Cross Talk Between Ceramide and Redox Signaling: Implications for Endothelial Dysfunction and Renal Disease

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Abstract Recent studies have demonstrated that cross talk between ceramide and redox signaling modulates various cell activities and functions and contributes to the development of cardiovascular diseases and renal dysfunctions. Ceramide triggers the generation of reactive oxygen species (ROS) and increases oxidative stress in many mammalian cells and animal models. On the other hand, inhibition of ROS-generating enzymes or treatment of antioxidants impairs sphingomyelinase activation and ceramide production. As a mechanism, ceramide-enriched signaling platforms, special cell membrane rafts (MR) (formerly lipid rafts), provide an

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important microenvironment to mediate the cross talk of ceramide and redox signaling to exert a corresponding regulatory role on cell and organ functions. In this regard, activation of acid sphingomyelinase and generation of ceramide mediate the formation of ceramide-enriched membrane platforms, where transmembrane signals are transmitted or amplified through recruitment, clustering, assembling, or integration of various signaling molecules. A typical such signaling platform is MR redox signaling platform that is centered on ceramide production and aggregation leading to recruitment and assembling of NADPH oxidase to form an active complex in the cell plasma membrane. This redox signaling platform not only conducts redox signaling or regulation but also facilitates a feedforward amplification of both ceramide and redox signaling. In addition to this membrane MR redox signaling platform, the cross talk between ceramide and redox signaling may occur in other cell compartments. This book chapter focuses on the molecular mechanisms, spatial–temporal regulations, and implications of this cross talk between ceramide and redox signaling, which may provide novel insights into the understanding of both ceramide and redox signaling pathways.

Keywords Sphingolipid • Membrane rafts • Free radicals • Signalosomes • Redoxosome • Caveolae • Acid sphingomyelinase • Lysosome • Methyl-β-cyclodextrin

1 Introduction

Redox signaling is a fundamental signaling mechanism in cell biology which importantly participates in a variety of cellular activities including cell proliferation (Burdon 1996; Cai 2006; Nicco et al. 2005), differentiation (Del Prete et al. 2008; Hansberg et al. 1993; Kusmartsev and Gabrilovich 2003; Sasaki et al. 2009; Sauer et al. 2001), and apoptosis (Hildeman 2004; Liu et al. 2009; Mates and Sanchez-Jimenez 2000; Perrone et al. 2008; Wolf 2005). Abnormal redox signaling is frequently involved in various pathophysiological processes such as senescence (Colavitti and Finkel 2005), inflammation (Azad et al. 2008; Muller-Peddinghaus 1989; Yamamoto et al. 2009), hypoxia (Bell and Chandel 2007; Guzy and Schumacker 2006; Kietzmann and Gorlach 2005; MacFarlane et al. 2008), and ischemia/reperfusion (Goswami et al. 2007; Szocs 2004; Toledo-Pereyra et al. 2004), which contribute to the progression of almost all diseases, from cardiovascular ones such as shock (Flowers and Zimmerman 1998; Gendzwill 2007a, b), hypertension (Delles et al. 2008; Hirooka 2008; Ong et al. 2008; Paravicini and Touyz 2008; Puddu et al. 2008; Zeng et al. 2009), and atherosclerosis (Kojda and Harrison 1999; Patel et al. 2000), to metabolic ones such as diabetes mellitus (Bagi et al. 2009; Ksiazek and Wisniewska 2001), to neurodegenerative ones such as Alzheimer's disease (Casadesus et al. 2004; Perry et al. 1998), infectious diseases (Jamaluddin et al. 2009; Mashimo et al. 2006; Ochsendorf 1998; Sun et al. 2008), and cancer (Azad et al. 2009; Oyagbemi et al. 2009; Weinberg and Chandel 2009).

Currently, it is of high interest to explore how redox signaling is regulated under both physiological and pathological conditions.

Despite extensive studies, the precise mechanisms for rapid activation of redox enzymes by different stimuli are still poorly understood. Redox enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, unlike G-protein-coupled enzymes, are not coupled with any specific receptors. Therefore, a previous undefined mechanism must exist to bridge receptor activation and redox signaling. Recent studies in non-phagocytes revealed that membrane raft (MR, formerly lipid raft) signaling platforms might be responsible for activation of death receptors, in particular CD95 and tumor necrosis factor receptor 1 (TNFR1). These death receptors were found to be localized in MRs, and these receptors in MRs can interact to stabilize the MRs and allow raft aggregation (i.e., MRs clustering), by which many raftophilic molecules are invariably recruited into the complex with the MRs, producing massive signaling and effector responses. Furthermore, it has been well documented that ROS or oxidative stress is a downstream mechanism of receptor clustering in MR platforms. This MR-associated ROS generation, downstream of CD95 and TNFR1, may be of high importance in the early alterations of cell functions during activation of death receptors and in induction of apoptosis (Dumitru et al. 2007; Morgan et al. 2007). Indeed, recently collected evidence support the view that MR signaling platforms are an important mechanism for initiating and transmitting redox signaling in mammalian cells (Li and Gulbins 2007; Oakley et al. 2009; Yang and Rizzo 2007; Zhang and Li 2010). Given the central role of ceramide and related signaling in the formation or regulation of MR redox platforms through clustering of ceramide-enriched microdomains, the cross talk between ceramide and redox signaling is emerging as an important cellular signaling mechanism that mediates the regulation of cellular activities.

Ceramide is generated by several enzymatic pathways in mammalian cells. Two major pathways are the sphingomyelinase (SMase) pathway that generates ceramide from sphingomyelin (SM) by the activities of SMase and the de novo synthesis pathway that synthesizes ceramide from serine and palmitoyl-CoA by the activity of ceramide synthase. The biophysical properties of ceramide molecules predict a tight interaction of ceramide molecules with each other, resulting in the formation of stable and tightly packed ceramide-enriched membrane microdomains that spontaneously fuse to form large ceramide-enriched membrane macrodomains or platforms. Among SMases, acid SMase (ASMase) has been considered as the major enzyme responsible for the formation of ceramide-enriched membrane platforms. Recently, we and others have reported that various death factors bind to their receptors in or around individual MRs and stimulate ASMase to produce ceramide from SM, leading to the formation of ceramide-enriched membrane platforms. In such platforms, NADPH oxidase subunits such as $gp91^{phox}$ and $p47^{phox}$ and other redox molecules are aggregated, clustered, and/or recruited, leading to signal transduction by increase or scavenging of $O_2^{\bullet -}$ or ROS. Such redox signaling associated with ceramide-enriched membrane platforms has been found to contribute to the regulation of a variety of cellular activities and organ functions and lead to pathological changes such as endothelial dysfunction, cell apoptosis, and phagosomal action in neutrophils or macrophages (Jin et al. 2008b; Li et al. 2007;

Zhang et al. 2006). Given the focus of this chapter on the cross talk between ceramide and redox signaling, we will discuss the role of ceramide in the regulation of MR or nonraft redox signaling and vice versa as well as summarize some evidence related to physiological and pathological relevances of this cross talk. Given that there are a lot of discussions about the basic knowledge of ceramide signaling in other chapters, here, we first provide some background information regarding current knowledge of redox signaling and regulation.

