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Invited Review

Physiological and pathophysiological aspects of ceramide

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Gulbins, Erich, and Pin Lan Li. Physiological and pathophysiological aspects of ceramide. *Am J Physiol Regul Integr Comp Physiol* 290: R11–R26, 2006; doi:10.1152/ajpregu.00416.2005.—Activation of cells by receptor- and nonreceptor-mediated stimuli not only requires a change in the activity of signaling proteins but also requires a reorganization of the topology of the signalosom in the cell. The cell membrane contains distinct domains, rafts that serve the spatial organization of signaling molecules in the cell. Many receptors or stress stimuli transform rafts by the generation of ceramide. These stimuli activate the acid sphingomyelinase and induce a translocation of this enzyme onto the extracellular leaflet of the cell membrane. Surface acid sphingomyelinase generates ceramide that serves to fuse small rafts and to form large ceramide-enriched membrane platforms. These platforms cluster receptor molecules, recruit intracellular signaling molecules to aggregated receptors, and seem to exclude inhibitory signaling factors. Thus ceramide-enriched membrane platforms do not seem to be part of a specific signaling pathway but may facilitate and amplify the specific signaling elicited by the cognate stimulus. This general function may enable these membrane domains to be critically involved in the induction of apoptosis by death receptors and stress stimuli, bacterial and viral infections of mammalian cells, and the regulation of cardiovascular functions.

signal transduction; acid sphingomyelinase; rafts; membrane platforms

RECEPTOR AGGREGATION/CLUSTERING

In recent years, the receptor-mediated activation of cells and signal transduction in cells has evolved to be determined by at least two principles: *1*) receptors regulate the activity of enzymes, and *2*) receptor molecules and intracellular signaling molecules are reorganized on stimulation. These two principles, i.e., activation/inactivation and a spatial reorganization of the cellular signalosom, determine the response of the cell to a stimulus. Many receptor molecules aggregate or cluster on stimulation; i.e., they are concentrated in a rather small area of the cell membrane, resulting in a very high density of the receptor molecules. Aggregation/clustering of cell surface receptors on binding their cognate ligands has been observed for many receptors, including the antigenic T cell receptor (TCR)-CD3 complex (19), the antigenic B cell receptor (62), the EGF receptor (3), CD40 (57), CD95 (11, 56), DR5 (C. Dumitru and E. Gulbins, unpublished observations), TNF (122), FcγRII (1), L-selectin (87), and integrins or leukoocyte function-associated antigen (LFA)-1 (136), to name a few. Clustering of receptor molecules correlates with a reassembly of intracellular signaling molecules. For instance, activation of the TCR-CD3 complex or the CD95 receptor results in an intracellular reorganization of the topology of CD4, Lck, Zap70, Ras, Rac-1, F-actin, and PKCθ (for comprehensive reviews, see Refs. 15, 96), which are key molecules in the transmission of signals via the TCR-CD3 complex, or of FADD, caspase 8, and caspase 3, central mediators of CD95-induced apoptosis (for a recent review, see Ref. 112). Although it is widely accepted that these receptors cluster, the mechanisms mediating clustering are largely unknown.

It is therefore of great interest to define mechanisms that determine the organization of receptors and intracellular signaling molecules on cellular stimulation.

BIOPHYSICAL PRINCIPLES OF RAFTS AND CERAMIDE-ENRICHED MEMBRANE DOMAINS

In 1972, Singer and Nicolson (149) suggested the classical fluid mosaic model of the cell membrane. However, this model was modified in recent years to integrate biophysical findings on the spontaneous organization of lipids into distinct microdomains of the cell membrane (4, 23, 148).

Somewhat simplified, biological membranes primarily consist of sphingolipids, cholesterol, and other (glycero)phospholipids. The most prevalent component of the sphingolipid fraction in the cell membrane is sphingomyelin, which is composed of a highly hydrophobic ceramide moiety and a hydrophilic phosphorylcholine headgroup. The amide ester of the sphingoid base D-erythro-sphingosine and a fatty acid of C_{16}–C_{26} chain length form the ceramide group in sphingomyelin.

Tight interactions between the cholesterol sterol ring system and the ceramide moiety of sphingomyelin, which are mediated by hydrogen bonds and hydrophobic van der Waal interactions as well as hydrophilic interactions between sphingolipid headgroups, promote the lateral association of sphingolipids and cholesterol and thus the separation from other phospholipids into distinct microdomains (23, 93, 148) (Fig. 1). These microdomains have been termed rafts because they seem to float in an "ocean" of other glycerophospholipids of

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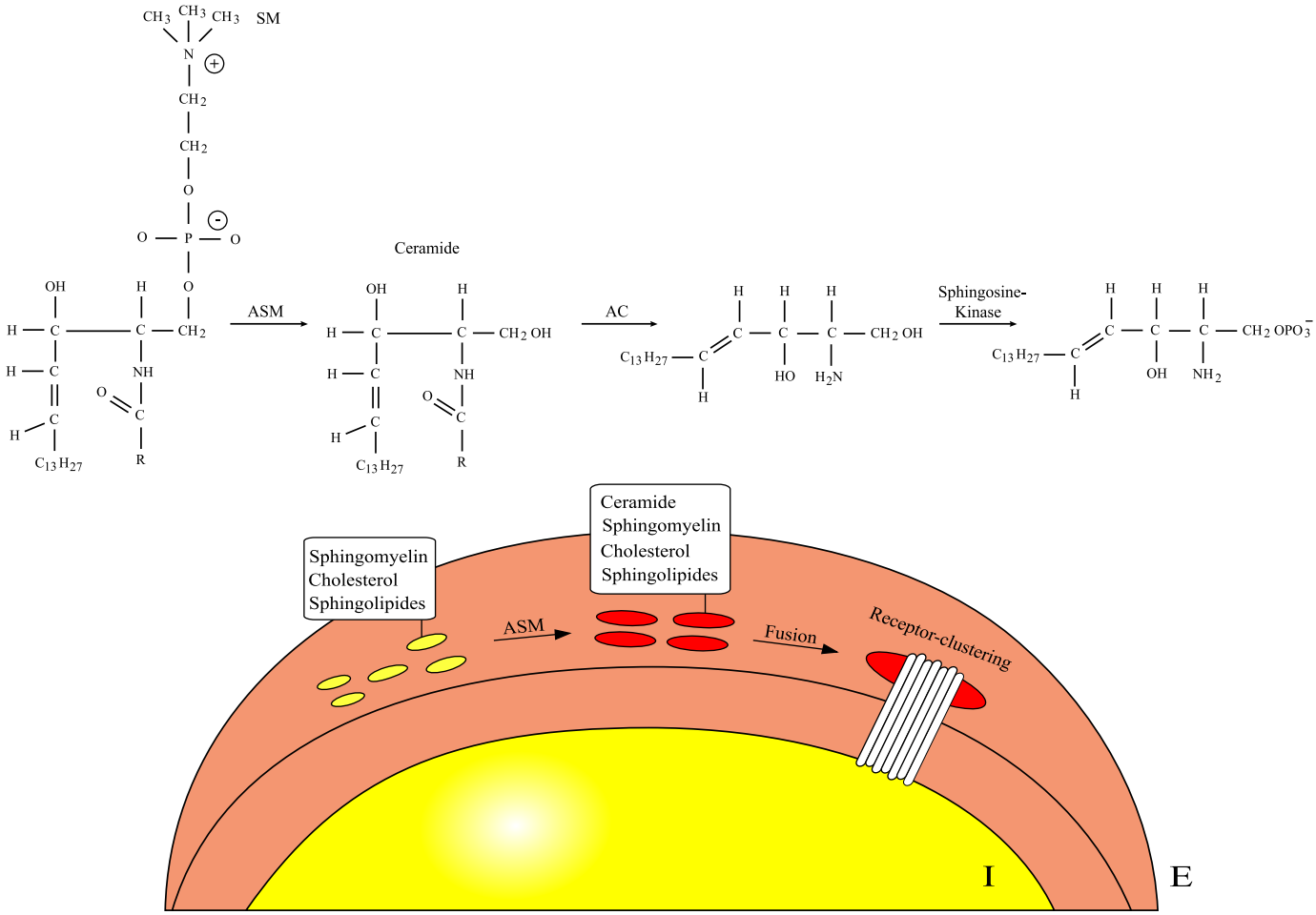

Fig. 1. Schematic scheme of the acid sphingomyelinase-ceramide pathway and the formation of ceramide-enriched membrane domains by transformation of rafts. Activation of the acid sphingomyelinase results in hydrolysis of sphingomyelin, the release of ceramide, and the transformation of rafts to small ceramide-enriched microdomains that spontaneously fuse to a large ceramide-enriched membrane platform. SM, sphingomyelin; ASM, acid sphingomyelinase; AC, acid ceramidase; I, intracellular; E, extracellular.

the cell membrane (148). Cholesterol stabilizes rafts by filling the voids between the large and bulky glycerosphingolipids, and, in accordance, pharmacological extraction of cholesterol destroys membrane rafts. It is assumed that the tight interactions between sphingolipids and cholesterol and the high local concentration of these lipids result in a transition of these microdomains into a liquid-ordered or even gel-like phase, whereas the other domains of the cell membrane exist in a more fluid liquid-disordered phase (23, 93). Because sphingomyelin is almost exclusively located in the anticytosolic leaflet of biological membranes (40), rafts seem to be present only in the outer leaflet of the cell membrane and the anticytosolic leaflet of intracellular membranes. It is unknown how rafts are translated into changes of the inner leaflet of the cell membrane, but it is very likely that the organization of the outer membrane leaflet into rafts is translated into a similar organization of the inner leaflet of the cell membrane.

