machinery is also involved in MR clustering to form redox signalosomes. In this regard, pre-treatment of coronary ECs with a specific inhibitor of vesicle-associated membrane protein

2 (VAMP2, a v-SNARE protein), almost completely blocked the formation of MR clusters. Using FITC-labelled anti-v-SNARE antibody and TRITC-labelled CTX-B, it was found that the aggregated v-SNARE was colocalized with CTX-B on the cell membrane and that both colocalized molecules produced FRET. FasL stimulation significantly increased the FRET efficiency between v-SNARE and GM1 when they aggregated on the cell membrane (unpublished data). This suggests that SNARE as a membrane fusion facilitator is also present in MR redox signalosomes.

Functionality of MR redox signalosomes

The functionality of MR redox signalosomes is to produce $O_2^{\cdot-}$ and thereby lead to redox regulation of cell and organ function. Although there are many methods that could be used to determine $O_2^{\cdot-}$ production or Nox activity, such as fluorescence microplate spectrometry with trapping dye, dihydroethidium (DHE), a chemiluminescence analysis using FCLA (3,7-dihydro-6-{4-[2-(N'-(5-fluoresceinyl) thioureido) ethoxy]phenyl}-2-methylimidazo-[1,2-a]pyrazin-3-one sodium salt) and HPLC analysis, to our knowledge the most definitive measurement is to analyse $O_2^{\cdot-}$ production in isolated MR fractions by membrane floatation in order to reflect the enzymatic activity of Nox associated with MRs. Electron spin resonance (ESR) spectrometry is considered to be the most accurate and sensitive method in detecting $O_2^{\cdot-}$ production within MRs. It has been demonstrated that Nox-dependent $O_2^{\cdot-}$ production was increased in isolated MR fractions from cells stimulated by FasL or other stimuli, as determined by ESR spectrometry [17]. In addition, this MR-associated $O_2^{\cdot-}$ production was also found to be stimulated or boosted in LM clusters in response to FasL, TNF- α , endostatin and angiotensin II in ECs or Fc γ activation in neutrophils [19,62,71]. This Nox-derived $O_2^{\cdot-}$ has been reported to be importantly implicated in the redox regulation of vascular endothelial and smooth muscle function [1,7]. These functionality analyses support the view that MR clustering contributes to the activation of Nox by aggregation and recruitment of its sub-units to MRs, which constitutes MR redox signalosomes, regulating downstream effector response and influencing cell function.

In addition, it has been reported that ROS may modulate the activity of many enzymes such as heme oxygenase, aconitase, tyrosine phosphatase, alkaline phosphatase, 5'-nucleotidase and ADP-ribosyl cyclase [72,73]. This redox regulation of enzyme activity is associated with the dimer formation of these enzymes due to oxidation of the cysteine residue in the enzyme molecule, which leads to the formation of one or several disulphide bonds in the enzyme molecule, resulting in the potentiation of enzyme activity. Based on a new model proposed by Dumitru et al. [74], ASMase

is activated by ROS because the free C-terminal cysteine of ASMase can be modified and lost by the actions of ROS, whereby a zinc coordination in this enzyme could be altered, constituting a feed forward regulation of ASMase activity and ROS production.

Mechanisms of MR redox signalosome formation

Role of ceramide and ASMase

As discussed above, ceramide and ASMase are important components of MR redox signalosomes. In particular, ceramide derived from ASMase is originally characterized as a lipid messenger in apoptotic signalling [75] and it is capable of forming highly ordered, gel-like domains in membranes [76]. Such ordered membrane domains may transiently exist in living cells, as recently demonstrated by the Ca^{2+} -stimulated co-recruitment of annexin-1-GFP to ceramide platforms [77]. Ceramide domains also appear to be essential for efficient clustering of Fas upon interaction with FasL [78]. In ECs, we have demonstrated that ceramide is accumulated to form MR signalling platforms in response to some agonists such as FasL or TNF- α [79,80]. In addition, it has been shown that SMases are enriched in vascular ECs and importantly mediate ceramide production, thereby leading to the formation of ceramide-enriched MR platforms with aggregation or recruitment of Nox sub-units. Ceramide, as a fusogen, facilitates the aggregation of rafts and the formation of signalling platforms, which cluster many proteins or lipid molecules to form redox signalosomes.