2 Redox Signaling and Oxidative Stress

2.1 Reactive Oxygen Species

Reactive oxygen species (ROS) is a collective term that often includes not only the oxygen radicals such as superoxide ($O_2^{\bullet -}$), hydroxyl (OH^-), peroxyl (RO_2), alkoxyl ($RO^{\bullet}$), and hydroperoxyl ($HO_2^{\bullet}$) but also non-radicals such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen (ΔgO_2), and peroxynitrite ($ONOO^-$). Since these oxygen derivatives, whether they are radicals or non-radicals, are very reactive, they can oxidize or reduce other molecules in living cells or tissues. Therefore, in general, redox signaling is often referred to as the signaling induced by ROS (Halliwell and Cross 1994; Stadtman 2004; Tang et al. 2002). The most important ROS is $O_2^{\bullet -}$, which is unstable and short-lived because it has an unpaired electron, and it is highly reactive with a variety of cellular molecules, including proteins and DNA. $O_2^{\bullet -}$ is reduced to H_2O_2 by superoxide dismutase (SOD), and both $O_2^{\bullet -}$ and H_2O_2 can diffuse from their sites of generation to other cellular locations. H_2O_2 is further reduced to generate the highly reactive $^{\bullet}OH$ through the Haber–Weiss or Fenton reaction under pathological conditions. In contrast to $O_2^{\bullet -}$ and H_2O_2, $^{\bullet}OH$ is highly reactive and, therefore, causes primarily local damage. In addition, $O_2^{\bullet -}$ can also interact with NO to form another reactive oxygen free radical, $ONOO^{\bullet -}$. Under physiological conditions, O_2^- preferably produces H_2O_2 via the dismutation reaction. However, when excess $O_2^{\bullet -}$ is produced, a substantial amount of $O_2^{\bullet -}$ reacts with NO to produce $ONOO^{\bullet -}$. Taken together, these ROS constitute a redox regulatory network that controls cellular activity and function.

2.2 Redox Signaling and Injury

It has been reported that ROS can be produced as a basic signaling messenger to maintain cell or organ functions or increasingly generated or released in response to various stimuli. Meanwhile, these active molecules are constantly scavenged by the endogenous antioxidant systems, mainly composed of the enzyme-mediated pathways as SOD, catalase, glutathione peroxidase, glutathione-*S*-transferase,

thioredoxin/thioredoxin reductase, and other peroxidases. In addition, direct reactions between ROS and different molecules may also result in antioxidant actions such as the interactions between ROS and NO, –SH, vitamin E, β-carotene, ceruloplasmin, ferritin, transferrin, hemoglobin, and ascorbates. Being tightly regulated under normal conditions, intracellular and extracellular ROS are maintained at very low levels (less than 1 % of produced ROS). If the generation of ROS exceeds its removal by scavengers, the intracellular and extracellular levels of ROS will increase, leading to oxidative stress and a progression of various pathophysiological processes and respective diseases. If the level of ROS increases to even higher levels, its damaging effects, to DNAs, proteins, lipids, and glycols, become inevitable. These damaging effects of ROS are often tightly correlated together and share a common redox system responsible for the generation and scavenging of ROS molecules.

2.3 ROS-Generating Systems

Among four common ROS including $O_2^{\bullet -}$, H_2O_2, HO^-, and $ONOO^-$, $O_2^{\bullet -}$ has been considered as the progenitor of other common ROS. The production of $O_2^{\bullet -}$ and related regulation in biological systems have been intensively studied. In mammalian cells, many pathways are involved in the production of $O_2^{\bullet -}$, including NADPH oxidase, xanthine oxidase, mitochondrial respiration chain, cytochrome P450, lipoxygenase, cyclooxygenase, peroxisomes, and NO synthase uncoupling. Some nonenzymatic derivatives of $O_2^{\bullet -}$ may be formed via photolysis, heme protein + Fe, and auto-oxidation reactions. Among these pathways, NADPH oxidase has been reported to be a major source of $O_2^{\bullet -}$ for the redox regulation in some cells such as vascular endothelial and smooth muscle cells (Griendling et al. 2000). It is estimated that this nonmitochondrial NADPH oxidase-derived $O_2^{\bullet -}$ constitutes more than 95 % of the production of $O_2^{\bullet -}$ in these cells, especially when they are stimulated (Mohazzab et al. 1994; Rajagopalan et al. 1996).

3 Interactions of Ceramide and Redox Signaling Pathways

There is accumulating evidence that ceramide induces the activation of ROS-generating enzymes, including NADPH oxidase, xanthine oxidase, NO synthase, and the mitochondrial respiratory chain (Corda et al. 2001; Lecour et al. 2006). In particular, ceramide has been shown to activate NADPH oxidase and to increase the production of $O_2^{\bullet -}$ in a variety of mammalian cells, including human aortic smooth muscle cells, endothelial cells (ECs), and macrophages (Bhunia et al. 1997; Zhang et al. 2007, 2008). Because many stimuli activate NADPH oxidase by translocation and aggregation of its subunits, it has been proposed that ceramide may mediate the fusion of small raft domains to ceramide-enriched membrane platforms, thereby clustering subunits of NADPH oxidase, assembling them into an active enzyme

complex and producing of $O_2^{\bullet -}$. In addition, ceramide has also been shown to interact with the mitochondrial electron transport chain leading to the generation of ROS (Corda et al. 2001). Given the crucial role of NADPH oxidase in the normal regulation of cell functions and as one of the most important redox signaling pathways (Dworakowski et al. 2006; Finkel and Holbrook 2000), we will focus on the cross talk between ceramide signaling pathway and NADPH oxidase-derived redox regulation.

3.1 Ceramide-Enriched Microdomains in MR Redox Signaling

Ceramide belongs to a highly hydrophobic lipid family, which consists of fatty acids with carbon chains in variable lengths (2–28 carbons) and sphingosine (Mathias and Kolesnick 1993). SMase is released to the cell surface or extracellular space in an autocrine or paracrine manner that hydrolyzes cell-surface SM, inducing cell–cell communications and exerting remote action through blood circulation. More importantly, SMase may act on SM incorporated in the MR area of cell membranes and thereby produce ceramide locally (Cremesti et al. 2002). Ceramide molecules spontaneously bind to each other to form microdomains that fuse to large ceramide-enriched membrane platforms (Brown and London 1998; Grassme et al. 2001). In such ceramide-enriched platforms, redox molecules such as NADPH oxidase subunits or cofactors can be aggregated to assemble into active NADPH oxidase complex, producing $O_2^{\bullet -}$ to conduct signaling (Jin et al. 2008b; Zhang et al. 2006).

