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## Ceramide: A Common Pathway for Atherosclerosis?

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### Abstract

Plasma sphingomyelin concentration is correlated with the development of atherosclerosis. It has been found to exist in significantly higher concentrations in aortic plaque. This appears to have clinical relevance as well as it has been shown to be an independent predictor of coronary artery disease. Ceramide, the backbone of sphingolipids, is the key component which affects atherosclerotic changes through its important second-messenger role. This paper sheds light on some of the current literature supporting the significance of ceramide with respect to its interactions with lipids, inflammatory cytokines, homocysteine and matrix metalloproteinases. Furthermore, the potential therapeutic implications of modulating ceramide concentrations are also discussed.

### Keywords

Ceramide; atherosclerosis; homocysteine; C-reactive protein; lipids

### 1. Introduction

Atherogenesis and its ensuing inflammatory changes result in some of the most common causes of mortality in the Western World, namely myocardial infarction, stroke and peripheral vascular disease. The disease ties hyperlipidemia, inflammatory cytokines as well as other traditional risk factors to its development. Evidence has also shown a probable implication of sphingolipids in atherosclerosis. Jiang et al. have shown that plasma sphingomyelin concentration is correlated with the development of atherosclerosis and furthermore was also shown to be an independent predictor of coronary artery disease [1]. The accumulation of sphingolipids noted in atherosclerosis appears to influence the atherogenic process by affecting lipoprotein metabolism and lipid efflux and by acting as a key signaling molecule. This fact is well illustrated by Schissel et al. [2], who found a 10 to 50-fold higher content of ceramide (the backbone of all sphingolipids) in low density lipoproteins (LDL) of atherosclerotic lesions when compared with plasma LDL. Additionally, Li et al. showed that feeding mice with a sphingolipid-rich diet produces significantly greater atherosclerotic lesions in aortic specimens [3]. How sphingolipids, oxidized-LDL (ox-LDL), inflammatory cytokines and endothelial cells interact is not entirely defined yet. However, it becomes relatively clear is that ceramide is an important second-messenger in multiple atherosclerotic processes. The objective of this review is to reveal some of the research supporting this key role of ceramide related to many of the well-known risk markers or factors for cardiovascular disease, such as lipoproteins, inflammatory cytokines, and homocysteine. Additionally, we reviewed preliminary studies in modification

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of the ceramide biosynthetic pathway which directly affects the atherosclerotic burden. Thus, ceramide could be a new therapeutic target for cardiovascular disease.

## 2. Lipoproteins and ceramide

A critical event in the formation of an atherosclerotic plaque is the aggregation of atherogenic lipoproteins. These lipoproteins thereafter play an important role in promoting a series of biological actions that are key to the initiation and progression of atherosclerosis. Aggregation of lipoproteins significantly increases the amount of lipoprotein retained in the foam cells [4]. For example, aggregated LDL is a potent inducer of foam cell formation from macrophages [5]. Clearly, lipoprotein aggregation could occur by sphingomyelin hydrolysis with sphingomyelinase (SMase) [6]. SMase is a sphingomyelin-specific form of phospholipase C and hydrolyzes the phosphodiester bond of sphingomyelin, yielding ceramide and phosphocholine [7-9]. Two distinct SMases have been described: acidic SMase (A-SMase) and neutral SMase (N-SMase). A-SMase is lysosomal while N-SMase is localized to the outer leaflet of the plasma membrane [10,11]. Experimental data support the role of lipoprotein sphingomyelin and arterial SMase in atherogenesis. Sphingomyelin, which is transported into the arterial wall by atherogenic lipoproteins, is in turn affected by arterial wall SMase, increasing in ceramide content and promoting lipoprotein aggregation [2]. LDL extracted from human atherosclerotic lesions is highly enriched in sphingomyelin compared with plasma LDL [12,13].

Sphingolipids containing sphingomyelin [12] are key components of cellular plasma membranes. They are not found in a free form in plasma, but rather are associated with lipoproteins and are particularly abundant in LDL [14]. Additionally, sphingolipids have not been found in lipoprotein deficient plasma, but have been associated with LDL in both normal subjects and patients with familial hypercholesterolemia [15,16]. Sphingolipid enrichment in vitro increased aggregation of mouse lipoproteins. Macrophage-derived SMase was also shown to stimulate the aggregation of very low density lipoproteins (VLDL) and LDL [12]. Thus, lipoprotein aggregation induced by SMase is dependent on ceramide increase.

