J. Biochem. **122**, 1080-1087 (1997)

Functional Roles of Sphingosine, Sphingosine 1-Phosphate, and Methylsphingosines: In Regard to Membrane Sphingolipid Signaling Pathways

Yasuyuki Igarashi[1]

Fred Hutchinson Cancer Research Center and Department of Pathobiology, University of Washington, 1124 Columbia Street, M621, Seattle, WA 98104, USA

Received for publication, August 29, 1997

The signaling roles of ceramide and sphingosine produced through the degrading processes of membrane sphingolipids are currently receiving hot attention in the biochemical and biomedical research fields. For these 9 years at the Biomembrane Institute in Seattle, we have studied functional roles of various sphingolipids such as ceramide, sphingosine, methylsphingosines, and sphingosine 1-phosphate in a variety of biomedical systems. In this article, first, the recent conceptual developments on sphingolipid signaling pathways is outlined. Next, our recent findings on the functional roles of sphingolipids are described focusing on (i) functional roles of sphingosine 1-phosphate in cell motility regulation and platelet activation (ii) involvement of sphingosine in cell signaling (iii) effects of methylsphingosines in cancer cell apoptosis induction and in the regulation of inflammatory processes. Based upon these findings from our studies and others, the perspective of future sphingosine research (sphingology or sphingophysiology) is briefly discussed.

Key words: apoptosis, ceramide, inflammation, methylsphingosines, sphingolipid signaling pathway, sphingosine, sphingosine 1-phosphate.

1. Introduction: Significance of sphingolipid signaling pathway

The signaling roles of ceramide, sphingosine, and their derivatives produced through the degrading processes of membrane sphingolipids are currently receiving hot attention in the biochemical and biomedical research field. Membrane sphingolipids, sphingomyelin (SM) and glycosphingolipids (GSLs), had long been regarded as metabolically inactive, and rather stable structural components of the membrane, compared with glycerolipids including inositolphospholipids which produce lipid-derived second messengers, diacylglycerol, IP_3 (*1, 2*) or D3-phosphoinositides (*3*). However, about 10 years ago, this concept of static membrane sphingolipids was challenged by Bell's group (*4*), first reporting the vitamin D_3-induced degradation and turnover of SM in HL-60 cells. Stimulated by this discovery, a number of studies regarding extracellular agents or insults such as TNFα, IL-1β, FAS ligand, or γ-radiation were reported to cause the activation of sphingomyelinase and the release of ceramide as a lipid second messenger or biomodulator of stress-related diverse responses such as cell-cycle arrest, apoptosis, and cell senescence (*5, 6*). This pathway is mostly designated as the "SM cycle" or "SM-ceramide pathway," and a number of comprehensive review articles have been already published focusing on the roles of ceramide in cellular regulation. But, as shown in the metabolic pathway of membrane sphingolipids (Fig. 1), it is now broadly recognized that not only ceramide, but other bioactive sphingolipids are produced through this degrading pathway, and that they also play a variety of roles in regulation of cellular activities as modulators or signaling molecules (*7-9*).

Therefore, the author would emphasize here that not only ceramide formation but the following four aspects of the membrane sphingolipid degradation pathway are equally important and must be considered to be of primary significance. Also, the possible involvement of GSLs in the signaling pathway should not be neglected (*8-10*), although it has not yet been greatly explored. (i) Alterations in ceramide concentration in cells and subsequent activation of ceramide-dependent enzymes or other activities. (ii) Alterations in sphingosine and other sphingosine derivatives arising from ceramide and subsequent changes in related activities. (iii) Structural changes of membranes or membrane micro-domains caused by the degradation and the resulting decrease of the membrane components SM and GSLs. (iv) Alterations in the balance among various sphingolipids and their localization in the cells.

As mentioned above, viewpoint (i) has been the major concern in the sphingolipid signaling pathway studies so far. In this article, this author will mainly discuss point (ii), presenting our own studies on the functional roles of sphingosine, sphingosine-1-phosphate, and methylsphingosines. The other two viewpoints, (iii) and (iv), will not be discussed here, although they are newly arising, important subjects to be seriously considered and explored [for example, see Ref. *11* for (iii) and Refs. *12* and *13* for (iv)].

2. Physiology of sphingosine 1-phosphate

The phosphorylated sphingoid base, sphingosine 1-phosphate (Sph-1-P), is the initial product of the catabolism of

[1] Tel: +1-206-667-2844, Fax: +1-206-667-6519, E-mail: yigarash@fhcrc.org

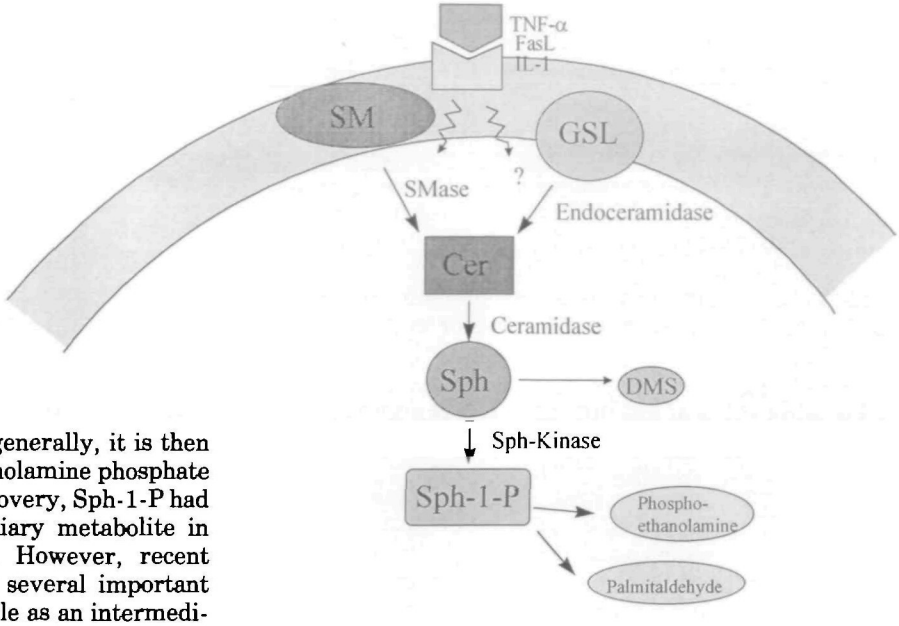

Fig. 1. **Membrane sphingolipid metabolism and signaling pathway.** The ceramide second messenger hypothesis solely emphasizes the production of ceramide through a sphingomyelin cycle and its roles in signal transduction. On the other hand, the figure illustrates the importance of sphingosine and other bioactive sphingosine derivatives derived from degrading pathways of membrane sphingolipids including both sphingomyelin and glycosphingolipids, in addition to the roles of ceramide (see text).