This ceramide-enriched redox signaling platform has been found to be formed in response to different death receptor ligands such as CD95 ligand, TNF-α, or endostatin (Jin et al. 2008a, b; Zhang et al. 2006). Furthermore, ultraviolet irradiation also induces the formation of ceramide-enriched platforms that mediate ROS production (Chatterjee and Wu 2001). We recently demonstrated that a rapid movement and consequent fusion of lysosomes to supply ASMase into the MR area of cell membranes occur in response to various stimuli (Jin et al. 2007, 2008a). This lysosome fusion is critical for the formation of ceramide-enriched platforms and therefore determines MR redox signaling in different cells, in particular in ECs (Jin et al. 2008a, b).

Further studies have revealed that sortilin, a glycoprotein responsible for transferring ASMase from the Golgi apparatus to lysosomes, is also important in initiating the movement of lysosomes and promoting their fusion to the cell membrane in ECs (Bao et al. 2010a, b). Sortilin is a 95-kDa glycoprotein, which has been reported to play an important role in targeting or transferring proteins to lysosomes (Ni and Morales 2006). Its Vps10p domain in the luminal region may be the binding site for the saposin-like motif of ASMase, while its cytoplasmic tail containing an acidic cluster-dileucine motif binds the monomeric adaptor protein GGA and is structurally similar to the cytoplasmic domain of M6P. The coupled sortilin-1 and ASMase work together to promote the movement of lysosomes toward the cell membrane, which, in turn, leads to MRs clustering and NADPH oxidase activation in ECs. This ASMase-dependent clustering of receptors was also

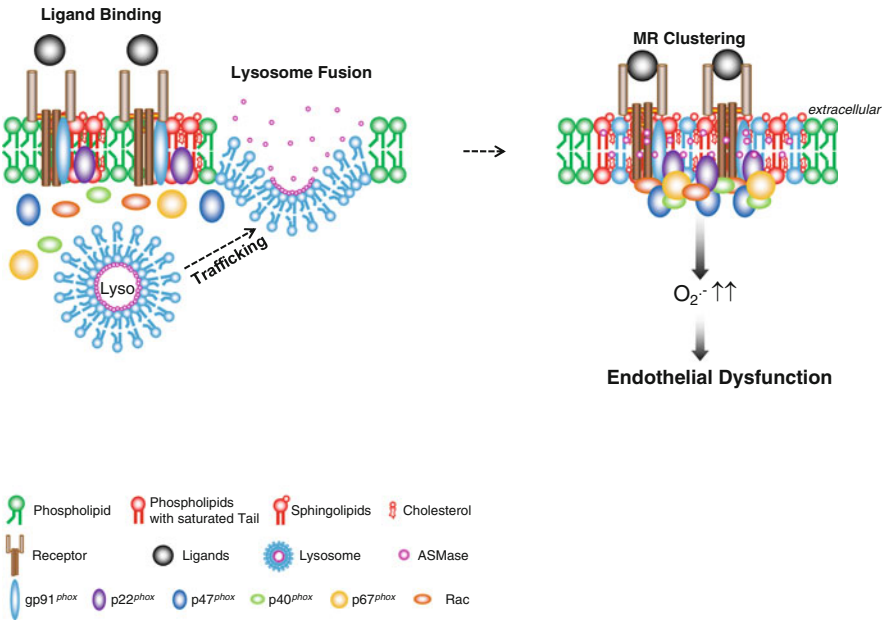

Fig. 1 Lysosome biogenesis and fusion to cell membrane to form ceramide-enriched redox signaling platforms. ASMase is synthesized from the ER and transported through Golgi apparatus to lysosomes. These lysosomes can be mobilized to traffic and fuse into cell membrane, where ASMase is activated and ceramide produced, resulting in MRs clustering and formation of ceramide-enriched platforms (adapted from Xia M, et al. *Cardiovasc Res* (2011) 89 (2): 401–409)

observed for other receptors such as CD20, CD40, TNFR, and epidermal growth factor receptors (EGFR) (Rodighiero et al. 2004). In addition, the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor)-centered exocytic machinery was found to be involved in MR clustering to form redox signaling platforms. SNAREs comprise a superfamily of small, mostly membrane-anchored proteins, which mediate membrane fusion between organelles or from organelles to cell plasma membranes (Gerst 1999). In particular, SNARE-mediated membrane fusion plays an essential role in the secretory pathway of various eukaryotic cells, which is named as the SNARE or SNARE-centered exocytic machinery (Blank et al. 2002). It seems that SNARE as a membrane fusion facilitator is also present in MR redox signaling platforms although its major function is to help lysosome fusion (Zhang and Li 2010).

A comprehensive working model for the mediation of MR clustering and the formation of MR signaling platforms in arterial ECs is presented in Fig. 1; this model emphasizes the derivation of membrane ASMase as being from lysosomes, which target ASMase when it is synthesized from ER and transported through Golgi apparatus. Many mature lysosomes with ASMase are proximal to the cell membrane. When a receptor such as death receptor is activated by a ligand binding

to it or by other stimulations, these lysosomes proximal to the cell membrane become mobilized to move and fuse with the cell membrane, activating ASMase and synthesizing ceramide, thereby resulting in MRs clustering and the formation of ceramide-enriched platforms. These MR platforms, in turn, recruit, translocate, and aggregate NOX and its subunits or cofactors and assemble them into an active enzyme complex, which produces $O_2^{\bullet -}$, promoting transmembrane signaling.

3.2 Redox Signaling Molecules Associated with Ceramide-Enriched Membrane Platforms

3.2.1 The NADPH Oxidase Family

NADPH oxidase, identified and characterized first in neutrophils, catalyzes the 1-electron reduction of oxygen producing $O_2^{\bullet -}$ using NADPH as the electron donor. This neutrophil oxidase consists of at least five subunits: two membrane-bound subunits gp91^{phox} (also known as NOX2) and p22^{phox} and three cytosolic subunits p47^{phox}, p40^{phox}, and p67^{phox}. NOX2 and p22^{phox} form an integral membrane complex termed cytochrome b_{558}, and the other four subunits, p47^{phox}, p67^{phox}, p40^{phox}, and the small GTPase Rac, localize in the cytosol in resting cells (Babior et al. 2002). In the classic model of phagocytic-type NADPH oxidase, activation involves translocation of the four cytosolic proteins to the cell membrane and interactions with the membrane spanning subunits p22^{phox} and NOX2, resulting in the transfer of the NADPH electron to oxygen molecules and the generation of $O_2^{\bullet -}$ (Babior et al. 2002; Dang et al. 2002).