Schissel et al. analyzed plaque harvested during standard reconstructive surgery for abdominal aortic aneurysms, and found that ceramide was elevated in the areas of aggregated LDL (10-50 fold higher than plasma LDL) and was 2-3 fold higher than that in early human atherosclerotic streaks of the aorta of a 43-year old heart donor [2]. More studies on the analysis of human atherosclerotic plaques revealed that the plaque glycolipids consisted mainly of glucosylceramide and also contained appreciable amounts of lactosylceramide which was completely absent in the unaffected intima [17]. In animal models of atherosclerosis, ceramide contents were significantly increased in atherosclerotic plaques than the normal vascular wall [2,18]. Modifications in LDL including oxidation to ox-LDL and sphingomyelin enrichment can alter the lipoprotein surface, which may in turn affect the atherosclerotic process. In an attempt to further define the relationships of these elements, it was noted that oxidized sphingomyelin-LDL was hydrolyzed 5-6 times more than native LDL [19]. This was not the case at neutral pH. At neutral pH, the hydrolysis by SMase yielded low levels of ceramide, which was below the threshold for ceramide-mediated aggregation in vitro [5]. It can be postulated that the situation may be very different in vivo, particularly in advanced lesions where the atherosclerotic plaque may be in a more acidic environment and the threshold for ceramide-mediated aggregation may be lower. Prior research has also shown that SMase treatment of LDL promoted uptake of lipoprotein and accumulation of cholesterol within macrophages [6]. Independently, it has been shown that sphingomyelin inhibits lipolysis of triglycerides by changing some of the properties of lipoprotein lipase [20].

### 3. Tumor necrosis factor- $\alpha$ and ceramide

The vascular endothelium is a major site for the action of TNF- $\alpha$ , which can cause endothelial dysfunction [21-23]. As more literature is emerging regarding the role of ceramide as a second messenger, it is important to define which factors affect the ceramide pathway. This will eventually become particularly important when assessing the clinical application of interfering with the ceramide pathway. Therefore, TNF- $\alpha$ , one of many cytokines, holds a very central role in arteriosclerosis. A direct relationship has been identified between TNF- $\alpha$  and ceramide, in which TNF- $\alpha$  has been shown to be responsible for increases in intracellular (vascular endothelial cells) ceramide content [24,25]. Zhang et al. found that TNF- $\alpha$  could trigger SMase and augment superoxide anion generation in endothelial cells. Furthermore, they found that, indeed, TNF- $\alpha$  caused a rapid increase of superoxide free radical within 2 minutes, but the effect was not sustained and dissipated after 60 minutes [24,25]. TNF- $\alpha$  can stimulate ceramide formation via the activation of both neutral and acid SMases, accompanied by ROS such as superoxide anion production [26]. TNF- $\alpha$  initiates the pathway through TNFR1 (55-kDa receptor) leading to phospholipase A2 activation, generation of arachidonic acid, and subsequent activation of SMase [27]. TNF- $\alpha$ -stimulated cells are redox-sensitive [28]. Some studies showed that ROS participate in TNF- $\alpha$ -mediated ceramide formation, suggesting that the ROS-sensitive pathway may be located upstream of ceramide formation [29]. While other studies demonstrated that ceramide plays a role as an intermediate in TNF- $\alpha$ -mediated ROS production [30].