In the past years, we and others elaborated a novel mechanism regarding how very small membrane rafts in resting cells are transformed into large membrane domains that mediate aggregation/clustering of receptor molecules and the reorganization of intracellular signaling molecules to transmit a signal into the cell. These data demonstrated that many receptors, including CD95 (33, 35, 56, 60, 65, 91, 129) , CD28 (21), TNF (50, 144, 176), CD40 (57), DR5 (C. Dumitru and E. Gulbins, unpublished observations), CD5 (146), LFA-1 (136), FcγRII (1), CD20 (12), the interleukin-1 receptor (116), the platelet-activating factor (PAF) receptor (51); as well as infection with *Pseudomonas aeruginosa* (58), *S. aureus* (42), *N. gonorrhoeae* (55), Sindbis virus (83), Rhinovirus (61); or treatment with gamma-irradiation (128, 138), UV light (30, 137, 182), doxorubicin (117), cisplatin (97), resveratrol (37), thalidomide (160), and developmental processes (117, 139) trigger the release of ceramide (Fig. 1 and Table 1). Most of these stimuli activate the acid sphingomyelinase (Fig. 1 and Table 1), which belongs to a class of enzymes that hydrolyze sphingomyelin and thus generate ceramide. Sphingomyelinases are characterized by their pH optimum, and an acid, neutral, and alkaline sphingomyelinase were described. Here, we focus on the cellular function of the acid sphingomyelinase. Activation of the acid sphingomyelinase by receptor molecules correlates with a translocation of the enzyme from intracellular stores onto the extracellular leaflet of the cell membrane (Fig. 1). Although not proven, we assume that the acid sphingomyelinase localizes within secretory vesicles, which are mobilized on stimulation to fuse with the cell membrane. This notion is supported by

Table 1. *Stimuli triggering the acid sphingomyelinase and ceramide*

Stimuli activating the acid sphingomyelinase and/or release ceramide and/or require acid sphingomyelinase for their biological effects
Receptors: CD95, CD28, TNF, CD40, DR5, CD5, LFA-1, FcγRII, CD20, interleukin-1 receptor, PAF receptor
Pathogens: *P. aeruginosa, S. aureus, N. gonorrhoeae,* Sindbis virus, rhinovirus
Stress stimuli: γ-irradiation, UV light, doxorubicin, cisplatin, resveratrol
Developmental processes: oocyte apoptosis, neutrophil apoptosis
Stimuli formally shown to induce ceramide-enriched membrane platforms
Receptors: CD95, CD40, DR5, CD20, FcγRII
Pathogens: *P. aeruginosa,* rhinoviruses
Stress stimuli: UV light, cisplatin
Developmental death of neutrophils

PAF, platelet-activating factor.

electron and fluorescence microscopy studies that suggest an intravesicular staining of the acid sphingomyelinase in resting cells and the fusion of vesicles with the membrane on stimulation via CD95 (56, 60). This fusion may result in exposure of the acid sphingomyelinase onto the outer leaflet of the cell membrane and brings the enzyme in close vicinity to its substrate sphingomyelin. Fluorescence microscopy suggests that surface acid sphingomyelinase localizes to membrane rafts, since the acid sphingomyelinase colocalizes with the β-subunit of cholera toxin (54, 56), which is often employed as a marker for rafts. Externalization and activation of the acid sphingomyelinase occur very rapidly after stimulation, and recent data on platelets demonstrate an activation of the enzyme within 5 s after stimulation. Although the pH optimum for the acid sphingomyelinase is in the acidic range, neutral pH values only increase the K_m value of the enzyme, whereas V_{max} is not altered (27). Furthermore, environmental and membrane factors such as LDL modify the substrate affinity of the acid sphingomyelinase (140, 141), which permits full activity of the enzyme on the cell surface. However, further molecular details of the activation of the enzyme on cellular activation are poorly characterized. Recently, Qui et al. (135) suggested a redox regulation of the acid sphingomyelinase. In vitro oxidation of the cysteine 629 regulated the activity of the acid sphingomyelinase, and it is attractive to speculate that at least some of the above-named stress stimuli stimulate the acid sphingomyelinase via redox processes.

The generation of ceramide within rafts dramatically alters the biophysical properties of these membrane domains, since ceramide molecules have the tendency to spontaneously self associate to small ceramide-enriched membrane microdomains (93). Furthermore, these microdomains spontaneously fuse to large ceramide-enriched macrodomains or platforms (79, 93, 124). The formation of ceramide-enriched membrane platforms was demonstrated for many of the above-named stimuli in vivo (1, 12, 30, 37, 42, 56–58, 60, 72, 97, 139) (Figs. 2 and 3) but was also shown upon local treatment of a phosphatidylcholine/sphingomyelin-composed unilamellar vesicle with sphingomyelinase that was immobilized to a bead attaching the vesicle (79, 124). This suggests that formation of ceramide-enriched membrane domains could, at least theoretically, also occur without the presence of rafts.

FUNCTION OF CERAMIDE-ENRICHED MEMBRANE DOMAINS

Ceramide-enriched membrane platforms serve the reorganization and clustering of receptor molecules, which has been formally proven for CD95 (56, 60), CD40 (57), FcγRII (1), and DR5 (Dumitru and Gulbins, unpublished observations), and CD20 (12). The fusion of very small rafts to larger platforms by the generation of ceramide might simply cluster receptor molecules that are constitutively present in membrane rafts. However, many receptor molecules are primarily outside of rafts but will be trapped in ceramide-enriched membrane platforms on activation. This might be mediated by a conformational change of the receptor molecule on binding of the ligand, resulting in a preferential interaction of the receptor with ceramide or ceramide-enriched membrane domains. However, it has to be pointed out that the molecular mechanisms of receptor clustering in general remain to be defined. Receptor clustering leads to a high receptor density that seems to be required for effective transmission of the signal into cells. Thus ceramide-enriched membrane platforms are not part of the specific signaling cascade of the activated receptor molecule; instead, these domains greatly facilitate and amplify signaling via a specific receptor. This notion is consistent with the

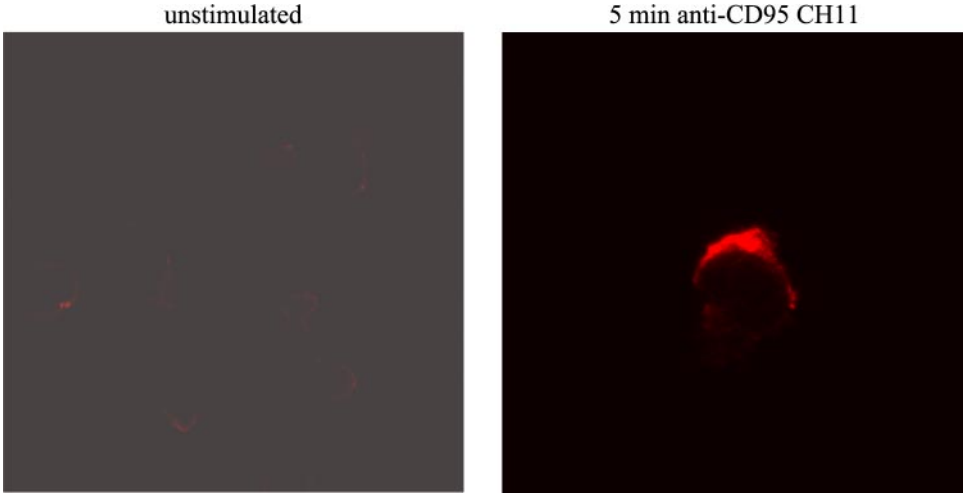

Fig. 2. CD95 induces the formation of ceramide-enriched membrane platforms. Fluorescence microscopy of Jurkat T lymphocytes reveals the formation of large ceramide-enriched membrane platforms after 5 min of stimulation via CD95. Cells were fixed and stained with Cy3-coupled anti-ceramide antibodies 15B4.

