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ROLE OF THE SPHINGOMYELIN/CERAMIDE PATHWAY IN DIABETIC RETINOPATHY

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ROLE OF THE SPHINGOMYELIN/CERAMIDE PATHWAY IN DIABETIC RETINOPATHY

By

Madalina Opreanu

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ABSTRACT

ROLE OF THE SPHINGOMYELIN/CERAMIDE PATHWAY IN DIABETIC RETINOPATHY

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Diabetic retinopathy (DR) stands as a sight threatening disease without effective therapeutic options. Early DR is recognized to be a persistent low-grade chronic inflammatory disease. Acid (ASMase) and neutral (NSMase) sphingomyelinases (SMases) are important early responders in inflammatory cytokine signaling and key regulatory enzymes of sphingolipid metabolism. Sphingolipids are a major component of plasma membrane microdomains and sphingomyelin hydrolysis by SMases to bioactive lipid ceramide represents a prerequisite for inflammatory cytokines signaling. This study addresses the role of SMases in retinal vascular endothelium inflammation and development of microangiopathic lesions in the retinas affected by retinopathy. We first demonstrated that inhibition/gene silencing of both SMases decrease cytokine-induced cellular adhesion molecules expression in human retinal endothelial cells (HREC); yet a more pronounced anti-inflammatory effect was observed when ASMase inhibition/gene silencing was attained. Similarly, docosahexaenoic acid (DHA), the major ω3 polyunsaturated fatty acid in the retina and well known anti-inflammatory agent, downregulated cytokine-induced inflammation in HREC. Interestingly, DHA decreased basal and cytokine-induced ASMase and NSMase expression and activity in HREC, further underlining the role of SMases as mediators of the inflammatory process. SMases pathway rather than ceramide *de novo* synthesis pathway was important for inflammatory

signaling in HREC. To further demonstrate the role of SMase pathway in inflammation, the caveolae/lipid microdomains sphingolipid composition was then characterized. In support of SMases inhibition and displacement of ASMase from caveolae microdomains by DHA, we found a significant decrease in ceramide/sphingomyelin (Cer/SM) ratio in the caveolae fraction isolated from HREC treated with DHA; moreover, DHA prevented the TNFα-induced increase in the Cer/SM ratio in caveolar membrane microdomains and intracellular inflammatory signaling. To address the role of SMases in retinal inflammation and vessel loss in DR, an *in vivo* model of streptozotocin (STZ)-induced diabetes in rat was employed. ASMase, but not NSMase was upregulated in the diabetic retinas with an inflammatory status and microangiopathy lesions. DHA-enriched diet restored ASMase in diabetic retina to the control levels and prevented retinal inflammation and vessel loss. More importantly, DHA supplementation specifically prevented vascular ASMase upregulation in the retinas of a rat model with vasodegenerative phase of DR. To directly investigate the role of ASMase in development of retinal acellular capillaries and ocular neovascularization, we next used accelerated models of retinopathy by inducing ischemia/ reperfusion (I/R) injury and oxygen-induced retinopathy (OIR) in wild type ($ASMase^{+/+}$) and $ASMase^{-/-}$ mouse retina. $ASMase^{-/-}$ mouse retina was significantly protected against retinal inflammation, vascular degeneration and ocular neovascularization. In summary, this is the first study to show that ASMase, a key regulatory enzyme of sphingolipid metabolism, is a novel and fundamental mediator and a promising therapeutic target for the prevention of retinal vascular inflammation and diabetic retinopathy.

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LIST OF ABBREVIATIONS

AGE	advanced glycation end product
ASMase	acid sphingomyelinase
BSA	bovine serum albumin
CAM	cell adhesion molecule
Cer	ceramide
COX2	cyclooxigenase 2
CRD	caveolae-related domains
DD	death domaain
DHA	docosahexaenoic acid (22:6n3)
DNA	deoxyribonucleic acid
DR	diabetic retinopathy
Elovl	elongase
FADD	Fas-associating protein with DD
FAN	factor associated with NSMase activation
HDL	high density lipoprotein
HMC	human Muller cells
HREC	human retinal endothelial cells
HRPE	human retinal pigment epithelial cells
HSL	hormone-sensitive lipase
ICAM-1	intercellular cell adhesion molecule -1
IL	interleukin
I/R	ischemia/reperfusion

LAMP1	lysosomal associated protein 1
LDL	low density lipoprotein
LPL	lipoprotein lipase
MCD	methyl-β-cyclodextrin
MMP	matrix metalloprotease
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NSMase	Neutral sphingomyelinase
NF-κB	nuclear-factor kappa B
OIR	oxygen-induced retinopathy
PKC	protein kinase C
PCR	polymerase chain reaction
RNA	ribonucleic acid
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SM	sphingomyelin
SMase	sphingomyelinase
STZ	streptozotocin
TNF	tumor necrosis factor
TRADD	TNF receptor associated DD
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein

Chapter I

Literature Review

1. Diabetic Retinopathy

1.1. Overview

Current estimates suggest that approximately 135 million individuals across the world have diabetes mellitus (1). In the U.S., over 23.6 million people are affected by diabetes with significant morbidity and mortality (2). Diabetic retinopathy (DR) stands as the most common and disabling microvascular complication of diabetes and results in blindness for over 12,000 people with diabetes each year (2). Moreover, DR is the primary cause for blindness in the working age population worldwide (3) and in 2004, vision loss from DR accounted for approximately $500 million in direct medical costs among Americans age 40 or older (4). To place this in perspective, it is estimated that the number of Americans 40 years or older with DR will triple during the period of time from 2005 to 2050, from 5.5 million in 2005 to 16.0 million in 2050 (5).

In conclusion, it is largely recognized that visual impairment among people with diabetic retinopathy is a major disability with a profound impact on patient independence and can result in depression, reduced mobility and overall, poor quality of life (6). Consequently, diabetic eye disease continues to represent an important socio-economic burden on society and remains a challenge for the ophthalmology services provision.

1.2. Histopathological and Clinical Manifestations

Diabetic retinopathy represents the ocular expression of diabetic microvascular complications. The natural history of the DR has been classified into an early, non-proliferative stage (background retinopathy) and a late, proliferative stage.

The early, non-proliferative stage of DR has distinct vascular lesions that are histologically characterized by the existence of saccular capillary microaneurysms, retinal hemorrhage, venous dilation and beading, retinal lipid exudates, pericyte degeneration, occlusion and degeneration of retinal capillaries (7-8). The mechanisms considered to be involved in diabetic retinal vascular lesions include obstruction of the vascular lumen by leukocytes or platelets; death of endothelial cells due to pathophysiological processes within the vascular cells themselves; or endothelial cell death secondary to products generated by other cells in the retina that are in close contact with these vascular cells (such as neurons or glial cells) (7).

During the early phase of DR, clinical signs include retinal microaneurysms and dot hemorrhages that are present in almost all patients with type 1 diabetes of a duration 20 years (9) and in approximate 80% of those affected by type 2 diabetes for the same duration of time (10). As the disease progresses, the number and size of intraretinal hemorrhages increases, and is accompanied by the appearance of cotton-wool spots on the retinal background, indicative of ischemia due to local failure of the retinal microvascular circulation. During the ophthalomoscopic investigation of the retina, retinal veins appear dilated, tortuous and with irregular caliber, whereas arteries may appear white; fluorescein angiography may reveal non-perfused retinal arteries (11).

The late, proliferative stage of DR is histologically characterized by the presence of aberrant new vessels, and fibrous tissue proliferation on the retinal surface that can extend into the vitreous. The newly synthesized blood vessels are abnormal and fragile. They tend to leak blood into the center of the eye inducing blurred vision and/or causing blindness. As DR progresses, macular edema develops in the retina (due to the breakdown of the blood-retinal barrier) resulting in leakage of plasma from the small retinal vessels into the maculaand leading to severe loss of central vision (11).

The clinical signs of late stage DR are due to the proliferating retinal vessels along with the glial tissue that anchor into the vitreous and lead to traction of the retina (3). In the most severe cases, the fibrovascular tissue proliferation leads to total retinal detachment and vitreous hemorrhage (12). Retinal neovascularization most commonly takes place along the temporal vascular arcades and the optic nerve head. Retinal neovascularization is an important contributor to the visual impairment and blindness in diabetes.

Current approaches to prevention of DR involves general medical measures such as control of blood glucose, blood pressure and serum lipids (11), whereas treatment of DR involves specific therapeutic strategies that target retinal abnormalities encountered during the course of the disease. The surgical options (laser panretinal photocoagulation and vitreo-retinal surgical interventions) applied locally at the retinal level are highly invasive, traumatic to the retinal tissue and do not prevent visual impairment.

Although the medical and surgical management of diabetes represent the mainstay of DR management, they are not fully protective against visual impairment and patients

suffering blindness from diabetes are still arising, with approximately 8,000 new cases reported annually. Therefore, new therapeutic approaches based on the etiology of early diabetic microvascular lesions need to be developed and evaluated in clinical trials to ultimately improve the outcome for patients with DR.

1.3. Epidemiology

Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR) was the most comprehensive study of retinal neovascularization within a diabetic population, where the prevalence and incidence of DR was investigated in more than 2000 patients with diabetes (9-10, 13-14). This study showed that the prevalence of all retinopathy was approximately 17% in patients with diabetes for less than 5 years, and 97.5% in patients with diabetes for more than 15 years. The increase in prevalence of proliferative retinopathy was strongly correlated with greater duration of diabetes and it varied from 1.2% in patients with diabetes mellitus for less than 10 years to more than 67% in patients with diabetes for 35 years or more (9). This study on DR concluded that there is a strong association between the frequency and severity of retinopathy and the duration of diabetes. Moreover, the severity of DR was also related to a diagnosis at a younger age, increased levels of glycosylated hemoglobin, the use of insulin, higher systolic blood pressure, proteinuria and a small body mass (10).

New clinical data identified dyslipidemia as a critical factor in the development of diabetic retinopathy. The Diabetes Control and Complication Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) identified a

strong association between the severity of DR in type 1 diabetes and lipid metabolism (15). This study was conducted on patients with type 1diabetes and revealed a strong association between severity of retinopathy in type 1 diabetes and the size of the particles of three major classes of serum lipoproteins, very low density, low density and high density lipoprotein (VLDL, LDL and HDL) as well as LDL concentration. Cross-sectional studies showed positive associations between the severity of retinopathy and total- and LDL-cholesterol levels and the LDL-HDL cholesterol ratio (16). The Early Treatment Diabetic Retinopathy Study has demonstrated that higher levels of serum lipids are associated with an increased risk of development of hard exudates in the macula and visual loss (17-18). Several studies showed that lipid-lowering dietary (19) and drug (20) therapy may lead to regression of retinal hard exudates, and that a diet high in polyunsaturated fatty acids may protect against retinopathy (21).

2. Dyslipidemia and Diabetic Retinopathy

Dyslipidemia is not only a major metabolic disorder of diabetes mellitus, but also a critical factor in the development of DR. Diabetic dyslipidemia with lipoprotein abnormalities become manifested during the asymptomatic diabetic prodrome and represent a high risk for vascular complications (22).

2.1. Insulin and Lipid Metabolism

Insulin, a polypeptide hormone secreted by β cells of the islets of Langerhans in the pancreas, exerts an essential metabolic regulation not only on carbohydrate metabolism, but also on lipid metabolism and protein synthesis. Insulin decreases triacylglycerol degradation and hence the level of circulating fatty acids by inhibiting hormone-sensitive lipase (HSL) activity in the adipose tissue (23). Moreover, insulin increases glucose transport and metabolism in adipocytes and provides the substrate glycerol 3-phosphate for triacylglycerol synthesis. In adipose tissue and muscle, insulin also enhances the activity of lipoprotein lipase (LPL) and thus provides fatty acids for esterification (24). LPL is then transported to the surface of endothelial cells where it hydrolyzes the lipid chore of chylomicrons and low/very low density lipoprotein particles and thus facilitate in the removal of triglyceride-rich lipoproteins from the blood stream. Patients with either type 1 or type 2 diabetes have low adipose LPL activity, a higher rate of HDL-metabolism and increase in plasma triglycerides level (25).

Fatty acid synthesis and remodeling is also under insulin control. Fatty acids are classified according to their structure by the number of carbons, double bonds and proximity of the first double bond to the methyl ($-CH_3$) end of the fatty acyl chain.

According to the number of the double bonds, fatty acids are classified as saturated (no double bonds), mono-unsaturated (MUFA, one double bond) and poly-unsaturated (PUFA, multiple double bonds) and are presented in Figure 1.1. Saturated, mono-unsaturated and poly-unsaturated fatty acids are synthesized from dietary precursors (glucose, 16:0, 18:1n9, 18:2n6, 18:2n3, 18:2n6 and 20:5n3) through a series of elongation (Elovl-2,-4,-5 and -6) and desaturation (Δ5-desaturase, Δ6-desaturase and Δ9-desaturase) reactions. Insulin is the most potent activator of Δ5, Δ6 and Δ9 desaturase, where Δ5 and Δ6 desaturases represent rate-limiting enzymes in fatty acid metabolism (25-30). Therefore, low insulin availability or insulin resistance will result in impaired desaturase expression, leading to substrate accumulation and product depletion. The major impact is observed on long chain polyunsaturated ω 3 fatty acids that are no longer synthesized in the desirable amounts. The lack of insulin in type 1 diabetes and insulin resistance in type 2 diabetes will promote a fatty acid profile with high saturation index and short fatty acyl chain and will lead to a significant increase in HSL activity and decrease in PLP activity, with a profound impact on plasma lipid levels, lipid profile and fatty acid composition.

2.2. Diabetic Dyslipidemia

2.2.1. Lipoproteins and Triglycerides

The natural course of development of type 2 diabetes (or insulin-resistant diabetes) affects virtually all lipids and lipoproteins (31-32). The metabolic milieu is characterized by accumulation of chylomicrons and VLDL remnants, triglyceride enrichment of HDL and LDL particles, resulting in decrease level of HDL particles and

formation and accrual of small, dense LDL particles (33). Increased triglyceride production along with decreased catabolism of triglyceride-enriched lipoproteins heightens hypertriglyceridemia. Studies have shown that LDL particles become small and dense in approximatively 90% of the patients whom triglycerides levels are > 200 mg/dL; when triglyceride levels are shifted 90 mg/dL, almost all LDL lipoprotein particles are of the larger, buoyant range (34).

The epidemiological data on the prevalence of dyslipidemia and phenotype distribution showed that, regardless of a poor glycemic control, total triglyceride and cholesterol levels are the same when compared patients with insulin-dependent type 1 diabetes and nondiabetic, control, healthy subjects age matched (35). However, increased LDL-cholesterol levels and decrease in HDL-cholesterol have been found in diabetic group compared to nondiabetic group (35). More importantly, the lipid abnormalities may improve with a good glycemic control, except for HDL-cholesterol levels (35). Kordonouri *et al.* also showed that HDL-cholesterol is the most important variable, tightly correlated to the development of retinal lesions in children with type 1 diabetes (36).

2.2.2. Long chain PUFAs

Over the past few years, there has been an increasing interest in exploring the role of long chain PUFAs in pathophysiology of diabetic metabolic complications. These PUFAs contains more than one *cis* double bond and are classified as ω6 or ω3-PUFAs according to the location of the first double bond compared to the fatty acid methyl end as shown in Figure 1.1. ω3- PUFAs are regarded as important anti-inflammatory mediators

(37-39), whereas ω6- PUFAs are considered to have pro-inflammatory properties (40). More importantly, diabetic dyslipidemia is characterized by an elevated ω6 to ω3-PUFA acid ratio (41-42) that can "incline the body physiologic equilibrium" toward an inflammatory state. Ferruci *et al.* studied the relationship between plasma PUFAs and circulating inflammatory markers in a community based-sample and found that increased total ω-3 PUFAs levels were associated with lower levels of inflammatory markers (IL-6, IL-1ra, TNFα, C reactive protein) and higher levels of anti-inflammatory markers (soluble IL-6r, IL-10) (43-44). The authors concluded that ω3-PUFAs are beneficial in patients affected by diseases with an active inflammatory component.

2.2.3. Sphingolipids

New research revealed the importance of another class of lipids that are dysregulated in diabetes, the sphingolipids. Sphingolipids represent a complex class of lipids and the prefix "sphingo-" that appears in the nomenclature of this lipid class was first attributed by J.L.W. Thudichum in 1884 because the enigmatic nature of these molecules reminded him of the riddle of the sphinx. In 1974, Herbert Carter and colleagues introduced the "sphingolipide" name. Sphingolipids are extremely versatile molecules. Relative to phospholipids, sphingolipids are enriched in saturated long fatty acyl chains, are more hydrophobic, have a higher melting temperature and can undergo a tighter packing in the cellular membrane due to the lack of unsaturated acyl chains with kinked structures (Figure 1.2) (45-46). Sphingolipids have a chemical structure composed of a 1,3-dihydroxy-2-aminoalkane backbone, also known as the sphingoid base. Sphingosine, the most prevalent backbone of mammalian sphingolipids is characterized

by a (2S,3R,4E)-2-amino-4-octadecene-1,3-diol chemical structure. Saturation of the sphingosine backbone results in sphinganine, which represents another sphingoid base frequently used to form mammalian sphingolipids. Attachment of a fatty acid to the sphingosine backbone via an amide bond results in formation of ceramides; attachment of a variety of polar headgroups to the ceramide structure results in formation of complex sphingolipids, such as sphingomyelin, gangliosides, sulfatides, globosides or cerebrosides.

Sphingolipids such as ceramide and glucosylceramide, while representing a relatively minor component of the lipid milieu in most tissues, may be among the most pathogenic lipids in the onset of diabetic metabolic complications, including insulin resistance, pancreatic β-cell failure and vascular dysfunction (47-48). Circulating factors associated with diabetes (e.g. saturated free fatty acids, inflammatory cytokines) selectively induce enzymes that promote ceramide synthesis and stimulate the accumulation of ceramide and various ceramide metabolites (49-52); these sphingolipids have been shown to amass in tissues from rodents (53-54) and humans (55-56) with type 2 diabetes. Extensive lipidomic profiling revealed ceramide accumulation in the muscle tissue from rats on a high fat diet for three weeks or from streptozotocin diabetic rats, one month after diabetes induction (54). Related to these findings, Straczkowski *et al.* have shown elevated ceramide levels in the skeletal muscle of obese men at risk of developing type 2 diabetes (57). Interestingly, a fish oil (DHA) enriched diet significantly reduced ceramide content in the mouse muscle tissue (58).

Dysregulation of sphingolipid metabolism in diabetes emerges as an important pathway for development of the deleterious clinical manifestations associated with this

disease. Importantly, sphingolipid metabolism was found to be significantly altered in diabetic retina (59). Therefore, pharmacologic inhibition or genetic ablation of key regulatory enzymes of sphingolipid metabolism may identify new therapeutic targets for combating insulin resistance, β-cell failure and vascular dysfunction of diabetic metabolic disease.

2.3. Retinal Fatty Acid Profile and Diabetic Retinopathy

The normal retina has a very specific fatty acid profile, with the highest level of long-chain PUFAs in the body. Various studies have identified DHA, an ω-3 PUFA as being enriched in the retinal tissue (60-61). Work from our group demonstrated that DHA represents 45.37 ± 1.32 mole % of total fatty acids identified in the retina (61). Omega 3-PUFAs and particularly DHA have the ability to modulate various biological processes involved in retinal vascular inflammation, retinal capillary structure and integrity alterations, and retinal neovascularization (62). Therefore, ω3 long-chain PUFAs and mainly DHA status in the retina is closely related to normal retinal structure and function (62).

Long chain PUFA synthesis and remodeling involves a series of desaturation (Δ5-desaturase, Δ6-desaturase, Δ9-desaturase) and elongation (Elovl1-7) reactions. The elongases Elovl2 and Elovl6 are ubiquitously present in most tissues; yet, the retinal tissue express very high levels of Elovl2 and this elongase is involved in several steps of DHA synthesis (61, 63). Elovl4 has a restricted tissue specificity, being highly expressed in the retina (64-66), thymus and skin (65), and to a lesser extent in the brain (65-66) and testis (66). Insulin deficiency or complete absence of insulin downregulates Elovl2 and

Elovl4 elongases specifically in the retina, and thus precludes the synthesis of various retinal PUFAs, and particularly ω3-PUFAs and DHA (61). We have found that retinas isolated from type 1 diabetic animals had a 28% reduction in DHA levels when compared to control (61). Previous studies have also demonstrated decreased levels of ω3-PUFAs, and mainly DHA in plasma of diabetic children (67), as well as in human retina of diabetic eyes (68). More importantly, decreased retinal DHA levels, and an increase in the ω6-to- ω3 PUFA ratio in diabetic retina was associated with significant upregulation of ICAM-1 and inflammatory cytokine (VEGF, TNFα and IL-6) gene expression, suggesting that DHA tissue insufficiency may create pro-inflammatory conditions in the retina, potentially contributing to the development of DR (61). Our study also demonstrates that retinal fatty acid metabolism is distinct from the metabolic changes that occur in diabetic plasma or diabetic liver (61).

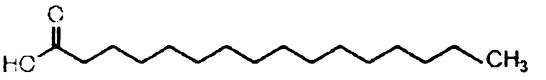
Several studies have identified DHA retinal insufficiency to be associated with alterations in retinal function (reviewed in (62)) and DHA deficiency has also been documented to be associated with certain retinal degenerative diseases, such as retinitis pigmentosa, retinopathy of prematurity and age-related macular degeneration (69-73). Taken together, all these demonstrate that DHA has a critical role in maintaining the normal structure and function of the retina.

A study by Connor *et al.* showed a beneficial effect of dietary DHA in reducing pathological retinal angiogenesis and thus preventing the development of retinopathy (74) in an oxygen-induced retinopathy (retinopathy of prematurity) animal model. Furthermore, it has been shown that DHA supplementation could ameliorate visual processing deficits (62). *In vitro* studies from our group demonstrated a significant

protective effect of DHA against cytokine induced inflammation in human retinal endothelial cells (39-40). However, the protective effect of DHA against diabetic retinal inflammation and microangiopathic lesions has not been addressed and represents an objective of my study.

➢ **Saturated Fatty Acids**

Palmitic Acid (16:0)

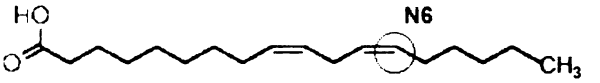


➢ **Mono Unsaturated Fatty Acids**

Oleic Acid (18:1,n9)

➢ **Poly Unsaturated Fatty Acids**

Linoleic Acid (18:2,n6) (n6-PUFA)



➢ **Poly Unsaturated Fatty Acids**

Docosahexaenoic Acid

(DHA, 22:6,n3) (n3-PUFA)

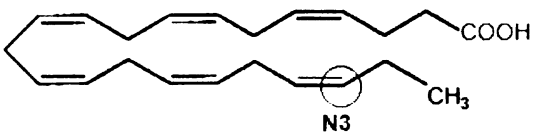


Figure 1.1. Fatty acids classification. Fatty acids classification is based on the number of carbons in the chain length, the number of double bonds and the localization of the first double bond related to the methyl end. Based on these considerations, fatty acids can be classified as saturated, monounsaturated and polyunsaturated.