In the clinical situation, the effects caused by TNF- $\alpha$  are more likely to be sustained given prolonged exposure. Inflammatory cytokines such as TNF- $\alpha$ , Interleukin-2 (IL-2) and endostatin stimulate ischemic reperfused myocardium to increase ceramide levels [31,32]. In animal studies, it has been shown that when exposed to inflammatory cytokines, the hepatic production of lipoproteins enriched in sphingomyelin and ceramides is significantly increased [33]. On the other hand, ceramide has additionally been found to promote IL-6 and consequently C-reactive protein (CRP), thereby having direct pro-inflammatory effects and participation in the atherosclerotic process [34]. Interestingly, Hirokawa et al. also showed that C2-ceramide, a highly cell-permeable ceramide analog, was able to stimulate human umbilical vein endothelial cells to express TNF- $\alpha$  [35]. These mutual inter-relationships between TNF- $\alpha$  and ceramide may determine a vicious cycle by which TNF- $\alpha$  and ceramide may potentiate their damaging effects during inflammation. Thus, we hypothesize that inflammation conditions including TNF- $\alpha$  may be the initiator of ceramide production and its signal functions. The goal should therefore be to prevent the ceramide cascade by decreasing the exposure of the endothelium to inflammatory cytokines. Several authors have been able to show reductions in TNF- $\alpha$  during ischemia with several different drugs including benazapril and amlodipine [36-39]. Furthermore, myocardial injury was actually shown to be smaller when there had been significant reductions in TNF- $\alpha$  [36]. In a septic shock mouse model, alpha-galactosyl ceramide sensitization was responsible for a rise in TNF- $\alpha$  which could be abolished by TNF- $\alpha$  antibody. Additionally, TNF- $\alpha$  deficient mice were not affected by the galactosyl ceramide [40]. Recent studies have shown that anti-TNF- $\alpha$  therapy could significantly improve endothelial functions in human subjects with vascular inflammation [41,42]. However, it is not known whether this therapy could be correlated with ceramide production in these individuals. Further studies are warranted.

### 4. Homocysteine and ceramide

Homocysteine, independent risk factor for arteriosclerosis and specifically coronary artery disease [43,44], has also been shown to increase levels of superoxide anion via the NADPH oxidase pathway [45]. However, it is not clear whether ceramide could be involved in homocysteine biological functions. Yi and colleagues investigated this possibility and found

that in a concentration-dependent fashion, homocysteine increased ceramide levels. At their highest dose of 80  $\mu\text{mol/L}$ , they saw a 47% increase in ceramide levels [46]. Interestingly, they also found that increased levels of ceramide were independent of SMase. It was a direct consequence of stimulation of ceramide synthase. In a more recent study, Yi et al. also showed that blocking the de novo synthesis of ceramide with myriocin significantly reduced homocysteine-induced ceramide production and glomerular injury in rats [47]. These studies demonstrate that the detrimental effects of homocysteine on many organ systems are mediated by ceramide signaling.

## 5. Matrix metalloproteinases and ceramide

Matrix metalloproteinases (MMPs) are a large family of zinc proteases which are involved in vascular remodeling, angiogenesis and atherosclerosis. MMPs are elevated in atherosclerotic lesions, and as such, it has been suspected that they are involved in the infiltration of inflammatory cells, smooth muscle cell migration/proliferation and plaque disruption [48-50]. It is known that ox-LDL and inflammatory cytokines affect MMPs. For example, ox-LDL enhances the expression of MMP-1 (collagenase) and MMP-9 as well as activating precursors to MMP-2 activators [51-53]. In light of this, Auge et al. were able to show that MMP-2 has a decisive role in triggering SMase and therefore increasing ceramide production. This was shown in two fashions: first by using anti-MMP-2 antibodies which effectively inhibited SMase activation and ceramide production, and second by using exogenous MMP-2 to activate the sphingomyelin/ceramide pathway as well as DNA synthesis [54]. This suggests that the high content of ox-LDL in the plaque is capable of triggering MMP-2, propagating the signaling of the ceramide pathway by activation of SMase. The exact mechanism by which MMP-2 activates SMase is not completely established yet as the nature and precise localization of this enzyme is not known [55].

## 6. Ceramide and reactive oxygen species

As already described, endogenous ceramide is generated primarily from sphingomyelin by SMase-induced hydrolysis, and it can also be generated de novo by the action of ceramide synthase [56]. Both ceramide production and ceramide function are involved in reactive oxygen species (ROS), which include  $\text{H}_2\text{O}_2$ , superoxide and hydroxyl radicals. As discussed above, inflammation cytokines could activate SMase and increase ceramide production mediated by increased ROS. Hernandez et al. showed that the generation of ceramide is indeed a downstream of ROS production [57]. This concept was further established by Pchejetski et al. who were recently able to show that the production of ROS was directly linked to an increase in ceramide.