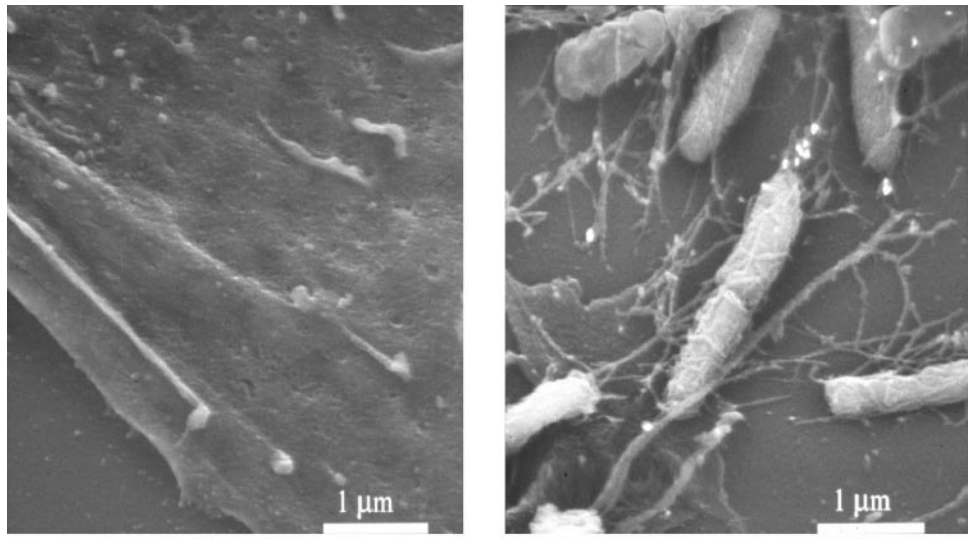

Fig. 3. *Pseudomonas aeruginosa* induces surface translocation and clustering of the acid sphingomyelinase at the bacterial infection site. Epithelial cells were infected with *P. aeruginosa*, fixed, stained with gold-coupled anti-acid sphingomyelinase antibodies, and analyzed by scanning electron microscopy. The gold particles appear as small white dots and indicate surface acid sphingomyelinase. The results demonstrate an accumulation of acid sphingomyelinase at the site of the infections, whereas uninfected cells do not display acid sphingomyelinase on the cell surface.

finding that ceramide-enriched membrane platforms amplify the signaling via CD95 ~100-fold (54).

In addition to an amplification of the signal strength, trapping of an activated receptor in rafts may also stabilize the interaction with the receptor ligand. This mechanism might be in particular operative in the directed interaction of two cells with each other, for instance in the immunologic synapse. Furthermore, the clustering of a receptor in a ceramide-enriched membrane domain alters the biophysical environment of the receptor molecule, which ultimately may lead to a change of receptor conformation. This might also stabilize the interaction of a receptor with its ligand. However, at present, it is unknown whether ceramide is able to alter the conformation of proteins in vivo.

As discussed above, it is very likely that the formation of ceramide-enriched membrane domains and the reorganization of rafts in the outer leaflet of the cell membrane are translated in alterations of the inner leaflet of the cell membrane. Therefore, ceramide-enriched membrane domains may serve to recruit and/or exclude intracellular signaling proteins. The recruitment of signaling molecules to ceramide-enriched membrane domains results in a close vicinity of the activated receptor and its intracellular signaling molecules, which permits the receptor to transmit the signal and greatly facilitates the transactivation of receptor-associating molecules. This model is supported by findings on CD95 (54). It was demonstrated that the modification of rafts and the generation of ceramide-enriched membrane platforms mediates the recruitment of FADD, caspase 8, and even caspase 3 to aggregated CD95 (6, 54). Furthermore, it was shown that ceramide recruits caveolin 1, which results in a block of phosphatidylinositol-3-kinase and a sensitization to apoptosis (186). Additional signaling molecules that might be recruited to the inner leaflet of ceramide-enriched membrane domains are Src-like tyrosine kinases and small G proteins.

Ceramide may also flip to the inner side of the cell membrane, although biophysical studies suggest that a spontaneous flip of ceramide through the membrane would occur slowly (34). However, intracellular ceramide might interact with phospholipase A_2 (80), kinase suppressor of Ras (identical to ceramide-activated protein kinase) (183), ceramide-activated protein serine-threonine phosphatases (38), protein kinase C isoforms (119), and/or c-Raf-1 (161). Ceramide has been described to activate these proteins, resulting in regulation of cellular transcription, proliferation, and survival. Furthermore, the TNF receptor is rapidly internalized on activation, and most of the ceramide is released by the acid sphingomyelinase within the endosomes (142). Endosomal ceramide binds to and activates cathepsin D (74, 142), which is released from endosomes into the cytoplasm by still unknown mechanisms to trigger apoptosis.

A novel target of ceramide and ceramide-enriched membrane platforms, respectively, has been recently identified, i.e., ion channels. In particular, it was demonstrated that ceramide inhibits the potassium channel Kv1.3 and calcium release-activated calcium channels in lymphocytes (32, 67, 100, 151). Both channels are central for activation, differentiation, proliferation, and regulation of apoptosis. At present, it is unknown how ceramide blocks these channels. Ceramide-enriched membrane platforms may organize signaling molecules, for instance, tyrosine kinases that regulate the activity of at least Kv1.3 (78, 151). However, it is also possible that the lipid environment regulates the function of these channels. The alteration of sphingomyelin and cholesterol-rich rafts by the generation of ceramide may change the conformation of Kv1.3 and calcium release-activated calcium, resulting in an inhibition of the channels. However, more experimental evidence is required to characterize the regulation of ion channels by the lipid environment, in particular ceramide.

The concept of receptor clustering and reorganization of the cellular signalosom may also explain the central function of ceramide-enriched membrane domains for the induction of cell death by gamma-irradiation, UV-A and UV-C light, and some chemotherapeutics (30, 37, 97, 117, 128, 138, 182). Although the initial events that mediate cellular activation by these stimuli are unknown, it has been shown that all of those stimuli activate the acid sphingomyelinase, induce the release of ceramide, and trigger the formation of ceramide-enriched membrane platforms. Genetic or pharmacological inhibition of the formation of ceramide-enriched membrane platforms pre-

vented biological effects of these stress stimuli, suggesting that ceramide-enriched membrane platforms cluster pro-apoptotic receptors, recruit pro-apoptotic, and exclude anti-apoptotic signaling molecules to mediate or at least facilitate cell death.

In summary, ceramide-enriched membrane platforms might be a central motif to reorganize the topology of a given signalosom, enabling stress stimuli and receptors to transmit a signal into the cell.

CERAMIDE-ENRICHED MEMBRANE PLATFORMS IN INFECTIOUS BIOLOGY

Recent studies have indicated an important function of membrane rafts and ceramide in infectious biology. Many pathogens target and employ rafts for infection of cells, including *Escherichia coli*, *Mycobacterium tuberculosis*, *Campylobacter jejuni*, *Vibrio cholerae*, *Clostridium difficile*, *Clostridium tetani*, *Salmonella typhi* and *typhimurium, Shigella flexneri*, influenza virus, HIV, measles virus, respiratory syncytial virus, *Ebolavirus, Papillomaviridae*, EBV, *Echovirus, Sindbisvirus*, *Plasmodium falciparum, Trypanosoma, Leishmania, Prions*, and *Toxoplasma gondii* (for reviews, see Refs. 115, 147). At present, the molecular mechanisms of raft-mediated infection of mammalian cells with pathogens are unknown. In general, rafts seem to be involved in the interaction of pathogens with their receptors, receptor clustering, interaction of the receptor with intracellular signaling molecules and/or the cytoskeleton, as well as internalization of pathogens. Whether these events require a modification of rafts is unknown, although studies have suggested that the formation of ceramide is required for cellular infection with at least some bacteria and viruses. These studies showed that ceramide-enriched membrane platforms mediated the infection of mammalian cells with *P. aeruginosa* (58)*, Staphylococcus aureus* (*S. aureus*) (42)*, Neisseriae gonorrhoeae* (*N. gonorrhoeae*) (55, 72), Rhinoviruses (61), and Sindbis virus (83).

Bacterial infections. Initial studies with *N. gonorrhoeae* described an activation of the acid sphingomyelinase and a release of ceramide on infection of human epithelial cells and macrophages (55, 72). Genetic deficiency or pharmacological inhibition of the acid sphingomyelinase prevented the infection of human epithelial cells with *N. gonorrhoeae* (55, 72). Ceramide-enriched membrane domains seem to cluster receptors of the CEACAM-family that function as receptors for Opa proteins of *N. gonorrhoeae*, suggesting that ceramide-enriched membrane platforms are required for the reorganization of *N. gonorrhoeae* receptors and intracellular signaling molecules that mediate internalization of the pathogens.