It is known that there are five types of SMase identified in different cells, including: (1) the lysosomal ASMase, (2) the cytosolic Zn^{2+} -dependent ASMase, (3) the membrane-bound magnesium-dependent neutral SMase, (4) the cytosolic magnesium-independent neutral SMase and (5) the alkaline SMase. With respect to cell signalling through MRs in ECs, ASMase has been extensively studied. There is increasing evidence that ASMase was accumulated in MR clusters when ECs were stimulated by FasL or endostatin and disruption or blockade of MR platform formation markedly attenuated agonist-induced activation of ASMase in these cells [16,18]. ASMase inhibitor and siRNA targeting ASMase blocked MR clustering and thereby restored endothelium-dependent coronary vasodilation that was inhibited by death factors such as FasL, TNF- α or endostatin. Correspondingly, increases in Nox activity and $O_2^{\cdot-}$ production induced by these death factors were also substantially attenuated by inhibition of ASMase. We assume that activation of 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 signalosomes [81]. During the formation of these ceramide-enriched membrane platforms, different membrane proteins, enzymes or signalling molecules can be aggregated, and some molecules in the cytosol are recruited to the membrane, which may result in a bulk amplification of the signals from related receptors.

Lysosome fusion and activation of ASMase

Given that ASMase is essential to the formation of MR redox signalling platforms but is mainly present in lysosomes, lysosome-plasma membrane fusion is an important mechanism in translocating ASMase into the MR cluster to reach its substrate, sphingomyelin, in the outer leaflet of the plasma membrane to produce ceramide. It is well known that lysosomes are membrane-bound organelles, which originate as membrane-bound vesicles from the Golgi apparatus (called Golgi vesicles) and exist in the cytoplasm of all eukaryotic cells. These cytoplasmic organelles contain several dozen acid hydrolases that are primarily responsible for intracellular digestion [82]. Based on different functions of lysosomes, they are divided into two types, namely conventional lysosomes and secretory lysosomes. Conventional lysosomes are the common lysosomes we are all very familiar with. These lysosomes are the digestive organelles of the cell. Another type of lysosome, the secretory lysosome, is able to fuse with the plasma membrane and secrete its content outside the cell. Many cells, including ECs, are found to have secretory lysosomes which mediate secretion of different substances by exocytosis. Recently, another type of lysosome has been reported to mediate membrane repair, which has conventional lysosome features, but works like secretory lysosomes to fuse to the plasma membrane and repair the damaged membrane areas [68,83].

Beyond autophagy, the intracellular digestion for cell defence, recent studies have extended lysosomal function to cellular signalling in different cells [84,85]. This organelle has been found to play an important role in receptor-mediated endocytosis and mediate events of receptor recycling. More recently, lysosomal vesicles have been reported to contribute to exocytosis in non-secretory cells, where these vesicles can fuse with the plasma membrane to excrete the contents of the vesicle and incorporate the vesicle membrane components into the cell membrane [67]. In addition, some studies have demonstrated that lysosomes as a Ca^{2+} store importantly participate in the regulation of cell functions in a variety of tissues or cells, where lysosome Ca^{2+} stores can be mobilized to mediate NAADP-induced Ca^{2+} release [86,87]. We, and others, recently demonstrated that lysosomes may rapidly fuse into the cell membrane, leading to ASMase translocation to the surface of ECs [14,17,67]. This lysosomal ASMase in MR clusters hydrolyses