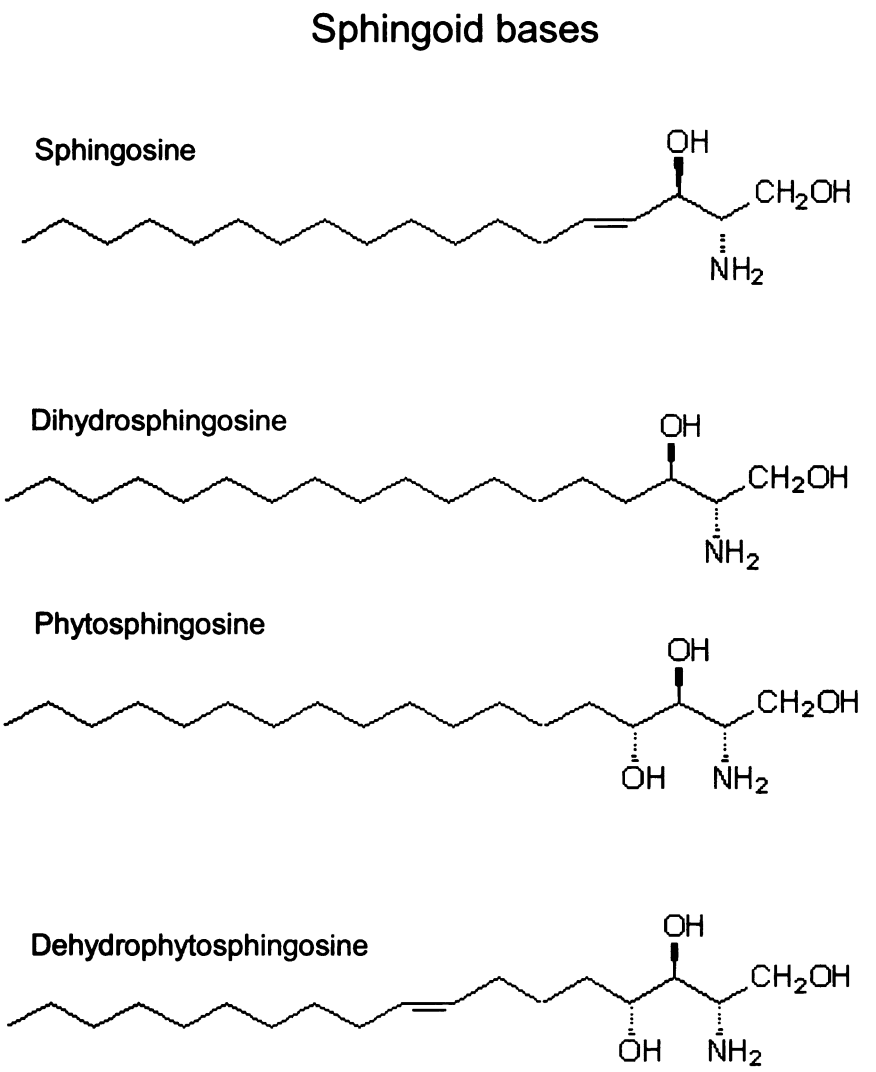


Figure 1.2. Sphingolipids chemical structures. Sphingoid bases are long-chain aliphatic amines, with two or three hydroxyl groups, and often a characteristic *trans*-double bond in position 4; they are 2-amino-1,3-dihydroxy-alkanes or alkenes with *(2S,3R)-erythro* stereochemistry, with various structural modifications.

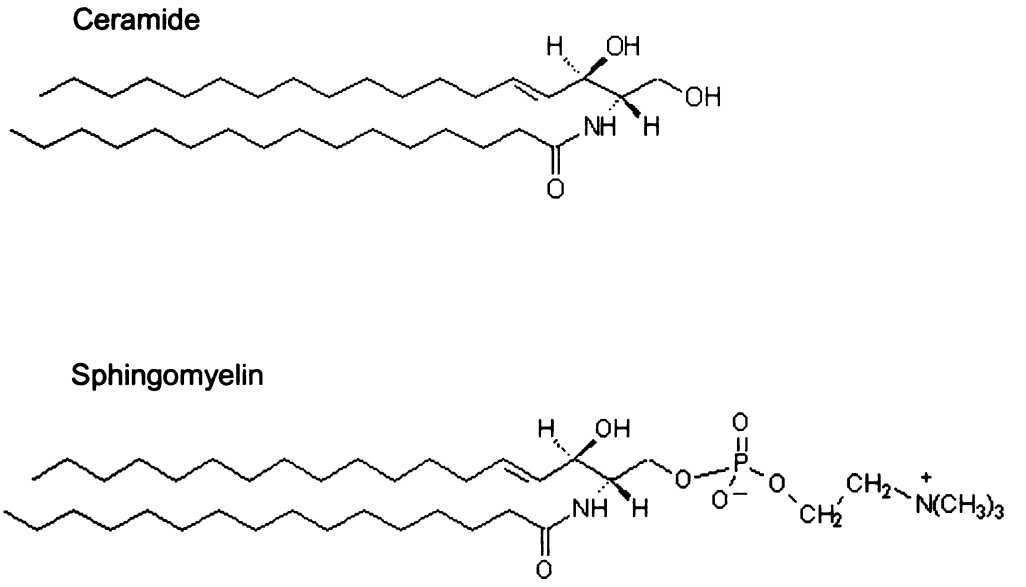


Figure 1.2 (continued). Sphingolipids chemical structures. Ceramide consists of a sphingoid base linked to a fatty acid via an amide bond. Sphingomyelin consists of a ceramide molecule with a phosphorylcholine moiety attached to position 1.

3. Inflammatory Mechanisms in Diabetic Retinopathy

An extensive body of evidence, including both preclinical and clinical findings, supports the concept that very early stage diabetic retinopathy represents a persistent low-grade chronic inflammatory disease that involves increased expression of adhesion molecules on endothelial cell surfaces, eventuating in leukocyte recruitment, retinal cytokine production and activation of transcription factors that result in up-regulation of retinal inflammatory genes. These pathophysiological changes will be separately discussed and evidence to support their role in DR will be presented.

3.1. Adhesion Molecules in Diabetic Retinopathy

The first line of evidence supporting a connection between inflammation and diabetic retinopathy came from an epidemiological data in 1964 showing a decrease in severity of DR in diabetic patients treated with high doses of aspirin for rheumatoid arthritis (75). In a rat model of diabetic retinopathy, aspirin and meloxicam (nonsteroidal anti-inflammatory agents) prevented the development of early diabetic retinopathy, a result that correlated with suppress of intercellular adhesion molecule-1 (ICAM-1) expression and leukocyte adhesion (76). Adhesion molecules, especially ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), are involved in leukocyte attachment and transmigration into the vascular intima (77-78) that could result in early blood retinal barrier breakdown, capillary non-perfusion and endothelial cell injury and death. Evidence supporting this view are provided by findings of significant increase in leukocyte density and retinal vascular ICAM-1 expression in human eyes with DR (78). Moreover, a role of CD18/ICAM-1-mediated leukocyte adhesion in the pathogenesis of

early diabetes-induced leukostasis and blood-retinal barrier breakdown has been established. Reduced expression of ICAM-1 on endothelial cells or its leukocyte counter-receptor CD18 significantly inhibits leukocyte adhesion, pericyte and endothelial cell loss and consequently the formation of acellular, degenerated capillaries in experimental models of diabetic retinopathy (79). Theoretically, an intervention at this level might carry an important role in designing future treatment strategies in DR.

3.2. Proinflammatory Cytokines in Diabetic Retinopathy

Proinflammatory cytokines (tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β), and vascular endothelial growth factor (VEGF) are increased in diabetic eyes and several inflammatory pathways are activated in the very early stages of DR (79-82).

TNFα is a proinflammatory cytokine implicated in a number of inflammatory diseases, including rheumatoid arthritis, Chron's disease, ankylosing spondylitis and psoriasis (83). In diabetes, TNFα has been involved in pancreatic β cell apoptosis (84) and insulin resistance (85), as well as in diabetic microvascular complications, including nephropathy (86) and diabetic retinopathy (76). TNFα acts through ligand-receptor interactions and initiate a number of inflammatory processes, such as upregulation of adhesion molecules expression, leukocyte recruitment and leukostasis, cellular apoptosis, chemoattraction of monocytes and plays a critical role in amplification of the immune response through upregulation of the expression of numerous transcription factors, growth factors and other inflammatory mediators (86). Clinical and preclinical studies indicate an important role of TNFα in the pathogenesis of DR. Correlative clinical studies with

genetic data have implicated a TNFα polymorphism in conferring susceptibility to the disease (87). Moreover, retinal levels of TNFα are significantly increased in diabetic rats compared to control (76) and increased amounts of TNFα can be found in the extracellular matrix, vessel walls, endothelium and vitreous of diabetic eyes affected by proliferative retinopathy (80, 88-89). Playing a central role in the pathogenesis of DR, TNFα has been targeted with specific medication aimed to counteract its inflammatory effects.

Systemic administration of eternacept, a soluble TNFα receptor/Fc fusion protein that acts as a competitive inhibitor to block the effects of TNFα on cells, resulted in reduction of the inflammatory process in DR, including leukocytes adhesion to the retinal vasculature, upregulation of ICAM-1, NF-κB activation and breakdown of the blood retinal barrier (76). The effects of eternacept were not evaluated on advanced histologic lesions in DR, but it was reported that mice genetically deficient in TNFα were protected against galactose-induced retinopathy (7). Administration of Infliximab, a monoclonal antibody directed against TNFα, for two months in patients with advanced DR showed positive responses, with improvement of visual acuity and reduction in macular edema and further improvements were seen after repeated infusions (90).

Increased intraocular VEGF expression, a growth factor recently recognized to have cytokine properties, has been identified in rat and human diabetic retina (82, 91-94) and has become a focal point of current research on the pathogenesis of DR. The VEGFs are a family of peptides produced by alternative splicing from a single gene; VEGF isoforms are particularly mitogenic for vascular endothelium and also amplify permeability at blood-tissue barriers (11). VEGF is synthesized by multiple cell types in

the retina, such as ganglion cells, Muller cells and pericytes (95-97). VEGF induces upregulation of ICAM-1 expression in endothelial cells (98), resulting in chronic retinal leukostasis that plays a key role in endothelial cell injury and death and blood-retinal barrier breakdown (94). VEGF is a key molecule that plays a well-recognized role in promoting vascular permeability (99) and pathological ocular neovascularization (100). VEGF expression is mainly regulated by hypoxic stimulus; yet there is evidences that it also accumulates in the retina early in diabetes, before any retinal hypoxia is present (101-102). Therefore, VEGF upregulation is strongly involved in the pathogenesis of both non-proliferative (background) and proliferative DR (81-82, 94, 103). Specific inhibition of VEGF hinders ICAM-1 upregulation, leukostasis, blood-retinal barrier breakdown and pathological ocular neovascularization in diabetic rats (98). Clinical trials using anti-VEGF therapeutic strategies intravitreously administered (pegaptanib, a VEGF inhibitor; ranibizumab and bevacizumab, monoclonal antibodies that bind all VEGF isoforms) showed promising results against advanced stages of DR (104-106). Among these, ranibizumab is the only anti-VEGF therapy that was approved by Food and Drug Administration for ophthalmologic use in neovascular (wet) age-related macular degeneration; yet, it's efficacy in DR remains to be investigated.

Pro-inflammatory cytokine IL-1β levels are also increased in retinas from diabetic rats (107-108). *In vivo* studies with intravitreal injection of IL-1β or exposure of retinal endothelial cells to IL-1β *in vitro* was able to induce endothelial cell inflammation and death, resulting in degeneration of retinal vascular endothelium and formation of acellular capillaries (109). Caspase-1, the enzyme involved in production of proinflammatory cytokine IL-1β from pro-IL-1β, is activated in the retina of diabetic patients and diabetic

animal models (110). The involvement of IL-1β in pathogenesis of DR has been recently defined in diabetic mice in which caspase-1 was inhibited or the IL-1β receptor was deleted (108). Inhibition of caspase-1 using minocycline or inhibition of IL-1β signaling using IL-1β receptor genetic deficient mice protected the mice from development of retinal pathology (108). These data suggests a major role for IL-1β and its receptor in mediating signaling pathways responsible for endothelial cell activation and death, and consequent retinal vessel loss.

Proinflammatory cytokines that are upregulated in diabetic retina (i.e. TNFα, IL-1β and VEGF) are likely to activate nuclear factor-kappa B (NF-κB) pathway (40). NF-κB proteins consist of a family of structurally related transcription factors widely expressed in mammalian tissues, which control a large number of cellular processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis (7). NF-κB transcription factors represent a prerequisite for various gene expressions, including adhesion molecules, inflammatory cytokines, cyclooxygenase 2 (COX2) and matrix metalloproteases (MMPs) (111-112). These transcription factors are persistently active in various disease states, including chronic inflammation, arthritis, asthma, cancer, and heart disease (113). DR is hypothesized to be a chronic inflammatory disease and activation of NF-κB is well documented in the retinal vasculature of diabetic patients and animals (114-115).

TNFα and IL-1β have been shown to activate cellular sphingomyelinases in various cell types (116-119). Stimulation of the sphingomyelin pathway by TNFα, and sphingomyelinases-induced membrane ceramide formation leads to activation of NF-κB and marked increase in nuclear NF-κB localization in human leukemia (HL-60) cells

(118). In the same way, activation of sphingomyelinases is shown to be an important signaling system for induction of IL-1β in the murine T helper cells EL-4 (117); IL-1β action in these cells is mediated through NF-κB activation (120). Sphingomyelinases may play an important function in mediating the inflammatory process in DR; their role in vascular retinal inflammation has not been addressed and represents the objective of my study.

4. Sphingomyelinases and Inflammation

Sphingomyelinases (sphingomyelin phosphodiesterase) are key regulatory enzymes in sphingolipid metabolism. They induce sphingomyelin hydrolysis to generate the pro-inflammatory and pro-apoptotic second messenger ceramide. Several isoforms of sphingomyelinases have been identified and further distinguished by their catalytic pH optimum, cellular localization, primary structure and co-factor dependence. Alkaline sphingomyelinase activity is confined to the intestinal mucosa, bile and liver and does not participate in signal transduction (121-123). Neutral (NSMase) and acid (ASMase) sphingomyelinases, however, are crucially involved in pathophysiology of metabolic disorders (47) and play an active role in cellular signaling (124).

4.1. Neutral Sphingomyelinase

Neutral sphingomyelinase (NSMase) activity was described for the first time by Schneider and Kennedy in 1967 when they found that spleens of patients with Niemann-Pick disease (deficiency of ASMase) contained a magnesium-dependent enzyme capable of promoting sphingomyelin hydrolysis to ceramide at an optimum pH of 7.4 (125). NSMase shows an absolute dependence on magnesium or manganese for its activation, with a best concentration ranging between 2 and 10 mM for magnesium and up to 5 mM for manganese (126-127). *In vitro* studies showed that NSMase requires neutral detergents for presentation of its substrate in mixed micelles (127).

NSMase has an estimated molecular mass of 92 kDa, although smaller isoenzymes have been described, which could result from either the proteolytic process or from an alternative processing of common transcripts (128). Subsequent research showed

NSMase activation can occur in response to a variety of stimuli, such as cytokines, cellular stress (UV light, chemotherapeutic drugs), amyloid β and lipopolysaccharide (124, 129). Activation of NSMase by such a wide array of stimuli further highlights the significance of this enzyme as an important regulator of ceramide production and ceramide-dependant signaling (130).

NSMase appears to be ubiquitously expressed in mammalian tissues, with a particular high activity in brain (128) and to a lesser extent, spleen and liver (130). NSMase has been reported to be localized to the plasma membrane in mammalian cells (128), has two putative transmembrane domains at the NH_2 terminus and palmitoylated on five cysteine residues via thioester bonds and it likely has affinity for caveolae/lipid rafts microdomains (124, 131-132). Moreover, Veldman *et al.* described a NSMase in caveolae isolated from human skin fibroblasts where the enzyme appeared to interact with the scaffolding domain of caveolin protein (133). Also, Czarny *et al.* have reported a caveolar form of NSMase in the caveolin-enriched membrane fractions isolated from bovine lung microvascular endothelial cells (134).

Programmed cell death is an essential and strongly regulated process for normal tissue development and homeostasis. The role of ceramide as a major mediator of apoptosis is well-established. Data in the literature indicate sphingomyelinases-induced ceramide production as a major pathway in this process, a theory supported by the activation of NSMase in response to various apoptotic stimuli, including cytokines, serum starvation and heat stress (135-136). Several studies have described a possible role for NSMase-mediated ceramide production in regulating cell cycle and growth arrest (137-138). There exist strong evidence for sphingolipids as mediators of inflammatory

responses such as prostaglandin production, upregulation of adhesion molecules and leukocyte recruitment (139) and the possibility has been raised that NSMase activation could be involved in the cytokine-induced inflammatory process (137).

TNFα and IL-1β activate cellular sphingomyelinases in different cell types (116-119). The mechanisms by which IL-1β activates NSMase remains to be elucidated. Majority of studies on sphingomyelinases activation have used a model system utilizing the p55 TNFα receptor. Kronke *et al.* have defined a region of p55 receptor that is adjacent to the death domain and is required for NSMase activation (50). This motif was named the NSMase activation domain. The same authors have also described a novel protein (factor associated with NSMase activation, FAN) that binds the NSMase activation domain on the TNFα receptor and is required for NSMase activation (140). Overexpression of full-length FAN increased NSMase activity in TNFα treated cells, whereas a truncated mutant of FAN exerted dominant negative effects. Similarly, a dominant negative form of FAN significantly decreased cell death induced by CD40 and TNFα in transformed human fibroblasts (141-142). Although all these studies begin to shed some light on NSMase mechanism of activation, the mechanisms coupling FAN to NSMase have not been determined.

4.2. Acid Sphingomyelinase

Acid sphingomyelinase (ASMase) is a soluble glycoprotein and the first described sphingomyelinase. The complete absence of this enzyme is responsible for Niemann-Pick syndrome, a major neurological disorder (128). The gene encoding ASMase is designated as sphingomyelin phosphodiesterase 1 (*SMPD1*), is located within an "imprinted"region

of the human genome (chromosomal region 11p15.4) and is preferentially expressed from the maternal chromosome. This form of genetic regulation is classical for genes that play a critical role in development (143-144).

The biosynthesis of ASMase begins with a 75 kDa pro-precursor that is proteolitically cleaved to a 72 kDa precursor in the endoplasmic reticulum and Golgi. The 72 kDa form is then processed in the endosome-lysosome compartment to a 70 kDa mature and fully active lysosomal ASMase. The mature forms of ASMase, as well as its precursors, possess six potential N-glycosylation sites and out of this, at least five are critical for proper folding, protection against proteolysis and enzymatic activity (145-146).

4.2.1. Tissue Distribution and Cellular Localization

ASMase is a sphingomyelin phosphodiesterase enzyme ubiquitously express in various mammalian tissues (128) and is involved in various metabolic processes and signal transduction pathways. However, vascular endothelium is well recognized to express 20 times as much ASMase as any other cell type in the body (147) and it has been shown that this large surplus of ASMase in endothelium may be involved in tissue remodeling and wound repair (148), processes that normally require waves of endothelial cell apoptosis and tissue neovascularization (149).

Although ASMase was initially described as a strictly lysosomal enzyme because of its optimum activity at pH 4.5-5.0, an ASMase isoform was recently shown to be localized into secretory vesicles in close proximity to the plasma membrane and to be

secreted extracellularly upon cell stimulation (150); moreover, ASMase activity has also been observed in caveolae/lipid rafts membrane microdomains.

The secreted form of ASMase is stimulated by zinc, whereas the intracellular, lysosomal isoform is already tight bound to this cation; therefore, both ASMase isoforms require zinc for their activity (151). The secretory and lysosomal forms of ASMase are encoded by the same gene and display differences in the oligosaccharide structure as well as in the N-terminal proteolytic processing (151-152). The lysosomal form is rich in mannose-type oligosaccharides which are phosphorylated for lysosomal targeting; the secretory form of ASMase has complex-type, N-linked oligosaccharides (151).

Liu and Anderson have described the plasma membrane form of ASMase present in caveolae and they have shown that IL-1β stimulates its activity in this compartment (116). The same authors were first to report that ASMase localize to the caveolae microdomains in human fibroblasts and its activity was zinc-independent, suggesting that this ASMase was not the zinc-dependent secretory form of ASMase. Another study brought out evidence of the existence of a small pool of zinc-independent ASMase in the plasma membrane microdomains purified from NIH-3T3 L1 fibroblasts and PC12 cells and more importantly, this pool of ASMase could undergo activation in response to neurotrophins, whereas the lysosomal ASMase did not respond to activation by this ligand (153).

Various immunohistochemistry studies have shown that cells exposed to different stress stimuli exhibit a change in ASMase location from intracellular compartment to the cell surface (154). For instance, ASMase translocates onto the extracellular leaflet of plasma membrane upon activation, a process that is assumed to be mediated by the fusion

of the vesicles containing ASMase with the plasma membrane (155). It was shown that this process occurs within seconds after cellular stimulation, for example after CD95 ligation (150, 156). A recent study described ASMase activation and translocation to the outer leaflet of the plasma membrane by a mechanism that involves ASMase phosphorylation at serine 508 by protein kinase Cδ (PKCδ) upon cellular stimulation with phorbol ester (157). Zeidan *et al.* demonstrated that activated PKCδ is translocated to vesicles containing ASMase, PKCδ then phosphorylates ASMase; the phosphorylated enzyme then trafficks to the extracellular leaflet of the cell membrane where it generates ceramide and promotes the formation of ceramide-rich membrane platforms.

4.2.2. Mechanisms of Regulation

The mechanisms that control ASMase activation are not yet completely elucidated; however, several factors have been described to regulate ASMase activity *in vitro* and *in vivo* settings.

pH and ions. As previously mentioned, the optimum pH for ASMase activity ranges between 4.5 and 5.5; however, a pH increase appears to affect only substrate affinity and not ASMase activity (158). Although ASMase present at the cell surface functions best at acid pH, it is also capable of hydrolyzing certain physiologic substrates, including caveolae/lipid raft associated sphingomyelin or atherogenic proteins, at neutral or slightly acidic pH (159-160). Divalent cations (cobalt, calcium, magnesium and manganese) up to 250 mM and EDTA and EGTA have no effect on ASMase activity; however, the enzyme requires tightly bound zinc for its activity (124).

Lipids. It has been shown that various lysosomally occurring lipids, including bis (monoacylglycero) phosphate and phosphatidylinositol were highly effective in ASMase activation (124). Several reports postulated 1,2 diacylglycerol (DAG)-mediated ASMase activation upon cell stimulation with pro-inflammatory cytokines (TNFα, IL-1β) (116, 161-162) or Fas receptors (163). A recent study showed ASMase inhibition by sphingosine-1-phosphate in macrophages isolated from apoptotic bone marrow (164). However, the mechanisms of ASMase regulation are not completely understood.