Most studies on the function of the acid sphingomyelinase and ceramide-enriched membrane domains were performed with *P. aeruginosa* (58). These studies demonstrated that *P. aeruginosa* induces an activation and surface translocation of the acid sphingomyelinase within a few minutes after infection of human and murine cells (58) (Fig. 3). Surface acid sphingomyelinase triggers the release of ceramide, which forms large ceramide-enriched membrane platforms on infection (Figs. 1 and 3). The pathogens attach to the cell within ceramide-enriched membrane platforms. Genetic studies employing acid sphingomyelinase-deficient epithelial cells and fibroblasts, in vivo studies on acid sphingomyelinase-deficient mice, and experiments that disrupted rafts by extraction of cholesterol revealed that rafts and ceramide-enriched membrane platforms are required for at least three central aspects of *P. aeruginosa* infection of mammalian cells (58, 59): infection with *P. aeruginosa* results in the release of cytokines from, the induction of apoptosis in, and the invasion of the bacteria into infected cells. Inhibition of the formation of ceramide-enriched membrane platforms prevented the induction of apoptosis of infected cells and internalization of the bacteria, whereas the release of cytokines was increased and uncontrolled from cells lacking the acid sphingomyelinase (58). The combination of apoptosis deficiency, internalization defect, and uncontrolled release of cytokines resulted in a very high sensitivity of acid sphingomyelinase-deficient mice to pulmonary infections with *P. aeruginosa*. The mice were unable to clear pulmonary *P. aeruginosa* infections and succumbed to a septic shock. Fluorescence microscopy studies revealed that ceramide-enriched membrane platforms serve to cluster at least two receptors that have been implied in internalization of *P. aeruginosa* and induction of apoptosis in infected cells. Those molecules are the CFTR and the CD95 receptor, which cluster within minutes after *P. aeruginosa* infection in ceramide-enriched membrane platforms. CFTR seems to be both constitutively present in and additionally recruited to rafts that are fused by ceramide to large platforms after infection (58, 94). The aggregation of CD95 in ceramide-enriched membrane platforms might be involved in the induction of apoptosis by *P. aeruginosa* (59). The function of CFTR clustering in ceramide-enriched membrane domains is less clear, but it might be involved in internalization of the bacteria and/or upregulation of CD95 on the cell surface of infected cells (28, 134).

A different pathway of ceramide formation seems to be employed by *Salmonella typhimurium*. These bacteria seem to generate ceramide in infected cells independent of the acid sphingomyelinase and appear to employ the acid ceramidase and/or ceramide synthase for the generation of ceramide (H. Grassmé, A. Riehle, and E. Gulbins, unpublished observations). A similar pathway was recently demonstrated in thrombocytes that are able to generate ceramide from sphingomyelin via the acid sphingomyelinase and from sphingosine-1-phosphate and sphingosine via the acid ceramidase (162).

An interesting novel function of ceramide and other sphingolipids such as sphingosine and sphingosine-1-phosphate was recently suggested by Anes et al. (5). Uptake of bacteria into mammalian cells results in the formation of phagosomes that mature and fuse with lysosomes to a phagolysosome, which serves to degrade the bacteria. The fusion requires the coordinated assembly of actin filaments on phagosomes, an event which is regulated by different lipids, including ceramide. Preliminary data from our laboratory support the notion of ceramide formation within phagosomes, suggesting that ceramide and other sphingolipids are actively involved in the maturation of phagosomes and/or the fusion of these vesicles with lysosomes.

Viral infections. The acid sphingomyelinase and ceramide-enriched membrane platforms are not only important for the infection of mammalian cells with bacteria but also for the infection with viruses. This is indicated by recent findings that demonstrate an activation of the acid sphingomyelinase, a release of ceramide, and the formation of large ceramide-enriched membrane platforms within 15 min after infection of human epithelial cells with rhinoviruses (61). Pharmacological

inhibition or genetic deficiency of the acid sphingomyelinase prevented infection with rhinoviruses, suggesting a central function of the acid sphingomyelinase and ceramide-enriched membrane platforms for the uptake of rhinoviruses. A similar function of the acid sphingomyelinase was demonstrated for the infection of neurons with *Sindbis* virus. *Sindbis* viruses activate the acid sphingomyelinase and trigger the release of ceramide, which is required for induction of apoptosis in infected cells (83).

Parasite infections. Finally, parasites also seem to employ ceramide-enriched membrane platforms for infection. In contrast to the activation of the mammalian acid sphingomyelinase by bacteria, *Plasmodium falciparum* seems to express its own sphingomyelinase for the generation of ceramide in infected cells (69). This sphingomyelinase functions as a sphingomyelin/lysocholinephospholipid-phospholipase C (69). The *P. falciparum* sphingomyelinase was shown to be required for the infection of erythrocytes with *P. falciparum*, and inhibition of the enzyme prevented intraerythrocytic proliferation (69). However, the exact roles of ceramide and possibly ceramide-enriched membrane platforms for the infection of erythrocytes with *P. falciparum* remain to be defined.

Ceramide in organ failure and sepsis. Several recent studies suggested a central role of ceramide in the response of human organs to infections or shock (39, 51). These experiments demonstrated that PAF, which is one of the most important factors of septic shock, induces an activation of the acid sphingomyelinase and a release of ceramide in the lung (51). Inhibition or genetic deficiency of the acid sphingomyelinase protected from pulmonary edema. Complete protection of PAF-induced lung injury was obtained by a combined inhibition of the acid sphingomyelinase and the cyclooxygenase, suggesting that PAF employs two independent pathways to trigger pulmonary edema. In addition, PAF has been recently shown to trigger ceramide in human erythrocytes, leading to phosphatidylserine exposure on the surface of these cells (98). The exposure of this lipid may result in an increased phagocytosis of those erythrocytes and, finally, anemia. The notion that the acid sphingomyelinase and ceramide are involved in the systemic response of the organism to shock is further supported by the finding that patients with sepsis display an increase in the plasma levels of ceramide (present most likely in the form of lipoproteins) in septic patients (39), although the pathophysiological significance of this finding remains to be determined.

REGULATION OF APOPTOSIS BY CERAMIDE

Apoptosis can be induced by at least three general modes. First, death receptors such as CD95, DR5, or TNF are activated and trigger apoptosis. Second, nonreceptor stimuli, such as irradiation, heat shock, cytotoxic drugs, H_2O_2, toxins, UV light, bacteria, and viruses, mediate death. Finally, third, the deprivation of cells from growth factors or the disruption of the cell's contact with its matrix results in the induction of cell death. Ceramide has been shown to be important in all three types of apoptosis.

Receptor-mediated apoptosis. CD95, which may serve as a paradigm for receptor-mediated apoptosis, has been shown to activate the acid sphingomyelinase and to trigger the release of ceramide and the formation of ceramide-enriched membrane platforms that mediate clustering of the receptor (33, 35, 56, 60, 65, 91, 129). Clustering was recently shown by six independent groups to occur within seconds in Jurkat cells, SKW 6.4 and JY B cell lymphoma, H9T cell lymphoma, epithelial and mouse granulosa cells, and primary murine splenocytes and hepatocytes (43). CD95 clustering in ceramide-enriched membrane platforms is required for the induction of apoptosis, which was evidenced by several in vivo and in vitro studies. Genetic studies demonstrated that acid sphingomyelinase-deficient cells fail to release ceramide on CD95 stimulation and resist apoptosis, which is restored by readdition of natural C_{16}-ceramide, suggesting that ceramide is central for CD95-triggered apoptosis (35, 56, 91, 129). In vivo studies on acid sphingomyelinase-deficient mice revealed a more than 10-fold reduction of apoptosis in hepatocytes of acid sphingomyelinase-deficient animals on stimulation via CD95 (91, 110, 129). Furthermore, neutralization of ceramide in ceramide-enriched membrane domains employing anti-ceramide antibodies or proteins that bind to ceramide or destruction of rafts employing reagents that interfere with cellular cholesterol prevent CD95-induced apoptosis (56, 97). Likewise, acid sphingomyelinase-deficient animals were protected from TNF-α-induced apoptosis of hepatocytes, hepatic failure, and death (50).

The following model might outline the function of ceramide-enriched membrane platforms for CD95-triggered apoptosis: CD95 molecules seem to exist in the cell even before binding of the ligand in a pretrimerized state (145). Ligand binding to pretrimerized CD95 molecules initiates a very weak recruitment of FADD and stimulation of caspase 8 (~1% of the maximal activity) in the cell that is sufficient to translocate and activate the acid sphingomyelinase but insufficient to trigger apoptosis (54). The generation of ceramide-enriched membrane platforms permits the receptor to cluster, to gain a high density within a small area of the cell membrane, and to fully activate caspase 8. Ceramide-mediated clustering of dispersed CD95 receptor trimers is thus the prerequisite for efficient transactivation of caspase 8 and, finally, induction of apoptosis by CD95 (54).

Stress stimuli-induced apoptosis. The functions of the acid sphingomyelinase and ceramide-enriched membrane platforms are not restricted to death receptor-induced apoptosis but also applies to stress stimuli-triggered cell death. In particular, the acid sphingomyelinase has been shown to be central for the induction of cell death by gamma-irradiation (128, 138) and UV-A and UV-C light (30, 182).