sphingomyelin to generate ceramide, resulting in the formation of ceramide-enriched platforms via MR clustering. Indeed, confocal microscopy showed that FasL as an agonist induced the formation of MR clusters in the plasma membrane of ECs, accompanied by aggregation of Nox sub-units and O_2^- production. When these cells were pre-treated with two structurally different lysosomal vesicle function inhibitors, bafilomycin A1 and glycyl-L-phenylalanine-beta-naphthylamide (GPN), the FasL-induced MR clustering was substantially blocked and corresponding ROS production significantly decreased. By using LysoTracker, a colocalization of MRs and lysosomal vesicles was found around the cell membrane, which was abolished by bafilomycin A1 or GPN. These results suggest that lysosomal vesicles importantly contribute to the formation of MR-redox signalosomes. Further studies have revealed that sortilin, a glycoprotein which is responsible for transferring ASMase from the Golgi apparatus to lysosomes, is essential in initiating the movement of lysosomes and promoting their fusion to the cell membrane [69].

Sortilin is a 95-kDa glycoprotein, which has been reported to play an important role in targeting or transferring proteins to lysosomes [88]. Its Vps10p domain in the luminal region may be the binding site for the saposin-like motif of ASMase, while the cytoplasmic tail of sortilin contains an acidic cluster-dileucine motif that binds the monomeric adaptor protein GGA and is structurally similar to the cytoplasmic domain of M6P. All of these structural features determine sortilin as an intracellular protein transporter responsible for the sorting of soluble hydrolases such as ASMase to lysosomes. These results about colocalization of sortilin with lysosome proteins during death receptor activation indicate that sortilin not only simply mediates the targeting of ASMase to lysosomes, but also functionally interacts with ASMase [69]. The coupled sortilin-1 and ASMase work together to promote the movement of lysosomes toward cell membrane, which leads to MR clustering and Nox activation in ECs. This ASMase-dependent clustering of receptors was also observed for other receptors such as CD20, CD40, TNFR and epidermal growth factor receptor (EGFR) [89,90].

In addition to the colocalization of lysosome markers with MR components, lysosome fusion to the plasma membrane was also dynamically observed in living cells using fluorescent dye FM1-43 under a confocal microscope. This dye can be reversibly quenched by bromide phenol blue (BPB), which is easier to enter or come out of the lysosomes than FM1-43. Therefore, this dye can be used to observe its quenching and dequenching. In quenching experiments, agonists such as FasL, an ASMase activator, were found to cause a decrease in the FM1-43 fluorescence, which was due to the FasL-stimulated lysosome fusion with the

plasma membrane, allowing BPB to enter lysosomes to quench FM1-43 fluorescence [70]. In contrast, in dequenching experiments, cells were loaded simultaneously with FM1-43 and BPB together for 2 h. FasL caused an increase in the FM1-43 fluorescence when lysosomes were fused to the cell membrane since BPB was moved out of cells [17]. All these results strongly suggest that the fusion of lysosomes into the cell membrane occurs in these ECs upon FasL stimulation, which leads to the formation of MR clustering and redox signalosomes.