Proinflammatory cytokines. Pro-inflammatory cytokines that are increased in diabetic eyes, TNFα and IL-1β have been demonstrated to activate cellular sphingomyelinases in various cell types (116-119). The mechanisms by which IL-1β stimulates ASMase activity have not yet been elucidated. p55 TNFα receptor was used as model system to study sphingomyelinases activation, showing that this receptor has particular domains for different sphingomyelinases. The intracellular C-terminal part of the p55 receptor, which contains a 75-amino acid motif, termed the death domain (DD), appears to be necessary for ASMase activation; the exact molecular mechanism is not known, but Schwandner *et al.* have reported ASMase activation (seconds to minutes) in response to TNFα through overexpression of TRADD (TNF receptor associated DD) and/or FADD (Fas-associating protein with DD) (165).

Other regulatory factors. Several studies have described a role for PI-kinase pathway (166), tumor suppressor p53 (167) and oxidants (168-169) in regulation of ASMase activity, but further studies are needed to elucidate the relationship between these pathways and ASMase activation.

4.2.3. Roles of ASMase

ASMase is considered to be an important mediator of inflammation and apoptotic effects due to various stimuli, including radiation (170-171), chemotherapy (172), ischemia (173), TNFα (174), Fas/CD95 (175). Lysosomal ASMase has a classical, important role is sphingomyelin storage and metabolism, as shown by sphingomyelin accumulation within the lysosomal compartment in Niemann-Pick disease (ASMase deficiency) (176). However, it is now believed that ASMase plays a key role in initiating cell signal transduction at the cell membrane level (and not in the intracellular compartment) (177) where it participates in formation of ceramide-enriched membrane microdomains that coalesce to form ceramide-enriched platforms, receptor clustering and amplification of signal transduction *via* a specific receptor (124, 178-180).

4.3. Sphingomyelinases, Plasma Membrane Microdomains and Inflammatory Signal Transduction

4.3.1. Plasma Membrane Microdomains

4.3.1.1. Classification and functions

Over the past decade, a number of membrane fractions could be isolated by their insolubility in the non-ionic detergent Triton X-100 at 4°C, conditions under which most cell membranes are disrupted. Due to their high lipid content, these detergent-resistant membrane fractions can be further isolated by flotation in sucrose gradients and density gradient centrifugation, due to their relatively low density (181). The detergent-resistant fractions enclose at least two kinds of structures: a) caveolae, bottle-shaped invaginations

of the plasma membrane that contain a cholesterol-binding protein named caveolin-1and b) non-caveolin containing membranous structures, also known as "rafts" or caveolae-related domains (CRD). Retinal endothelial cells contain both lipid rafts and caveolae. In spite of initial doubts, strong evidence supports the hypothesis that detergent-resistant, lipid-enriched domains do exist in cell plasma membrane in vivo and are not an artifact of detergent extraction (181-183). In addition, caveolae structures have been visualized in vascular endothelial cells since 1953 as 50-500 nm cell surface invaginations (184) and caveolae fractions have been isolated and purified in the absence of detergents (185).

Caveolae were initially believed to play an important role in regulation of transcytosis in endothelial and epithelial cells (186). However, at present, a more complex role for caveolae has been revealed, including regulation of vascular permeability (187), lipid trafficking, cholesterol homeostasis (188-189) and, in particular, signal transduction (190-191).

Caveolae are stabilized by particular membrane spanning proteins, the caveolins, a family of palmitoylated hairpin-like membrane proteins, which organize Ω-shaped cell-surface invaginations (192-193). Caveolin-1, the primary structural protein constituent of caveolae, is a biochemical marker for establishing the putative existence of caveolae (193).

Both types of detergent-resistant membrane fractions are enriched in cholesterol, sphingolipids and glycerophospholipids (194). The lipid core seems to play an important role in attracting lipid-modified membrane proteins to caveolae; many peripheral and integral molecules associated with caveolae/lipid rafts are dually lipidated with saturated acyl chains that readily group into this ordered lipid environment (195-197). The acyl

chains of these proteins intercalate in the lipid bilayer, collect in caveolae due to a slow lateral mobility (upon encountering the liquid-ordered phase that characterize the caveolae) and determine protein-protein and protein-lipid interaction at this site (198-200). The membrane surrounding the caveolae/lipid rafts is more fluid since is mainly composed of unsaturated phospholipids.

4.3.1.2. Sphingolipids and Plasma Membrane Microdomains

Sphingolipids represent a major component of the plasma membrane microdomains (122). Compared to phospholipids, sphingolipids have saturated long fatty acyl chains in their structure and can undergo a tighter packing in the cellular membrane due to the lack of unsaturated acyl chains with kinked structures (Figure 1.2) (45-46). In these membrane microdomain fractions, sphingolipids interact with each other *via* hydrophilic interactions between the sphingolipid headgroups (201-202). Moreover, sphingomyelin, the predominant sphingolipid in these membrane microdomains, interacts with cholesterol *via* hydrogen bonds with the hydroxyl group in the cholesterol structure and *via* hydrophobic van der Waal interactions between the ceramide moiety and sterol ring structure (155). This provides an extra element of stability of these membrane structures, which are said to exist in the "liquid-ordered state".

Sphingolipids may have vital roles in caveolae/lipid rafts structure and are now known to act as messengers in signaling pathways mediating inflammation, apoptosis, cell differentiation and proliferation (203). Lipid analysis showed that approximately 70% of total cellular sphingomyelin is found in caveolae/lipid rafts domains (122, 202). New evidence suggests that enrichment in sphingomyelin content in caveolae/lipid rafts make

them potential substrate pools for cellular sphingomyelinases to produce a high local concentration of ceramide.

4.3.2. Sphingomyelinases and Plasma Membrane Microdomains

Caveolae/lipid rafts appear to be definite sites for ceramide production in response to different agonists and stress signals. Activation of various forms of sphingomyelinases can hydrolyze the phosphodiester bond of sphingomyelin to ceramide and phosporylcholine (Figure 1.3) (122). Both NSMase and ASMase are rapidly and transiently activated by various exogenous stimuli, leading to a significant increase in ceramide levels in a time frame of seconds to minutes (204-206). Liu and Anderson showed for the first time elevated ceramide levels with consequent decrease in sphingomyelin content of caveolae compartment of human fibroblasts in response to proinflammatory cytokine IL-1β (116). Bilderback *et al.* showed that sphingomyelin hydrolysis takes place in caveolae-rich domains in NIH 3T3 fibroblasts in response to nerve growth factor (NGF) treatment (207). As a second messenger, ceramide plays a role in various biological processes (122, 208); yet, the exact mechanism by which ceramide completes its biologic functions is not completely understood. However, important findings in the last decade suggest that changes in cell membrane structure promoted by ceramide accumulation are essential for its biologic function (209).

Ceramide generation within the plasma membrane significantly alters the properties of the cell membrane since ceramide molecules spontaneously self-aggregate (201, 210). In membrane models, as little as five mol% ceramide is enough for spontaneous formation of ceramide-rich membrane domains (211). Furthermore, these

ceramide-rich domains spontaneously self-associate to generate ceramide-rich macrodomains with a diameter up to 5μm (201, 210, 212). The formation and existence of ceramide-rich membrane domains was visualized *in vivo* by the use of confocal microscopy employing fluorescent-labeled anti-ceramide antibody (150, 213-215). Changes of the biophysical properties of the membrane domains may result in selective trapping of proteins, and in particular various receptors and signaling molecules, within these ceramide-rich membrane platforms. Clustering of receptors may lead to an increase in receptor density, a spatial connection of activated receptors with downstream signaling molecules, a conformational modification of the receptor and a possible stabilization of the receptor-ligand interaction. Thus, the release of ceramides alters the dynamics of these rafts and may drive signal transduction processes by allowing oligomerization of specific cell surface molecules, such as ligated receptors. Among these are the TNFα receptor (216-217), IL-1 receptor (117), CD95 (150, 156), CD40 (178), LFA-1 (218), CD5 (219), FcγRII (213), CD20 (220), platelet-activating factor (PAF) receptor, insulin receptor, epidermal growth factor receptor, and T cell receptor are most characterized (180). Clustering is shown to be an important feature used by several receptors that mediate inflammatory signaling pathways in DR, such as TNFα and IL-1β pathways (221-222). Thus, ceramide-rich macrodomains may have a primarily role to reorganize receptors and signaling molecules at the plasma membrane level in order to facilitate and augment signaling processes *via* a particular receptor (Figure 1.4) (155).

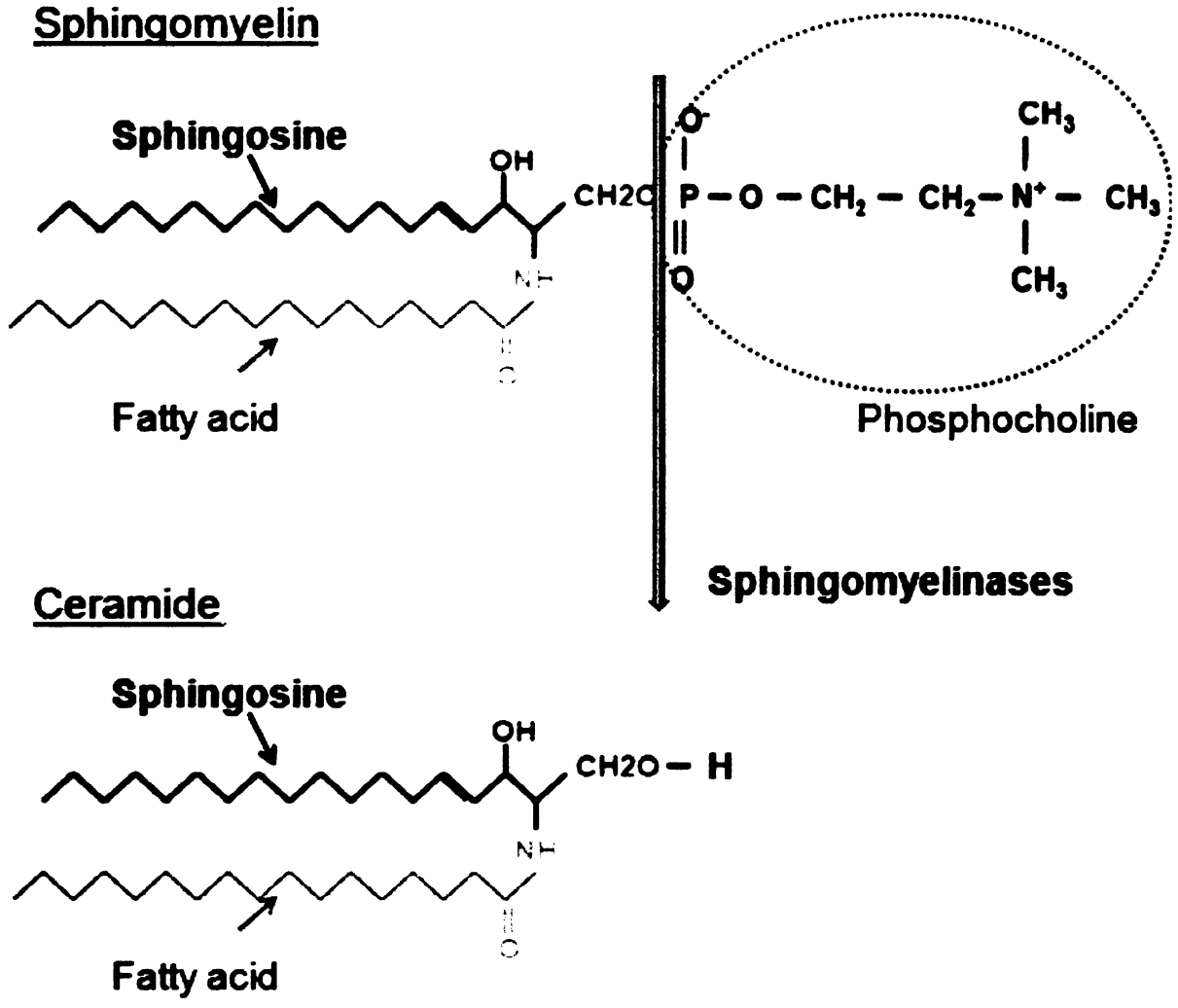


Figure 1.3. Ceramide generation by sphingomyelinases-mediated sphingomyelin hydrolysis. Sphingomyelin consists of a sphingosine backbone, a fatty acid linked by an amide bond to the sphingoid base and a phosphocholine head group. Ceramide consists of a sphingosine backbone with the fatty acid attached to the sphingoid base. Sphingomyelin hydrolysis by sphingomyelinases generates ceramides.

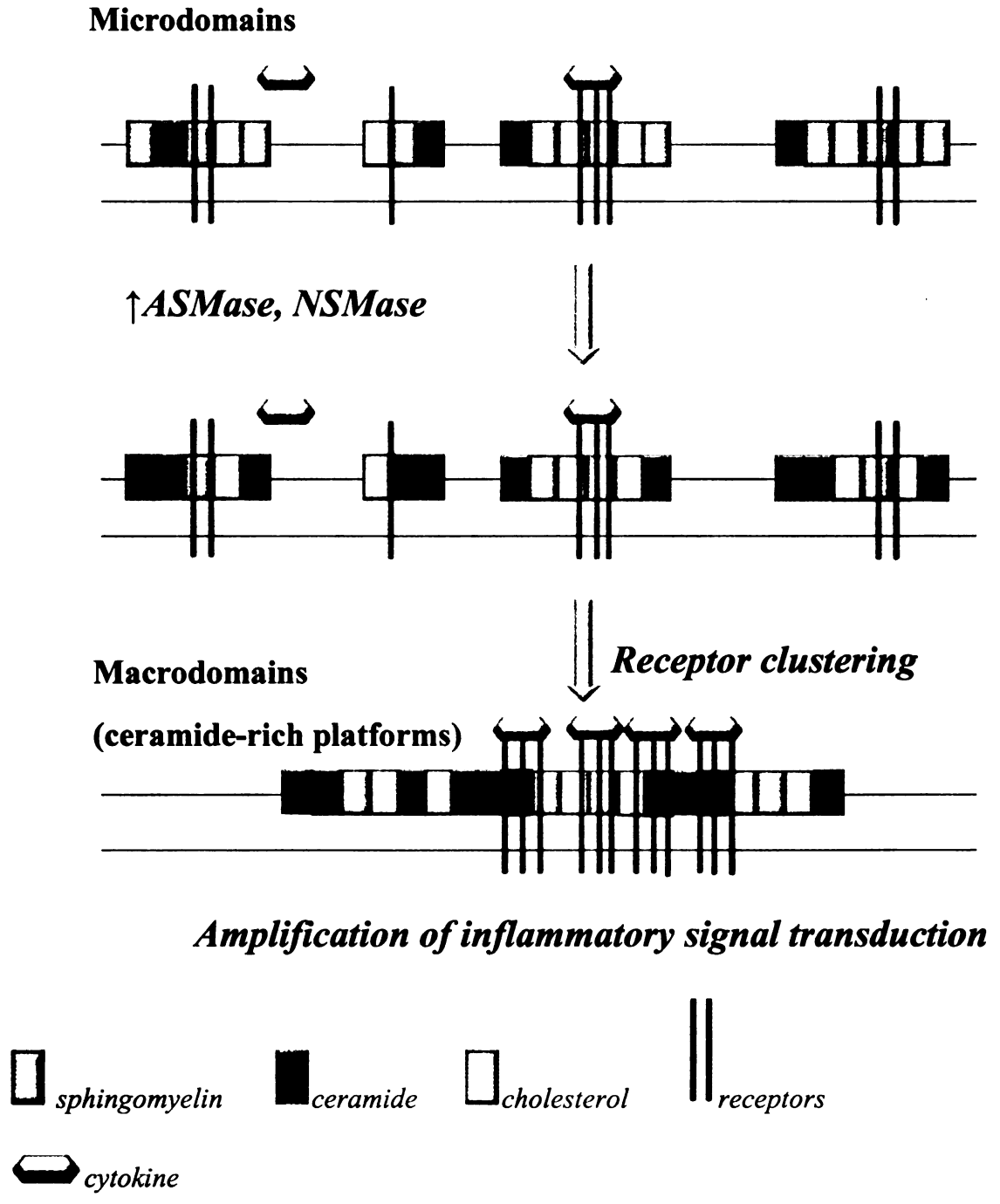


Figure 1.4. Receptor clustering and amplification of signal transduction by ceramide-enriched macrodomains. Stimulation of cells *via* a receptor, for example p55TNFα receptor, activates ASMase and/or NSMase with ceramide-release from sphingomyelin that results in formation of ceramide-enriched macrodomains. These membrane platforms induce further receptors clustering, re-organization of the intracellular signalosome and amplification of the initial signal.

5. Retinal Vascular Endothelium

5.1. Overview

Vascular endothelium forms the inner blood-retinal barrier, a metabolically active interface responsible for fatty acids uptake from the blood and delivery to the retina. Moreover, retinal vasculature may also actively remodel fatty acids, thus providing the retina with long chain highly unsaturated fatty acids such as DHA (223). Several studies have shown that retinal microvessels are highly enriched in DHA (60). In retinal bovine vasculature, DHA accounts for approximately 10% of the total fatty acids (60). Therefore, DHA may have vital role in maintaining retinal capillary structure and integrity (62). Moreover, DHA supplementation in cytokine-stimulated human retinal endothelial cells has a pronounced anti-inflammatory effect (39-40).

Retinal vascular endothelial cells are the resident vasculature affected in DR. Although diabetes may also result in damage to nonvascular retinal tissue, retinal microangiopathy lesions represent the key pathologic feature of DR. Diabetic retinal endothelial dysfunction includes a number of functional alterations in the vascular endothelium, including inflammatory activation, cellular apoptosis and altered blood-retinal barrier function. Many of the molecular and functional changes characteristic of inflammation have been detected in retinas from diabetic animal models and humans and are recognized to be an early trigger for endothelial cell activation and apoptosis and further development of degenerated, non-perfused capillaries. The inflammatory mechanisms in retinal endothelial cells appear to be mediated at the plasma membrane level where the integrity of membrane microdomains are essential for inflammatory signal transduction (39).

5.2. Sphingomyelinases and Vascular Endothelium

Vascular endothelial cells, such as human retinal endothelial cells, represent one of the cell type that possess lipid rafts and numerous caveolae structures with high levels of caveolin-1 (224). Membrane domains, by virtue of their unique lipid composition, serve as signaling platforms that regulate various cellular events (225). Sphingolipids (sphingomyelin, ceramide) represent an essential component of membrane microdomains and, as previously described, sphingomyelinases-induced ceramide enrichment within caveolae/lipid rafts would promote receptors' clustering that is a prerequisite for the signalosome organization and amplification of intracellular inflammatory cascade.

Although sphingomyelinases are ubiquitously expressed in various tissues and organs (128, 226), vascular endothelial cells are well recognized to be an important source of ASMase (119, 135), containing as much as 20 times more ASMase than other cell types in the body (147). Moreover, human coronary artery and human umbilical vein endothelial cells, as well as bovine and murine aortic endothelial cells secrete copious amounts of ASMase in response to inflammatory cytokines (119). Importantly, an ASMase isoform has been identified in the caveolar fractions isolated from pulmonary vascular endothelial cells (227), suggesting a major role for ASMase in local ceramide production and amplification of cellular inflammatory transduction in pulmonary vascular endothelium. Several studies have identified ASMase activation as a triggering mechanism and driving force for membrane microdomain clustering in coronary endothelial cells and consequent endothelial dysfunction (228-230).

Retinal vascular inflammation and degeneration is the hallmark microangiopathic lesion characteristic for DR. Therefore, in this study, we first examined *in vitro* the role of

sphingomyelinases in mediating cytokine-induced inflammatory signaling in human retinal endothelial cells; then we assessed sphingomyelinases involvement in retinal inflammation and vascular degeneration in *in vivo* animal models of retinopathy.

6. Hypothesis and Objective of the Thesis

Sphingomyelinases, key regulatory enzymes of sphingolipid metabolism, are important early responders in inflammatory cytokine signaling that catalyze the hydrolysis of sphingomyelin to the proinflammatory bioactive second messenger ceramide. SMases are critically involved in pathophysiology of various metabolic disorders (47) and play an active role in cellular signaling (124). ASMase is shown to be significantly upregulated in plasma (231), skeletal muscle (57) or adipose tissue (232) of diabetic humans and animal models, whereas increased NSMase has been described in diabetic skeletal muscle and adipose tissue (57, 232-233). However, their role in mediating retinal inflammation and vascular degeneration in diabetic retinopathy has not been described.

Omega 3-PUFAs and particularly DHA have the ability to modulate various biological processes involved in retinal vascular inflammation, retinal capillary structure and integrity alterations, and retinal neovascularization. The major ω3-PUFA DHA is decreased in diabetic retina and associates with the inflammatory status in the diabetic retinal tissue (61). DHA is well recognized to have anti-inflammatory properties and our previous study demonstrated that DHA inhibits cytokine-induced retinal endothelial cells activation through modification of caveolae membrane microdomain (39-40). The role of DHA in sphingolipid methabolism is not known and will represent one of the goals of this dissertation.

Various inflammatory signaling pathways involved in the pathogenesis of diabetic retinopathy, such as TNFα and IL-1β pathways, are initiated at the receptors located in the caveolae/lipid rafts and ceramide enrichment in these membrane microdomains

promotes receptors' clustering that is a prerequisite for the intracellular inflammatory cascade. Therefore, we hypothesize in this study that sphingomyelinases play an important role in modulating intracellular inflammatory signaling in retinal vascular endothelial cells and inhibition of sphingomyelinases will render protection against cytokine-induced endothelium activation.

Taken all together, in this dissertation we set our goal to determine the effect of inflammatory cytokines on sphingomyelinases activation; the role of sphingomyelinases in mediating cytokine-induced inflammation; the caveolae microdomains lipid composition with regards to sphingomyelin and ceramide molecular species in cells where sphingomyelinases activation and inhibition is attained and the subsequent impact on cytokine-induced inflammatory signaling. The studies will be conducted in primary vascular endothelial cells isolated from human retina as a cell culture model. Furthermore, the role of sphingomyelinases in mediation of inflammatory process obtained in the cell culture setting will be confirmed and characterized in *in vivo* studies using three complementary animal models of retinopathy: 1) streptozotocin (STZ)-induced diabetes rat model, 2) retinal ischemia-reperfusion mouse and rat models and 3) oxygen-induced retinopathy mouse model.

Overview of chapters

Chapter I is dedicated to a literature review of diabetic retinopathy, the role of dyslipidemia and ω-3 PUFAs (especially DHA) in mediating retinal inflammation, the inflammatory mechanisms in diabetic retinopathy that result in endothelial cell

dysfunction and death, and involvement of sphingomyelinases (sphingomyelin/ceramide pathway) in inflammatory signal transduction.

Chapter II establishes the anti-inflammatory role of DHA by decreasing basal and cytokine-induced activation of ASMase and NSMase, whereas inhibition and/or gene silencing of both SMases recapitulates the DHA protective effect against cytokine-induced inflammatory signaling in human retinal endothelial cells.