sphingosine (Sph) by Sph kinase and, generally, it is then cleaved by Sph-1-P lyase to yield ethanolamine phosphate and a fatty aldehyde (*14*). Since its discovery, Sph-1-P had been simply regarded as an intermediary metabolite in sphingolipid metabolism in the cell. However, recent studies showed that Sph-1-P exhibits several important biological functions in addition to its role as an intermediary lipid metabolite. The first observation of such a biological function was reported in 1990 by Ghosh *et al.*, showing that a phosphorylated product of sphingosine (presumably Sph-1-P) in cultured muscle cells mobilized intracellular Ca^{2+} concentration through an IP_3-independent pathway (*15*). Expanding this work further, in 1991, Spiegel's group found that exogenously added Sph-1-P stimulates proliferation of Swiss3T3 fibroblast cells by mobilizing intracellular Ca^{2+} (*16*). They further showed that PDGF (platelet derived growth factor) activates Sph kinase and causes transient Sph-1-P production in the Swiss3T3 cells. Based upon these studies, they claimed the novel notion that Sph-1-P is a mitogenic second messenger in fibroblast cell proliferation induced by PDGF and serum factors (*17*) as well as in the prevention of ceramide-induced apoptosis of HL-60 cells (*12*). This second messenger notion of Sph-1-P was further supported by the recent work of Kinet *et al. indicating* that Sph-1-P mediates FcεRI antigen receptor signaling resulting in histamine release in human mast cells (*18*).

Cell motility regulation. Independent from those works, in 1992, we discovered that exogenously added Sph-1-P, a pure D-*erythro* isomer synthesized chemically (*19*), inhibits chemotactic and haptotactic cell motility of mouse melanoma and other various cancer cells as demonstrated by *in vitro* assay systems using a Boyden chamber or a gold colloid-coated plate (phagokinetic assay) (*20, 21*). Furthermore, in collaboration with Prof. Ross's group, University of Washington, we expanded these studies and showed that PDGF-induced cell motility of human aorta smooth muscle cells is regulated by this lipid through its effects on actin filament reorganization in pseudopodia (*22, 23*). Very recently, introducing Sph-1-P-immobilized glass beads (*24*), we found that Sph-1-P regulates melanoma cell motility through binding to its cell surface receptor protein(s), and we newly identified original Sph-1-P binding proteins (4.1 and 7.9 kDa) on the melanoma cell surface.

Physiological and pathophysiological roles in platelets and blood vessels. Additionally, we found that Sph-1-P acts as an autocrine stimulator of platelets, being abundantly stored in platelets and released extracellularly upon stimulation, and that its exogenous addition induces platelet activation (*25*) through a specific receptor on the surface of human platelets (*26*). Furthermore, we identified Sph-1-P as a normal constituent of human plasma and serum (*27*) by developing a new facile, but sensitive method for quantifying the lipid (*28*). The source of discharged Sph-1-P during blood clotting is most likely platelets, since they abundantly store Sph-1-P as compared to other blood cells. The Sph-1-P released from activated platelets might be involved in a variety of physiological processes, including hemostasis, thrombosis, atherosclerosis, and wound healing. Moreover, recent studies suggested that Sph-1-P affects heart functions by stimulating K^+-channels in rat myocytes (*29*). Therefore, it is not unlikely that deregulated Sph-1-P in blood vessels could cause various adverse effects under certain pathological conditions. As an example, we recently revealed that Sph-1-P content in the plasma from stored platelet concentrates correlated with poor platelet increments after transfusion and with occurrences of transfusion reactions in patients (*30*). These studies suggest further that variations in the plasma level of Sph-1-P may be related to some clinical disorders, and measurement of the plasma Sph-1-P level may provide information on the implications of pathophysiological roles of this lipid in certain diseases (*30*).

First messenger and/or second messenger? As mentioned above, Sph-1-P was first proposed as a second messenger in PDGF- or serum-induced Swiss3T3 fibroblast cells through intracellular Ca^{2+} mobilization and subsequent protein kinase activation (*16*). On the other hand, as shown here, we investigated the action mode of Sph-1-P in the activation of human platelets, as well as in the motility regulation of mouse melanoma F10 cells, and found that Sph-1-P acts extracellularly and plays a role as an intercellular signaling molecule (first messenger) in these particular cells and phenomena. Interestingly, effective doses of Sph-1-P required for the proliferation enhancement and the motility inhibition were entirely different: μM orders for proliferation and nM orders for motility inhibition. Other biological functions of Sph-1-P have also

been reported, such as the activation of muscarinic K^+ currents in atrial myocytes (*29*) and the Rho-dependent neurite retraction in neuronal cells (*31*), each through a G-protein coupled receptor mechanisms, and nM orders of Sph-1-P concentrations were shown to be effective concentrations for these functions when added exogenously (summarized in Table I). Two conceptually different messenger roles have been claimed for this single lipid molecule, in a cell type- and phenomenon-specific manner, making Sph-1-P a unique bioactive signal molecule. Further extensive studies including those of intra- and intercellular signaling mechanisms as well as Sph-1-P receptor purification and cloning are needed.

3. Possible roles of sphingosine in cell signaling

Sphingosine (Sph) is a lipid amine base produced from ceramide by ceramidases (acidic, basic, and neutral). Since *de novo* synthesis of this lipid (but not sphinganine) was experimentally disproved (*32*), Sph is now recognized to be solely produced through the degrading pathway of membrane sphingolipids. The roles of Sph seem to be cell type- or phenomenon-specific and in some extent even contradictory effects have been reported. Sph was first identified as an endogenous PKC inhibitor (*33, 8*). However, it was reported later that in Swiss3T3 fibroblast cells it shows strong mitogenic effects, probably through its conversion to Sph-1-P (*7*). In most cells, Sph has been known to be a rather adverse or toxic molecule affecting PKC or other activities. In this chapter, our recent studies on the role of Sph in cancer cell apoptosis induction, as an example of that, will be described.