NOX protein family consists of six other homologues of NOX2 (gp91^{phox}) catalytic subunits, namely, NOX1, NOX3, NOX4, NOX5, DUOX1 (dual oxidases1), and DUOX2, which determine ROS production in non-phagocytes (Cheng et al. 2001). NOX2 is also expressed in non-phagocytes, including neurons, cardiac cells, skeletal muscle cells, liver cells, ECs, B lymphocytes, epithelial cells, and hematopoietic cells (Piccoli et al. 2005). The structure and function of non-phagocytic NOX are very similar to NOX2. They can also catalyze a single-electron reduction of molecular oxygen, generating $O_2^{\bullet -}$ and other ROS. It is interesting to note that almost all NOXs were demonstrated to have some structural or functional link to or relationship with MRs (Ushio-Fukai et al. 2001; Zhang et al. 2006; Zuo et al. 2004, 2005). Given that NOX activation requires many cofactors to work together, MRs provide a wonderful platform for NOX and the other NADPH oxidase subunits and cofactors to assemble and then work as an active enzymatic complex. However, the driving force or actual physical platform for NADPH oxidase assembly as an active enzyme complex is still unknown. As noted above, the ceramide-enriched membrane macrodomains or platforms may represent an important mechanism mediating this assembly or activation process of NADPH oxidase.

3.2.2 Superoxide Dismutase

Recently, proteomic analysis demonstrated that membrane SOD (SOD1) is present in MR fractions (Zhai et al. 2009), a fact consistent with previous reports that SOD1 is detectable in MRs (Siafakas et al. 2006). Reported SOD1 levels, for example, in MR fractions were much higher than that in other areas of the plasma membrane. These results support the view that aggregation of MRs may play an important role for SOD1 actions. It is assumed that localization and subsequent aggregation of SOD1 in MRs could affect cellular functions as well as the interplay between different cell types, as MRs are rich in receptors and the signaling molecules necessary for cell–cell communications (Zhai et al. 2009). Indeed, a more recent study has reported that H_2O_2, generated extracellularly by extracellular SOD, anchored to ECs surface via the heparin-binding domain (HBD), enhances VEGF-induced VEGF receptor 2 (VEGFR2) autophosphorylation in caveolin-enriched MRs, but not in non-caveolar MRs. The HBD of endothelial SOD is required for its localization in plasma membrane MRs, suggesting that localization of endothelial SOD in caveolae/MRs via HBD can serve as an important mechanism by which SOD-derived extracellular H_2O_2 efficiently promotes VEGFR2 signaling in ECs and postnatal angiogenesis (Oshikawa et al. 2010).

3.2.3 Catalase

In neutrophils, recent proteomic analysis (Feuk-Lagerstedt et al. 2007) has also found catalase in MR fractions that play critical roles in redox signaling by cleavage of H_2O_2. Although some studies have demonstrated that MR-associated catalase may be related to peroxisome biogenesis, the function of this catalase association with MRs remains still largely unknown. It is possible that MRs in hepatic peroxisomal membrane cells are able to help catalase sorting and distribution to different compartments of these cells, assigning them an important role in hepatocyte proliferation and lipid metabolism. Given that hepatic caveolin-1 plays an important role in liver regeneration and lipid metabolism, caveolae with catalase may be critically involved in this liver regeneration and lipid metabolism. However, recent studies found that the absence of caveolin-1 did not affect the peroxisomal location of catalase in mouse liver. It seems caveolin-1 is not required for peroxisome biogenesis, whereas other types of peroxisomal MRs are required (Woudenberg et al. 2010). Obviously, more research and thinking need to be invested into the formation and function of MR-associated catalase complexes.

3.2.4 Thioredoxin

Although it is not yet extensively studied, thioredoxin (TRX) has also been reported as a MR-associated protein. In some reports, MRs have been shown to mediate the effects of TRX. There is convincing evidence that MRs may mediate the actions of

TRX on leukocyte–EC interaction related to redox regulation during inflammation. TRX is a ubiquitous protein with a redox-active disulfide that functions in concert with NADPH and TRX reductase to control the redox state of cysteine residues of different oxidant-targeted proteins. Given the antioxidant role of TRX, the MR-mediated role of TRX in the interaction between leukocytes and ECs may importantly regulate inflammatory responses through counteracting oxidative stress and ROS. In addition, TRX can be internalized into the cells through MR-mediated endocytosis. In particular, a TRX mutant, TRX-C35S (with replacement of cysteine 35 by serine), was found to bind rapidly to the cell surface and be internalized into the cells through MRs in the plasma membrane. This indicates that the cysteine at the active site of TRX is important for the internalization and signal transduction of extracellular TRX through MRs (Hara et al. 2007; Kondo et al. 2007).

3.2.5 Transient Receptor Protein C3 and C4 (TRPC3 and TRPC4)-Redox Sensors

MRs have also been reported to promote molecules aggregation, gating, or activation producing their downstream impact on redox sensing or enhancement of effector responses to redox signaling. Among these molecules, a currently identified redox-sensitive protein-transient receptor protein (TRP) is particularly noteworthy. TRPs are a family of voltage-independent nonspecific cation-permeable channels. Evidence exists that TRPC3 and TRPC4 localize or relocalize in MRs and can form a TRPC3–TRPC4 complex with different properties from their respective homomeric channels, which are redox sensitive (Poteser et al. 2006). Perhaps these TRP channels are directly gated or influenced by the formation of MR platforms, and therefore, their redox-sensing function is altered. Indeed, the TRPC3 channel activity is increased by cholesterol loading of the cell membrane when TRPC3 is overexpressed. This increased channel activity may lead to enhanced redox sensitivity of the channels, exerting an important redox regulation or resulting in pathologic consequences in different cells (Poteser et al. 2006).

3.3 Redox Regulation of Ceramide-Enriched Membrane Platform Formation

There is increasing evidence that the formation of MR-derived signaling platforms can also be regulated by redox molecules. For example, the formation of ceramide-enriched membrane platforms in the membrane of coronary arterial ECs can be reduced by SOD but increased by $O_2^{\bullet -}$ donor or generating systems (Zhang et al. 2007). H_2O_2 was also found to activate pro-survival signaling pathways, including activation of PI3 kinase/Akt and extracellular signal-regulated kinases (ERK)1/2 by changes in MRs behaviors (Yang et al. 2006a). Exogenous administration of

xanthine/xanthine oxidase, a $O_2^{\bullet -}$ generating system, has demonstrated a dramatic increase in MRs clustering and ceramide-enriched membrane platform formation in the membrane of ECs (Qiu et al. 2003; Zhang et al. 2007). Furthermore, ROS in T lymphocytes were also shown to enhance MR signaling, and blockade of ROS production by the SOD-mimic MnTBAP reduced the localization of several signaling molecules such as LAT, phospho-LAT, and PLC-gamma in MRs fractions. Treatment of T cells with the ROS synthesizer, *tert*-butyl hydrogen peroxide (TBHP), greatly enhanced MR formation and the distribution of phospho-LAT into MRs. Moreover, lipid peroxides were found to promote the formation of larger rafts or platforms on the membrane, and photooxidation, at the lipid double bonds, caused raft enlargement (Ayuyan and Cohen 2006). These observations corroborate and reinforce the conclusion that ROS are able to enhance MR clustering or formation of macrodomains and must contribute to the formation of MR platforms (Lu et al. 2007). In addition to the direct regulation of SMase/ceramide pathway, various ROS were found to influence MR signaling or function through their actions on many other MR constituents such as caveolin-1, cholesterol, and related raft proteins (Dumitru et al. 2007; Morgan et al. 2007).