Chapter III determines the anti-inflammatory effect of regulation of caveolar ASMase expression by DHA and the impact on caveolae/lipid membrane microdomains sphingomyelin and ceramide molecular species that results in decreased cytokine-induced inflammatory signaling in human retinal endothelial cells.

Chapter IV demonstrates ASMase downregulation by DHA as a protective, anti-inflammatory effect in diabetic retinopathy that prevents retinal vessel loss in *in vivo* model of type 1 diabetic rat.

Chapter V characterizes the decreased inflammatory profile and reduced retinal vessel loss in the ASMase deficient ($ASMase^{-/-}$) when compared to wild type ($ASMase^{+/+}$) mouse model of retinal ischemia-reperfusion, as well as the protective role of ASMase genetic deficiency against retinal pathological neovascularization in the oxygen-induced retinopathy mouse model.

The results of this work will add to our understanding the role of sphingomyelin/ceramide pathway in mediating retinal vascular inflammation, vessel loss and aberrant angiogenesis and facilitate novel therapeutic and preventive strategies for development and progression of diabetic retinal microangiopathic lesions.

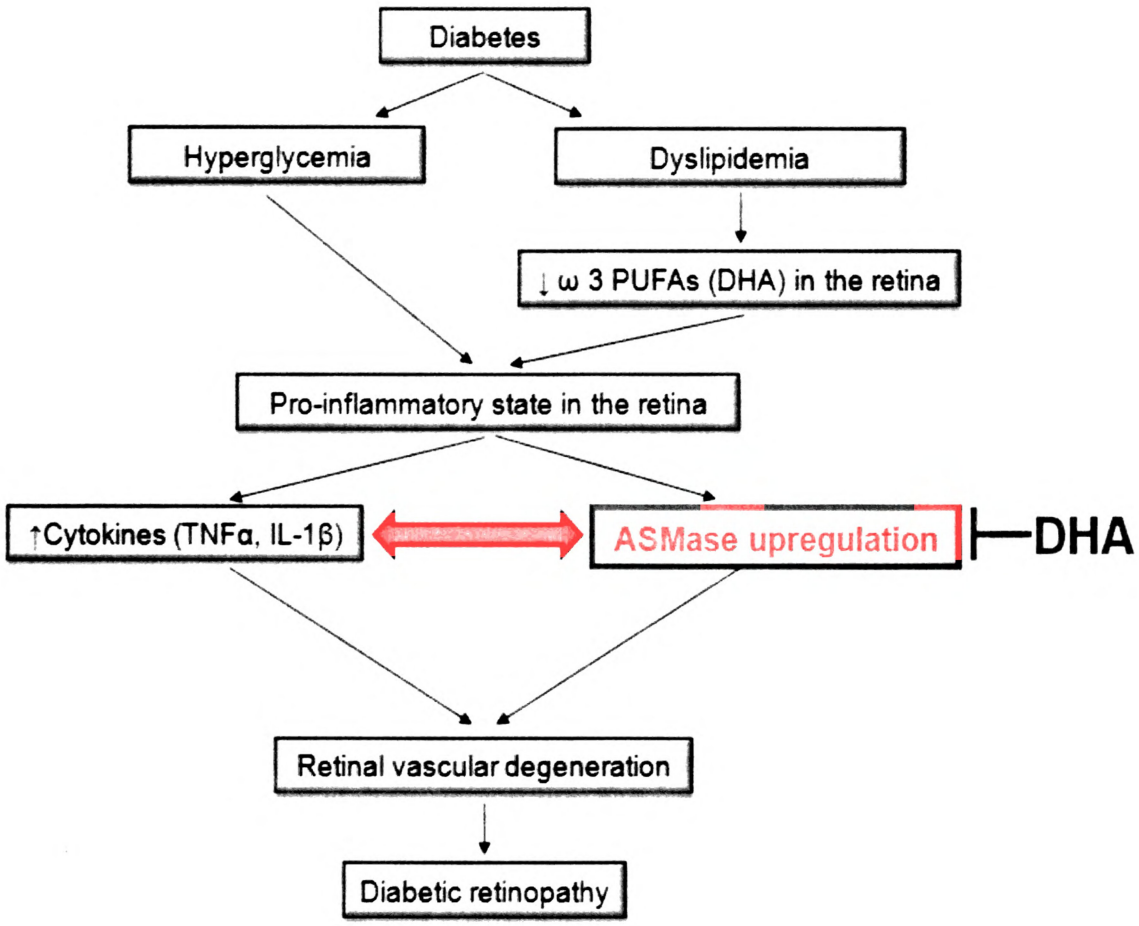


Figure 1.5. Diagram of working hypothesis. Potential impact of decreased retinal ω3-PUFA DHA on retinal inflammatory status, ASMase upregulation, microvascular complications and progression of diabetic retinopathy.

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Chapter II

Docosahexaenoic Acid Inhibits Cytokine Signaling by Downregulating Sphingomyelinases in Human Retinal Endothelial Cells

1. Abstract

The anti-inflammatory mechanism of docosahexaenoic acid (DHA) is well accepted; however, the molecular mechanism(s) of DHA action are still not well defined. We have previously demonstrated that DHA modifies the caveolar membrane microdomain and inhibits cytokine-induced inflammation in human retinal endothelial cells (HREC), the resident vasculature affected by diabetic retinopathy. Sphingolipids represent a major component of plasma membrane microdomains and ceramide-enriched microdomains appear to be a prerequisite for inflammatory cytokines signaling. Acid and neutral sphingomyelinase (ASMase and NSMase) are key regulatory enzymes of sphingolipid metabolism, promoting sphingomyelin hydrolysis to pro-inflammatory ceramide. In this study, we propose to test the hypothesis that DHA inhibits cytokine-induced inflammatory signaling in HREC by downregulating sphingomyelinases.

Primary cultures of HREC were maintained in the presence or absence of DHA. ASMase and NSMase activity was determined by sphingomyelinase assay. The expression of ASMase, NSMase, ICAM-1 and VCAM-1 was assessed by quantitative PCR and Western blotting. Gene silencing of ASMase and NSMase was obtained by siRNA treatment. Inflammatory cytokines TNFα and IL-1β induced cellular adhesion molecules (CAMs) expression and rapid increase in ASMase and NSMase activity in

HREC. Growth of HREC in media supplemented with DHA decreased basal and cytokine-induced ASMase and NSMase expression and activity, as well as upregulation of CAMs expression. Anti-inflammatory effects of DHA on cytokine-induced CAMs expression were mimicked by inhibition/gene silencing of ASMase and NSMase. Inhibition of sphingomyelinases activity was mediated by DHA-induced cholesterol displacement from caveolae/lipid microdomains. Sphingomyelinases pathway rather than ceramide *de novo* synthesis pathway was important for inflammatory signaling in HREC.

This study provides a novel potential mechanism for anti-inflammatory effect of DHA in HREC. DHA downregulates basal and cytokine-induced ASMase and NSMase activity and expression level in HREC and inhibition of sphingomyelinases in endothelial cells prevents cytokine-induced inflammatory response.

2. Introduction

The retina has a unique fatty acid profile, with the highest level of polyunsaturated fatty acids (PUFAs) in the body, especially DHA (1). Deficiency in DHA has been documented to be associated with a number of retinal degenerative diseases, including retinitis pigmentosa, retinopathy of prematurity and age-related macular degeneration (reviewed in (2)). DHA is also significantly decreased in plasma of diabetic children (3), as well as in the human retina of diabetic eyes (4). Recent data showed a beneficial effect of dietary DHA in reducing pathological retinal angiogenesis and thus preventing the development of oxygen-induced retinopathy (5). Yet, the DHA protective mechanism in retinopathy remains poorly understood.

The aim of our study was to investigate the anti-inflammatory mechanism of DHA in human retinal endothelial cells (HREC), the target tissue affected by diabetic retinopathy. Very early stage diabetic retinopathy is hypothesized to represent a low-grade chronic inflammatory disease that involves leukocyte adhesion to the retinal vasculature, a process mediated by adhesion molecules expressed on the endothelial cell surface, especially intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (6-8). Pro-inflammatory cytokines, including TNFα and IL-1β are increased in diabetic eyes (9-11) and induce upregulation of adhesion molecule expression (12). Our group has previously shown that DHA inhibits cytokine-induced cellular adhesion molecule expression in HREC through cholesterol displacement from caveolae/lipid rafts membrane microdomains (12).

HREC contain lipid rafts and specific plasma membrane microdomains known as caveolae that are considered to have an important role in regulating vascular permeability

(13), lipid trafficking, cholesterol homeostasis (14-15) and signal transduction (16-17). The caveolae are stabilized by membrane spanning proteins, the caveolins, a family of palmitoylated hairpin-like membrane proteins (18-21). Caveolin-1, the primary structural protein constituent of caveolae in endothelial cells, is a biochemical marker for caveolae identification (22).

Caveolae/lipid rafts are compositionally and functionally specific domains which are not static but are rather in equilibrium with the rest of membrane lipids; they are lateral assemblies of cholesterol, sphingolipids (sphingomyelin, ceramide) and glycerophospholipids (23). Sphingolipids play essential roles in membrane microdomain structure and are now known to also act as messengers in signaling pathways mediating inflammation, apoptosis, cell differentiation and proliferation (24). Ceramides can be generated at the membrane level by catabolism of sphingomyelin by either neutral or acid sphingomyelinases or by *de novo* synthesis in the endoplasmic reticulum.

NSMase and ASMase are rapidly activated by diverse stress stimuli and promote hydrolysis of sphingomyelin into ceramide and phosphorylcholine (25-27). NSMase is considered one of the major candidates for mediating stress-induced production of ceramide; it is a membrane-bound protein and palmitoylated on five cysteine residues via thioester bonds (28-30). ASMase was initially described as a lysosomal enzyme because of its optimal pH at 4.5–5.0. Nevertheless, an ASMase isoform was recently shown to be enclosed into secretory vesicles in close proximity to the plasma membrane and to be secreted into the extracellular space upon cell stimulation (31-33). Liu and Anderson described the plasma membrane form of ASMase in caveolae and showed that the pro-inflammatory cytokine IL-1β may induce ASMase activation in this compartment (27).

Lipid analysis demonstrated that approximately 70% of total cellular sphingomyelin is found in membrane microdomains (20, 34-37). Enrichment of sphingomyelin content in membrane microdomains makes them potential substrate pools for cellular sphingomyelinases to produce a high local concentration of ceramide; ceramide-enriched membrane microdomains have the ability to self-associate to form ceramide rich macrodomains (38-41) that mediate receptor clustering and downstream signaling (42-43). Clustering is shown to be an important feature used by several receptors that mediate inflammatory signaling pathways in diabetic retinopathy, such as the TNFα and IL-1β pathways (44-45).

In this study, we tested whether the anti-inflammatory mechanism of DHA is mediated through downregulation of ASMase and NSMase, as manifested by decreased cytokine signaling in human retinal endothelial cells.

3. Materials and methods

3.1. Reagents and Supplies

DMEM and F12 culture medium, antibiotics, fetal bovine serum and trypsin were obtained from Invitrogen (Carlsbad, CA). Amplex Red Sphingomyelinase Assay Kit, Amplex Red Cholesterol Assay Kit, NuPAGE Novex 10% Bis- Tris gels, Platinum SYBR Green qPCR SuperMix-UDG w/ROX were purchased from Invitrogen (Carlsbad, CA). GW4869 was purchased from Calbiochem (San Diego, CA). Desipramine, methyl-cyclodextrine and commonly used chemicals and reagents were purchased from Sigma (St. Louis, MO). Caveolin-1 and LAMP1 antibodies (BD Bioscience, San Jose, CA), ICAM-1, VCAM-1 and NSMase antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used. ASMase antibody was a generous gift from Dr. Richard Kolesnick. TNFα and IL-1β were from R&Dsystems (Minneapolis, MN). ON-TARGET plus SMART pool SMPD1 (ASMase), ON-TARGET plus SMART pool SMPD2 (NSMase) and ON-TARGET plus siCONTROL (non-targeting pool, against luciferase) were purchased from Dharmacon (Chicago, IL). For lipid extraction and mass spectrometry, all solvents used were HPLC grade. Methanol (MeOH), and water were purchased from J.T. Baker (Phillipsburg, NJ). Ammonium hydroxide (NH_4OH) and chloroform ($CHCl_3$) were from EMD Chemicals (Gibbstown, NJ). Isopropanol was from Fisher Scientific (Pittsburgh, PA). Lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL).

3.2. Cell Culture

Primary cultures of HREC were prepared from tissue (National Disease Research Interchange, Philadelphia, PA) cultured as previously described (46). Passages 1 to 5 were used in the experiments. For experimental treatment, cells were transferred to serum-free medium for 14 to 24 hours before stimulatory agents were added.

3.3. Fatty Acid Treatment

Fatty acid stocks were prepared by dissolving fatty acids (NuCheck Prep, Inc., Elysian, MN) in ethanol to a final concentration of 100 mM fatty acid, as described previously (12, 47). The fatty acid stock solutions were diluted to 50 to 100 μM in serum-free medium containing 10 to 20 μM charcoal-treated, solvent-extracted, fatty acid–free bovine serum albumin (BSA; Serologica Inc., Norcross, GA) as a fatty acid carrier. The fatty acid/albumin molar ratio was maintained at 5:1 (48). The final concentration of ethanol in the media was less than 0.1%. Cells were incubated for the times indicated in Results. Equivalent amounts of BSA and ethanol were added to control plates. Linoleic acid, a ω6 polyunsaturated fatty acid, was used as a lipid control for the following considerations. The most abundant polyunsaturated fatty acid classes in the retina microvessels are ω3 (especially DHA) and ω6 (linoleic acid, arachidonic acid) fatty acids (1). In this study we wanted to compare the effects of the principal ω3 PUFA (DHA) with a ω6 PUFA (linoleic acid).

3.4. Sphingomyelinase Assay

Fatty acids (DHA and linoleic acid) were added to HREC in serum-free medium to a concentration of 100 μM fatty acid with 20 μM charcoal-treated, solvent extracted, fatty-acid-free bovine serum albumin (BSA) as a fatty acid carrier. Cells were incubated with fatty acids for specified times at 37°C; equivalent amounts of BSA and ethanol were added to the control plates. Then cells were lysed in the acid lysis buffer (50 mM Sodium Acetate, pH=5; 1% TritonX-100; 1 mM EDTA) or neutral lysis buffer (20 mM Tris-HCl, pH=7.5; 1% TritonX-100; 1 mM EDTA) with freshly added protease inhibitor cocktail (Sigma, St. Louis, MO). For the sphingomyelinase assay in a cell-free system, 0.5mU of bacterial sphingomyelinase was incubated with 5-80 μM fatty acids (DHA and linoleic acid), while equivalent amounts of ethanol were added to the control wells. Sphingomyelinase activity was measured using the Amplex Red Sphingomyelinase Assay Kit (Molecular Probes, Eugene, OR) as described in the manufacturer's protocol.

3.5. Methyl-β-cyclodextrin (MCD) and Cholesterol Treatment

To deplete cholesterol from the membranes, cells were treated with 8 mM MCD for 30 minutes. To replenish cholesterol, cells were treated with 25 μM water-soluble cholesterol (25 μM cholesterol complexed with 250 μM MCD; Sigma) for 30 minutes. After MCD or water-soluble cholesterol treatment, the cells were washed twice with PBS containing 40 μM BSA before membrane microdomain isolation, and cell lysate and plasma membrane preparation for the sphingomyelinase assay.

3.6. Cholesterol Measurement

Cholesterol levels of membrane microdomains isolated from HREC with or without MCD treatment (previously described) were measured by the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR) as described in the manufacturer's protocol.

3.7. Western Blot Analysis

Cells were lysed in the lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100 and 10% glycerol) with freshly added protease inhibitor cocktail (Sigma) and phosphatase inhibitors (1 mM Na_3VO_4, 100 μM glycerophosphate, 10 mM NaF, 1mM Na_4PPi). Protein concentration was measured by Qubit fluorometer (Invitrogen, Eugene, OR) as described in the manufacturer protocol. Proteins were resolved by NuPAGE on Novex 10% Bis-Tris gels, transferred to nitrocellulose membrane, and then immunobloted using appropriate primary antibodies followed by secondary horseradish peroxidase-conjugated antibody (Bio-Rad, Hercules, CA) or IRDye infrared secondary antibodies (Invitrogen, Molecular probes, Eugene, OR). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ) or the Odyssey program. Blots were quantified by scanning densitometry (ImageJ software).

3.8. Gene Silencing of ASMase and NSMase

For silencing ASMase and NSMase expression, cultured HREC were detached with trypsin, centrifuged at 100 g for 5 minutes and resuspended in electroporation solution (Amaxa, Gaithersburg, MD) to a final concentration of 4-5x10^5 cells/100 μL. The 100 μL of cell suspension was mixed with 100 nM ASMase/NSMase siRNA into the electroporation cuvette. HREC were then electroporated with the Nucleofactor program M-030 (Amaxa Biosystems). The electroporated cells were maintained in supplemented medium in 37°C/5% CO_2 incubator for 48 hours prior to TNFα (10 ng/ml) and IL-1β (5 ng/ml) treatment. To determine the efficiency of gene silencing, RNA was extracted from 100 nM siRNA treated HREC and used as template for real time-PCR as described (49). ASMase/ NSMase siRNA treatment of HREC induced gene silencing of 94 % for ASMase and 86 % for NSMase.

3.9. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from HREC with or without DHA pretreatment(50). Specific primers for each gene were designed using IDT DNA PrimerQuest software (Coralville, IA): for NSMase: forward –GAGCGCAATCATGGCAGT and reverse –CCCACTGA ACCAGTCACCAT; for ASMase: forward –CAACCTCGCGCTGAAGAA and reverse– TCCACCATGTCATCCTCAAA. First strand cDNA was synthesized using the SuperScript II RNase H-Reverse Transcriptase (Invitrogen Carlsbad, CA). Synthesized cDNA was mixed with 2x SYBR Green PCR Master Mix (Invitrogen Carlsbad, CA) and different sets of gene-specific forward and reverse primers, and then subjected to real-

time PCR quantification using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). All reactions were performed in triplicates. The relative amounts of mRNAs were calculated by using the comparative CT method (User Bulletin #2, Applied Biosystems). Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA.

3.10. Isolation of Caveolae/Lipid Rafts Membrane Domains

Isolation of caveolae/lipid raft-enriched detergent-resistant membrane domains were prepared using a slightly modified sucrose gradient ultracentrifugation protocol(47). Briefly, 5 X 10^6 HREC were washed twice with cold PBS, lysed in 0.8 ml MNE buffer (25 mM MES, pH 6.5, 0.15 M NaCl, and 5 mM EDTA) containing 1% Triton X-100, fresh protease and phosphatase inhibitors, and kept on ice for 20 minutes. Lysates were then homogenized with 10 strokes of a tight fitting Dounce homogenizer and spun down at 4000*g* at 4°C for 10 minutes. Supernatant (0.8 ml) was mixed with the same volume of 80% sucrose prepared in MNE buffer and was placed at the bottom of an ultracentrifuge tube. Then 1.6 ml of 30% sucrose and 0.8 ml of 5% sucrose were overlaid on top of the sample to form a 5 % to 30 % discontinuous sucrose gradient. After 18 hours of centrifugation at 200,000 *g* and 4°C in a swinging bucket rotor, 0.4 ml samples were collected carefully from the top of each fraction. A band confined to fractions 2 through 4 was designated as caveolae/lipid raft–enriched membrane domains.

3.11. Isolation of Plasma Membrane Domains

Cells were lysed in hypotonic buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM $MgCl_2$ freshly added with protease inhibitors) for 30 minutes on ice, then homogenized with 20 strokes in a nonstick (Teflon; DuPont, Wilmington, DE) glass homogenizer and spun down at 500*g* for 5 minutes. Supernatants were subjected to centrifugation (16,900*g*, 60 minutes, 4°C) and pellets were collected as plasma membrane–enriched fractions.

3.12. Lipid Extraction

3 x 10^6 HREC were washed twice with cold PBS, collected in 500 µL cold 40% methanol, and then homogenized in a glass homogenizer on ice. The cell homogenate was transferred to a screw-cap glass tube and lipids were extracted as previously described (51). The samples were dried under a stream of nitrogen and re-extracted twice to remove non-lipid contaminants. The lipid film was dried under nitrogen, then further dried overnight in a speedvac, and resuspended in an appropriate amount of isopropanol : methanol : chloroform (4 : 2 : 1) based on the protein concentration of each sample.

3.13. Mass Spectrometry Analysis

Synthetic lipid standards (Sphingolipid Mix I, Avanti) were added to lipid extracts used in mass spectrometry analysis at 20 nmol/mg protein and 5 nmol/mg protein, respectively. Prior to analysis, lipids were appropriately diluted in isopropanol/methanol/chloroform (4 : 2 : 1 v /v/ v) containing 20 mM ammonium

hydroxide, centrifuged, then loaded into Whatman Multichem 96-well plates (Fisher Scientific, Pittsburgh, PA) and sealed with Teflon Ultra-Thin Sealing Tape (Analytical Sales and Services, Pompton Plains, NJ). Lipids were introduced to a Thermo model TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA) as previously described (51). Ceramide (Cer) molecular species were monitored as their $[M+H]^+$ ions by tandem mass spectrometry (MS/MS) precursor ion scanning for the formation of characteristic product ions at m/z 264.4 (Cer) in positive ionization mode. All MS/MS spectra were acquired automatically for 5-10 minutes at a rate of 500 m/z sec^{-1} by methods created using Xcalibur software (Thermo, San Jose, CA). Correction for ^{13}C isotope effects and quantitation of lipid molecular species against appropriate internal standards was performed using the Lipid Mass Spectrum Analysis (LIMSA) software (52) peak model fit algorithm in conjunction with an expanded user-defined database of hypothetical lipid compounds. Ceramide molecular species were quantitated ratiometrically by comparison with the peak area of a synthetic lipid internal standard. The LIMSA software peak model fit algorithm was used as previously described for peak finding and isotope correction to arrive at an "absolute" value for each identified lipid species. All identified ceramide species were summed in order to quantify the total ceramide amount in the samples. MS/MS collision energies, CID gas pressure, and Q1 and Q3 settings were optimized using synthetic lipid standards corresponding to the individual lipid classes of interest.

3.14. Statistical Analysis

Data are expressed as the mean ± SD. Factorial ANOVA with the post hoc Tukey test (GraphPad Prism5, GraphPad Software, San Diego, CA) was used for comparing the data obtained from independent samples. Significance was established at $P<0.05$.

4. Results

4.1. Dose Response of TNFα and IL-1β Induced CAMs Expression in HREC

To determine the dose of cytokines necessary to induce an inflammatory response in HREC, dose response curves for TNFα (0-20ng/ml) and IL-1β (0.5-10ng/ml) were established using CAMs (ICAM-1 and VCAM-1) expression as a measure. The induction of ICAM-1 and VCAM-1 by TNFα and IL-1β was assessed by immunoblot analysis (Figure 2.1). Based on these data, we used 10 ng/ml TNFα and 5 ng/ml IL-1β in the further studies.