Several studies by Kolesnick, Fuks, and Haimovitz-Friedman demonstrated that gamma-irradiation induces a very rapid activation of the acid sphingomyelinase and a release of ceramide (49, 113, 128, 130, 131, 138). Acid sphingomyelinase-deficient T and B lymphocytes, murine embryonic fibroblasts, oocytes, and endothelial cells failed to release ceramide on irradiation and were shown to be resistant to gamma-irradiation-induced cell death (49, 113, 130, 131). The physiological significance of these data was demonstrated by the finding that acid sphingomyelinase-deficient mice were resistant to gamma-irradiation (128, 138). These mice failed to generate ceramide in endothelial cells in the lung, small intestine, or brain and were resistant to radiation-induced tissue damage, events readily observed in normal mice (138). Studies on the effects of gamma-irradiation in the gastrointestinal tract revealed that deficiency of the acid sphingomyelinase rendered endothelial

cells of small blood vessels resistant to radiation-induced cell death and protected acid sphingomyelinase-deficient mice from development of the gastrointestinal syndrome (128), a major limiting toxicity for use of chemotherapy and irradiation to the abdomen. The resistance of acid sphingomyelinase-deficient endothelial cells to gamma-irradiation was confirmed in studies that determined the development of endothelial leakage and edema in the central nervous system after gamma-irradiation (108, 131). These studies showed that acid sphingomyelinase-deficient mice were protected from the development of an edema up to a radiation dose of 50 Gy (108, 131). Finally, Kolesnick and colleagues (49) demonstrated that tumors implanted into acid sphingomyelinase-deficient mice are resistant to gamma-irradiation, whereas the same tumor implanted into acid sphingomyelinase-positive mice was sensitive to gamma-irradiation. The sensitivity of the tumor correlated with induction of apoptosis in acid sphingomyelinase-positive endothelial cells, whereas the endothelial cells in tumor vessels of acid sphingomyelinase-deficient mice resisted gamma-irradiation. Subsequent studies excluded a defect in tumor immunity in acid sphingomyelinase-deficient mice (48). These data suggest that endothelial cells and not the tumor cells are the primary targets of gamma-irradiation, at least in this model, and indicate a central role of the acid sphingomyelinase in the regulation of radiation sensitivity.

Very similar results were described for the induction of cell death by UV light (30, 137, 182). UV-A and UV-C light activate the acid sphingomyelinase and induce an externalization of the acid sphingomyelinase and the formation of ceramide-enriched membrane platforms (30, 137, 182). Expression of the acid sphingomyelinase was shown to be required for UV-light-induced cell death and cells lacking the acid sphingomyelinase were resistant to UV-light-triggered apoptosis (30, 137, 182). Furthermore, neutralization of surface ceramide and destruction of rafts prevented cells from clonogenic death upon UV light treatment (137). Recent studies employed platelets to discriminate whether nuclear or cytosolic events activate the acid sphingomyelinase on UV-C radiation (30). The studies revealed that platelets respond to UV-C light with an externalization and activation of the acid sphingomyelinase, suggesting an extranuclear effect of UV light on the acid sphingomyelinase. Likewise, Haimovitz-Friedman et al. (68) reported an activation of the acid sphingomyelinase in the cell membrane independent of nuclear events after gamma-irradiation.

Finally, at least some chemotherapeutic reagents kill tumor cells by activation of the acid sphingomyelinase and formation of ceramide-enriched membrane platforms, in particular cisplatin (97) and doxorubicin (117). Cisplatin was shown to stimulate the acid sphingomyelinase, which triggers the release of ceramide and the formation of ceramide-enriched membrane platforms that served to cluster CD95 and to execute death (97).

Growth factor-deprivation-mediated apoptosis. Detachment of cells from the matrix and interruption of integrin signaling results in detachment-induced apoptosis, also termed anoikosis. It was recently shown that inhibition of $\alpha_v\beta_3/\alpha_v\beta_5$ integrins results in an activation of the acid sphingomyelinase and a release of ceramide, which was prevented by pharmacological inhibition of the acid sphingomyelinase employing desipramine and imipramine (41). Both drugs also prevented apoptosis induced by inhibition of integrins, indicating that integrin activation protects cells from death by suppressing the acid sphingomyelinase and the release of ceramide.

Developmental death. The first evidence for a role of the acid sphingomyelinase in developmental death was provided by Tilly, Kolesnick, and coworkers (117, 133), who demonstrated that acid sphingomyelinase-deficient ovaries in mice were protected from developmental cell death. In mice, already ~80% of all oocytes undergo cell death until birth and a similar process occurs in human females. Genetic deficiency of the acid sphingomyelinase prevented and delayed developmental apoptosis of oocytes, resulting in oocyte hyperplasia at birth, which demonstrates a fundamental function of the acid sphingomyelinase in oocyte apoptosis (117). Recent studies demonstrated that cumulus cells that surround the oocyte are the producers of ceramide, which is transferred to the oocytes via a gap junction-dependent communication (133).

Furthermore, upon release of neutrophils from the bone marrow, the cells undergo spontaneous apoptosis within a few days. In vitro-isolated neutrophils die within 24–48 h. Lord and coworkers (139) demonstrated that deficiency of the acid sphingomyelinase significantly delayed developmental death of neutrophils. These investigators indicated that activation of the acid sphingomyelinase, release of ceramide, and formation of ceramide-enriched membrane domains occur as the earliest steps in the spontaneous death of neutrophils. Although very likely, at present it is unknown whether the acid sphingomyelinase is also involved in developmental death of other cells and tissues, respectively.

In summary, many recent studies have demonstrated a central role for the acid sphingomyelinase, ceramide, and ceramide-enriched membrane platforms in regulation of apoptosis.

CARDIOVASCULAR ACTIONS OF CERAMIDE

General aspects of the function of ceramide on microvessels. There is accumulating evidence that sphingolipid-mediated signaling exists in cardiovascular cells and plays an important role in the regulation of cardiovascular function (31, 70, 81, 84, 90, 102, 116, 143, 156, 175, 178). Ceramide and other sphingolipids are involved in the modulation of the cell membrane and intracellular ion channels, cell proliferation and apoptotic cell death, neutrophil adhesion to the vessel wall, and vascular tone. All of these biological actions are importantly implicated in the control of cardiovascular functions.

With respect to vasomotor responses, ceramide has been reported to either induce or inhibit contraction of vascular smooth muscles, depending on the species, vascular beds, or artery size used in different studies. For example, cell-permeable ceramides and/or sphingomyelinase treatment have been shown to induce relaxation of phenylephrine-contracted rat thoracic aorta, an effect correlated with decreased intracellular calcium mobilization, but produced contraction in canine cerebral arteries, rat mesenteric resistance and capacitance vessels, and bovine coronary resistance arteries (85, 86, 107, 180, 184, 185). Other ceramide-associated sphingolipids such as sphingosine-1-phosphate and sphingosylphosphocholine have also been reported to induce contraction of mesenteric and intrarenal microvessels and coronary arterial strips (17). In addition, there are several lines of evidence indicating that ceramide and glycosphingolipids accumulate in atherosclerotic lesions (31) and may serve as potential mediators of the

atherogenic process. First, these sphingolipids may participate in the proliferation of vascular wall cells, favoring vessel wall thickening and plaque formation (7). Second, through an inflammatory response initiated by cytokines or oxidized LDL, ceramide, lactosylceramide, or sphingosine-1-phosphate could upregulate expression of adhesion molecules and induce adhesion and migration of monocytes, which are crucial events in initiation and progression of atherogenesis. In this regard, one of the antiatherogenic effects of HDL was attributed to the inhibition of TNF-stimulated sphingosine kinase in endothelial cells (157). Third, ceramide or sphingosine derivatives may promote cell death (mostly by apoptosis) in the vascular wall, a process implicated in plaque erosion and associated thrombosis (114). Fourth, by modulating platelet activation and aggregation, ceramide and glycosphingolipids favor thrombosis by affecting tissue factor or plasminogen activator inhibitor-1 release (77, 150). Fifth, atherosclerotic lesions contain an extracellular sphingomyelinase, which is secreted from cells, in particular macrophages, that is able to hydrolyze sphingomyelin of LDL (47, 103, 140). Hydrolysis of sphingomyelin results in an increased efflux of cholesterol from LDL (47, 103, 140), a process that seems to contribute to subendothelial LDL retention, foam cell formation, and alterations of macrophage function.

Recent studies also demonstrated that ceramide importantly participates in the regulation of cardiac function. It has been reported that the sphingomyelin-ceramide signaling pathway was activated by myocardial ischemia/reperfusion in vivo (16, 53, 173) and that hypoxia/reoxygenation in vitro activated sphingomyelinase to produce ceramide (75, 175). Exogenous cell-permeable ceramide has been shown to induce cardiomyocyte apoptosis in vitro, which contributes to myocardial ischemia/reperfusion injury (16). In addition, the ceramide metabolite sphingosine was found to exhibit negative inotropic effects in adult mammalian cardiac myocytes by inhibition of intracellular Ca^{2+} mobilization, thereby facilitating the development of ischemia/reperfusion injury (127).

Many recent studies have intensively addressed the biological actions of ceramide on coronary endothelial function (82, 85, 89, 99, 132, 168–175). The results indicated that ceramide-mediated signaling is a novel mechanism underlying endothelial dysfunction associated with overproduction of cytokines during ischemic heart disease, which could be directed toward the development of therapeutic strategies to improve endothelial dysfunction during myocardiac ischemia and reperfusion. These results and their perspectives are discussed below.