Ceramide=second messenger hypothesis. When ceramide appeared as a novel signaling molecule, it was claimed, by Hannun's and Kolesnick's groups, to be a second messenger in TNF-α, FAS ligand, or γ-radiation-induced apoptosis in leukemic cells or lymphocytes, possibly mediated through ceramide activated protein kinase (CAPK), or ceramide activated protein phosphatase (CAPP) as its target effector molecules (*5*). But even now the underlying mechanism and the precise roles of effector enzymes remain unclear. More recently, doubt regarding the quick formation of ceramide upon stress stimulation has been expressed by many researchers, including Hannun himself, who has now claimed ceramide to be a long term coordinating regulator, playing "biostat" roles in stress-induced phenomena, rather than as a second messenger (*6*). Ceramide-induced NF-$\varkappa$B activation, which was considered to be strong evidence indicating its second messenger role, also remains controversial (*5, 34*). Thus, the roles of ceramide (and sphingolipid signaling pathways) in apoptosis induction remain unclear and more intensive studies from various angles are required for further elucidation of precise contributions from each sphingolipid.

TABLE I. **Roles of sphingosine 1-phosphate (Sph-1-P).**

Intermediary catabolite of Sph degradation (Stoffel, 1973; Ref. *14*)
Bioactive lipid messenger
A. Intracellular messenger
PDGF-induced fibroblast proliferation (Spiegel, 1993; Ref. *17*)
IgE-induced histamine release in mast cells (Kinet, 1996; Ref. *18*)
B. Intercellular messenger
Regulation of cancer cell motility and invasiveness (Igarashi and Hakomori, 1992, 1997; Refs. *20* and *24*)
Platelet activation and discharge from activated human platelets (Yatomi and Igarashi, 1995, 1997; Refs. *25* and *26*)
Retraction of neurite in neuronal cells (Moolenaar, 1996; Ref. *31*)
Activation of myocyte IK(Ach)-potassium channel (Jakobs and Pott, 1996; Ref. *29*)
Activation (H_2O_2 generation) of thyroid FRTL-5 cells (Okajima and Kondo, 1997; Ref. *64*)

Involvement of sphingosine in apoptosis induction. As a possible mechanism for signaling, we have addressed, in the past three years, the question that Sph which is derived from ceramide, may play a crucial role in some apoptotic systems, cooperatively with or independently from ceramide signaling. (i) We first studied the sphingolipid changes and their roles during the TNFα-induced apoptosis in human neutrophils (*35*) and found that TNFα causes a 3-fold increase of endogenous Sph content during the 30-60 min incubation, prior to the apoptotic morphological changes observed. The increased levels of Sph reach 5-10 μM in the cells, and the corresponding concentrations of exogenously added Sph induce apoptosis in neutrophils, mimicking TNFα, whereas no effect is observed with similar concentrations of cell-permeable ceramide (C8) or Sph-1-P, both primary metabolites of Sph, when exogenously added. (ii) Similar results were obtained with human leukemic HL-60 cells treated with PMA. PMA treatment causes terminal differentiation of HL-60 cells into macrophages and induces subsequent apoptosis (after 24-48 h incubation). Endogenous Sph content increases 3.3-fold during the 30 min incubation upon PMA treatment. Furthermore, we observed the elevation of ceramidase activity in PMA-treated differentiated cells by examining the conversion of [^{3}H]C-8 ceramide to [^{3}H]sphingosine (*36*). These results together strongly suggest that, in some cells, Sph may function as an endogenous modulator mediating the apoptotic signal triggered by extracellular stimulants.

The notion of Sph involvement in apoptotic signaling was recently supported by several works from other labs including Sabbadini's and Mann's groups, in which enhancement of endogenous Sph and its involvement in the signaling of TNFα-induced apoptosis and negative inotropic effects in mammalian myocytes were reported (*37, 38*).

Mechanisms of sphingosine-induced apoptosis. Exogenously added sphingosine and its methylated derivative *N,N*-dimethylsphingosine (DMS), metabolically a more stable lipid than sphingosine as will be mentioned later, both cause apoptosis (5 μM, 6 h) in a variety of solid cancer cell lines, in addition to leukemic cells, but not in normal fibroblasts or HUVECs (*39*). In the presence of serum, ceramide analogs failed to cause apoptosis, whereas induction of apoptosis by sphingosine is independent from serum (*39*). The mechanisms underlying these phenomena were next investigated, and we found: (i) Induction of apoptosis by sphingosine is strongly correlated with inhibition of MAPK which is highly expressed in some solid cancer cells. EGF-induced apoptosis in EGFR-overexpressed cells also supports the idea that inhibition of MAPK cascade mediates apoptotic signaling in this type of cancer cell (*40*). (ii) Some mechanisms of MAPK inhibition by sphingosine were studied: (a) there is no direct inhibi-

tion on MAPK, (b) PKC inhibition by Sph/DMS is not involved, (c) tyrosine phosphatase is stimulated 2-4-fold. (iii) Sph (2-5 μM) down-regulates Bcl-2 expression in HL-60 cells (which contain a high concentration of this anti-apoptotic protein) (*41*) as well as Bcl-X_L expression in human prostatic carcinoma DU-145 cells (*42*). However, ceramide does not affect any of these processes. Effect of ceramide on apoptosis was reported to be abolished in Bcl-2 overexpressing cells (*43*). On the other hand, ceramide strongly activates SAPK, but Sph does not or does very weakly. (iv) We found a Sph/DMS dependent protein kinase which phosphorylates 14-3-3 proteins in A31 fibroblast cells (*44*), although any relation with apoptosis induction remains to be studied. Very recently, inhibition of DNA primase by Sph/DMS (but not by ceramide) in HL-60 cells was also reported (*45*). In summary, the underlying mechanisms in apoptosis induction by each sphingolipid, ceramide and sphingosine, are obviously distinct (summarized in Fig. 2). These sphingolipids act cooperatively in some cells (*46*), but in other cells they act oppositely or independently (*e.g.*, Ref. *12*). It should be noted here that many sphingolipid-related phenomena are expressed in a cell type-specific manner and that, in a certain cell, the balance among these sphingolipids (including Sph-1-P) may determine the fates of cells such as apoptosis, cell cycle arrest, cell senescence, and cell proliferation (*12, 47*).