4.2. TNFα and IL-1β Induce ASMase and NSMase Activation in HREC

The effect of proinflammatory cytokines TNFα and IL-1β known to be increased in diabetic eyes on ASMase and NSMase activity in HREC was first determined. Cells were stimulated with either 5ng/ml IL-1β or 10 ng/ml TNFα for 15sec – 2min. The cells were then collected for ASMase and NSMase activity analysis using the Amplex Red Sphingomyelinase assay kit. IL-1β treatment increased ASMase activity in HREC as early as 15 seconds of stimulation and ASMase activity remained significantly elevated during 30 seconds of stimulation (Figure 2.2A). Similar results were obtained for NSMase activity, where IL-1β stimulation induced a maximum activation after 45 seconds of treatment (Figure 2.2 C). HREC treatment with pro-inflammatory cytokine TNFα also significantly induced ASMase and NSMase activation after 45 seconds of stimulation (Figure 2.2, B and D).

4.3. Inhibition of Cytokine-Induced ASMase and NSMase Activation by DHA in HREC

DHA, the most abundant ω3-PUFA in the retina, has been shown to have a pronounced anti-inflammatory effect, inhibiting cytokine-induced CAMs expression in HREC and endothelial cell activation (12). To determine the effect of DHA on basal ASMase and NSMase activity, HREC were treated with DHA or linoleic acid (lipid control) for 24 hours. Pretreatment of HREC with 100 μM of BSA-bound DHA significantly decreased both ASMase (Figures 2.3 A, B, E) and NSMase (Figures 2.3 C, D, F) mRNA expression and activity level. Pretreatment of HREC with 100 μM of BSA-bound linoleic acid had no effect on ASMase or NSMase basal activity when compared to vehicle control (BSA). We next determined the effect of DHA on TNFα and IL-1β induced ASMase and NSMase activity. As shown in Figure 2.3, DHA, but not linoleic acid pretreatment of HREC, significantly down regulated TNFα and IL-1β-induced ASMase (Figure 2.3 A and B) and NSMase (Figure 2.3 C and D) activity. To validate the effect of DHA on cytokine-induced ASMase and NSMase activation, HREC were treated with 15 μM desipramine (53-54) and 25 μM GW4869 (55) prior to addition of stimulatory agents. Inhibition of cytokine-induced ASMase activity by desipramine is shown in Figure 2.4 A and inhibition of cytokine-induced NSMase activity by GW4869 is shown in Figure 2.4 B.

4.4. Effect of ASMase and NSMase Inhibition and/or Gene Silencing on TNFα and IL-1β Induced CAMs Expression

To establish the role of ASMase and NSMase in cytokine-induced inflammatory signaling in HREC, ASMase and NSMase activity was inhibited using desipramine (Sigma) and GW4869 (Sigma), respectively. HREC were treated with 15 μM desipramine (53-54) for one hour and 25 μM GW4869 (55) for 30 minutes prior to TNFα (10 ng/ml) stimulation. Six hours after cytokine stimulation the activation of inflammatory pathways was analyzed using ICAM-1 and VCAM-1 protein expression levels as a measure. As shown in Figure 2.5, inhibition of ASMase significantly reduced the TNFα induced adhesion molecules expression, similarly to results achieved with DHA treatment. Inhibition of ASMase also reduced IL-1β induced ICAM-1 expression (Figure 2.11 A, B and C). Interestingly, inhibition of NSMase did not have a significant effect on TNFα and IL-1β induced ICAM-1 expression, and although the effect on TNFα induced VCAM-1 expression was significant, it was less pronounced that the effect of ASMase inhibition or DHA.

Gene silencing of ASMase and NSMase (100 nM siRNA for ASMase and NSMase) significantly decreased TNFα and IL-1β-induced ICAM-1 and VCAM-1 expression in HREC (Figure 2.6) as compared to untreated or control siRNA (100 nM) treated cells. Similarly to the inhibitors study, ASMase gene silencing had more pronounced inhibitory effect compared to NSMase gene silencing. No additive effect on cytokine induced CAM expression was observed in the samples where both ASMase and NSMase gene silencing was attained.

4.5. Inhibition of ASMase and NSMase Activity by DHA

To determine the time course of DHA effect on ASMase and NSMase activity, HREC were treated with BSA-bound DHA, or BSA alone. First, the dose of DHA to be used in this experiment was selected based on dose–response experiment for ASMase (Figure 2.7 A) and NSMase (Figure 2.7 C) activity. The time course for DHA effect on ASMase and NSMase activity in HREC showed a gradual decrease in ASMase (Figure 2.7 B) and NSMase (Figure 2.7 D) activity with an early time point of 1 hour and 2 hours, respectively, and a maximum effect on both enzymes activity after 24 hours of DHA treatment. To determine whether the effect of DHA on sphingomyelinases activity is due to a direct interaction with the enzymes, we performed the sphingomyelinase assay in a cell-free system where 0.5 mU of bacterial sphingomyelinase (homolog of NSMase) was incubated with 5 to 80 μM fatty acid (DHA, linoleic acid). In this cell free system fatty acids were used without a carrier molecule. Free fatty acid concentration of 5 mM without a carrier in cell free system corresponds to 25 mM in cell culture experiments where fatty acids are complexed to BSA at 1:5 molar ratio. No fatty acids were added to the control samples. We found no direct effect of DHA or linoleic acid on SMase activity (Figure 2.8), strongly suggesting that direct binding of DHA to sphingomyelinases does not play a role in the observed inhibitory effects.

4.6. Inhibition of ASMase and NSMase Activity by MCD

DHA incorporation into the fatty acyl chains of phospholipids in caveolae/lipid rafts causes cholesterol displacement from these specialized membrane microdomains

(47). To prove that lipid composition of caveolae/lipid rafts controls sphingomyelinase activity, we determined the effect of cholesterol depletion on ASMase and NSMase in HREC. Caveolae/lipid raft fractions were isolated by sucrose discontinuous gradient ultracentrifugation based on their insolubility in Triton-X-100 at 4°C. Caveolin-1, a caveolae marker, was used to identify the lipid raft fractions in density gradient fractions 2 to 4 (Figure 2.9 A). MCD and DHA pretreatment of HREC dramatically decreased cholesterol content of the raft fractions (Figure 2.9 B) when compared to cholesterol content of caveolae/lipid raft fractions isolated from BSA and linoleic acid pretreated HREC.

Pretreatment of HREC with 8 mM MCD for 30 min decreased NSMase activity by 42%, similarly to DHA effect on NSMase activity (Figure 2.9 C). Cholesterol replenishment with 25 μM water-soluble cholesterol reversed the inhibitory effect on NSMase activity (Figure 2.9 C). NSMase activity was only assessed in the whole cell lysate since NSMase is only localized to the plasma membrane and activity in the whole cell lysate represents the quantification of NSMase activity in the plasma membrane compartment.

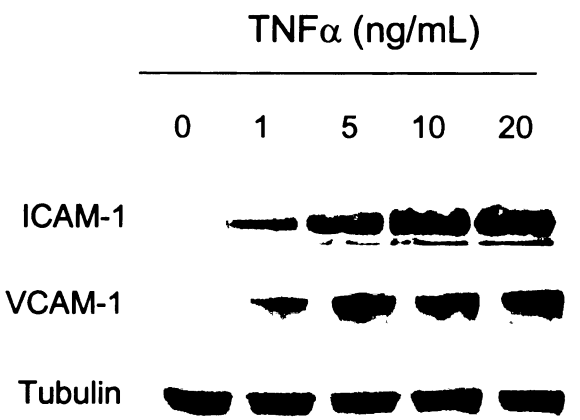
MCD treatment of HREC had no effect on whole cell lysate ASMase activity and cholesterol replenishment did not restore ASMase activity in DHA treated HREC (Figure 2.9 D). As a significant portion of cellular ASMase is in lysosomal compartment that would not be affected by MCD and cholesterol treatments used, we next analyzed ASMase activity in plasma membrane fraction. ASMase activity was significantly decreased in plasma membrane fraction isolated from MCD treated HREC and

cholesterol replenishment in DHA treated cells restored ASMase activity in this cellular compartment (Figure 2.9 E).

4.7. No Effect of Ceramide *de Novo* Synthesis Inhibition on TNFα and IL-1β Induced CAMs Expression in HREC

Ceramides can be synthesized in the cells at the membrane level from sphingomyelin hydrolysis by ASMase and/or NSMase or in the endoplasmic reticulum by *de novo* synthesis. We next determined whether ceramide *de novo* synthesis pathway is involved in mediating pro-inflammatory cytokine signaling in HREC. Fumonisin B1was used to inhibit ceramide synthase, the enzyme that catalyzes the final step of *de novo* pathway of ceramide synthesis. Cells were treated for 16 hours with 50 μM Fumonisin B1 prior to TNFα (10 ng/ml) and IL-1β (5 ng/ml) stimulation. The expression levels of ICAM-1 and VCAM-1 were analyzed by western blot (Figure 2.10, A and D). Fumonisin B1 pretreatment of HREC did not affect TNFα and IL-1β induced adhesion molecules expression. In contrast, as shown above, inhibition of ASMase significantly decreased TNFα and IL-1β induced ICAM-1 and VCAM-1 expression in HREC. These data suggest that ceramide production at the membrane level, rather than total cellular ceramide level, plays an essential role in modulating cytokine induced inflammatory signaling in HREC. Decreased ceramide levels in Fumonisin B1 treated HREC were confirmed by tandem mass spectrometry analysis. Inhibition of ceramide synthase by incubation of HREC with Fumonisin B1 resulted in a 26.55±9.45% decrease in HREC total ceramide levels, as expected (Figure 2.10 I).

A.



B.

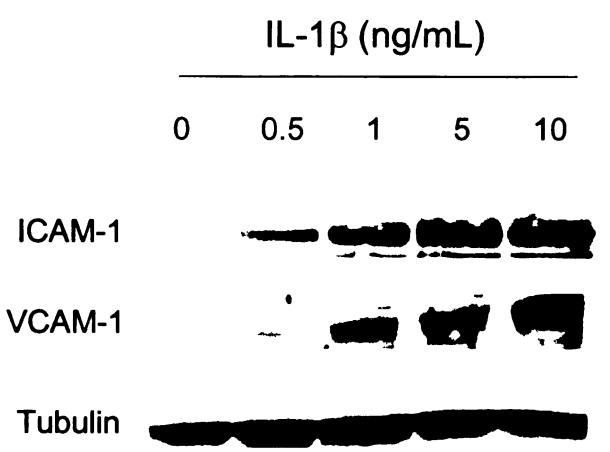
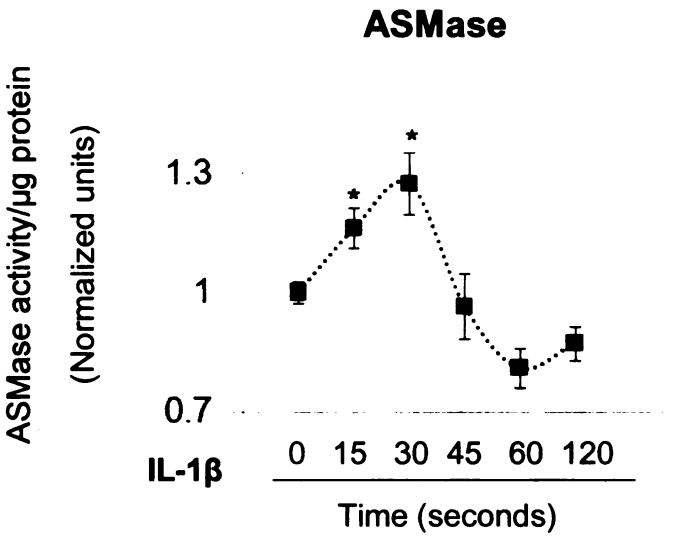


Figure 2.1. Dose response of cytokine-induced CAMs expression in HREC. HREC maintained in serum free media overnight were stimulated with 0-20 ng/ml TNFα and 0-10 ng/ml IL-1β for six hours. The induction of ICAM-1 and VCAM-1 by TNFα **(A)** and IL-1β **(B)** was assessed by immunoblot analysis. Equal amounts of protein were added to each lane as confirmed by tubulin level.

A.



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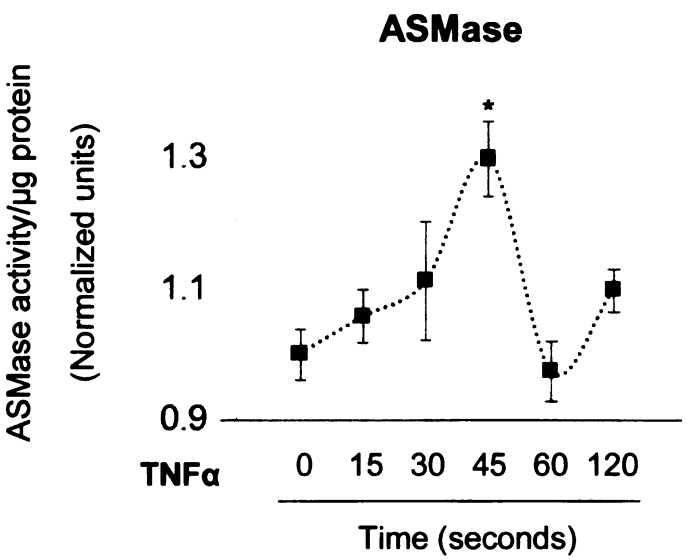
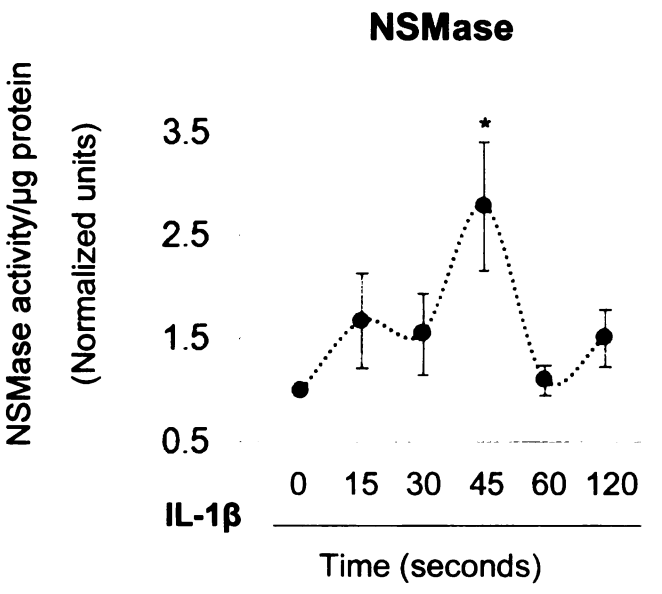


Figure 2.2. Cytokine induced ASMase and NSMase activation in HREC. HREC cultured on 10cm plates were stimulated with 5 ng/mL IL-1β (**A,C**) and 10 ng/mL TNFα (**B,D**) for 0-120 seconds. Each time point represents an individual plate. The cells were collected for ASMase (**A,B**) and NSMase (**C,D**) activity analysis using the Amplex Red Sphingomyelinase assay kit. IL-1β significantly induced ASMase (**A**) activation after 15 seconds of stimulation, with a maximal activation after 30 seconds of stimulation. TNFα induced ASMase (**B**) activation after 45 seconds of stimulation.

C.



D.

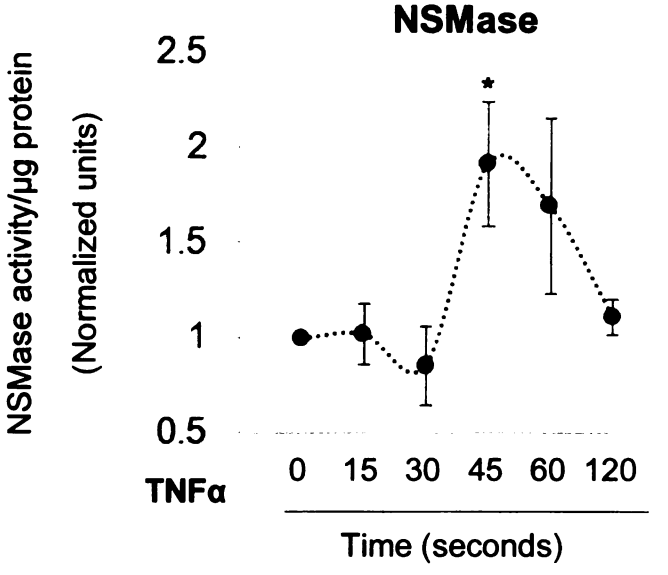


Figure 2.2 (continued). Cytokine induced ASMase and NSMase activation in HREC. IL-1β **(C)** or TNFα **(D)** induced maximal NSMase activation after 45 seconds of stimulation. Results are presented as mean ± SD of 3 independent experiments. *P<0.05 compared to basal expression level.

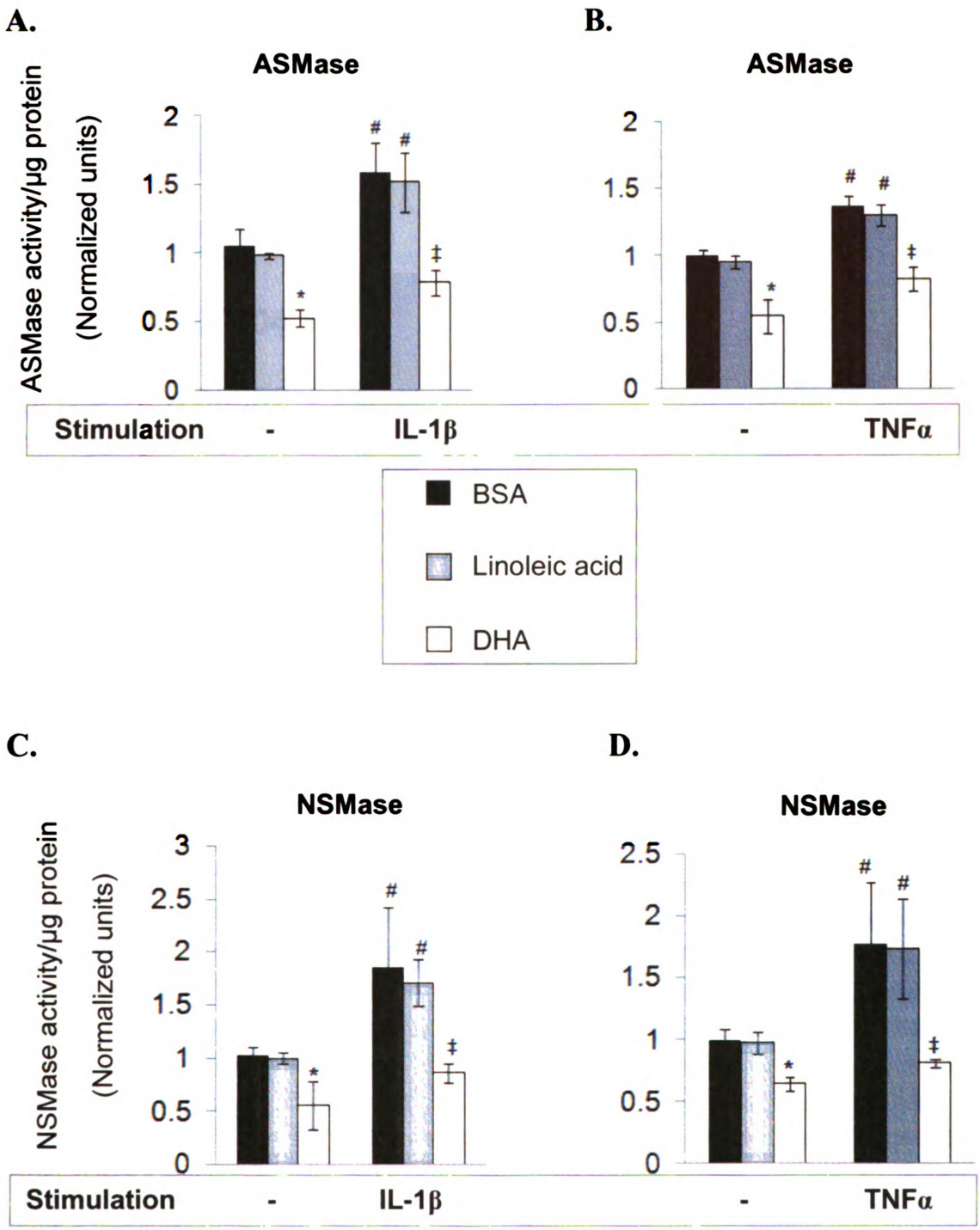


Figure 2.3. Inhibition of cytokine induced ASMase and NSMase activation, as well as ASMase and NSMase expression by DHA in HREC. HREC treated with 100 µM fatty acid (DHA or linoleic acid) complexed to BSA, or BSA alone (vehicle control) overnight (24 hours) were stimulated with either 5 ng/mL IL-1β (**A**) for 30 seconds to assess the activation of ASMase and for 45 seconds to assess the activation of NSMase;

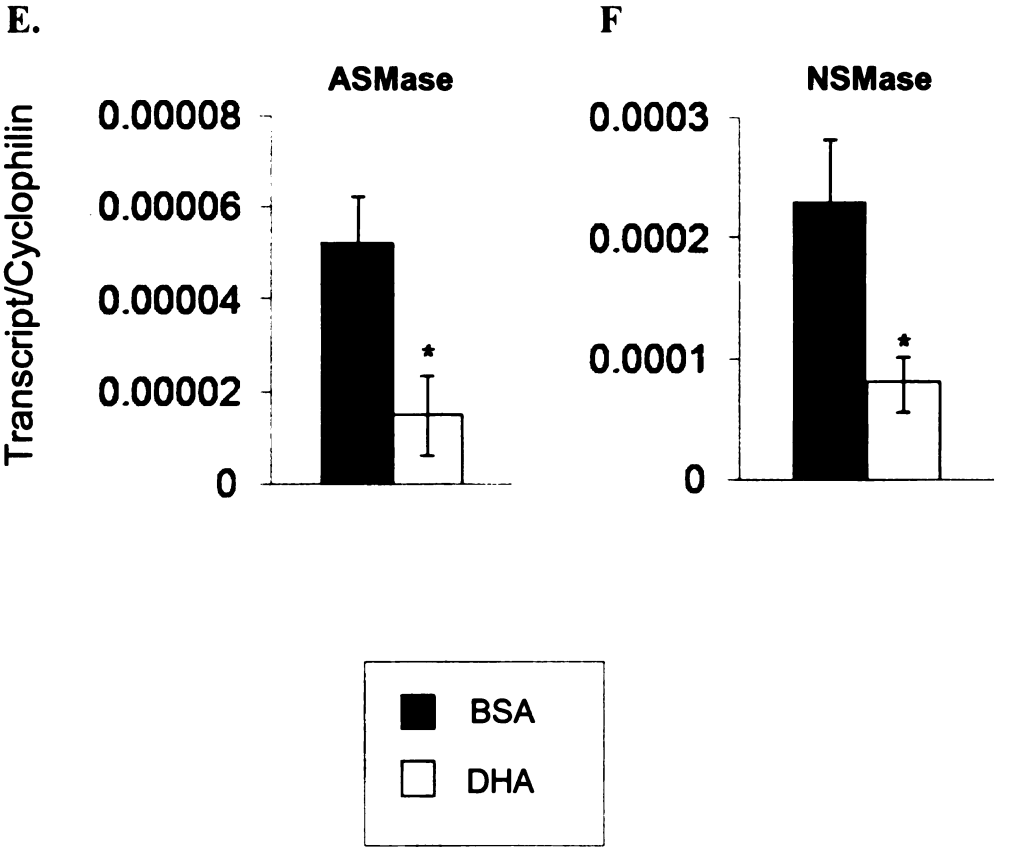
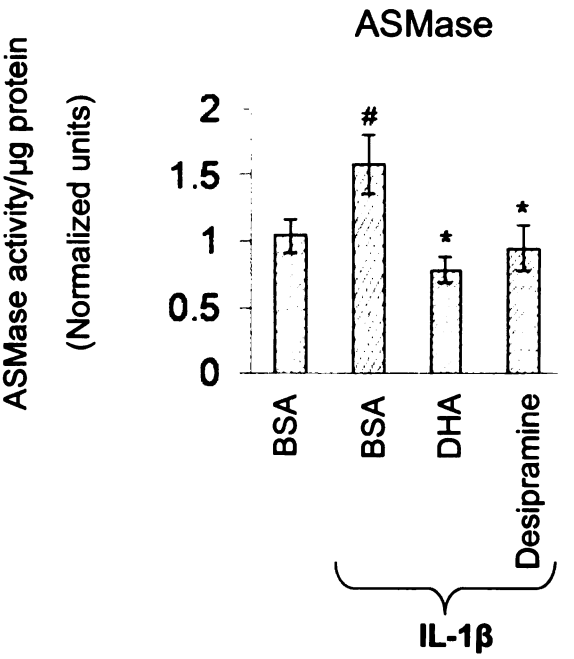


Figure 2.3 (continued). Inhibition of cytokine induced ASMase and NSMase activation, as well as ASMase and NSMase expression by DHA in HREC. HREC treated with 100 μM fatty acid (DHA or linoleic acid) complexed to BSA, or BSA alone (vehicle control) overnight (24 hours) were stimulated with with 10 ng/mL TNFα for 45 seconds to assess both ASMase and NSMase activation. The cells were then collected for ASMase **(A,B)** and NSMase **(C,D)** activity analysis using the Amplex Red Sphingomyelinase assay kit. DHA decreased both ASMase **(A,B)** and NSMase **(C,D)** basal activity and down-regulated cytokine-induced sphingomyelinases activity. RT-PCR analysis of ASMase **(E)** and NSMase **(F)** expression in HREC with or without DHA pretreatment. Results are presented as mean ± SD of 3 independent experiments. *$P<0.05$ compared to basal expression level in BSA and linoleic acid treated HREC, $^{\#}P<0.05$ compared to non-stimulated HREC, $^{\ddagger}P<0.05$ compared to cytokine stimulated HREC.