Ischemia, stroke, and the acid sphingomyelinase. Myocardial infarction and stroke caused by tissue ischemia are important clinical problems and are major causes of death, at least in Western countries. Induction of stroke by experimental ischemia of the brain was shown to correlate with an activation of the acid sphingomyelinase and a release of ceramide (166). Most importantly, acid sphingomyelinase-deficient mice were protected from tissue damage caused by focal cerebral ischemia. Acid sphingomyelinase-deficient mice displayed smaller infarct sizes and less behavioral changes than their wild-type littermates after induction of a middle cerebral artery stroke. Likewise, deficiency of the acid sphingomyelinase conferred hippocampal neurons resistant to hypoxic and excitotoxic stress, suggesting that activation of neuronal acid sphingomyelinase is central to stress-induced apoptotic death of hippocampal neurons. It will be of great interest to test whether the acid sphingomyelinase is also involved in ischemic tissue damage of other organs, e.g., the heart.

Ceramide-mediated regulation of endothelium-dependent vasorelaxation. Many studies have been undertaken to demonstrate the production of ceramide in endothelial cells. Using thin-layer chromatography assays, our laboratory reported that basal concentration of ceramide in bovine coronary arterial endothelial cells is 72.8 pmol/10^5 cells or 5.32 nmol/mg protein (176). As shown in Fig. 4, ceramide can be also detected in cultured single bovine coronary arterial endothelial cell by immunofluorescence microscopy with a specific anti-ceramide antibody. This ceramide production was accompanied by acid sphingomyelinase activation. Functional studies investigated the effects of pharmacological ceramide species on the function of microvessels. Using an isolated, perfused, and pressurized small coronary arterial preparation, we demonstrated that C_2-ceramide inhibits L-NAME-sensitive vasodilator responses to the endothelium-dependent vasodilators bradykinin (receptor dependent) and A-23187 (receptor independent). In the presence of L-NAME, ceramide had no further inhibitory effect on the responses to these vasodilators (180). However, ceramide was without effect on vasodilator responses to the endothelium-independent vasodilator DETA NONOate. By direct measurement of nitric oxide (NO) levels in the intact endothelium of coronary arteries employing real-time fluorescence microscopy, endothelial NO concentrations during stimulation of bradykinin and A-23187 were significantly attenuated by C_2-ceramide. These results provide direct evidence that ceramide inhibits NO-mediated endothelium-dependent relaxation and thereby may lead to endothelial dysfunction in coronary microcirculation. However, we did not observe any significant action of C_2-ceramide on the basal vascular tone, although it produced a marked vasoconstriction when the arteries were pretreated with BAY K 8644. This

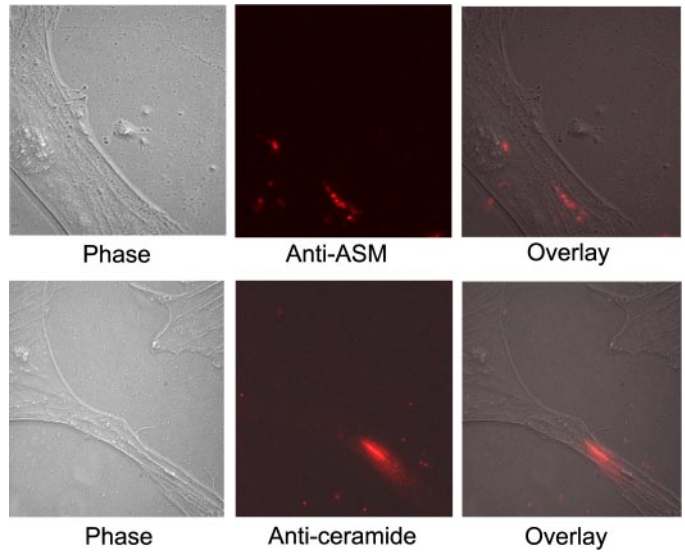

Fig. 4. Immunofluorescent microscopic detection of ceramide and acid sphingomyelinase in bovine coronary arterial endothelial cell. Bovine endothelial cells were stimulated with CD95 ligand, fixed, and stained with Cy3-coupled anti-acid sphingomyelinase or anti-ceramide antibodies. Surface translocation of the acid sphingomyelinase (ASM) and formation of ceramide-enriched membrane domains in endothelial cells on stimulation are shown.

action is similar to that of another sphingolipid, sphingosine (9, 118), which has been shown to contract PGF_2-prestimulated rings, although it was without effect on quiescent pig coronary rings. It is likely that the action of C_2-ceramide on basal vascular tone varies with species, vascular beds, and artery sizes.

Consistent with these findings, recent studies have shown that ceramide reduces the release of bioactive NO in human umbilical vein endothelial cells (24, 82).

It is indicated that ceramide decreases endothelial NO levels and thereby inhibits NO-mediated endothelium-dependent relaxation in response to different endothelium-dependent vasodilators. Therefore, ceramide may be a pathogenic factor resulting in endothelial dysfunction in coronary circulation.

Endogenous ceramide regulates actions of cytokines. It has been suggested that cytokines, such as TNF-α, are involved in the regulation of multiple cellular functions, and are important mediators of circulatory changes associated with the development of various cardiovascular diseases, e.g., atherosclerosis, sepsis-associated cardiovascular dysfunction, and myocardial ischemia/reperfusion injury (9, 31, 81, 90, 102, 116, 126, 143, 156, 175, 178). In regard to the action of TNF-α in myocardial ischemia/reperfusion, previous studies have shown that increased TNF-α may contribute to decreased coronary vascular tone at a late phase of myocardial ischemia and reperfusion (25). These actions of TNF-α on vascular tone are often attributable to the induction of inducible nitric oxide synthase (NOS) in macrophages and cardiomyocytes and usually occur with a lag-time of 4–6 h after ischemia/reperfusion. Acutely, TNF-α (a 2-h incubation) was found to selectively impair ACh-induced relaxation in cat left anterior descending coronary artery rings (99). We also demonstrated that a short-term incubation (~1 h) of coronary arteries with TNF-α attenuated L-NAME-sensitive, endothelium-dependent relaxation (174, 176). The cytokine-induced impairment of endothelium-dependent vasodilation was observed in cat carotid artery, rat aorta, rat and bovine pulmonary artery, and human forearm resistance artery and vein (13, 92, 95, 111, 121, 154, 158).

The role of ceramide in mediating coronary endothelial dysfunction associated with cytokines such as TNF-α during myocardial ischemia and reperfusion was defined in experiments that determined the cardiovascular effects of TNF-α, bradykinin, and A-23187 in isolated coronary arterial preparations (176). Pretreatment of the arteries with desipramine prevented TNF-α-induced impairment of endothelium-dependent relaxation in coronary arteries, indicating that endogenously produced ceramide mediates most of the actions of TNF-α on endothelium-dependent vasorelaxation (176). In agreement with the results from pharmacological experiments in isolated coronary arteries, biochemical assays demonstrated that TNF-α induced a rapid increase in endogenous ceramide in coronary endothelial cells (176). TNF-α rapidly activated the acid sphingomyelinase in endothelial cells, whereas the neutral sphingomyelinase was not affected (174, 180). However, no stimulation of sphingomyelinases in cerebral endothelial cells was observed after a long-term treatment with TNF-α (16 h), and the increase in ceramide at that time was attributed to de novo ceramide biosynthesis via ceramide synthase (159). This suggests that the acute exposure of endothelial cells to TNF-α and other cytokines releases ceramide via the acid sphingomyelinase, whereas a chronic treatment produces ceramide through synthetic pathways.

Mechanisms underlying ceramide-induced NO decrease. There are several possible mechanisms that may mediate a decrease of NO and thus endothelial dysfunction elicited by exogenous or endogenous ceramide. First, a selective loss of receptor-mediated, endothelium-dependent vasodilatation, as described in various vascular disease states (20, 109, 152) may be one of the mechanisms. It has been reported that lysolipids are readily incorporated into the lipid bilayer of the cell membrane, thus altering membrane fluidity and receptor/G protein coupling (44, 45, 120). These lipid-mediated membrane changes may result in receptor-mediated signaling disturbances, for instance, impaired agonist-induced release of NO. For example, work by Flavahan (45) demonstrated that lysophosphatidylcholine modifies G_i-protein-dependent signaling in porcine endothelial cells. However, ceramide decreases the NO response to both receptor-dependent (bradykinin) and -independent (A-23187) stimuli in small coronary arteries (179, 180); therefore, it seems to be likely that the action of ceramide in arterial endothelial cells is not due to changes in receptor function but rather due to a common signaling pathway downstream from receptor activation.

Second, ceramide-induced decreases in NO levels could be caused by inactivation of NO, as shown previously for other lipids. For example, Galle et al. (47) have shown that NO released from endothelial cells was inactivated by oxidized LDL in a bioassay system, suggesting that LDL may directly mediate degradation of NO and thus attenuate vasodilatation to agonists. However, we did not find any significant effects of ceramide on NO degradation, as indicated by a lack of a direct effect on PAPA-NONOate-induced NO increases using the 4,5-diaminofluorescein-2 fluorescence assay in intact endothelium. In addition, we found that the impaired vasorelaxation to bradykinin and A-23187 was still present 1–2 h after ceramide removal. Therefore, ceramide does not seem to cause degradation of NO in endothelial cells (174, 177, 181).