3.3.1 ROS Interact with Caveolin-1

Biochemical and morphological experiments have shown that at least two subtypes of lipid microdomains are present in mammalian cells: caveolar and noncaveolar MRs. The size of noncaveolar MRs is 50–100 nm or even smaller, and each of them contains 10–30 protein molecules. Caveolae, cave-like plasma membrane subdomains, are considered as another subtype of lipid domains. Caveolin-1 is the major protein component of caveolae, and its polymerization forms a rigid scaffold that maintains the characteristic cave-like morphology. In addition to its structural function, caveolin-1 has several important regulatory activities through direct interaction with other functional proteins and signaling molecules. Caveolin-1 is subject to two types of posttranslational modification that might be critical for regulating its intracellular activity and localization, namely, phosphorylation and palmitoylation. Recent studies have indicated that both phosphorylation and palmitoylation of caveolin-1 can be regulated by ROS and ultimately affect caveolar functions. In vascular ECs, H_2O_2 causes increased tyrosine phosphorylation of caveolins (Brown and London 1998). In addition, Parat and colleagues showed that exogenous H_2O_2 did not alter the intracellular localization of caveolin-1 in ECs, but it inhibited the trafficking of newly synthesized caveolin-1 to MRs (Brown et al. 1998). They further demonstrated that H_2O_2 did not alter the rate of caveolin-1 depalmitoylation but rather decreased the "on-rate" of palmitoylation (Brown et al. 1998). Functional studies substantiated that caveolin-1 is a sensitive target of oxidative stress and that the oxidation of caveolar membrane cholesterol causes the translocation of caveolin-1 from the plasma membrane to the Golgi apparatus (Grassme et al. 2001). In a separate study, treatment of ECs with ROS caused a release of caveolin-1 from membranes and

also a decrease in the number of caveolae detected by electron microscopy (van den Elzen et al. 2005). In summary, these results suggest that oxidative stress modulates caveolin-1 function and cellular levels, which may ultimately affect caveolar function and plasma membrane composition, namely, alteration in the ratio of caveolar vs. noncaveolar MRs or membrane signaling platforms.

3.3.2 ROS Interact with Cholesterol

It has been known that the formation of MRs is driven by tight packing between cholesterol and sphingomyelin and other sphingolipids. Oxysterols are derivatives of cholesterol that contain a second oxygen atom as a carbonyl, hydroxyl, or epoxide group (Morita et al. 2004). Cytotoxic oxysterols formed by nonspecific oxidative mechanisms can affect many cellular processes that contribute to the pathogenesis of disease. According to their biophysical properties, which can be distinct from those of cholesterol, oxysterols can promote or inhibit the formation of membrane microdomains or MRs (Byfield et al. 2006; Scheel-Toellner et al. 2004). For instance, the activities of receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor and insulin receptors, which are found in MR/caveolae, can be modulated by changes in cellular cholesterol content. EGF stimulation-induced phosphatidylinositol 4,5-bisphosphate turnover is inhibited by depletion of cholesterol, but the effects of repletion with different oxysterols varied according to their structure. Turnover was not restored by 25-hydroxycholesterol, while 7-ketocholesterol and 5α, 6α-epoxycholesterol restored turnover (Morita et al. 2004). Likewise, desmosterol, another oxysterol, impaired raft-dependent signaling via the insulin receptor, while nonraft-dependent protein secretion was not affected (Morita et al. 2004). Therefore, these studies suggest that ROS may oxidize cholesterol to various oxysterols, which affect the stability of MRs and formation of MR-derived signaling platforms.

3.4 Redox Regulation of SMase Activity

The role of SMase in the formation of ceramide-enriched membrane platforms has been extensively studied. Recently, several studies have indicated that the generation of ROS may be involved in the activation of the enzyme in response to various stimuli (Charruyer et al. 2005; Dumitru and Gulbins 2006; Malaplate-Armand et al. 2006; Scheel-Toellner et al. 2004). Scheel-Toellner and colleagues demonstrated that ASMase activation, ceramide generation, and CD95 clustering play a crucial role in the spontaneous apoptosis of neutrophils; apoptosis was substantially delayed in Asm-deficient mice (Scheel-Toellner et al. 2004). Based on the observations that the intracellular redox balance changes in aging neutrophils, the authors investigated the possibility that ROS may be involved in ASMase activation. They demonstrated that pretreating neutrophils with the antioxidants

N-acetylcysteine (NAC) and desferrioxamine substantially inhibited the events downstream of ASMase, such as ceramide generation and CD95 clustering, indicating that ROS release is required for ASM activation (Scheel-Toellner et al. 2004). Similarly, pretreatment with the antioxidant, pyrrolidine dithiocarbamate (PDTC), abolished ASMase activation by ultraviolet (UV)-C light in U937 cells (Charruyer et al. 2005).

In other studies, neuronal stimulation with soluble oligomers of amyloid-beta peptide was found to result in the release of ROS and the subsequent activation of SMases (Malaplate-Armand et al. 2006). Treatment of these neurons with antioxidant molecules and a cPLA2-specific inhibitor or antisense oligonucleotide led to inhibition of SMase activation and subsequent apoptosis, suggesting that amyloid-beta oligomers induce neuronal death by activating both NSMase and ASMase via a redox-sensitive cPLA2-arachidonic acid pathway (Malaplate-Armand et al. 2006). In addition, Dumitru and colleagues have also demonstrated the involvement of ROS in TRAIL-induced activation of ASMase and apoptosis (Dumitru and Gulbins 2006). Stimulation with TRAIL/DR5 led to the activation of ASMase and the subsequent formation of ceramide-enriched membrane platforms, DR5 clustering, and consequent apoptosis. Pretreatment with antioxidants NAC and Tiron substantially inhibited TRAIL-induced ASMase activation, ceramide/DR5 clustering, and apoptosis, demonstrating that ROS play a crucial role in TRAIL-associated signaling pathway (Dumitru and Gulbins 2006). Finally, studies investigating the cellular effects of Cu^{2+} showed that Cu^{2+} also promotes the ROS-dependent activation of ASMase and leads to the death of hepatocytes (Dumitru and Gulbins 2006). It was shown that the accumulation of Cu^{2+}, as occurred in Wilson disease, activates ASMase in hepatocytes and triggers the release of ceramide in these cells. This process results in Cu^{2+}-induced hepatocyte death, which can be prevented by a deficiency in ASMase (Lang et al. 2007).

One of the mechanisms by which ASMase activity is regulated by ROS has been described by Qiu and coworkers (Qiu et al. 2003). It has been proposed that C-terminal cysteine (Cys629) plays a crucial role in the enzymatic activity of recombinant human ASMase (rhASM). The loss of the free sulfhydryl group on this amino acid results in activation of the enzyme, and this loss of free sulfhydryl group may be due to copper-promoted dimerization of rhASM by C-terminal cysteine, thiol-specific chemical modification of this cysteine to form a mixed disulfide bond or a sulfur-carbon linkage, deletion of this cysteine by carboxypeptidase or recombinant DNA technology, and site-specific mutation to change the cysteine to a serine residue. Because zinc is required for ASMase activity, the effect of C-terminal cysteine modification on the activation of ASMase may be associated with zinc coordination. It is known that zinc coordinates with a water molecule to produce an optimal structure for catalysis of ASMase. This zinc coordination model is essentially identical to the "cysteine switch" activation mechanism described previously for the matrix metalloproteinase family (Van Wart and Birkedal-Hansen 1990).