A.



B.

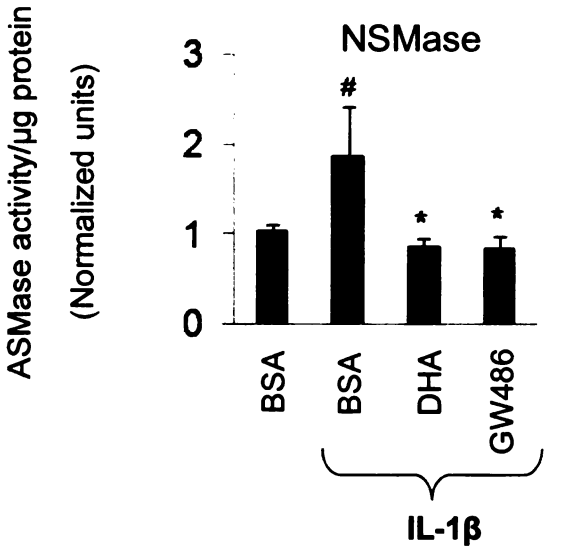
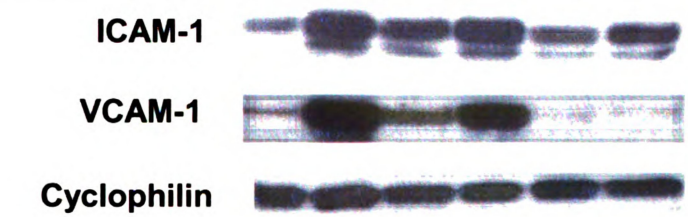


Figure 2.4. Inhibition of ASMase and NSMase cytokine-induced activation by DHA and sphingomyelinases inhibitors. HREC treated with 100 µM DHA complexed to BSA overnight and inhibitors for ASMase (Desipramine, 15 µM for 1 hour) or NSMase (GW4869, 25 µM for 30 min) down-regulated IL-1β-induced ASMase (**A**) and NSMase (**B**) activity below the basal level, respectively. Results are presented as mean ± SD of 3 independent experiments. $^{\#}P<0.05$ compared to basal expression level in BSA treated HREC, $^{*}P<0.05$ compared to IL-1β stimulated, HREC.

A.

BSA	+	+	+	+	+	+
TNFα	-	+	+	+	+	+
DHA	-	-	+	-	-	-
GW 4869	-	-	-	+	-	+
Desipramine	-	-	-	-	+	+



B.

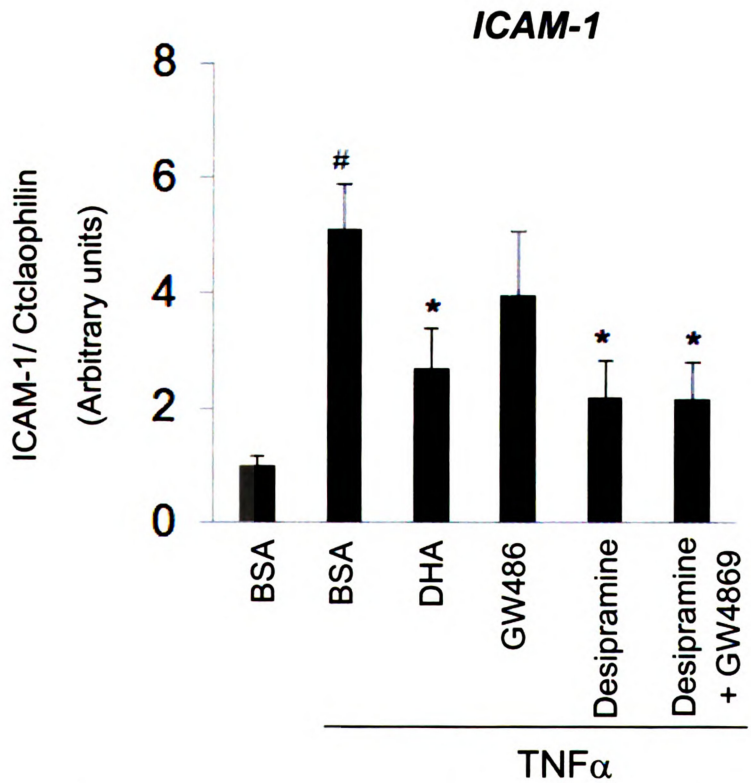


Figure 2.5. Decrease in TNFα induced CAMs expression by ASMase and NSMase inhibition in HREC.

C.

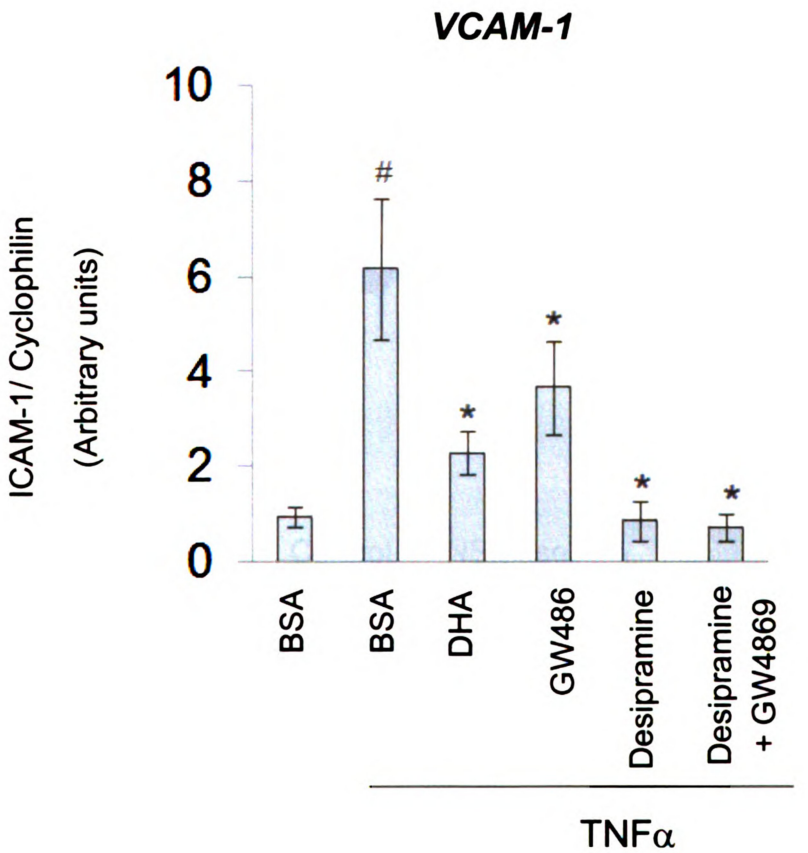


Figure 2.5 (continued). Decrease in TNFα induced CAMs expression by ASMase and NSMase inhibition in HREC. HREC were treated with 100 μM DHA complexed to BSA, or BSA alone (vehicle control) for 24 hours, or the inhibitors for ASMase (Desipramine, 15 μM for 1 hour) or NSMase (GW4869, 25 μM for 30 min) prior to 10 ng/mL TNFα stimulation. The induction of ICAM-1 and VCAM-1 was assessed 6 hours later by immunoblot analysis **(A)**. DHA, as well as Desipramine significantly reduced the TNFα induced CAM expression. GW4869 significantly inhibited TNFα induced VCAM-1 expression. Quantitative analysis of the data presented in panel **(A)** for ICAM-1 **(B)** and VCAM-1 **(C)** expression. The results are mean±SD of 4 independent experiments. $^{\#}P<0.05$ compared to control cells; *P<0.05 compared to TNFα stimulated cells.

A.

B.

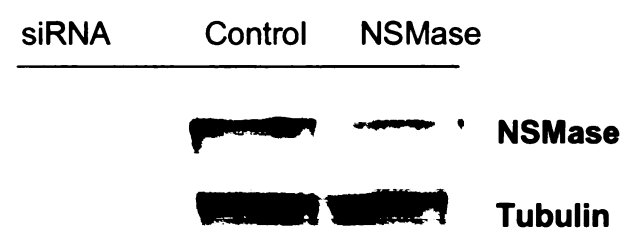
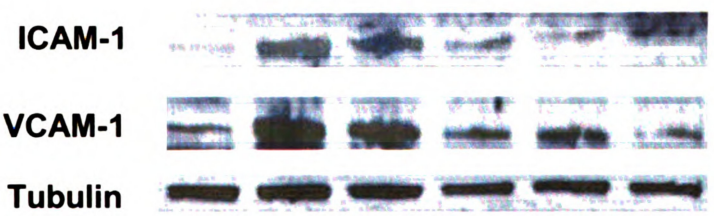


Figure 2.6. Inhibition in cytokine-induced CAMs expression by ASMase and NSMase gene silencing in HREC. ASMase **(A)** and NSMase **(B)** protein level in control siRNA, ASMase siRNA and NSMase siRNA treated HREC was assessed by immunoblot analysis.Gene silencing of ASMase and NSMase (100 nM siRNA for ASMase and NSMase) significantly decreased TNFα **(C)** and IL-1β **(F)** induced ICAM-1 and VCAM-1 expression in HREC. In contrast, control siRNA treatment (100 nM) had no effect. Quantitative analysis of the data presented in **C** and **F** is shown in **D,E** and **G,H** respectively. Equal amounts of protein were added to each lane, as confirmed by tubulin levels. Results are presented as mean±SD of 5 independent experiments. $^{\#}$ $P<0.05$ compared to control cells; *$P<0.05$ compared to TNFα and IL-1β stimulated cells.

C.

electroporation	+	+	+	+	+	+
TNFα	-	+	+	+	+	+
siRNA control	-	-	+	-	-	-
siRNA ASMase	-	-	-	+	-	+
siRNA NSMase	-	-	-	-	+	+



D.

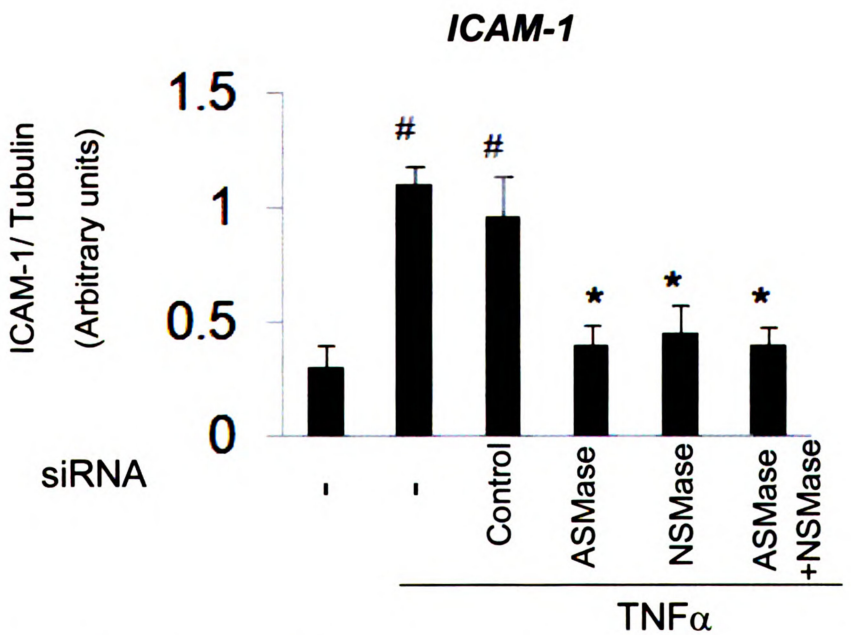
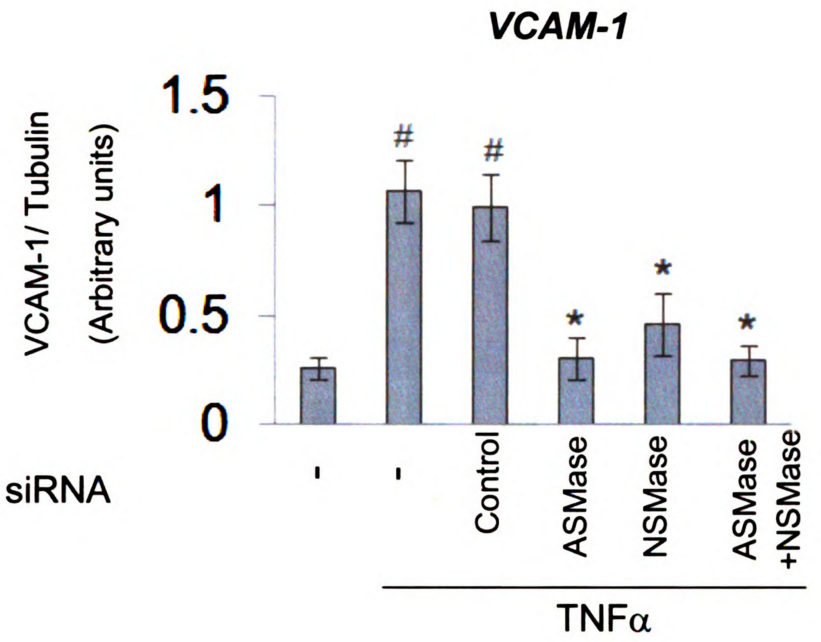


Figure 2.6 (continued). Inhibition in cytokine-induced CAMs expression by ASMase and NSMase gene silencing in HREC.

E.



F.

electroporation	+	+	+	+	+	+
IL-1β	-	+	+	+	+	+
siRNA control	-	-	+	-	-	-
siRNA ASMase	-	-	-	+	-	+
siRNA NSMase	-	-	-	-	+	+

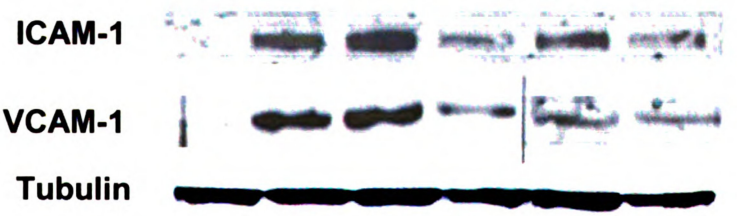
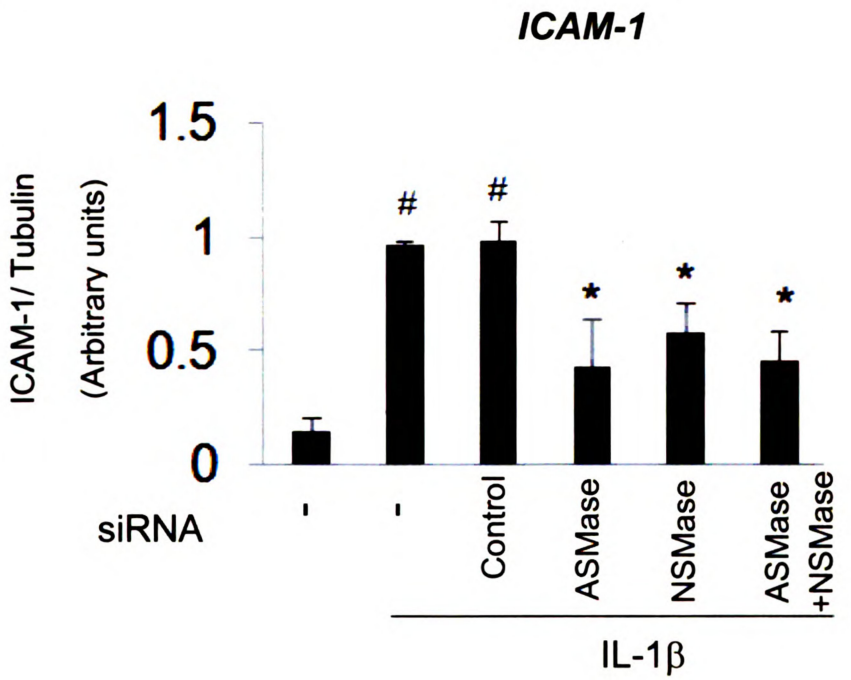


Figure 2.6 (continued). Inhibition in cytokine-induced CAMs expression by ASMase and NSMase gene silencing in HREC.

G.



H.

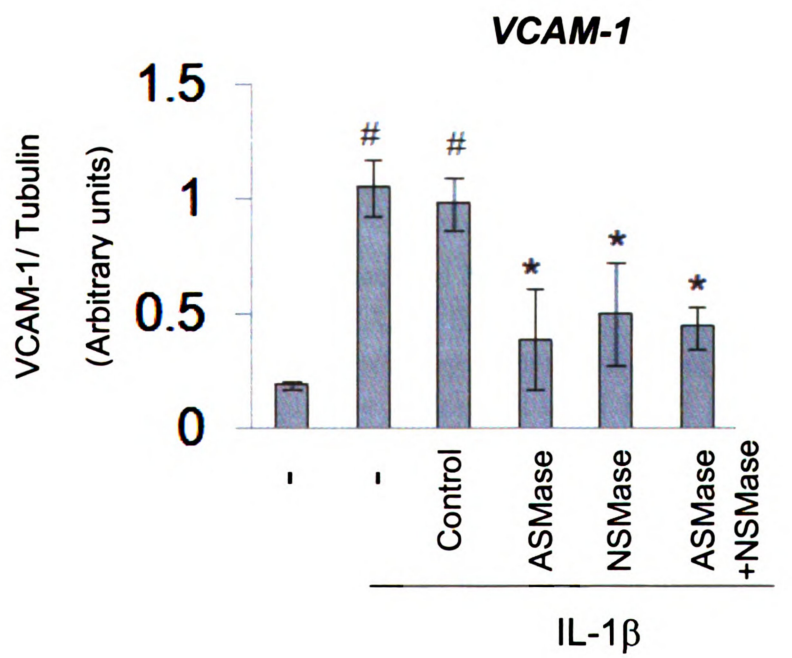


Figure 2.6 (continued). Inhibition in cytokine-induced CAMs expression by ASMase and NSMase gene silencing in HREC.

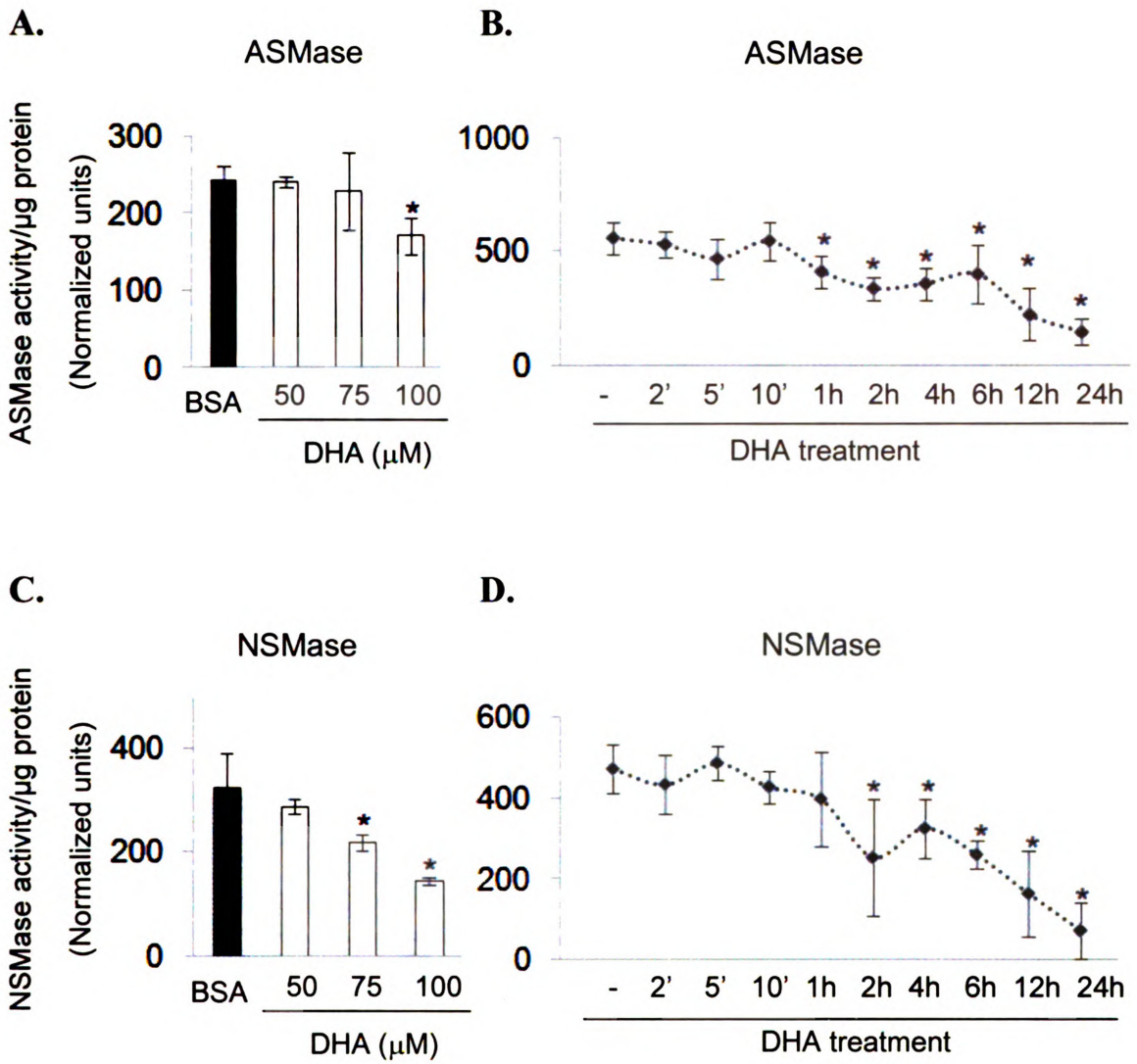


Figure 2.7. Inhibition of ASMase and NSMase activity by DHA. HREC treated with 100 µM DHA complexed to BSA, or BSA vehicle control overnight (24 hours) were collected for ASMase **(A)** and NSMase **(D)** activity analysis using the Amplex Red Sphingomyelinase assay kit. The optimal dose of DHA to inhibit ASMase **(A)** and NSMase **(C)** activity in HREC was 100 µM. The time course for DHA effect on ASMase and NSMase activity in HREC showed a gradual decrease in ASMase **(B)** and NSMase **(D)** activity with an early time point of 1 hour and 2 hours, respectively and a maximum effect on both enzymes activity after 24 hours of DHA treatment. The results are mean±SD of 3 independent experiments. *P<0.05 compared to BSA vehicle control.