On the other hand, ceramide has many biological functions mediated by ROS as signal molecules. Recent studies have shown that ceramide stimulates the production of ROS in human endothelial cells [58]. Superoxide production was noted to be stimulated 4-fold when aortic smooth muscle cells were incubated with lactosylceramide (LacCer) [14]. Similarly, Yeh et al. noted that the only analogue of ceramide to induce superoxide generation was LacCer, via a plasma membrane associated NADPH oxidase [59]. Zhang and colleagues found that ceramide caused a time-dependent rise in superoxide anion relative to controls and that this was attenuated by treatment with NADPH oxidase inhibitor. The activation of NADPH oxidase and the ensuing increase in superoxide anion are likely important clinical events as they are implicated in the pathophysiology of cardiovascular diseases such as atherosclerosis [60].

Ceramide may play an important role in ischemia/reperfusion injury, which is responsible for provoking cell injury [61,62]. There is significant evidence to support an important role for ROS in the reperfusion myocardial damage [63,64]. This role is facilitated by the

monoamine oxidases [65]. It has long been known that ceramide accumulation leads to cell death/apoptosis, what is somewhat paradoxical though, is the fact that one of its downstream metabolites sphingosine-1-phosphate (S1P) is a growth promoter and support cell survival [66]. Indeed, S1P preincubation has been shown to prevent mortality induced by hypoxia [67,68]. S1P has independently been shown to positively influence cardiac cell survival and is enhanced during ischemic periods [69].

## 7. Ceramide, SMase and nitric oxide (NO)

Nitric oxide (NO), produced by eNOS, is one of the most important regulatory mechanisms in vascular homeostasis such as endothelium-dependent vasorelaxation. Loss of NO bioavailability due to reduced synthesis and increased scavenging by ROS is a major cause of endothelial dysfunction in vascular disease states. Several lines of investigations have showed that ceramide plays an important role in NO related endothelial functions in the vascular system although controversies exist. Treatment with C2-ceramide significantly reduced endothelium-dependent vasorelaxation in small bovine coronary arteries in response to bradykinin. This effect was NO specific because C-ceramide had no further inhibitory effect on the vasorelaxation in response to bradykinin in the presence of eNOS inhibitor (L-NAME) [70]. However, Mogami et al. found that exogenous N-SMase induced endothelium-dependent relaxation via eNOS in bovine coronary arteries and that C8 ceramide also had vasodilatory effects, albeit less profound. Interestingly, they found that this was independent of calcium ( $Ca^{2+}$ ), which is unusual as most activation of eNOS is accompanied by  $Ca^{2+}$  elevation [64]. It also appears that SMase-induced increases in ceramide may have two effects on eNOS: 1). dissociation of eNOS from the plasma membrane and calveolin; and 2). activation of eNOS via phosphorylation [71,72]. The exact reasons for these controversial results from different reports are not clear. It may be related to differences in experimental conditions, model systems and reagents used. Recently, we have found that lactosylceramide at clinically relevant concentrations could significantly reduce endothelium-dependent relaxation in response to bradykinin in porcine coronary arteries. It is clear that ceramide can reduce NO bioavailability from endothelial cells. Zhang et al. directly measured NO levels in the intact endothelium of coronary arteries by using realtime fluorescence microscopy and found NO levels were reduced in ceramide-treated vessels [70]. Cell culture studies have shown that ceramide reduces the release of bioactive NO in human umbilical vein endothelial cells [73,74]. Overwhelming evidence demonstrates that ceramide-induced oxidative stress may be the major reason for its effects on the reduction of NO bioavailability and endothelial dysfunction. Indeed, ceramide and/or other sphingolipids stimulate the production of superoxide anion in vascular cells [58,75,76]. Accordingly, pretreatment of the arteries with tiron, achemical mimetic of SOD that is capable of removing superoxide anion could prevent ceramide-induced decreases in NO levels and endothelial dysfunction in small coronary arteries [77]. However, there are no data linking ceramide levels to vasomotor functions in humans yet. Thus, further studies of ceramides on clinical situations regarding to the vascular system are warranted.

## 8. Ceramide and potential therapeutics

Having described the multifaceted actions of ceramide in the development of atherosclerotic lesions, it seems evident that it would be a very attractive focus of potential pharmacologic intervention. For the sphingolipid biosynthetic pathway, multiple possible intervention sites are noted (Fig. 1). The first rate-limiting step in the sphingolipid biosynthetic pathway is catalyzed by palmitoyltransferase. It is known that this enzyme plays a significant role in the metabolism of sphingolipids, but its role in further lipid metabolism and the pathogenesis of atherosclerosis has not previously been explicitly shown. It has been noted that increases in palmitoyltransferase activity also increases sphingolipid production in liver [60] and lung



[78]. Likewise, the activity of palmitoyltransferase is increased in the aortas of rabbits fed a high cholesterol diet [79]. These facts give a great opportunity for medicinal intervention.