Lysosome fusion during the MR clustering process is demonstrated to be associated with SNARE-centred exocytic machinery, as discussed above. SNAREs comprise a superfamily of small, mostly membrane-anchored proteins, which mediate membrane fusion between organelles or from organelles to cell plasma membrane [91]. In particular, this SNARE-mediated membrane fusion plays an essential role in the secretory pathway of various eukaryotic cells, which is named the SNARE or SNARE-centred exocytic machinery [92]. It has been shown that three SNARE proteins constitute the minimal machinery for vesicle fusion, which includes vesicle membrane protein synaptobrevin, namely VAMP and the two plasma membrane proteins, syntaxins and SNAP-25/23. For fusion to occur, the four-helix bundle assembly of a VAMP, a syntaxin and two SNAP 25/23 has to be formed and coupled to the transmembrane region^(TM) [93]. After v-SNARE and target membrane-associated SNARE (*t*-SNARE) proteins zipper up into an α -helical bundle, another protein, SM (Sec1/Munc18-like) protein, will bind to SNARE complexes to direct their fusogenic action [94]. When the fusion process is completed, the SNAREs will be reused or recycled for repetitive rounds of exocytosis. Recycling is mediated by a machinery that dissociates the SNARE complexes, which is mediated by the hexameric ATPase N-ethylmaleimide-sensitive factor (NSF), a member of the ATPases associated with different cellular activities. The N-terminal domain of NSF binds the SNAP/SNARE complex to lead to ATP-hydrolysis, which dissociates the SNAP-SNARE complex (*cis*-form) and the four-helix SNARE bundle. Dissociated SNAREs can be used again. This SNARE-mediated lysosome fusion was observed in our recent studies. In addition to the demonstration of SNARE protein occurrence in the MR cluster by confocal microscopy and FRET detection, direct fusion response of lysosome to the cell membrane has been documented. It was found that in ECs stimulated by FasL, FM1-43 quenching or dequenching occurred due to lysosome fusion. In the presence of v-SNARE inhibitor, tetanus toxin, this lysosome fusion observed by FM1-43 was abolished (unpublished data). It is clear that SNARE-mediated lysosome fusion is essential for MR clustering and the formation of MR redox signalosomes.

Cytoskeletal components and MR clustering

Another potential mechanism modulating MR signalosome formation and function is the dynamic organization of the cytoskeleton. It has long been proposed that MRs are defined as a membrane structure enriched with cholesterol and associated with the cytoskeleton. The relationship between cytoskeletal elements and MRs is still emerging; however, it seems that microtubules and actin filaments are the primary interacting partners of MRs. It has been demonstrated that tubulin is present in MRs and can be co-immunoprecipitated with caveolin-1 in rat forebrain extracts [95]. One possible mechanism for the contribution of MRs to alterations in microtubules is indicated by experiments in smooth muscle cells [96]. In these cells, caveolins may stabilize microtubules by interfering with the interaction between the microtubule-destabilizing protein stathmin and tubulin. It has also been reported that treatment of glial cells or cardiac myocytes with microtubule-disrupting agents, such as colchicines, results in the loss of many signalling molecules from MR, in particular those involved in adrenergic receptor signalling [97,98].

The actin cytoskeleton is also found to have a bidirectional relationship with MRs. As an actin binding lipid, phosphoinositide lipids such as PtdIns(4,5)P₂ and PtdIns(3,4)P₂ can be accumulated in MRs and these lipids are also known to direct actin assembly into filaments [99,100]. In addition to binding to these lipids, actin also helps cluster signalling molecules in MRs. For example, small G proteins clustering in MRs is dependent on the actin cytoskeleton [101] and these GTPases may change their raft localization in response to the external signals that modify the actin cytoskeleton [102]. Therefore, agents that modify the raft association of actin can utilize small G proteins and other signalling molecules to form signalosomes in cell membrane. It should be noted that all evidence discussed above are from either neuronal or smooth muscle cells, and so far there is no direct evidence showing the regulatory role of tubulin and actin cytoskeleton in regulating the MR signalosomes in ECs. In addition, to our knowledge, little is known about whether the cytoskeleton is involved in the formation of MR redox signalosomes, which could be an interesting topic for further studies.

Death receptor signalling via MR redox signalosomes

Death factors like FasL, TNF- α , APO-3L and APO-2L can exert their actions through a group of receptors called the death receptors, such as Fas, TNFR1, TNFR2, DR3 and DR4/5. There is evidence that these receptors can be activated by their ligands, leading to apoptosis or other functional changes in a