Another redox regulatory mechanism of various SMases has been described in a number of studies (Bezombes et al. 2002; Hernandez et al. 2000; Mansat-de Mas

et al. 1999), which is related to the effect of glutathione (GSH) (Liu and Hannun 1997; Martin et al. 2007). It has been demonstrated that the GSH/GSSG ratio is critical to such redox regulation of SMase activity. In this regard, the influence of glutathione, its analogs, and individual fragments on the activity of various SMase isoforms have been studied (Liu and Hannun 1997; Liu et al. 1998). In particular, the inhibitory effect of GSH on the neutral Mg^{2+}-dependent SMase was shown to be associated with the g-glutamyl-group of GSH. Since the effect of GSH is accompanied by decrease in diene conjugate and diene ketone levels, the ability of GSH to inhibit oxidative processes in the cell due to its antioxidative properties may be mainly responsible for its effect to inhibit SMase activity. In other words, ROS-mediated oxidation of NSMase or AMSase may enhance their activity (Tsyupko et al. 2001). In addition, the intracellular GSH concentration is thought to be involved in the regulation of SMase activity by increase in its expression (Yoshimura et al. 1999).

3.5 Feedforward Amplifying Mechanism

MRs signaling platforms usually contain different proteins including different signaling molecules and cross-linkers or enzymes (Simons and Ikonen 1997; Simons and Toomre 2000). The formation of MR platforms activates, facilitates, and/or amplifies signal transductions. As mentioned above, if MR clustering forms a ceramide-enriched membrane platform, the ceramide production or enrichment is mainly from SMase-catalyzed cleavage of SM cholines in individual MRs (Gulbins and Kolesnick 2003; Hoekstra et al. 2003). Redox regulation of SMase, MRs clustering, and ceramide-enriched platform formation suggest that MRs and ROS may constitute an amplification system of redox signals and ceramide signaling cell membranes, which insures the efficiency of signal transduction. The formation of such feedforward amplifying loop for MR redox and ceramide signaling may also be responsible for the tempospatial regulation of a complex signalosome that precisely and efficiently control cell function. If the activity of this regulatory loop is excessively enhanced, excessive production of both ROS and ceramide may result in the progress and development of different diseases or pathological processes.

It should be noted that ceramide-enriched membrane platforms might also be formed without the presence of classically defined MRs simply through a fusion of several ceramide molecules. These ceramide molecules can come from MRs or other membrane fractions. The clustering of receptor molecules within ceramide-enriched membrane platforms might well have several important functions such as the aggregation in close proximity of many receptor molecules (Gulbins and Grassme 2002), the facilitation of the transactivation of signaling molecules associating or interacting with a receptor, and the amplification of the specific signals generated by activated receptors. However, in some studies the formation of ceramide or ceramide platforms may not play roles in signaling, but contribute to

the scrambling of the cell membrane as shown at the erythrocyte surface. It is shown that the eryptosis may be linked to apoptotic pathways via ceramide, which may be causally cross talked to local oxidative stress. This may represent another type of MR redox signaling in erythrocytes (Lang et al. 2006, 2010).

4 Functional Relevance of the Cross Talk

It has been known that the biological responses to cellular or tissue ROS levels are very different and vary from physiological to pathological reactions. Therefore, the cross talk between ceramide and redox signaling may be implicated in various cell and organ functions, depending upon the amount of ROS and ceramide. With respect to ROS, when small amounts of ROS are produced, they may mediate physiological redox signaling, but when large amounts of ROS are produced, which refer to increased oxidative stress, cell/tissue damage may occur, resulting in cellular apoptosis and necrosis and ultimately causing various systemic or organ-based diseases. In regard to ceramide, largely increased ceramide production through such feedwarding mechanism may also contribute to the development of different diseases. This section will focus on the functional relevance of ceramide–redox signaling to the regulation of endothelial function and renal glomerular and tubular functions.

4.1 Regulation of Endothelial Function

Ceramide was demonstrated to increase endothelial $O_2^{\bullet -}$ in the endothelium of isolated small coronary arteries, which was blocked by different NADPH oxidase inhibitors such as *N*-vanillylnonanamide, apocynin, and diphenyleneiodonium. By analysis of the enzyme activity, ceramide was found to significantly stimulate the activity of NADPH oxidase in ECs, which was prevented by NADPH oxidase inhibitors, but not by inhibitors of NOS, xanthine oxidase, and mitochondrial electron transport chain enzymes. In addition, inhibition of NADPH oxidase by different NADPH oxidase inhibitors largely prevented ceramide-induced and $O_2^{\bullet -}$-mediated impairment of endothelium-dependent relaxation to agonists in small bovine coronary arteries (Zhang et al. 2001). These studies very clearly indicate that NADPH oxidases mediate dysfunction of ECs induced by ceramide. In additional studies, ceramide-induced activation of NADPH oxidase was associated with a rapid translocation of $p47^{phox}$ to the cytoplasmic membrane. As discussed above, $p47^{phox}$ translocation is a crucial step leading to activation of NADPH oxidase in phagocytes, these data suggest that $p47^{phox}$ translocation may initiate ceramide-induced activation of NADPH oxidase in coronary ECs. However, the signaling mechanisms that initiate $p47^{phox}$ translocation are unclear. It has been suggested that TNF-α activates PKC-ζ, which in turn phosphorylates $p47^{phox}$,

thereby inducing the translocation of this subunit to the membrane where it associates with gp91^{phox} to form the active enzyme complex (Frey et al. 2002). It is possible that ceramide employs this kinase to regulate NADPH oxidase in ECs. On the other hand, it might be also possible that ceramide-enriched membrane platforms recruit the subunits of NADPH oxidase to assemble and activate the oxidase at the cell membrane after treatment with TNF-α.