*Isaria sinclairii* is a fungus from which myriocin has been isolated. This fungus has been used in traditional Chinese medicine in an effort to attain eternal youth [80]. Myriocin was found to have a similar structure to sphingosine. Myriocin binding proteins, LCB1 and LCB2, are responsible for palmitoyltransferase activity [81]. Park et al. have shown that giving myriocin significantly decreased the levels of sphingomyelin in a dose dependent manner. Furthermore, they were able to show that myriocin also lowered total cholesterol, triglycerides, VLDL and LDL [82,83]. This is not very surprising, though, as myriocin has been found to cause suppression of HMG CoA reductase. Ultimately, these effects of myriocin may have impact on the atherogenic process. Indeed, this same group was able to show a reduction in the atherosclerotic lesion area by more than 98% in cases treated with myriocin. Hojjati et al. similarly found that myriocin administration caused a decrease in plasma sphingomyelin, ceramide, sphingosine, and sphingosine-1-phosphate levels, as well as an increase in plasma phosphatidylcholine levels. However, they did not show the decrease in total cholesterol, triglycerides, VLDL and LDL. This was attributed to the mode of administration, which was oral in Park et al's study and intraperitoneal for Hojjati et al. Myriocin did nevertheless cause a decrease in atherosclerotic lesions in mice on both chow and high fat, high cholesterol diets [84]. On a western diet, Hojjati et al. were able to show a mean decrease of 37% in atherosclerotic lesion [84]. Myriocin is also able to reduce levels of inflammatory proteins, suggesting a very significant role in reducing the extent of atherosclerotic lesions [83]. Furthermore, treatment with myriocin resulted in less lesion necrosis. This is important as acute cardiovascular events are generally dependent on rupture, necrosis, and thrombosis of unstable plaques. This has been well documented for carotid plaques [85]. Park et al. used an *ApoE*<sup>-/-</sup> mouse model, which may be a good model for early and advanced lesions resembling human plaques. However, it is a poor model for rupture and thrombosis and therefore pathological changes may not reflect what actually causes the cardiovascular events in humans [86].

HMG-CoA reductase inhibitors (Statins) have been extensively used to inhibit cholesterol synthesis and increases LDL clearance. Statins also have potent anti-oxidative and anti-inflammatory properties [87,88]. Thus, statins may have effects on ceramide pathways since ceramide production, biological functions, and signal pathways are closely linked to oxidative stress and inflammation. However, such interesting studies are limited. Only one study demonstrates that pravastatin attenuates ceramide-induced cytotoxicity in mouse cerebral endothelial cells with HIF-1 activation and VEGF upregulation [89]. More investigations are warranted in this aspect.

## 9. Conclusions

A ceramide is composed of sphingosine and a fatty acid. Ceramide is mainly generated through SMase pathway, which breakdowns sphingomyelin in the cell membrane and releases ceramide. It can be also generated through the de novo pathway which creates ceramide from less complex molecules. Generation of ceramides can be significantly enhanced in many inflammation conditions including atherosclerosis and ischemia/reperfusion injury mainly through several key cytokines such as TNF- $\alpha$  and interleukins. Several cardiovascular risk factors/markers such as oxidized LDL and homocysteine can increase the production of ceramides. In addition to their role in the membrane structure, ceramides can act as a signaling molecule regulating many cell responses and functions including the differentiation, proliferation, apoptosis, ROS production and gene expression such as cytokines. Some of these roles are directly involved in the molecular mechanisms of cardiovascular disease. Ceramide pathway has been involved in the activation of several

signaling molecules including protein phosphatase, protein kinase C, small G-protein Ras, Rac1, Rho and Jun N-terminal kinase (JNK)/p38 kinases (p38-K) [90-92]. Stimuli of ceramide production and effects of ceramides are listed in Table 1. Since ceramide pathway is fundamental to mediate endothelial damage after exposure to damaging agents such as homocysteine and TNF- $\alpha$ , blocking ceramide pathways might prevent homocystein- and TNF- $\alpha$ -mediated endothelial damage. Thus, further investigations in both molecular pathways and therapeutic targets of ceramides-induced vascular pathogenesis are warranted.