variety of cells. Although it remains poorly understood how these receptors are aggregated and thereby recruit intracellular signalling components to initiate cell dysfunction or apoptosis, three major pathways are proposed to mediate the post-receptor processes after activation of various death receptors, including the actions of caspase-mediated execution, SMase activation and mitochondrial uncoupling [103,104]. Recent studies have highlighted the crucial role of sphingolipid- and cholesterol-enriched MRs for clustering or aggregation of these receptors and signalling molecules [105]. For example, Fas-associated death domain-containing proteins, procaspase-8, procaspase-10 and c-Jun amino-terminal kinase were detected in MRs, which may be related to their translocation or recruitment. In addition to these classic cytosolic apoptotic factors, other signalling molecules such as the actin-linking proteins ezrin, moesin, RhoA and RhoGDI were found to aggregate into Fas-enriched rafts in anti-tumour drug-treated leukaemic Jurkat cells [106,107]. These results support the view that MR-mediated signalling plays an important role in the mediation or regulation of death receptor conformation and recruitment of downstream signalling molecules, which activate or launch the apoptotic processes.

For some cells, such as ECs, that are relatively resistant to cell apoptosis due to some endogenous protective mechanisms [108–110], subtle pathological changes could be detectable at an early stage when ECs were incubated with these factors [111–113]. It appears that some functional disturbances may precede cell death in these cells 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 [114]. Recent studies have indicated that this endothelial dysfunction may be an early response to death receptor activation [115,116]. In this regard, work in our laboratory and by others has shown that death factors such as TNF- α attenuated endothelium-dependent vasodilation in a variety of vascular beds [116,117]. Although several mechanisms are proposed to contribute to this death receptor-mediated endothelial dysfunction, including inhibition of NOS activation and decrease in antioxidant enzymes such as superoxide dismutase (SOD), enhancement of Nox activity to produce $O_2^{\cdot-}$ is now considered to be a major mechanism that importantly contributes to the decrease in NO bioavailability and thereby to the impairment of endothelium-dependent vasodilation [54,118].

In recent studies, we have systematically examined the role of MR redox signalosomes associated with Nox in endothelial dysfunction induced by various

death factors including FasL, TNF- α and endostatin. It was found that FasL markedly impaired BK-induced endothelium-dependent vasodilation, which could be recovered by Nox inhibition using apocynin, suggesting the involvement of Nox-derived $O_2^{\cdot-}$. Interestingly, when the arteries were pre-treated by the disruptors of MRs, such as nystatin and M- β -CD, this FasL-induced impairment of endothelium-dependent vasodilation was also significantly attenuated. These results suggest that MR clustering, formation of MR redox signalosomes, and the following activation of Nox may be a contributing mechanism for FasL-mediated endothelial injury and dysfunction. Similar results were also obtained in endostatin-induced impairment of vasodilator response and recovery effects by apocynin and M- β -CD [16]. Therefore, the formation of these MR redox signalosomes seems to represent a common mechanism mediating death receptor activation-associated endothelial dysfunction or injury. In this regard, this redox signalosome formation has also been demonstrated in kidney cells such as glomerular capillary ECs and podocytes [12]. Eum et al. [119] also reported that upon the exposure to polychlorinated biphenyl, MR-dependent Nox/JAK/EGFR signalling mechanisms regulate the expression of cell adhesion molecules (CAMs) in brain ECs and adhesion of leukocytes to endothelial monolayers. Another study showed that high-density lipoprotein (HDL) inhibits Nox activation probably by interrupting the assembly of Nox sub-units at the MRs. This effect may contribute to the vascular protective actions of HDL against inflammation-mediated oxidative damage [120].

Despite these evidences, the functional significance of MR redox signalosomes is still not fully understood. Based on the findings discussed above, it is proposed that the formation of MR redox signalosomes may importantly contribute to the normal regulation of endothelial function and 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 signalosomes on the membrane of ECs in response to activation of death receptors may also be implicated in the development of these diseases.