4. Functional roles of *N*-methylsphingosines

The existence of a sphingosine methylation pathway which produces DMS was first discovered in our laboratory almost 8 years ago, using metabolic labeling methods with [^{3}H]serine or [^{3}H]AdoMet in some cancer cells and in animal brain tissues (*48-50*). We also identified the existence of tiny amounts of DMS in human brain extracts with FAB-mass spectrometry (*51*). Very recently, with newly-developed LC/ionspray ionization mass spectrometry, Mano *et al.* (*52*) identified and quantified the amount of DMS in HL-60 cells as about 3 pg/10^6 cells, similar levels as psychosine, and 1/10, 1/100 of Sph-1-P and Sph levels, respectively. Since the amount of DMS detected in HL-60 cells or human brain was so minute, the physiological significance of cellular DMS is not clear at present and remains to be studied in the future.

In the meantime, Sph added to the cells is rapidly metabolized to other compounds, whereas, DMS is metabolically very stable in the cell (*20*), although we recently found that DMS is phosphorylated to DMS-1-P in human platelets upon stimulation (*53*). Thus, it exhibits stronger or exaggerated properties of Sph as a "metabolically stable sphingosine" in the cell. Separated from its physiological relevance, therefore, we took advantage of DMS as a metabolically stable Sph, in the following studies.

Regulation of enzymatic activities by DMS. The concept of a "SM cycle" or "sphingolipid signaling pathway" was evoked from the findings of Sph, the degradation product of this pathway, as an endogenous lipid inhibitor of PKC ($KI_{50}=50\ \mu M$), and its possible involvement in the regulation of cellular activities through PKC regulation (*8, 33*). We demonstrated that D-*erythro*-DMS is a cell permeable, more potent inhibitor of PKC ($KI_{50}=10$-$15\ \mu M$) than Sph *in vitro* (*54*) and *in vivo* (*55*), exhibiting strong inhibitory effects on *in vivo* cancer cell growth in nude mice (*56*) and on agonist-induced platelet activation (*55, 57*). Very recently, we revealed that DMS is the strongest inhibitor of sphingosine kinase ($KI_{50}=2.5\ \mu M$) so far known, in a competitive manner with Sph (Yatomi *et al.*, unpublished observation), showing inhibitory effects on the synthesis and release of Sph-1-P from human platelets (*58*). Additional effects on other enzymes such as MAPK, tyrosine-phosphatase, and Sph/DMS dependent protein kinase were already mentioned in the previous chapter.

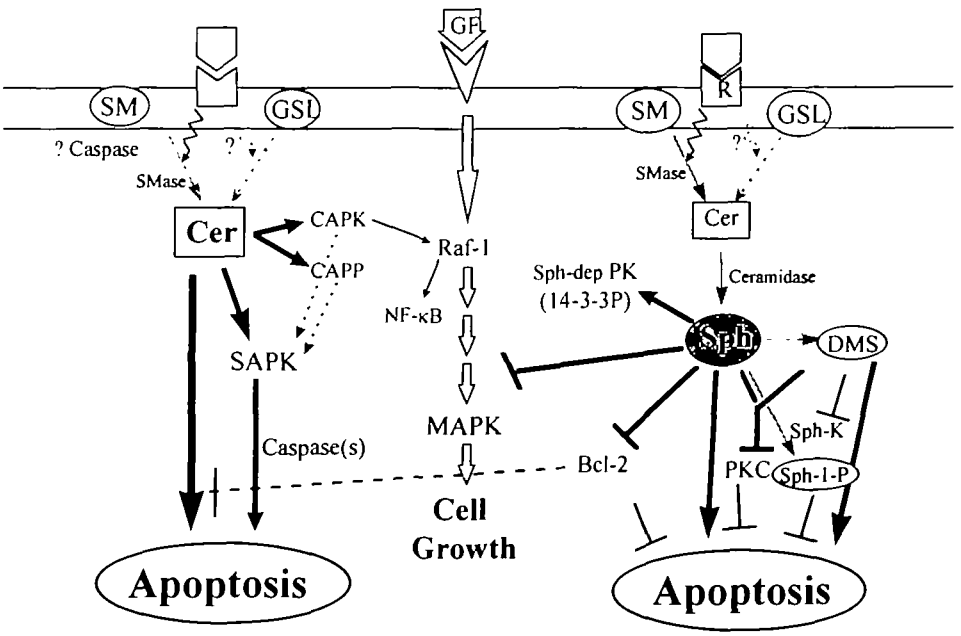

Fig. 2. **Working hypothesis on roles of sphingosines in cancer cell apoptosis induction.** Ceramide (Cer) activates stress-activated protein kinase (SAPK) probably through CAPK or CAPP activation, resulting in final caspase(s) activation. Cer is also reported to activate Raf-1/MAPK pathway which is usually stimulated by various growth factors (GF), and to activate NF-κB which is now considered to prevent apoptosis, although it remains controversial. On the other hand, sphingosine (Sph) inhibits bcl-2 expression, PKC and MAPK activation (each is regarded to prevent apoptosis) through as yet unknown mechanisms (possibly involving PKC, tyrosine-phosphatase, or Sph-dependent protein kinase). DMS inhibits Sph kinase and subsequent Sph-1-P production, which is proposed now to prevent apoptosis in some cells. The distinct modes of action for Cer and Sph may well explain the different dependence on serum factors in *in vitro* apoptosis induction by the two sphingolipids. The causal relationship of SMase activation and various caspase activation is not clear at present.

DMS as a potent apoptosis inducer in solid cancer cells. Based upon these bioregulative properties of DMS, we further pursued the applicability of this lipid as a possible therapeutic agent in a variety of disease model systems. The most promising one might be apoptosis induction for various solid cancer cells (*36, 39, 40, 56*). In these studies, DMS was shown to effectively induce apoptosis, not only in leukemia cells, but also in various solid cancer cells which show relatively higher activity of MAPK. Very recently, we further demonstrated that DMS significantly inhibits the *in vivo* growth of human epidermoid carcinoma KB-3-1 and even its multidrug-resistant subline, KB-C2 cells injected into nude mice, by inducing *in vivo* apoptosis (*59*). In agreement, Spiegel *et al.* also observed that DMS is a strong apoptosis inducer for cancer cells, attributing the apoptosis-inducing ability of DMS to its inhibition of sphingosine kinase and Sph-1-P production, preventing ceramide-induced apoptosis (*12*).