Third, ceramide-induced NO decrease could be associated with alterations in endothelial NOS (eNOS) expression/activity or eNOS activation. In this regard, Higashi et al. (76) recently reported that sphingolipids directly bind to calmodulin and thereby inhibit the activity of calmodulin-dependent enzymes such as eNOS. Ceramide has been shown to induce an activation of ceramide-activated protein phosphatases in a number of cells (31, 38, 102, 125), which may mediate inhibition of eNOS as previously reported for endostatin (127). Thus it is possible that ceramide inhibits activation or activity of eNOS by a calmodulin-dependent mechanism or by stimulating ceramide-activated protein phosphatases. However, we found that ceramide had no direct effect on eNOS activity when either intact cells or cell homogenates were incubated with ceramide. In addition, eNOS expression was unaffected by ceramide treatment. Moreover, eNOS phosphorylation, including bradykinin-induced phosphorylation of eNOS at Ser^{1177}, a crucial step in the activation of eNOS by agonists, was not impaired in coronary endothelial cells treated with ceramide, indicating that the inhibitory effect of ceramide on endothelial NO concentrations is not caused by changes in the activity/expression and activation of eNOS (178, 179, 181).

Fourth, there is overwhelming evidence that oxidative stress alters many functions of the endothelium, including modula-

tion of the vascular tone. Inactivation of NO by $O_2^-\cdot$ or other reactive oxygen species (ROS) has been suggested to be involved in the pathogenesis of various cardiovascular diseases, including hypertension, hypercholesterolemia, diabetes, atherosclerosis, and heart failure. Thus it was demonstrated that ceramide and/or other sphingolipids stimulate the production of $O_2^-\cdot$ in vascular cells (14, 71). Several lines of evidence were provided to support the view that the ceramide-induced NO decrease in response to bradykinin and A-23187 in coronary endothelial cells may be associated with enhanced $O_2^-\cdot$ production. Thus pretreatment of the arteries with tiron, a chemical mimetic of SOD that is capable of removing $O_2^-\cdot$ from both the intracellular and extracellular environment (73), prevented ceramide-induced decreases in NO levels and endothelial dysfunction in small coronary arteries. Furthermore, ceramide significantly increased $O_2^-\cdot$ in the endothelium, as measured by hydroethidine fluorescence microscopy. Finally, exogenous $O_2^-\cdot$ produced by xanthine/xanthine oxidase inhibited an increase in NO observed in the endothelium (177, 178, 180).

Similar to ceramide, TNF-α also induces $O_2^-\cdot$ production, which might be an important mechanism to decrease NO and consequently impair endothelium-dependent relaxation in small coronary arteries. Indeed, the SOD mimetic, tiron, and polyethylene glycol-SOD prevented TNF-α-induced impairment of endothelium-dependent vasorelaxation. These studies suggest that the decrease of NO on TNF-α treatment is mediated by a ceramide-induced $O_2^-\cdot$ production (174, 180).

Ceramide-induced $O_2^-\cdot$ production. The cardiovascular system contains several potential enzymatic sources of $O_2^-\cdot$ or other ROS, including NAD(P)H oxidase, xanthine oxidase, the mitochondrial respiratory chain, and NOS (64, 63). Recently, overwhelming evidence indicated that nonmitochondrial NAD(P)H oxidases are primarily responsible for the production of $O_2^-\cdot$ in vascular cells on stimulation (10, 52, 64, 105). In a series of studies, we found that NAD(P)H oxidase is also expressed in endothelial cells of small coronary arteries and that this enzyme is functionally active in producing $O_2^-\cdot$ in these cells, as measured by NAD(P)H-dependent $O_2^-\cdot$ production in the intact coronary endothelium or endothelial homogenates. Ceramide was demonstrated to increase endothelial $O_2^-\cdot$ in the endothelium of isolated small coronary arteries, which was blocked by different NAD(P)H oxidase inhibitors such as *N*-vanillylnonanamide, apocynin, and diphenyleneiodonium. By analysis of the enzyme activity, ceramide was found to significantly stimulate the activity of NAD(P)H oxidase in endothelial cells, which was prevented by NAD(P)H oxidase inhibitors, but not by inhibitors of NOS, xanthine oxidase, and mitochondrial electron transport chain enzymes. In addition, inhibition of NAD(P)H oxidase by different NAD(P)H oxidase inhibitors largely prevented ceramide-induced and $O_2^-\cdot$-mediated impairment of endothelium-dependent relaxation to agonists in small bovine coronary arteries (179, 180). These studies very clearly indicate that NAD(P)H oxidases mediate dysfunction of endothelial cells induced by ceramide. These actions of ceramide and related mechanisms are summarized in Fig. 5.

In additional studies, ceramide-induced activation of NAD(P)H oxidase was found to be associated with a rapid translocation of $p47^{phox}$ to the cytoplasmic membrane. Because $p47^{phox}$ translocation leads to the recruitment of other cytosolic subunits of NAD(P)H oxidase to the plasma membrane, resulting in activation of this enzyme in phagocytes (8, 101), these data suggest that $p47^{phox}$ translocation may initiate ceramide-induced activation of NAD(P)H oxidase in coronary endothelial cells. Other recent studies confirmed that $p47^{phox}$ phosphorylation and subsequent translocation to the membrane are critical in activation of endothelial NAD(P)H oxidase induced by TNF-α (46, 104). 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 (46). Because ceramide is able to activate PKC-ζ (22, 119, 155), it is possible that ceramide employs this kinase to regulate NAD(P)H oxidase in endothelial cells. On the other hand, it might be also possible that ceramide-enriched membrane platforms recruit the subunits of NAD(P)H oxidase to assemble and activate the oxidase at the cell membrane after treatment with TNF-α.

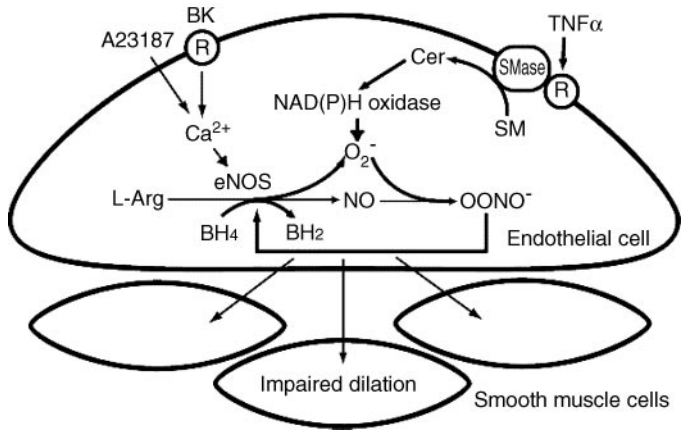

Fig. 5. Cytokines such as TNF-α induce endothelial dysfunction through ceramide (Cer) signaling pathway in coronary arteries. TNF-α activates the acid sphingomyelinase to trigger the release of ceramide, which induces the formation of reactive oxygen species and thereby dysfunction of endothelial cells by interference with cellular nitric oxide (NO). eNOS, endothelial nitric oxide synthase; L-Arg, L-arginine; R, receptor; SMase, sphingomyelinase.

Ceramide and membrane platforms in endothelial cells. Although it is very attractive to speculate that rafts and ceramide-enriched membrane platforms are involved in the homeostasis of endothelial cells 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 lipid raft clustering and trafficking on the cell membrane of endothelial cells are associated with ceramide production and action (106, 165, 167–172). It was found that acid sphingomyelinase and ceramide are of importance in CD95 ligand-induced formation of lipid raft clusters on the endothelial cell membrane. In cells treated with a small inhibitory RNA (siRNA) duplex of the acid sphingomyelinase gene, mRNA and protein levels of this enzyme were significantly reduced, and correspondingly acid sphingomyelinase activity and ceramide level in these cells were markedly inhibited. Under this condition, CD95 ligand no longer stimulated the formation of ceramide-enriched membrane platforms, indicating a central role of acid sphingomyelinase activation and ceramide production for CD95 ligand-