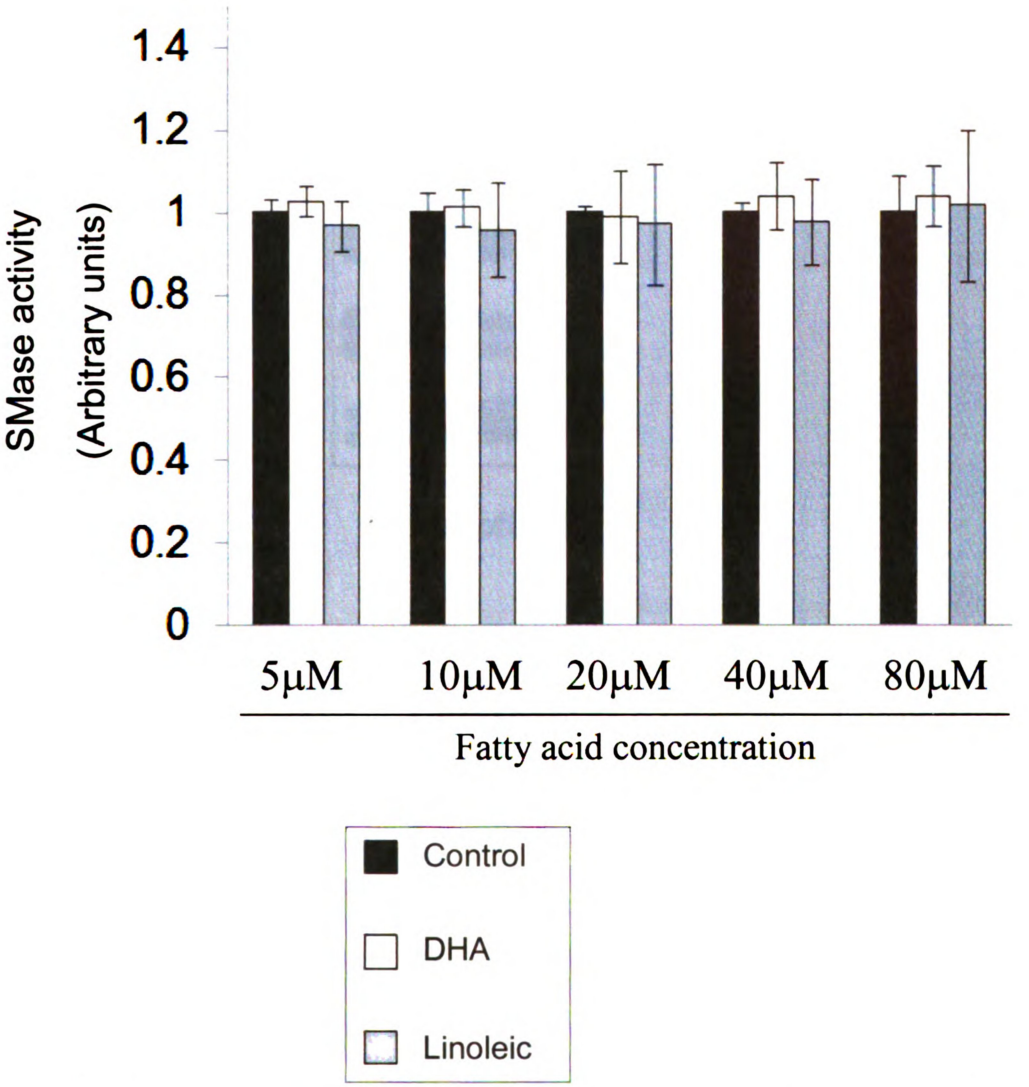
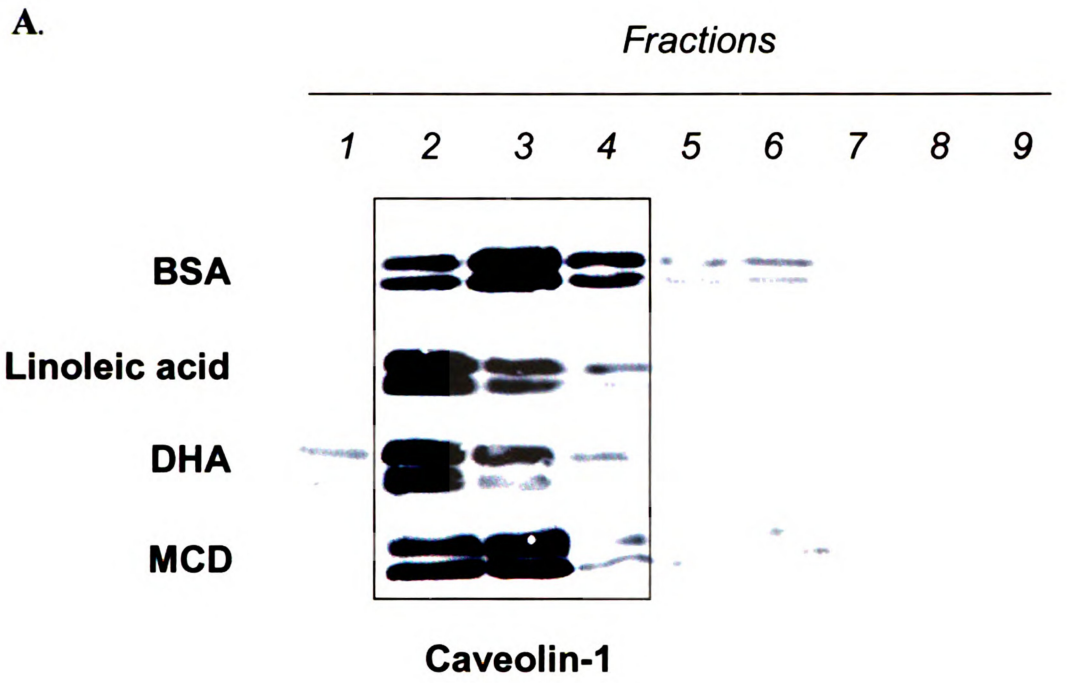


Figure 2.8. No direct effect of DHA on sphingomyelinase (SMase) activity in a cell-free system. To determine whether DHA decreases SMase activity through direct interaction with the enzyme, we measured SMase activity in a cell free system, where 0.5 mU of bacterial SMase (provided in the Amplex Red Sphingomyelinase assay kit) were incubated with 5 to 80 μM fatty acids (DHA, linoleic acid). A concentration of 20 μM free fatty acid corresponds to a concentration of 100 μM fatty acid complexed to BSA in 5:1 molar ratio. No fatty acids were used for the control wells. The assay was performed as described in the Methods. No effect of DHA or linoleic acid on SMase activity was observed when compared to control.



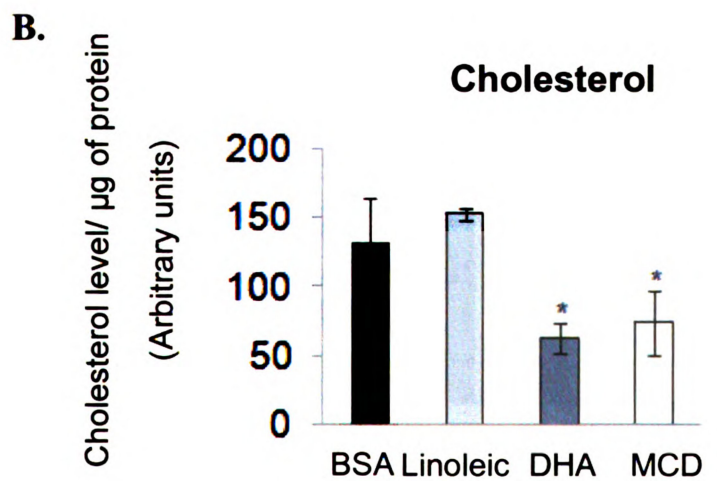
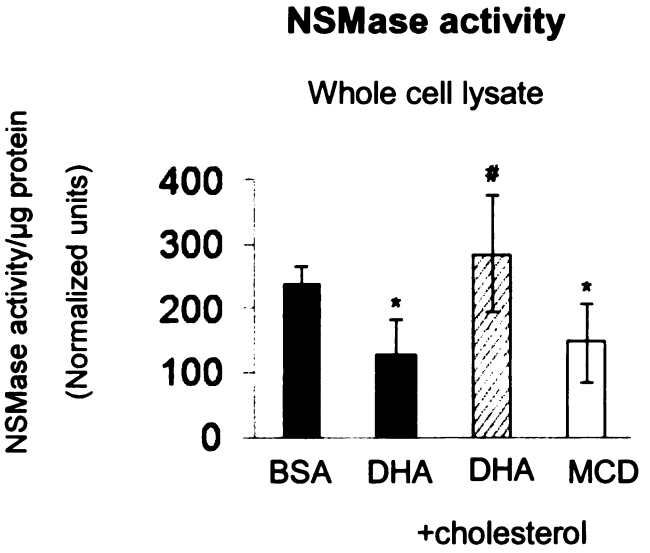


Figure 2.9. Effect of cholesterol depletion on ASMase and NSMase activity in HREC. Caveolae/lipid raft fractions were isolated by sucrose discontinuous gradient ultracentrifugation based on their insolubility in Triton-X-100 at 4°C and identified by western blotting as fractions 2-4 based on the caveolin-1 distribution **(A)**. MCD (white bars) and DHA (dark gray bars) pretreatment of HREC significantly decreased cholesterol content of the caveolae fractions when compared to BSA (carrier control) and linoleic acid (lipid control) **(B)**.

C.



D.

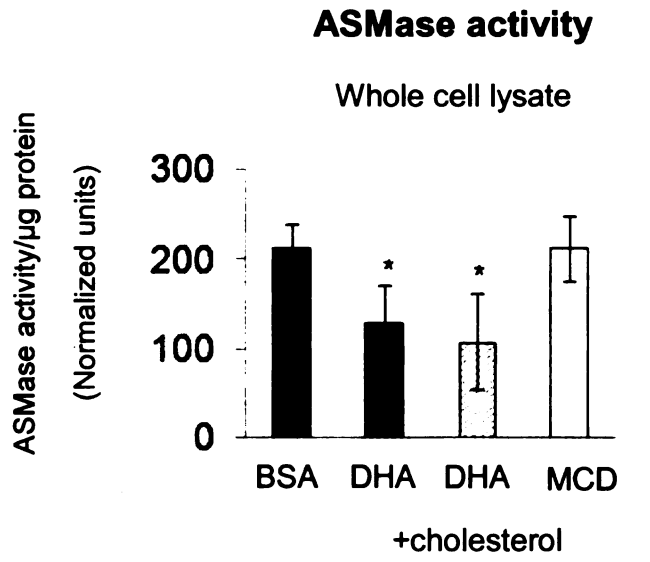


Figure 2.9 (continued). Effect of cholesterol depletion on ASMase and NSMase activity in HREC. NSMase activity was inhibited by cholesterol depletion caused by the treatment with 100 µM DHA overnight (*gray bar*) or 8 mM MCD for 30 min (*white bar*)**(C)**. The inhibitory effect of DHA on NSMase activity was reversed by cholesterol replenishment using 25 µM water-soluble cholesterol for 30 minutes (*hatched gray bar*, **C**). The inhibitory effect of DHA on ASMase activity was not mimicked by MCD and cholesterol replenishment did not restore ASMase activity in DHA treated HREC in whole cell lysates (**D**).

E.

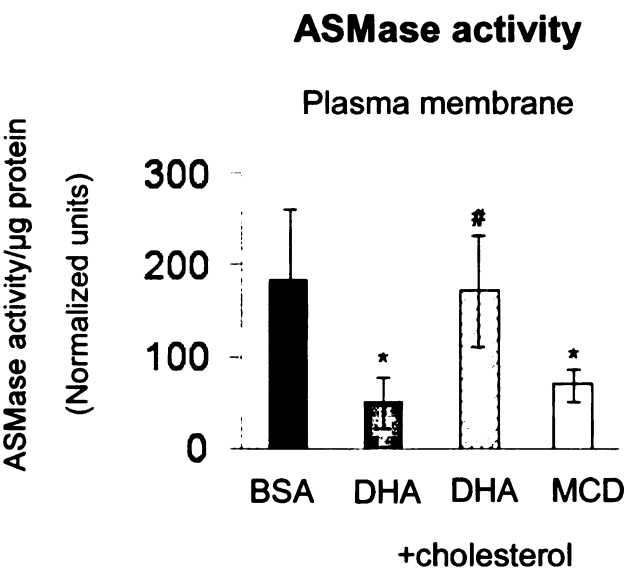
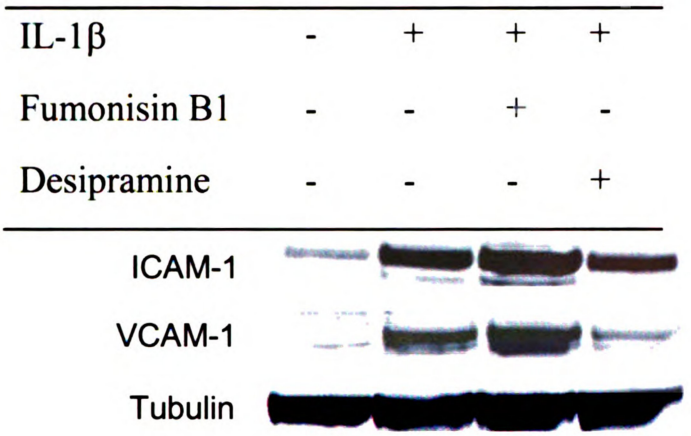
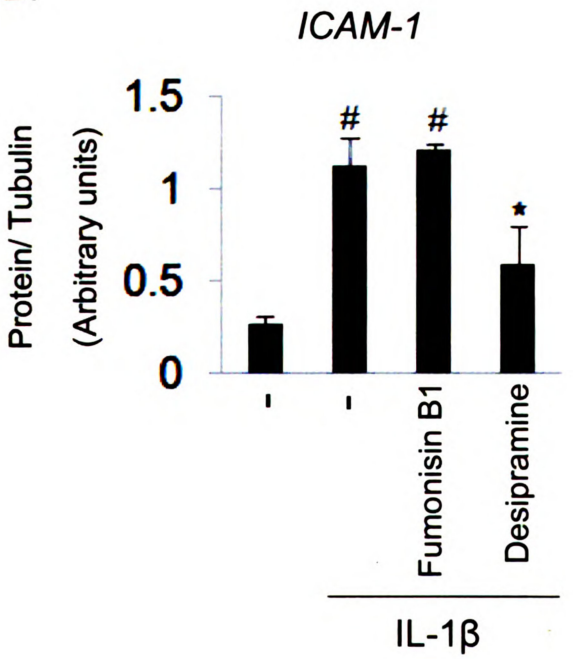


Figure 2.9 (continued). Effect of cholesterol depletion on ASMase and NSMase activity in HREC. However, cholesterol depletion inhibited ASMase activity and cholesterol replenishment reversed the inhibitory effect of DHA on ASMase activity in the plasma membrane fraction (**E**). Results are presented as mean±SD of 3 independent experiments. *$P<0.05$ compared to BSA control. $^{\#}P<0.05$ compared to DHA.

A.



B.



C.

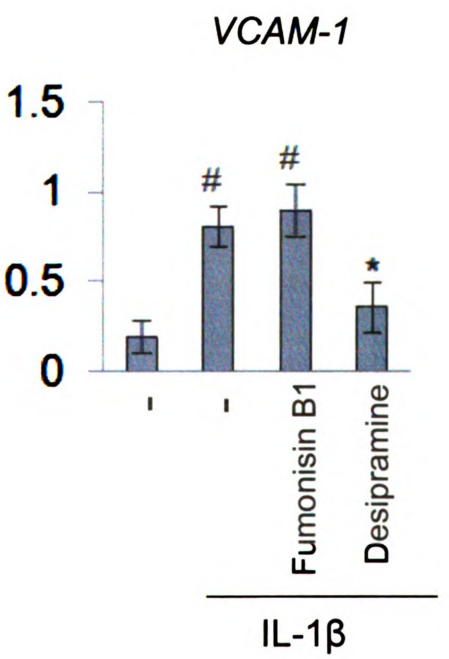
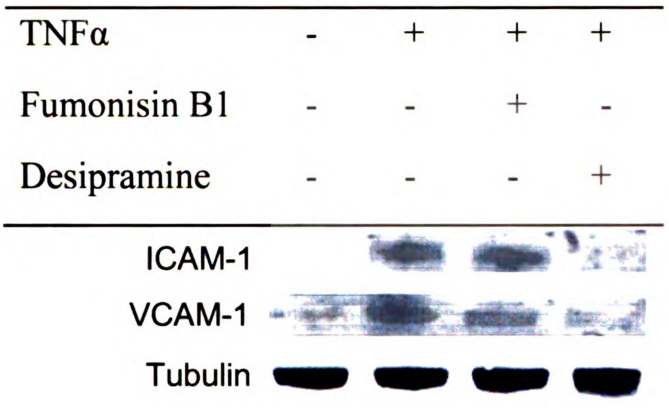


Figure 2.10. No effect of *de novo* ceramide production pathway inhibition on cytokine induced CAMs expression in HREC. Fumonisin B1 was used to inhibit ceramide synthase, the enzyme that catalyzes the final step in the *de novo* pathway of ceramide synthesis. Cells were treated for 16 hours with 50 μM Fumonisin B1 prior to TNFα (10 ng/ml) and IL-1β (5 ng/ml) stimulation. The expression levels of ICAM-1 and VCAM-1 were analyzed by western blot.

D.



E. **F.**

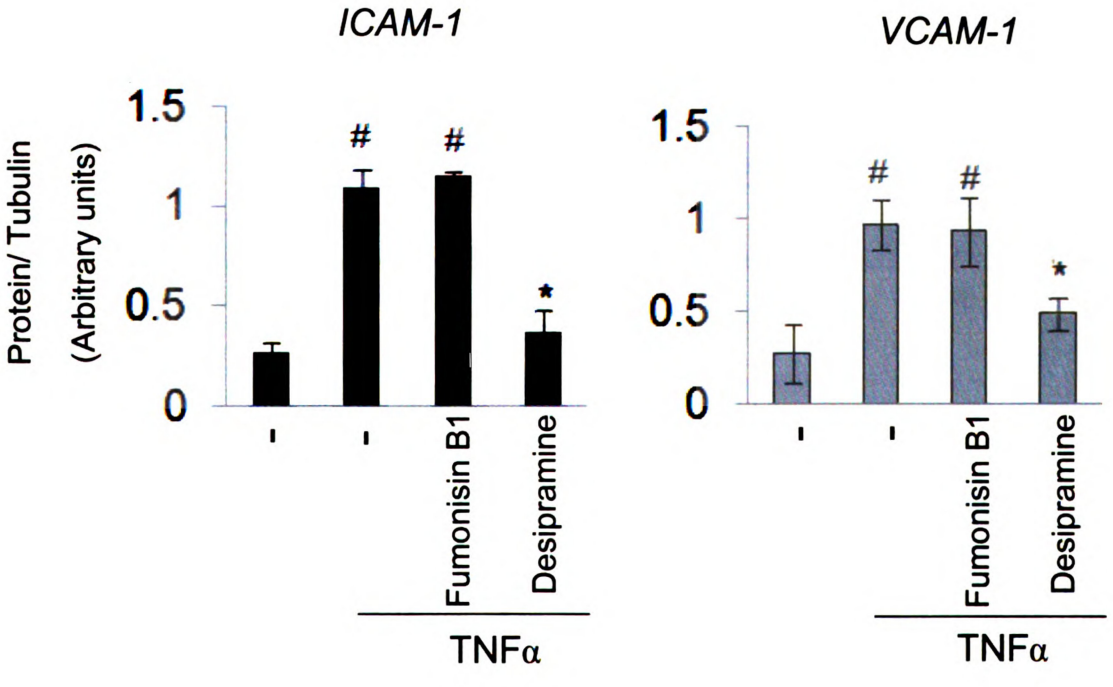
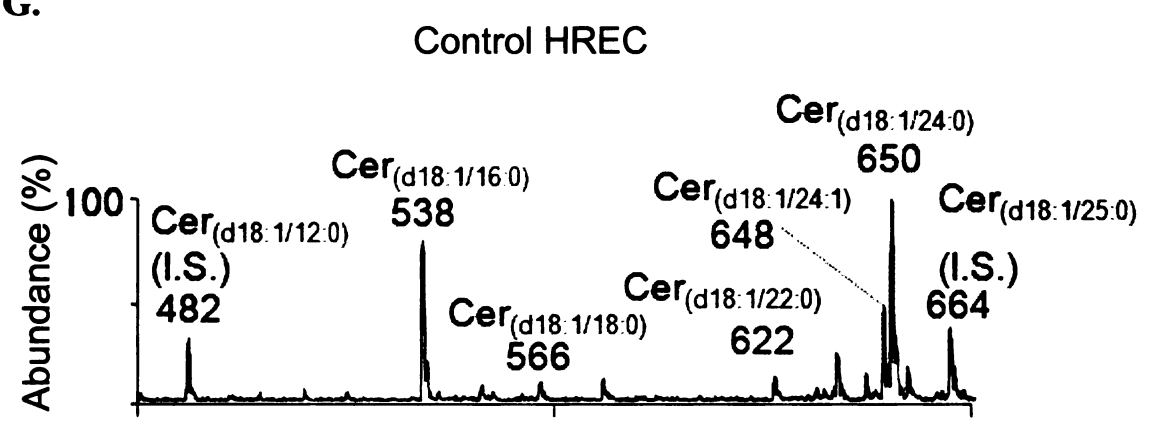


Figure 2.10 (continued). No effect of *de novo* ceramide production pathway inhibition on cytokine induced CAMs expression in HREC. Representative blots are shown in **A and D,** and quantitative analysis of 4 independent experiments is shown in **B,C,E and F**. Fumonisin B1 pre-treatment of HREC did not affect TNFα and IL-1β induced adhesion molecule expression. In contrast, inhibition of ASMase significantly decreased TNFα and IL-1β induced ICAM-1 and VCAM-1 expression.