***N,N,N*-trimethylsphingosine (TMS) as a potent anti-inflammatory agent.** TMS is a synthetic sphingosine analogue, which was designed and introduced to reduce the cytotoxicity of DMS and to enhance water solubility for easier *in vivo* administration (Fig. 3). It possesses a strong PKC inhibitory activity like DMS (*55, 56*). In the early period of our study, based on this fact, we investigated and demonstrated the applicability of TMS in cancer metastasis prevention and platelet inactivation (*e.g.*, Refs. *55* and *57*). However, our recent studies revealed that these compounds have distinct properties for other enzymes. For example, TMS does neither induce apoptosis (*39*) nor activate Sph/DMS dependent protein kinase (*44*). TMS inhibits Sph kinase, but very weakly, compared to DMS (*58*). On the other hand, TMS does strongly inhibit IL-1β-induced NF-κB activation in HUVEC, yet DMS does not (*60*).

In collaboration with other groups, we recently demonstrated that TMS shows remarkable anti-inflammatory activities, especially on prevention of reperfusion injury in various ischemia animal model experiments (*61-63*). These studies clearly indicate that TMS inhibits neutrophil adhesion to endothelial cells in injured blood vessels, through inhibiting acute P-selectin expression on the endothelial cells and/or, as mentioned above, E-selectin synthesis *via* suppressing NF-κB activation. A hypotheti-

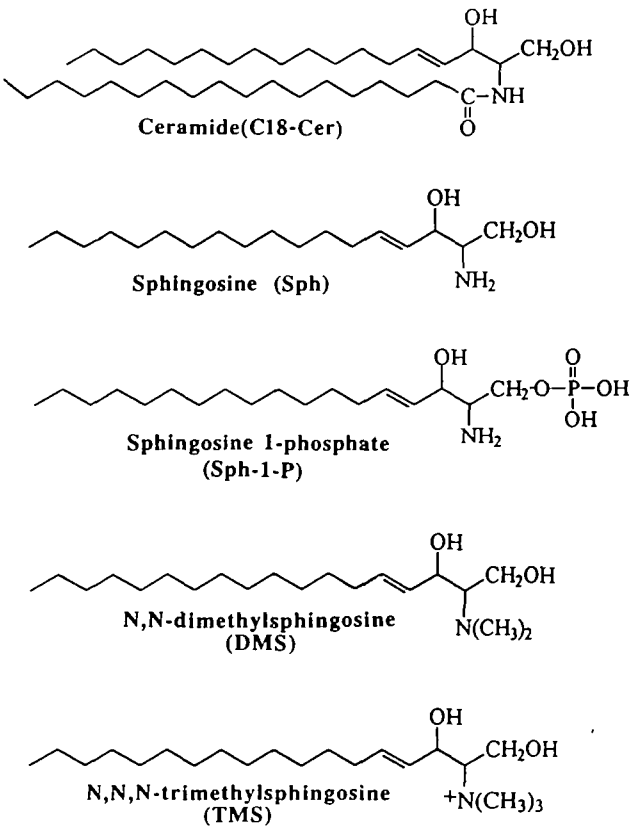

Fig. 3. **Structures of sphingosine-related derivatives.**

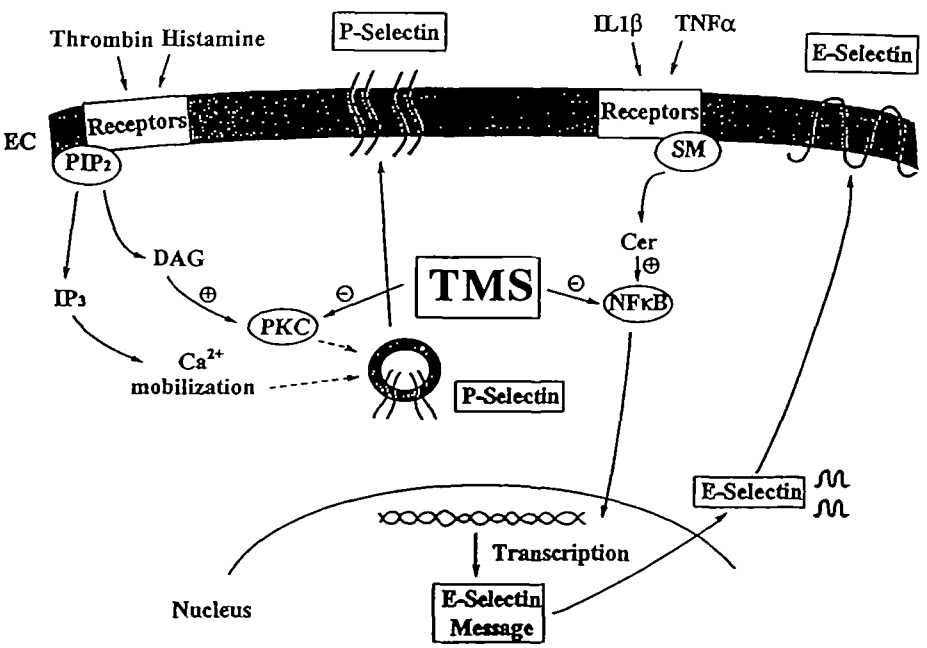

Fig. 4. **Conceivable mechanisms of TMS effects on prevention of reperfusion injury through inhibition of P- and E-selectin expression on endothelial cells.** P- or E-selectin expression on the endothelial cell surface is considered essential for neutrophil adhesion which mainly causes reperfusion injury. Various factors including thrombin and histamine activate endothelial cells through PIP2 breakdown pathways (IP_3-Ca^{2+}, DAG-PKC). PKC and Ca^{2+} cause acute P-selectin expression on the cell surface. Inflammatory cytokines such as IL-1β and TNF-α activate a transcription factor, NF-κB, which triggers *de novo* synthesis of E-selectin and later (in 4-24 h period) its cell surface expression. TMS may prevent P- and E-selectin expression through its inhibitory actions on PKC and NF-κB in the stimulated endothelial cells, thereby preventing reperfusion injury following ischemic insults.

cal mechanism of TMS effects on prevention of reperfusion injury, principally through inhibition of P- and E-selectin expression on endothelial cells, is summarized in Fig. 4.