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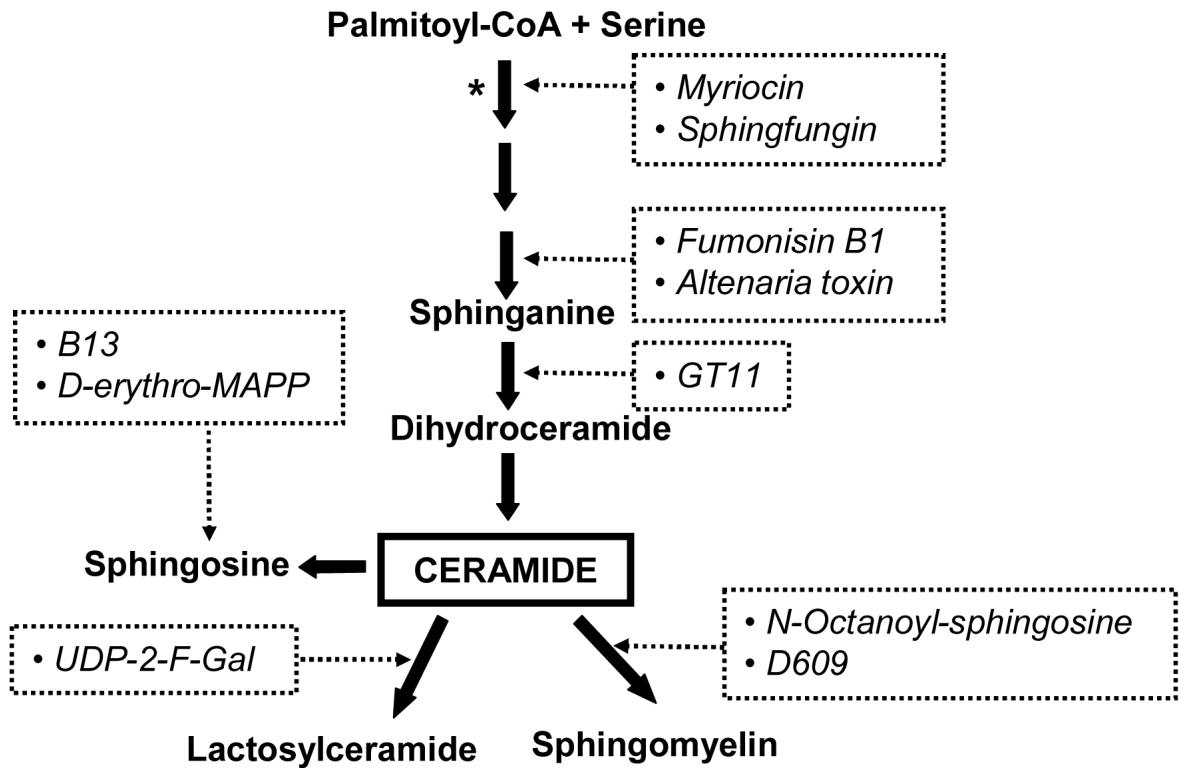
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**Fig. 1.** Sphingolipid biosynthetic pathway and potential inhibitors. Multiple compounds are outlined, but the most convincing data is that for myriocin and is therefore the only one discussed in the text. \* First rate-limiting step in the sphingolipid biosynthetic pathway. Myriocin is isolated from *Isari Sinclairii* (fungal species) and is a potent and specific inhibitor of serine palmitoyltransferase, the enzyme which is the catalyst in this step.

**Table 1**

## Stimuli of ceramide production and effects of ceramides

<u>Stimuli of ceramide production:</u>
Cytokines (TNF- $\alpha$ , IL-2, endostatin, etc)
OxLDL
Oxidative stress
Homocysteine
Matrix Metalloproteinases (MMPs)
<hr/>
<u>Effects of endogenous and exogenous ceramides:</u>
Increaseing lipoprotein aggregation
Increasing uptake of lipoprotein and accumulation of cholesterol within macrophages
Increasing production of superoxide anion and other ROS
Increasing the experssion of IL-6, CRP, and TNF- $\alpha$
Increasing ischemia/reperfusion injury
Causing endothelial dysfunction
Causing cell apoptosis
Deceasing NO bioavailability
Acting as second signal to activate phosphatases and protein kinases