Although it is very attractive to speculate that MRs and ceramide-enriched membrane platforms are involved in the homeostasis of ECs and the response of these cells to cytokines, little is known about the role of these domains for the regulation of vascular endothelial functions. Recently, work in our laboratory has tested whether MR clustering and trafficking on the cell membrane of ECs are associated with ceramide production and action (Jin et al. 2007, 2008a; Zhang et al. 2006, 2007). It was found that ASMase and ceramide are of importance in CD95 ligand-induced formation of MR clusters on the EC membrane. We also demonstrated that ceramide-mediated clustering of MRs is involved in the regulation of $O_2^{\bullet -}$ production in coronary ECs via NADPH oxidase. This effect was associated with the recruitment and aggregation of the NADPH oxidase subunits gp91^{phox} and p47^{phox} in MRs. It was shown that silencing the ASMase gene by siRNA reduced CD95 ligand-induced gp91^{phox} aggregation in MR clusters and p47^{phox} translocation and completely inhibited CD95 ligand-induced $O_2^{\bullet -}$ production in these cells. In isolated small bovine coronary arteries, transfection of ASMase siRNA markedly attenuated CD95 ligand-induced inhibition of endothelium-dependent vasorelaxation (a response to bradykinin) by 60 % (Zhang et al. 2007). The results suggest that ASMase, the release of ceramide, and MR-derived ceramide-enriched membrane platforms are involved in the activation of NADPH oxidase in response to cytokines in coronary ECs, consequently leading to endothelial dysfunction (Jin et al. 2007; Zhang et al. 2006, 2007).

Recently, the MR redox signaling platform associated with NADPH oxidase has been demonstrated to be responsible for endothelial dysfunction induced by various stimuli such as death receptor activation, homocysteine, cytokines, or adipokines (Jin et al. 2008b; Xia et al. 2011; Zhang et al. 2006). As a commonly used functional study, endothelium-dependent vasodilation (EDVD) response in isolated perfused arteries was intensively tested. It was found that various stimulations which led to the formation of MR redox signaling platforms such as CD95 ligand, endostatin, homocysteine, and visfatin all led to impairment of EDVD. This impairment was homeostatically recovered by NADPH oxidase inhibition using apocynin, M-β-CD, filipin, or ASMase siRNA, suggesting that MR redox signaling platforms with NADPH oxidase participate in the impairment of endothelial function (Jin et al. 2007, 2008b; Zhang et al. 2006, 2007). This MR redox enhancement in endothelial injury and dysfunction may be intimately involved in the pathophysiology of diverse cardiovascular diseases such as atherosclerosis, hypertension, shock, and ischemia/reperfusion injuries.

In addition, the formation or enhancement of MR redox signaling platforms may contribute to macrophage reprogramming, foam cell formation, and cell

deformability. Induction of lipid oxidation through ROS was found to amplify foam cell formation through oxidized low-density lipoprotein (Ox-LDL) uptake and a subsequent clustering of ceramide-enriched lipid domains (Morita et al. 2004). In addition, Ox-LDL was found to affect cell-surface turnover of ceramide-backbone sphingolipids and apoE-mediated uptake, by low-density lipoprotein receptor-related protein (MRP) family members, leading, in turn, to cell-surface expansion of ceramide-enriched domains and activation of apoE-/MRP1-/CD1-mediated antigen presentation (van den Elzen et al. 2005). On the other hand, high-density lipoprotein (HDL)-mediated lipid efflux can disrupt MRs and prevent foam cell formation. It has been suggested that MR redox signaling or regulation plays an important role in the formation of foam cells and thus in the progression of atherosclerosis (Schmitz and Grandl 2007).

In addition to the role in alterations of macrophage behavior, MR redox signaling may also play an important role in cell deformability, thereby initiating or promoting atherogenesis (Levitan and Gooch 2007). Studies have demonstrated that disruption of MRs by oxidants such as Ox-LDL altered the cytoskeletal structure, including the extent of polymerization, stabilization, cross-linking, and membrane association (Byfield et al. 2006). These molecular alterations may increase the force generated by the cytoskeleton, resulting in a stiffening of the cytoskeleton and hence stiffening of the cell and plasma membrane. Increased force in the cytoskeleton and its downstream increased stiffness may also elevate membrane tension and thereby influence the activity of various mechanosensitive ion channels. Direct evidence suggests that Ox-LDL can disrupt MRs, resulting in a series of pathological changes in the biomechanical properties of vascular ECs and ultimately induce endothelial dysfunction and atherogenesis (Blair et al. 1999; Byfield et al. 2006).

As summarized in Fig. 2, ASMase-mediated ceramide signaling and ROS-based redox signaling interact through a MR signaling platforms. As a molecular cross talk, this interaction of ceramide and ROS may play an important role in the regulation of endothelial function. When various death factors or other stimuli act on ECs, ASMase located in situ or translocated from lysosomes or lysosome-like vesicles is 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 $O_2^{\bullet -}$. $O_2^{\bullet -}$ reacts with NO to decrease NO bioavailability and to produce peroxynitrite ($ONOO^-$). Increased $ONOO^-$ uncouples NOS to produce more $O_2^{\bullet -}$ but less NO. $O_2^{\bullet -}$ or ROS may feedforward enhance MRs clustering by enhancement of ASMase and alteration of MR clustering process, forming positive amplifications. All these together constitute a redox signaling network resulting in endothelial dysfunction and impairment of endothelium-dependent vasodilation, which may be the basis for difference cardiovascular diseases such as atherosclerosis, coronary artery disease, hypertension, and peripheral arterial disease.

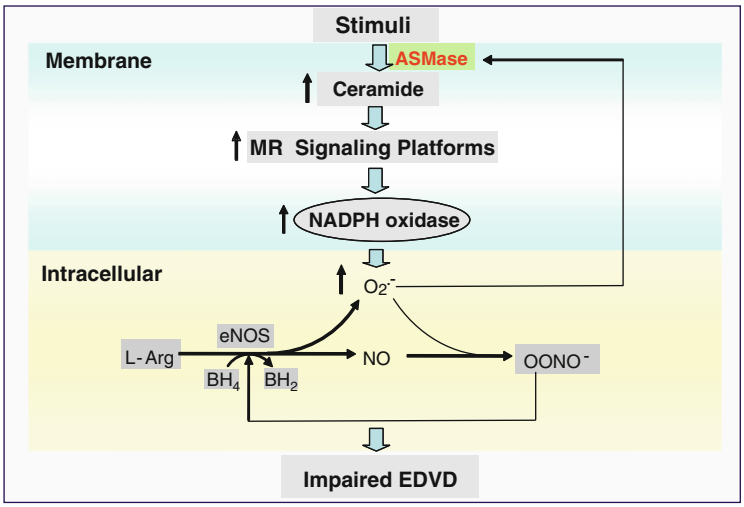

Fig. 2 Ceramide-enriched redox signaling platforms associated with NADPH oxidase in endothelial dysfunction. Upon ASMase stimulation, ceramide is released to promote MR clustering and form ceramide-enriched MR platforms, with aggregation and assembling of NADPH oxidase subunits and other proteins such as Rac GTPase. Then, NADPH oxidase is activated to produce $O_2^{\bullet -}$, which reacts with NO to produce $ONOO^-$ resulting in endothelial dysfunction in coronary arteries. Further, NADPH oxidase-derived $O_2^{\bullet -}$ regulates ASMase activation and ceramide production in a feedforward manner