5. Future perspectives for sphingo(physio)logy

The author discussed in this article the functional roles of Sph, Sph-1-P, and methylsphingosines (DMS and TMS), in regard to sphingolipid signaling pathways, based mainly upon our own findings in our recent studies. In the past 10 years, the research in this field has developed with an enormous speed. Although most principal enzymes or effectors involved in these signaling pathways have yet to be purified and cloned (except the acid SMase and the acid ceramidase), in the next few years the rapid development of these studies is expected to be accomplished, thus promoting further our understanding of the precise roles of sphingolipid signaling pathways in cellular functions. However, contradictory to the common thoughts often expressed, this author strongly believes that this accomplishment will not be the goal, but rather a prelude for future sphingolipid research, sphingology, and its successful application in medical fields such as diagnosis or therapeutics of various human diseases (called sphingophysiology), the beginnings of which were discussed briefly here.

The original studies mentioned here were supported by funds from The Biomembrane Institute, in part under research contracts with Otsuka Pharmaceutical Co. and Seikagaku Corp. The author expresses appreciation to Prof. S. Hakomori, Department of Pathobiology University of Washington, for his encouragement throughout this study and many other researchers who worked in The Biomembrane Institute in collaboration with these projects. The author also thanks Dr. Y. Nagai, Life Science Institute of Mitsubishi Kasei, Tokyo, for his advise on writing this review article and Dr. E.A. Sweeney, Fred Hutchinson Cancer Research Center, for her scientific editing of the manuscript.

REFERENCES

1. Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**, 693-698
2. Berridge, M.J. and Irvine, R.F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315-321
3. Toker, A. and Cantley, L.C. (1997) Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* **387**, 673-676
4. Okazaki, T., Bell, R.M., and Hannun, Y.A. (1989) Sphingomyelin turnover induced by vitamin D3 in HL-60 cells: Role in cell differentiation. *J. Biol. Chem.* **264**, 19076-19080
5. Kolesnick, R. and Golde, D.W. (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell* **77**, 325-328
6. Hannun, Y.A. (1996) Functions of ceramide in coordinating cellular responses to stress. *Science* **274**, 1855-1859
7. Spiegel, S. and Merrill, A.H., Jr. (1996) Sphingolipid metabolism and cell growth regulation. *FASEB J.* **10**, 1388-1397
8. Hakomori, S. (1990) Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. *J. Biol. Chem.* **265**, 18713-18716
9. Hakomori, S. and Igarashi, Y. (1993) Gangliosides and glycosphingolipids as modulators of cell growth, adhesion, and transmembrane signaling. *Adv. Lipid Res.* **25**, 147-162
10. Hakomori, S. and Igarashi, Y. (1995) Functional role of glycosphingolipids in cell recognition and signaling. *J. Biochem.* **118**, 1091-1103
11. Fourcade, O., Simon, M.F., Viode, C., Rugani, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L., and Chap, H. (1995) Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell* **80**, 919-927
12. Cuvillier, O., Pirianov,G., Kleuser, B., Vanek, P.G., Coso, O.A., Gutkind, S., and Spiegel, S. (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* **381**, 800-803
13. Zhang, P., Liu, B., Jenkins, G.M., Hannun, Y.A., and Obeid, L.M. (1997) Expression of neutral sphingomyelinase identifies a distinct pool of sphingomyelin involved in apoptosis. *J. Biol. Chem.* **272**, 9609-9612
14. Stoffel, W., Heimann, G., and Hellenbroich, B. (1973) Sphingosine kinase in blood platelets. *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 562-566
15. Ghosh, T.K., Bian, J., and Gill, D.L. (1990) Intracellular calcium release mediated by sphingosine derivatives generated in cells. *Science* **248**, 1653-1656
16. Zhang, H., Desai, N.N., Olivera, A., Seki, T., Brooker, G., and Spiegel, S. (1991) Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. *J. Cell Biol.* **114**, 155-167
17. Olivera, A. and Spiegel, S. (1993) Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature* **365**, 557-560
18. Choi, O.H., Kim, J.-H., and Kinet, J.-P. (1996) Calcium mobilization via sphingosine kinase in signaling by the FcεRI antigen receptor. *Nature* **380**, 634-636
19. Ruan, F., Hakomori, S., and Igarashi, Y. (1992) Chemical synthesis of D-*erythro*-sphingosine-1-phosphate, and its inhibitory effect on cell motility. *Biomed. Chem. Lett.* **2**, 973-978
20. Sadahira, Y., Ruan, F., Hakomori, S., and Igarashi, Y. (1992) Sphingosine-1-phosphate, a specific endogenous signaling molecule controlling cell motility and tumor cell invasiveness. *Proc. Natl. Acad. Sci. USA* **89**, 9686-9690
21. Sadahira, Y., Zheng, M., Ruan, F., Hakomori, S., and Igarashi, Y. (1994) Sphingosine-1-phosphate inhibits extracellular matrix protein-induced haptotactic motility but not adhesion of B16 mouse melanoma cells. *FEBS Lett.* **340**, 99-103
22. Bornfeldt, K.E., Graves, L.M., Raines, E.W., Igarashi, Y., Wayman, G., Yamamura, S., Yatomi, Y., Sidhu, J.S., Krebs, E. G., Hakomori, S., and Ross, R. (1995) Sphingosine-1-phosphate inhibits PDGF-induced chemotaxis of human arterial smooth muscle cells: spatial and temporal modulation of PDGF chemotactic signal transduction. *J. Cell Biol.* **130**, 193-206
23. Yamamura, S., Sadahira, Y., Ruan, F., Hakomori, S., and Igarashi, Y. (1996) Sphingosine-1-phosphate inhibits actin nucleation and pseudopodium formation to control cell motility of mouse melanoma cells. *FEBS Lett.* **382**, 193-197
24. Yamamura, S., Yatomi, Y., Ruan, F., Sweeney, E.A., Hakomori, S., and Igarashi, Y. (1997) Sphingosine 1-phosphate regulates melanoma cell motility through a receptor-coupled extracellular action and in a pertussis toxin insensitive manner. *Biochemistry* **36**, 10751-10759
25. Yatomi, Y., Ruan, F., Hakomori, S., and Igarashi, Y. (1995) Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. *Blood* **86**, 193-202
26. Yatomi, Y., Yamamura, S., Ruan, F., and Igarashi, Y. (1997) Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J. Biol. Chem.* **272**, 5291-5297
27. Yatomi, Y., Igarashi, Y., Yang, L., Hisano, N., Ruomei, Q., Asazuma, N., Satoh, K., Ozaki, Y., and Kume, S. (1997) Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent in plasma and serum. *J. Biochem.* **121**, 969-973
28. Yatomi, Y., Ruan, F., Ohta, H., Welch, R.J., Hakomori, S., and Igarashi, Y. (1995) Quantitative measurement of sphingosine 1-phosphate in biological samples by acylation with radioactive acetic anhydride. *Anal. Biochem.* **230**, 315-320
29. Bunemann, M., Brandts, B., Meyer zu Heringdorf, D., van Koppen, C.J., Jakobs, K.H., and Pott, L. (1995) Activation of muscarinic K^+ current in guinea-pig atrial myocytes by sphingosine-1-phosphate. *J. Physiol.* **489**, 701-707