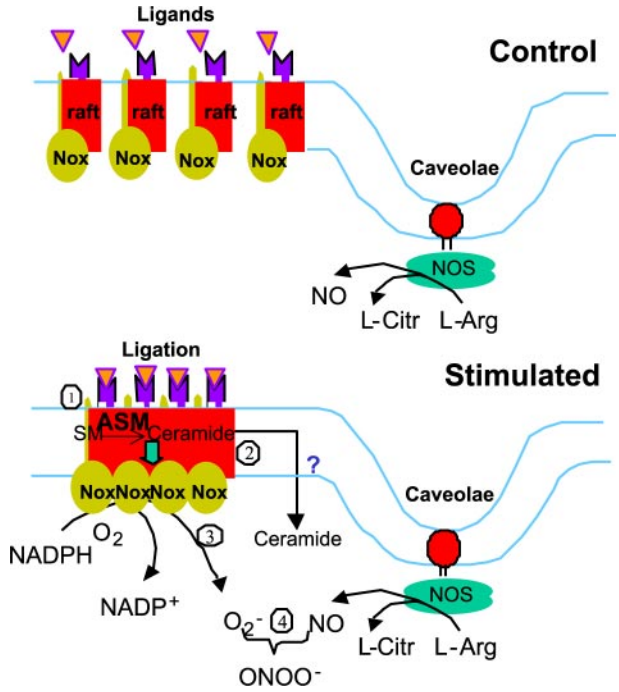

Fig. 6. Ceramide-mediated platform formation on coronary endothelial cell membrane. Under resting condition, individual lipid rafts with attached receptors, including TNF superfamily receptors such as CD95 and TNFR1 or TNFR2, are present on the membrane of endothelial cells (control). These lipid rafts are dynamic microdomains and carry several different membrane-bound or attached proteins or enzymes such as G proteins, protein kinases, or the subunit complexes of NADPH oxidase (Nox). When the ligands bind to their receptors in individual lipid rafts (*1*), sphingomyelinases (*2*) located in situ or translocated from lysosomes will be activated to produce ceramide from sphingomyelin (SM). Ceramide triggers lipid raft clustering on the cell membrane to form a signaling platform, in which acid sphingomyelinase, Nox, and other proteins are aggregated and activated, resulting in a prominent amplification of transmembrane signal (stimulated) (*3*). The activation of Nox produces $O_2^-\cdot$, thereby leading to NO decrease, peroxynitrite ($ONOO^-$) formation, and consequent endothelial dysfunction (*4*). L-Citr, L-citrulline.

induced clustering of lipid rafts in coronary arterial endothelial cells.

We also demonstrated that ceramide-mediated clustering of lipid rafts is involved in the regulation of $O_2^-\cdot$ production in coronary endothelial cells via NAD(P)H oxidase. This effect was associated with the recruitment and aggregation of the NAD(P)H oxidase subunits $gp91^{phox}$ and $p47^{phox}$ in lipid rafts. It was shown that silencing the acid sphingomyelinase gene by siRNA reduced CD95 ligand-induced $gp91^{phox}$ aggregation in lipid raft clusters and $p47^{phox}$ translocation by 85% and 68%, respectively, and completely inhibited CD95 ligand-induced $O_2^-\cdot$ production in these cells. In isolated small bovine coronary arteries, transfection of acid sphingomyelinase siRNA markedly attenuated CD95 ligand-induced inhibition of endothelium-dependent vasorelaxation (a response to bradykinin) by 60%. The results suggest that the activation of NAD(P)H oxidase in response to cytokines in coronary endothelial cells, consequently leading to endothelial dysfunction (168–172), is regulated by the acid sphingomyelinase, the release of ceramide, and lipid raft-derived ceramide-enriched membrane platforms. This hypothesized endothelial lipid rafts clustering or platform formation is illustrated in Fig. 6.

Together, a ceramide-mediated signaling cascade in endothelial cells has been demonstrated, which includes the mechanisms by which cytokines activate acid sphingomyelinase to release ceramide and the stimulation of $O_2^-\cdot$ production by ceramide through activation of NAD(P)H oxidase in endothelial cells. $O_2^-\cdot$ reduces NO bioavailability, resulting in endothelial dysfunction in the coronary circulation. In perspective, ceramide-mediated signaling and its role in endothelial dysfunction through activation of NAD(P)H oxidase is becoming an important theme in vascular biology (25, 26). Recent studies have indicated that uncoupled eNOS produces $O_2^-\cdot$ in endothelial cells. It will be interesting to determine whether the ceramide-induced $O_2^-\cdot$ increase and consequent $ONOO^-$ production uncouples eNOS, resulting in a reduced production of NO and an increased production of $O_2^-\cdot$. Measurement of tetrahydrobiopterin oxidation, conversion of L-arginine to L-citrulline in situ in endothelial cells, and eNOS uncoupling-induced $O_2^-\cdot$ production on ceramide or cytokine stimulation may help to solve this question. Furthermore, the physiological or pathological significance of ceramide-enriched membrane platforms and ceramide-mediated signaling remains to be determined. Because oxidative stress and endothelial dysfunction are involved in the pathogenesis of many cardiovascular diseases, including hypercholesterolemia, artherosclerosis, hypertension, diabetes, ischemic heart disease, and heart failure, it is possible that activation of ceramide signaling is an important mechanism responsible for the pathogenesis or development of these cardiovascular diseases.

Renal actions of ceramide. Ceramide has also been implicated in the regulation of kidney function and seems to be involved in renal glomerular and tubular pathology (88, 153, 163, 165). In this section, we focus on ceramide in chronic renal failure and the regulation of renal ROS. Recently, our group (163, 164) 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.

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 (88, 153, 159, 165). 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 of ceramide. Although both acid sphingomyelinase and neutral sphingomyelinase were found in normal kidneys, increased production of ceramide from the kidney during hyperhomocysteinemia or mesangial cells exposed to L-homocysteine were not associated with changes in the activity of these sphingomyelinases (163, 164). 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 (7, 29, 36, 163, 164). 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 (163, 164). These data provide direct evidence that the ceramide pathway is critically involved in L-homocysteine-induced glomerular injury and glomerular sclerosis (163, 164).

Ceramide and oxidative stress in the kidney. As discussed above, ceramide has been reported to stimulate the $O_2^-\cdot$ production in endothelial cells. It seems that ceramide-induced oxidative stress also occurs in the kidney on stimulation. Using glomerular mesangial cells, L-homocysteine or ceramide was found to increase $O_2^-\cdot$ production, which was associated with an activation of NAD(P)H oxidase. Blockade of ceramide de novo synthesis in hyperhomocysteinimic rats substantially inhibited the enhancement of NAD(P)H oxidase and production of $O_2^-\cdot$ in the kidney (163, 164).

However, the mechanism by which NAD(P)H oxidase is activated in renal cells seems different from that described above for endothelial cells. In contrast to endothelial cells, the abundance of NAD(P)H oxidase subunits $p47^{phox}$ and $p67^{phox}$ in cell membrane fractions was even higher than the cytosolic levels in mesangial cells under control condition. Neither L-homocysteine nor ceramide changed the abundance in either the cell membrane or the cytosol fraction. It appears that translocation of $p47^{phox}$ seen in endothelial cells is not involved in L-homocysteine- or ceramide-induced activation of NAD(P)H oxidase in rat mesangial cells (163). However, it might be possible that the transformation of small rafts to ceramide-enriched membrane platforms results in a clustering of NAD(P)H oxidase molecules that are constitutively present in small rafts.

In addition, NAD(P)H oxidase might be regulated via the small G protein Rac (18, 159). Rac is a member of the Rho family of GTP-binding proteins, and it is regulated by binding GTP for activation and hydrolyzing GTP to GDP for inactivation. Thus Rac "activity" has been generally viewed as being synonymous with GTP binding. In previous studies, the role of Rac as a regulator of NAD(P)H oxidase complex was described in phagocytes as well as in vascular smooth muscle and endothelial cells (118). Both L-homocysteine- and ceramide-stimulated Rac, as well as the stimulatory effect of L-homocysteine, was prevented by fumonisin B1, suggesting that ceramide mediates the activation of Rac by L-homocysteine. At present, it is unknown how ceramide activates Rac in glomerular mesangial cells. Rac might be recruited to ceramide-enriched membrane domains, which may also serve to concentrate proteins that activate Rac, for instance tyrosine kinases or the guanine nucleotide exchange factor Vav, which accelerates exchange of GDP by GTP (2, 66). L-Homocysteine-induced ceramide de novo synthesis and Rac activation may stimulate NAD(P)H oxidase and thereby increase $O_2^-\cdot$ production in glomerular mesangial cells. In summary, the signaling pathway initiated by ceramide seems to play a critical role in the development of chronic glomerular injury associated with hyperhomocysteinemia, which was recently considered as an important pathogenic factor of chronic glomerular injury and a novel risk factor for chronic renal failure and uremic cardiovascular diseases.

Perspective

Many recent studies have evolved a critical function of membrane rafts and ceramide-enriched membrane domains in the induction of apoptosis by death receptors, stress stimuli, and during development; the infection of mammalian cells by bacteria, viruses, and parasites; and the regulation of endothelial functions. The concept that the generation of ceramide in rafts transforms these membrane domains into an "active" signaling unit provides a rationale regarding why ceramide and ceramide-enriched membrane platforms are central in many biological processes. However, for future therapeutic interventions, it is very important to target the acid sphingomyelinase and ceramide specifically in the tissue or cell, which is supposed to be manipulated. A manipulation of ceramide could be envisioned to prevent tissue damage in ischemic insults of the brain and heart, to prevent renal failure at least in some instances, to trigger apoptosis of tumor cells and/or endothelial cells of the tumor vasculature, and to prevent the infection of mammalian cells with *P. aeruginosa*, *N. gonorrhoeae*, and possibly other pathogens.

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