4.2 Regulation of Renal Function

Recent studies have indicated that ceramide may be implicated in the regulation of kidney function and seems to be involved in renal glomerular and tubular pathology (Kaushal et al. 1998; Ueda et al. 2000; Yi et al. 2004; Yin et al. 1997). More recently, our group demonstrated that ceramide importantly contributes to the development of chronic glomerular injury associated with hyperhomocysteinemia, and thereby ceramide may serve as an important mechanism of end-stage renal disease (Yi et al. 2004, 2007, 2009b). Several studies that employed TLC and HPLC analysis reported the detection of ceramide in the kidney, leading to the hypothesis that ceramide might be involved already in the regulation of normal renal function (Kaushal et al. 1998; Ueda et al. 2000; Yi et al. 2004; Yin et al. 1997). To determine whether ceramide also participates in the development of chronic renal failure, we employed a model of hyperhomocysteinemia-induced renal injury. These studies revealed that hyperhomocysteinemia significantly increased ceramide levels in the renal cortex from rats. Likewise, treatment of cultured mesangial cells with L-homocysteine resulted in a concentration-dependent increase in ceramide. Evidence for a de novo synthesis of ceramide by L-homocysteine was provided in studies that employed fumonisin B1 and myriocin, inhibitors of the de novo synthesis pathway of ceramide. These inhibitors prevented

L-homocysteine-induced ceramide formation in mesangial cells as well as in vivo in the kidney and attenuated glomerular injury and proteinuria (Yi et al. 2004, 2009b). These data provide direct evidence that the ceramide pathway is critically involved in L-homocysteine-induced glomerular injury and glomerular sclerosis.

Further mechanistic studies have demonstrated that L-homocysteine stimulated ceramide production in different glomerular cells such as glomerular capillary ECs, podocytes, and mesangial cells and that ceramides appear to be an important regulator of the function of glomerular filtration membrane, which is consistent with previous results that ceramide may be involved in the regulation of normal renal function (Kaushal et al. 1998; Ueda et al. 2000). It was also found that blockade of ceramide production in hyperhomocysteinimic rats substantially inhibited the enhancement of NADPH oxidase activity and production of $O_2^{\bullet -}$ in the kidney (Yi et al. 2004). Although translocation of $p47^{phox}$, seen in ECs, was not shown to occur in L-homocysteine- or ceramide-induced activation of NADPH oxidase in rat mesangial cells (Yi et al. 2004), in podocytes and glomerular capillary ECs, homocysteine was shown to induce the formation of MR redox signaling platforms associated with NADPH oxidase (Yi et al. 2009a; Zhang et al. 2010). Perhaps, the transformation of small MRs to ceramide-enriched membrane platforms results in a clustering of NADPH oxidase molecules, producing redox signaling or injury in these glomerular cells, resulting in local oxidative stress and ultimate glomerular injury. This oxidative stress mediated by NADPH oxidase has been indicated to play an important role in progressive glomerular injuries or glomerulosclerosis associated with hyperhomocysteinemia and other diseases such as diabetes and hypertension (Eid et al. 2009; Fujimoto et al. 2008; Yi et al. 2004). It is now known that the formation of MR redox platforms and ROS production is a major mechanism responsible for hyperhomocysteinemia-induced enhancement of glomerular permeability, thereby producing glomerular injuries and consequent sclerosis, which is associated with the regulation of microtubule stability. It seems that the early injurious effects of hyperhomocysteinemia and other pathogenic factors acting on NADPH oxidase are associated with the formation of redox signaling platforms via MR clustering and consequent increases in glomerular permeability due to disruption of microtubule networks in the glomerular filtration membrane (Yi et al. 2007; Zhang et al. 2010).

In addition to their role in the regulation of renal glomerular function, MRs-associated NADPH oxidase may maintain an inactive state of this enzyme in human renal proximal tubule (RPT) cells. Disruption of such inactive MRs may result in their activation (Han et al. 2008). Different cells use MRs to conduct redox signaling in different ways. As Li et al. have reported, NADPH oxidase-dependent ROS production is differentially regulated in MRs and non-MR compartments of RPT epithelial cells (Yi et al. 2009b). This differential regulation or MR-associated inactive NADPH oxidase is mainly attributed to the action of the neurotransmitter dopamine. Dopamine is an essential neurotransmitter involved, mainly via its peripheral receptors, in the control of blood pressure, sodium balance, and various renal and adrenal functions (Jose et al. 2002). As G-protein-coupled receptors,

dopamine receptors are associated with both caveolar and noncaveolar MRs (Allen et al. 2007; Lingwood et al. 2009; Simons and Ikonen 1997; Yu et al. 2004). It has been shown that D_1-like receptors can exert an inhibitory action on ROS production in VSM and RPT cells (White and Sidhu 1998; Yang et al. 2006b; Yasunari et al. 2000). However, the molecular mechanisms involved still remain unknown. By sucrose density gradient ultracentrifugation and analysis of NADPH oxidase isoforms and subunits in MRs, it was found that the majority of membrane proteins was in non-MR fractions; only a small portion of proteins were in MR fractions. The D_1-like receptor agonist, fenoldopam, decreases NOX2 and Rac1 proteins in MRs, albeit to a greater extent in hypertensive than normotensive rats. Fenoldopam decreased the amount of NOX2 that co-immunoprecipitated with $p67^{phox}$ in cells from normotensive rats. These observations suggest that fenoldopam causes a redistribution of NOX2, NOX4, and Rac1 from MRs and to non-MR fractions. Further studies have shown that disruption of MRs results in the reactivation of NADPH oxidase that was destroyed by antioxidants and the silencing of NOX2 or NOX4. Perhaps this explains why in human RPT cells, MRs maintain NADPH oxidase in an inactive state (Han et al. 2008).

5 Concluding Remarks

In summary, there is no doubt that redox signaling through NADPH oxidase and other ROS producing or scavenging systems is correlated with the unique membrane structures known as MRs, where MRs serve as platforms to aggregate the membrane spanning or cytosolic components of the enzymes subunits or cofactors. In particular, MRs clustering may be a major mechanism for the assembly of NADPH oxidase subunits and cofactors into an active enzyme complex. Such MR redox platforms produce $O_2^{\bullet -}$ and thereby conduct redox signaling with compartmentalization and amplifications in response to different receptor bindings or other stimuli. It is well known that the formation of this MR redox signaling platform associated with NADPH oxidase is associated with activation of ASMase and production of ceramide. On the other hand, ROS production may enhance ASMase activity or alter MR components to promote MRs clustering. It is clear that ROS-based redox signaling and ceramide producing system and consequent signaling interact under control condition or upon different stimuli, constituting a temperospatial cross talk between two signaling pathways. Such cross talk may be significantly implicated in the regulation of organ functions. As an example, the regulation of endothelial function and renal glomerular or tubular functions is closely associated with ceramide–redox cross talk, and their interplay, if excessively enhanced, may result in endothelial dysfunction and renal glomerular or tubular dysfunction, leading to various cardiovascular and renal diseases. In perspective, it is imperative to develop new in vivo research strategies that are able to address the contribution of ceramide–redox interaction to organ functions and related regulatory mechanisms. More studies may also be needed to translate

experimental results related to MR redox signaling platforms and ceramide–redox cross talk to clinical use.

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