30. Igarashi, Y., Yatomi, Y., and Kickler, T.H. (1997) Sphingosine-phosphate content in platelet plasma samples correlates with poor platelet increments after transfusion and occurrences of platelet transfusion reactions in patients. *Am. J. Hematol.* in press
31. Postma, F.R., Jalink, K., Hengeveld, T., and Moolenaar, W.H. (1996) Sphingosine-1-phosphate rapidly induces Rho-dependent neurite retraction: action through a specific cell surface receptor. *EMBO J.* **15**, 2388-2395
32. Rother, J., van Echten, G., Schwarzmann, G., and Sandhoff, K. (1992) Biosynthesis of sphingolipids: dihydroceramide and not sphinganine is desaturated by cultured cells. *Biochem. Biophys. Res. Commun.* **189**, 14-20
33. Hannun, Y.A. and Bell, R.M. (1989) Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science* **243**, 500-507
34. Gamard, C.J., Dbaibo, G.S., Liu, B., Obeid, L.M., and Hannun, Y.A. (1997) Selective involvement of ceramide in cytokine-induced apoptosis. Ceramide inhibits phorbol ester activation of nuclear factor kappa B. *J. Biol. Chem.* **272**, 16474-16481
35. Ohta, H., Yatomi, Y., Sweeney, E.A., Hakomori, S., and Igarashi, Y. (1994) A possible role of sphingosine in induction of apoptosis by tumor necrosis factor-α in human neutrophils. *FEBS Lett.* **355**, 267-270
36. Ohta, H., Sweeney, E.A., Masamune, A., Yatomi, Y., Hakomori, S., and Igarashi, Y. (1995) Induction of apoptosis by sphingosine in human leukemic HL-60 cells: A possible endogenous modulator of apoptotic DNA fragmentation occurring during phorbol ester-induced differentiation. *Cancer Res.* **55**, 691-697
37. Krown, K.A., Page, M.T., Nguyen, C., Zechner, D., Gutierrez, V., Comstock, K.L., Glembotski, C.C., Quintana, P.J., and Sabbadini, R.A. (1996) Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. *J. Clin. Invest.* **98**, 2854-2865
38. Oral, H., Dorn, G.W. 2nd, and Mann, D.L. (1997) Sphingosine mediates the immediate negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian cardiac myocyte. *J. Biol. Chem.* **272**, 4836-4842
39. Sweeney, E.A., Sakakura, C., Shirahama, T., Masamune, A., Ohta, H., Hakomori, S., and Igarashi, Y. (1996) Sphingosine and *N,N*-dimethylsphingosine induce apoptosis in a variety of human cancer cell lines. *Int. J. Cancer* **66**, 358-366
40. Sakakura, C., Sweeney, E.A., Shirahama, T., Solca, F., Kohno, M., Hakomori, S., Fischer, E.H., and Igarashi, Y. (1997) Inhibition of MAPKinase by sphingosine and its methylated derivative *N,N*-dimethylsphingosine; a correlation with induction of apoptosis in solid tumor cells. *Int. J. Oncol.* **11**, 31-39
41. Sakakura, C., Sweeney, E.A., Shirahama, T., Hakomori, S., and Igarashi, Y. (1996) Suppression of Bcl-2 gene expression by sphingosine in the apoptosis of human leukemic HL-60 cells during phorbol ester-induced terminal differentiation. *FEBS Lett.* **379**, 177-180
42. Shirahama, T., Sakakura, C., Sweeney, E.A., Takemoto, M., Ozawa, M., Ohi, Y., and Igarashi, Y. (1997) Sphingosine induces apoptosis in androgen-independent human prostatic carcinoma DU-145 cells by suppression of bcl-X_L gene expression. *FEBS Lett.* **407**, 97-100
43. Zhang, J., Alter, N., Reed, J.C., Borner, C., Obeid, L.M., and Hannun, Y.A. (1996) Bcl-2 interrupts the ceramide-mediated pathway of cell death. *Proc. Natl. Acad. Sci. USA* **93**, 5325-5328
44. Megidish, T., White, T., Takio, K., Titani, K., Igarashi, Y., and Hakomori, S. (1995) The signal modulator protein 14-3-3 is a target of sphingosine- or *N,N*-dimethylsphingosine-dependent kinase in 3T3(A31) cells. *Biochem. Biophys. Res. Commun.* **216**, 739-747
45. Tamiya-Koizumi, K., Murate, T., Suzuki, M., Simbulan, C.M., Nakagawa, M., Takemura, M., Furuta, K., Izuta, S., and Yoshida, S. (1997) Inhibition of DNA primase by sphingosine and its analogues parallels with their growth suppression of cultured human leukemic cells. *Biochem. Mol. Biol. Int.* **41**, 1179-1189
46. Jarvis, W.D., Fornari, F.A., Traylor, R.S., Martin, H.A., Kramer, L.B., Erukulla, R.K., Bittman, R., and Grant, S. (1996) Induction of apoptosis and potentiation of ceramide-mediated cytotoxicity by sphingoid bases in human myeloid leukemia cells. *J. Biol. Chem.* **271**, 8275-8284
47. Coroneos, E., Martinez, M., McKenna, S., and Kester, M. (1995) Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines. Implications for cellular proliferation and differentiation. *J. Biol. Chem.* **270**, 23305-23309
48. Igarashi, Y. and Hakomori, S. (1989) Enzymatic synthesis of *N,N*-dimethylsphingosine: Demonstration of the sphingosine; *N*-methyltransferase in mouse brain. *Biochem. Biophys. Res. Commun.* **164**, 1411-1416
49. Igarashi, Y., Kitamura, K., Toyokuni, T., Dean, B., Fenderson, B., Ogawa, T., and Hakomori, S. (1990) A specific enhancing effect of *N,N*-dimethylsphingosine on epidermal growth factor receptor autophosphorylation: Demonstration of its endogenous occurrence (and its virtual absence of unsubstituted sphingosine) in human epidermoid carcinoma A431 cells. *J. Biol. Chem.* **265**, 5385-5389
50. Felding-Habermann, B., Igarashi, Y., Fenderson, B.A., Park, L. S., Radin, N.S., Inokuchi, J., Strassmann, G., Handa, K., and Hakomori, S. (1990) A ceramide analogue inhibits T cell proliferative response through inhibition of glycosphingolipid synthesis and enhancement of *N,N*-dimethylsphingosine synthesis. *Biochemistry* **29**, 6314-6322
51. Nudelman, E.D., Levery, S.B., Igarashi, Y., and Hakomori, S. (1992) Plasmalopsychosine: A novel plasmal (fatty aldehyde) conjugate of psychosine with cyclic acetal linkage: Isolation and characterization from human brain white matter. *J. Biol. Chem.* **267**, 11007-11016
52. Mano, N., Oda, Y., Yamada, K., Asakawa, N., and Katayama, K. (1997) Simultaneous quantitative determination method for sphingolipid metabolites by liquid chromatography/ionspray ionization tandem mass spectrometry. *Anal. Biochem.* **244**, 291-300
53. Yatomi, Y., Ozaki, Y., Satoh, K., Kume, S., Ruan, F., and Igarashi, Y. (1997) *N,N*-Dimethylsphingosine phosphorylation in human platelets. *Biochem. Biophys. Res. Commun.* **231**, 848-851
54. Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K., and Racker, E. (1989) Effect of chemically well-defined sphingosine and its *N*-methyl derivatives on protein kinase C and src kinase activities. *Biochemistry* **28**, 6796-6800
55. Okoshi, H., Hakomori, S., Nisar, M., Zhou, Q., Kimura, S., Tashiro, K., and Igarashi, Y. (1991) Cell membrane signaling as target in cancer therapy II: Inhibitory effect of *N,N,N*-trimethylsphingosine on metastatic potential of murine B16 melanoma cell line through blocking of tumor cell-dependent platelet aggregation. *Cancer Res.* **51**, 6019-6024
56. Endo, K., Igarashi, Y., Nisar, M., Zhou, Q., and Hakomori, S. (1991) Cell membrane signaling as target in cancer therapy: Inhibitory effect of *N,N*-dimethyl and *N,N,N*-trimethylsphingosine derivatives on in vivo and in vitro growth of human tumor cells in nude mice. *Cancer Res.* **51**, 1613-1618
57. Handa, K., Igarashi, Y., Nisar, M., and Hakomori, S. (1991) Down-regulation of GMP-140 expression on platelets by *N,N*-dimethyl and *N,N,N*-trimethyl derivatives of sphingosine. *Biochemistry* **30**, 11682-11686
58. Yatomi, Y., Ruan, F., Megidish, T., Toyokuni, T., Hakomori, S., and Igarashi, Y. (1996) *N,N*-Dimethylsphingosine inhibition of sphingosine kinase and sphingosine 1-phosphate activity in human platelets. *Biochemistry* **35**, 626-633
59. Shirahama, T., Sweeney, E.A., Sakakura, C., Nishiyama, K., Akiyama, S., Hakomori, S., and Igarashi, Y. (1997) *In vitro* and *in vivo* induction of apoptosis by sphingosine and *N,N*-dimethylsphingosine in human epidermal carcinoma KB-3-1 and its multidrug resistant cells. *Clin. Cancer Res.* **3**, 257-264
60. Masamune, A., Hakomori, S., and Igarashi, Y. (1995) *N,N,N*-Trimethylsphingosine inhibits interleukin-1-induced NF-κB activation and consequent E-selectin expression in human umbil-