Conclusion

This review has presented the existing information on MRs-associated redox signalosome formation and their functional relevance, particularly in ECs. As depicted in Figure 1, when death factors bind to their receptors on individual MRs, ASMase translocated from lysosomes or lysosome-like vesicles are

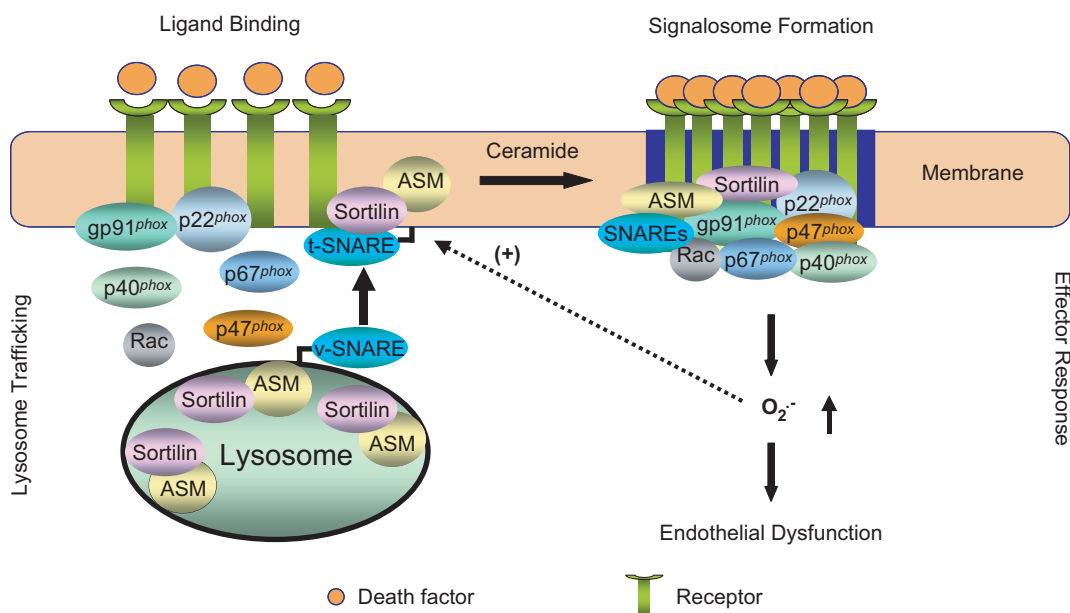


Figure 1. Formation and function of MR redox signalosomes in response to death factors in endothelial cells. When death factors, such as FasL, bind to their receptors on individual MRs, lysosomes are driven to fuse to the cell membrane through SNARE proteins. Then, acid sphingomyelinase is released to produce ceramide from sphingomyelin to form a number of membrane signalling platforms, in which acid sphingomyelinase, SNARE proteins and Nox sub-units, such as gp91^{phox} and p47^{phox}, are aggregated and activated, producing O₂⁻. O₂⁻ may feed-forward to enhance MR clustering, forming positive amplifications. All these together constitute a redox signalling network or signalosome, resulting in endothelial dysfunction and impairment of endothelium-dependent vasodilation in coronary arteries. ASM: acid sphingomyelinase; SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor; MR: Membrane raft.

activated to produce ceramide from sphingomyelin, resulting in the formation of a number of ceramide-enriched membrane signalling platforms in the cell membrane. In these platforms, Nox sub-units, such as gp91^{phox} and p47^{phox}, are recruited and other molecules aggregated to form MR redox signalosomes, a multiple protein complex in the cell membrane. Such MR redox signalosomes possess activated Nox and related molecules to promote production of O₂⁻. O₂⁻ or ROS in turn exert a feed-forward action to enhance MR clustering, forming positive amplifications. All these together constitute a redox signalling network resulting in cell dysfunction and even death. Considering the wide involvement of death factors in diseases, new therapeutic strategies targeting MR redox signalling can be expected for the prevention or treatment of multiple vascular diseases, such as atherosclerosis, hypertension and ischemia-reperfusion injury. In addition, more studies on the functional relevance and the pathophysiological implications of these MR redox signalosomes in diseased conditions are imperative at the whole animal level or in human diseases.

Declaration of interest: Many studies from our laboratory cited in this review were supported by grants from the National Institute of Health (HL-57244, HL-75316, and DK54927). The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 20 May 2010.