ical vein endothelial cells. *FEBS Lett.* **367**, 205-209
61. Murohara, T., Buerke, M., Margiotta, J., Ruan, F., Igarashi Y., Hakomori, S., and Lefer, A.M. (1995) Myocardial and endothelial protection by *N,N,N*-trimethylsphingosine in feline myocardial ischemia and reperfusion injury. *Am. J. Physiol.* **269** (*Heart Circul. Physiol.*) **38**, H504-514
62. Scalia, R., Murohara, T., Delyani, J.A., Nossuli, O., and Lefer, A.M. (1996) Myocardial protection by *N,N,N*-trimethylsphingosine in ischemia reperfusion injury is mediated by inhibition of p-selectin. *J. Leukoc. Biol.* **59**, 317-324
63. Vedder, N.B., Han, K.T., Igarashi, Y., Singhal, A.K., Hakomori, S., and Winn, R.K. (1994) Trimethylsphingosine blocks transmembrane signaling in neutrophils/endothelial cells/platelets and inhibits subsequent neutrophil-mediated reperfusion injury and inflammatory response. *39th Annual Meeting of Plastic Surgery Research Council* 16-19
64. Okajima, F., Tomura, H., Sho, K., Kimura, T., Sato, K., Im, D.S., Akbar, M., and Kondo, Y. (1997) Sphingosine 1-phosphate stimulates hydrogen peroxide generation through activation of phospholipase C-Ca^{2+} system in FRTL-5 thyroid cells: possible involvement of guanosine triphosphate-binding proteins in the lipid signaling. *Endocrinology* **138**, 220-229