G.



H.

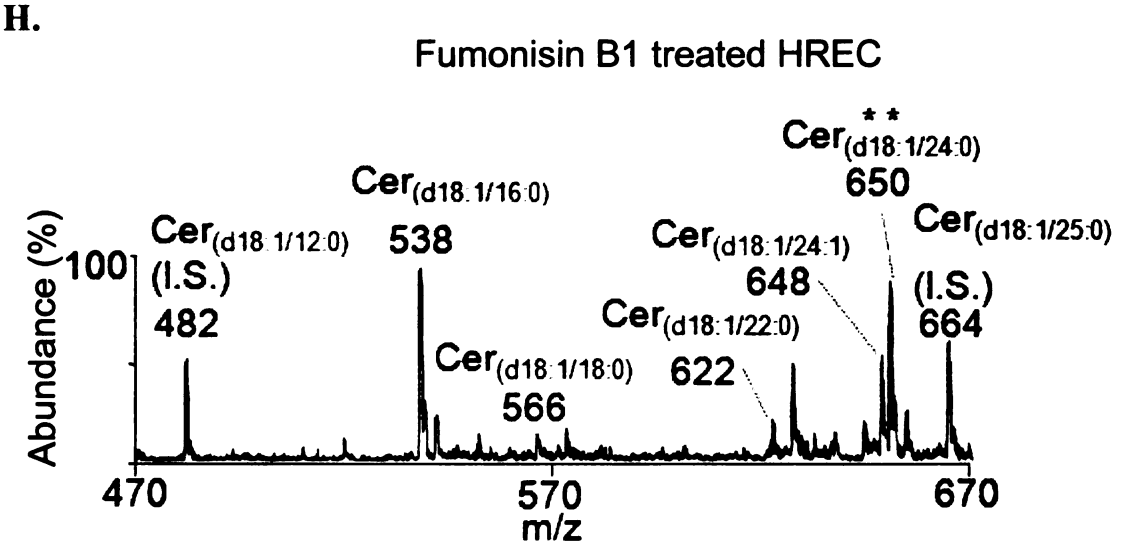


Figure 2.10 (continued). No effect of *de novo* ceramide production pathway inhibition on cytokine induced CAMs expression in HREC. Ceramide levels determined by tandem mass spectrometry precursor ion mode scanning for the characteristic ceramide product ion at m/z 264.4 in control and Fumonisin B1 treated HREC are presented in **G** and **H** respectively.

I.

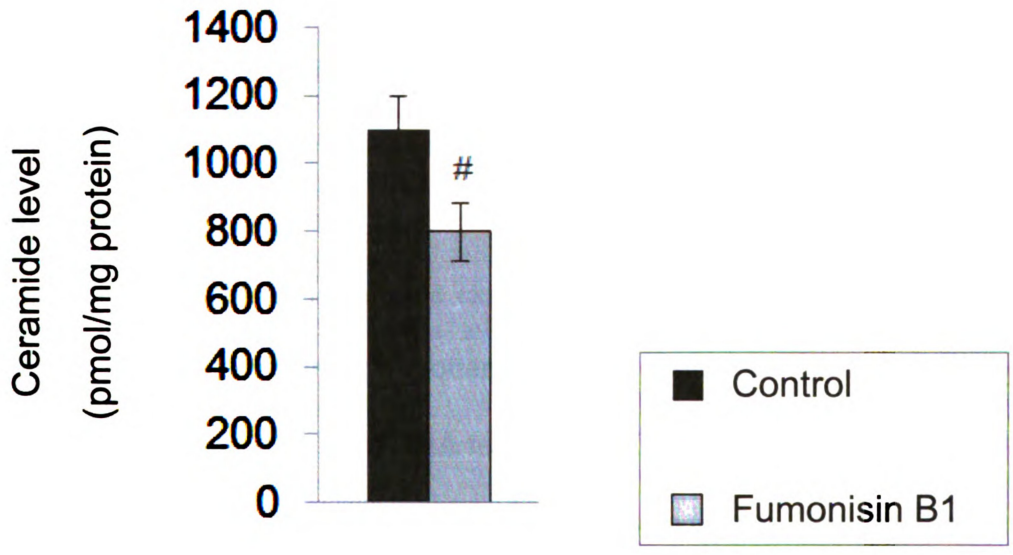


Figure 2.10 (continued). No effect of *de novo* ceramide production pathway inhibition on cytokine induced CAMs expression in HREC. Quantification of ceramide levels presented in **I** demonstrates significant decrease in ceramide levels in Fumonisin B1 treated compared to control HREC. Results are presented as mean±SD of 3 independent experiments. *$P<0.05$ compared to cytokine stimulated level. $^{\#}P<0.05$ compared to control.

5. Discussion

Omega 3 PUFAs have been long accepted to modulate inflammatory processes and are now widely used in the clinic as an adjuvant immunosuppressant in the treatment of various diseases with an inflammatory component (56). In a recent article, Connor *et al.* showed that increasing omega 3 PUFA retinal levels by diet or genetic means may be of benefit in preventing retinopathy and the DHA protective effect was mediated, in part, by suppression of retinal TNFα gene expression and protein level in a mouse model of oxygen-induced retinopathy (5). We have previously shown that DHA suppresses cytokine induced adhesion molecule expression in endothelial cells through cholesterol depletion and displacement of important signaling molecules from caveolae/lipid rafts (47). We now demonstrate that DHA treated HREC exhibit decreased basal and TNFα and IL-1β induced activity of ASMase and NSMase enzymes. The time course showed maximum effect of DHA on sphingomyelinase activity after more than 12 hours of treatment. This effect can be explained by our previously published observation that DHA incorporates into caveolae/lipid rafts phospholipids at a rate of 10% in 1.5 hours and 90% in 24 hours and dramatically alters the lipid environment of these specialized microdomains, decreasing their cholesterol content by approximately 70% (47).

Both ASMase and NSMase have been described to localize in the caveolae/lipid raft compartment of various cell types. Although ASMase was initially described as a strictly lysosomal enzyme because of its optimum activity at pH 4.5-5.0, Liu and Anderson have described the plasma membrane form of ASMase in caveolae and they have shown that IL-1β stimulates its activity in this compartment (27). Veldman *et al.*

described a NSMase in caveolae isolated from human skin fibroblasts where the enzyme appeared to interact with the scaffolding domain of caveolin protein (57). NSMase has two putative transmembrane domains and five palmitoylation sites (29) and thus likely has affinity for caveolae/lipid rafts microdomains. Cholesterol depletion as well as displacement of signaling molecules essential for sphingomyelinases activation from caveolae/lipid rafts by DHA may be the mechanism of DHA inhibitory effect on ASMase and NSMase activity. Indeed, in this study, cholesterol depletion using MCD decreased NSMase activity to the level similar to that observed with DHA. In contrast to NSMase, ASMase has a major intracellular lysosomal component. The function and control of ASMase activity in the plasma membrane and in lysosomal compartments are likely to be different. Thus, it is not surprising that whole cell ASMase activity, which represents lysosomal and plasma membrane ASMase combined, was not affected by short term MCD and cholesterol treatments the same way as NSMase, which has only plasma membrane localization. Plasma membrane ASMase activity was however, significantly decreased by MCD, and cholesterol replenishment in DHA treated cells restored ASMase activity in the plasma membrane.

Pro-inflammatory cytokines TNFα and IL-1β have been shown to activate cellular ASMase in various cell types (27, 58-60) and our results are in agreement with these studies. We have found that IL-1β treatment of HREC induced rapid activation of ASMase after only 15 seconds of stimulation, while TNFα increased both ASMase and NSMase activity after 45 seconds of stimulation. This rapid and transient time course of the effect is in agreement with the fact that induction of the inflammatory response is always transient and the extent of the initial event is always small as it is later amplified

several fold with each signal transduction step. For instance, as demonstrated in numerous studies of the NFκB pathway, IκBα is phosphorylated within a minute from cytokine stimulation and is dephosphorylated back to the control state by 5 min; in GPCR signaling ERK phosphorylation occurs within 30 seconds of receptor activation and it is back to normal level by 2 minutes. Indeed, a recent study described the importance of transient *versus* sustained ERK phosphorylation in inducing different signaling pathways (61). Moreover, sphingomyelinase literature is replete with demonstration of the very fast (within a minute) and transient nature of sphingomyelinase activation, and the fact that it is so fast is often cited to promote the notion that sphingomyelinase is an important first responder (58-59, 62). Although the induction of sphingomyelinases is transient, the effect of the sphingomyelinase activation, e.g., conversion of sphingomyelin to ceramide and further activation of the NFκB pathway and inflammatory genes, such as ICAM-1 and VCAM-1, is long lasting. Indeed, in this study inhibition and/or gene silencing of ASMase and NSMase decreased pro-inflammatory cytokine TNFα and IL-1β induced adhesion molecules expression in HREC.

The majority of studies on sphingomyelinase activation have used as a model system the p55 TNFα receptor, showing that this receptor has a modular structure, with particular domains for different sphingomyelinases. The intracellular C-terminal part of the p55 receptor, which contains the death domain (DD), appears to be necessary for ASMase activation; the exact molecular mechanism is not known, but Schwandner *et al.* have reported ASMase activation (seconds to minutes) in response to TNFα through overexpression of TRADD (TNF receptor associated DD) and/or FADD (Fas-associating protein with DD) (63). Kronke *et al.* have defined a region of this receptor that is adjacent

to the death domain and is required for NSMase activation (64). This motif was named the NSMase activation domain. The same authors have also described a novel protein (factor associated with NSMase activation, FAN) that binds the NSMase activation domain on the TNFα receptor and is required for NSMase activation (65). The mechanism by which IL-1β stimulates ASMase and NSMase activity is not yet elucidated.

Inhibition and/or gene silencing of ASMase and NSMase decreased pro-inflammatory cytokine TNFα and IL-1β induced adhesion molecules expression in HREC. Both ASMase and NSMase are specific enzymes with phospholipase C activity, which hydrolyze the sphingomyelin phosphodiester bond to produce ceramide and phosphocholine. The ability of ceramide to self-aggregate and their fusagenic properties may mediate membrane microdomain reorganization into large platforms required for receptor clustering (66-68). As TNFα and IL-1β have receptors that appear to be localized in the caveolae/lipid rafts fractions (58, 68) and protein oligomerization is an almost universal requirement for transmembrane signal transmission, release of ceramides alters the dynamics of these rafts and may drive and amplify inflammatory signal transduction processes.

Moreover, there is evidence that stimulation of the sphingomyelin pathway by TNFα with sphingomyelinases-induced membrane ceramide leads to activation of nuclear factor κB (NF-κB) and marked increase in nuclear NF-κB binding in human leukemia (HL-60) cells (59). Similarly, activation of sphingomyelinases is shown to be an important signaling system for IL-1β in the murine T helper cell, EL-4 (58), and IL-1β

action in these cells is mediated through NF-κB activation (69). NF-κB is a major transcription factor controlling the expression of an array of inflammatory response genes including adhesion molecules (70).

There are two different pathways of ceramide generation in cells. The first takes place at the membrane level by hydrolysis of sphingomyelin by sphingomyelinases; the second is localized to the endoplasmic reticulum by *de novo* synthesis involving the enzyme ceramide synthase. The results of this study demonstrate that plasma membrane ceramide production by sphingomyelinases rather than *de novo* synthesis is important for inflammatory signaling in HREC.

Several recent studies demonstrated that oxidized products of DHA formed either through lypoxygenase (5, 71-72), cyclooxygenase (72-73) or non-enzymatic pathways (74) possess potent anti-inflammatory properties. Although anti-inflammatory mediators formed by DHA oxidation are not likely to explain the results of this study, they might represent an important component of anti-inflammatory action of DHA in other cell types or under different experimental conditions. Endothelial cells are rich in caveolae and ASMase is preferentially expressed in endothelia (75), thus our findings may represent an identification of an endothelial-specific anti-inflammatory mechanism of DHA action.

In conclusion, this study provides a novel mechanism for the anti-inflammatory effect of DHA in the primary tissue affected by retinopathy, human retinal endothelial cells. We demonstrated that DHA-induced cholesterol depletion from membrane microdomain leads to inhibition of ASMase and NSMase activity. Sphingomyelinase downregulation by inhibitors and/or gene silencing recapitulated the DHA protective

effect against cytokine-induced inflammation in HREC. Importantly, preclusion of cytokine-induced adhesion molecules in HREC was more pronounced in HREC when ASMase rather than NSMase inhibition/gene silencing was attained, supporting the general hypothesis that ASMase may play a more important role in active reorganization of plasma membrane microdomains and receptor clustering (28). Furthermore, Veldman *et al.* showed that after TNF stimulation, NSMase exited the caveolae and was followed by a significant increase in ceramide content in this compartment, suggesting that ASMase might mediate sphingomyelin hydrolysis (57). Grassme *et al.* demonstrated that cells derived from Niemann-Pick disease patients (with ASMase deficiency) were defective in receptor clustering and signal transduction in response to Fas, underlining the importance of ASMase in oligomerization of cell surface proteins and transmembrane signal transmission. Moreover, vascular endothelium is well recognized to express 20 times as much ASMase as any other cell type in the body (76) and this large excess of ASMase in endothelium may be involved in tissue remodeling and wound repair (77), processes that normally require waves of endothelial cell apoptosis and tissue neovascularization (78).

Therefore, in the next chapter we characterized ASMase expression and cellular localization in HREC when compared to other retinal tissues affected by diabetic retinopathy, such as human retinal pigment epithelial cells and human Muller cells. Moreover, we tested the DHA effect on caveolar *versus* intracellular ASMase expression and we analyzed the sphingolipid composition of caveolae/lipid microdomains in control, DHA treated and/or cytokine stimulated HREC.

6. References

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Chapter III

Docosahexaenoic Acid Regulates Caveolar Acid Sphingomyelinase Expression and Promotes Sphingomyelin Enrichment of Membrane Microdomains in Human Retinal Endothelial Cells

1. Abstract

Retinal endothelial cells represent the resident vasculature affected by diabetic retinopathy. In addition to the retinal vasculature, diabetes may also result in damage to nonvascular retinal tissue; yet microangiopathy represents the key pathologic feature of diabetic retinopathy. As demonstrated in the previous chapter, acid sphingomyelinase (ASMase) represents a key molecule in the cytokine mediated cellular inflammatory cascade. To test the hypothesis that docosahexaenoic acid (DHA) has an anti-inflammatory effect in diabetic retina by modulating ASMase expression specifically in the retinal endothelial cells, we used three retinal cell types known to play a role in diabetes-induced pathology: human retinal endothelial cells (HREC), adult human retinal pigment epithelial cells (HRPE) and human Muller cells (HMC). We established that ASMase has the highest activity and protein level in HREC, when compared to HRPE and HMC. Immunohistochemistry assessment of ASMase distribution demonstrated that, although ASMase was ubiquitously expressed in the perinuclear intracellular compartment of all three cell types, HREC was the only cell type with pronounced plasma membrane ASMase expression. Moreover, DHA decreased ASMase activity only in HREC, when compared to HRPE and HMC. Importantly, DHA displaced ASMase from the caveolae membrane

microdomains in HREC and inhibited pro-inflammatory cytokine IL-1β induced ICAM-1 expression. ASMase gene silencing in HREC recapitulated DHA effects on cytokine-induced endothelial cell activation. The effect of DHA on membrane microdomain sphingolipid composition in HREC was analyzed by tandem mass spectrometry. In support of ASMase inhibition and displacement of ASMase from caveolae membrane microdomains by DHA, we found a significant decrease in ceramide/sphingomyelin ratio in the caveolae fraction isolated from HREC treated with DHA. We have previously demonstrated that DHA prevents TNFα-induced ASMase activation and now we show that DHA prevents the TNFα-induced increase in the ceramide/sphingomyelin ratio in caveolar membrane microdomains. Taken together, these *in vitro* data provide a mechanism for the anti-inflammatory effect of DHA in endothelial cells, which we show to occur through regulation of caveolar ASMase expression.

2. Introduction

Diabetic retinopathy is recognized as a microvascular complication of diabetes and there is evidence that diabetes compromises the survival of vascular cells (1). Very early stage diabetic retinopathy is regarded as a low-grade chronic inflammatory disease with leukocyte adhesion to the retinal vasculature, a process mediated by adhesion molecules expressed on the endothelial cell surface, especially intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (2-4). Proinflammatory cytokines, including TNFα and IL-1β are increased in diabetic eyes (5-7) and induce upregulation of adhesion molecule expression in vascular cells (8). Retinal endothelial cells become activated and then degenerate as diabetic retinopathy progresses (9, 10).

Vascular endothelial cells are not the only cell type affected by diabetes in the retina. Muller cells, the main glial cells of the retina, are also dysfunctional in diabetes. Muller cells, analogous to brain astrocytes, synthesize factors that participate in formation of tight junction (11) and maintain the proper functioning of the inner blood-retinal barrier under normal conditions (11, 12); they also synthesize and store various growth factors with trophic and regulatory functions for a variety of cell types in the retina (13). Mizutani *et al.* provided evidence for selective biosynthetic modifications of Muller cells isolated from diabetic human eyes (13).

Another retinal cell type affected by diabetes are the retinal pigment epithelial (RPE) cells that form the outer blood-retinal barrier. The RPE separates the neural retina from the fenestrated choriocapillaries and have a major role in controlling the flow of

solutes, nutrients and fluid from the choroidal vasculature to the outer retina (14, 15). Diabetes induces important morphological and biochemical changes in RPE cells (16-18).

HREC contain lipid rafts and particular plasma membrane microdomains known as caveolae (19, 20) that have important roles in various physiological processes, especially in regulating vascular permeability (21) and signal transduction (22, 23). Caveolae are stabilized by the caveolins (24-27) and caveolin-1 is the primary structural protein in endothelial cells caveolae (28).

Caveolae/lipid rafts microdomains are active assemblies of cholesterol, sphingolipids (sphingomyelin, ceramide) and glycerophospholipids (29). Sphingolipids are active components of membrane microdomains structure and are recognized to act as messengers in signaling pathways mediating inflammation, apoptosis, cell differentiation and proliferation (30). Sphingomyelinases are important early responders in inflammatory cytokine signaling that catalyze the hydrolysis of sphingomyelin to the bioactive second messenger ceramide (31-33). Acid sphingomyelinase (ASMase) is considered a major candidate for mediating stress-induced production of ceramide. ASMase is best known as a lysosomal enzyme where it plays an important role in lipid storage and sphingomyelin metabolism. Recent studies, however, identified an important role of ASMase in ceramide-mediated signal transduction. This unexpected function involves the translocation of ASMase from intracellular compartments to the outer leaflet of cell membranes. Hydrolysis of sphingomyelin into ceramide initiates a reorganization of the membrane which facilitates formation and coalescence of lipid microdomains (34, 35). Specifically, ASMase activity was localized to caveolae membrane microdomains (33).

Caveolae are enriched in sphingomyelin (26, 36-39) and thus represent a target for sphingomyelinase translocation and an important pool for ceramide production. Ceramide generation within the outer leaflet of the plasma membrane facilitates receptor clustering and enhances inflammatory signal transduction by several receptors that mediate the pathological changes typically associated with diabetic retinopathy, such as IL-1β and TNFα (40, 41).

In this study, we examined a potential anti-inflammatory mechanism of DHA in HREC through differential regulation of caveolar *versus* lysosomal ASMase localization and promotion of sphingomyelin-enriched membrane microdomain formation with important consequences on cellular inflammatory signaling.

3. Materials and Methods

3.1. Reagents and Antibodies

DMEM and F12 culture medium, antibiotics, fetal bovine serum and trypsin were obtained from Invitrogen (Carlsbad, CA). Amplex Red Sphingomyelinase Assay Kit, NuPAGE Novex 10% Bis- Tris gels, (Carlsbad, CA). GW4869 was purchased from Calbiochem (San Diego, CA). Commonly used chemicals and reagents were purchased from Sigma (St. Louis, MO). Caveolin-1 and LAMP1 antibodies (BD Bioscience, San Jose, CA), ICAM-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used. ASMase antibody was a generous gift from Dr. Richard Kolesnick. TNFα and IL-1β were from R&Dsystems (Minneapolis, MN). ON-TARGET plus SMART pool SMPD1 (ASMase) and ON-TARGET plus siCONTROL (non-targeting pool, against luciferase) were purchased from Dharmacon (Chicago, IL). For lipid extraction and mass spectrometry, all solvents used were HPLC grade. Methanol (MeOH), and water were purchased from J.T. Baker (Phillipsburg, NJ). Ammonium hydroxide (NH_4OH) and chloroform ($CHCl_3$) were from EMD Chemicals (Gibbstown, NJ). Isopropanol was from Fisher Scientific (Pittsburgh, PA). Lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL).

3.2. Cell Culture

Primary cultures of HREC, HRPE and HMC were prepared from postmortem human tissue (National Disease Research Interchange, Philadelphia, PA) cultured as previously described (42). Passages 1 to 5 were used in the experiments. For experimental treatment,

cells were transferred to serum-free medium for 14 to 24 hours before stimulatory agents were added. Only early passage cells were used in the experiments.

3.3. Fatty Acid Treatment

The method has been previously described in chapter II. BSA was used as a carrier control and linoleic acid, a ω6 polyunsaturated fatty acid was used as a lipid control.

3.4. Sphingomyelinase Assay

The method has been previously described in chapter II.

3.5. Western Blot Analysis

The method has been previously described in chapter II.

3.6. Gene Silencing of ASMase

The method has been previously described in chapter II.

3.7. Isolation of Caveolae/Lipid Rafts Plasma Membrane Microdomains

a. The detergent-based method of caveolae/lipid raft isolation from HREC was previously described in chapter II. *b. Non-detergent method of isolation of caveolae/lipid rafts*. Caveolae/lipid raft-enriched membrane microdomains were prepared using a slightly modified non-detergent OptiPrep gradient ultracentrifugation protocol (43). Briefly, 5 x 10^6 HREC were washed twice with cold PBS, collected into the base buffer (20 mM Tris-HCl, pH 7.8, 250 mM sucrose) to which has been added 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Cells were eluted by centrifugation for two minutes at 250 g and resuspended in 0.33 ml of base buffer containing 1 mM CaCl2 and 1 mM MgCl2 and protease inhibitors. The cells were then lysed by passage through a 22gx3" needle 20 times. Lysates were centrifuged at 1,000*g* for 10 minutes. The resulting postnuclear supernatant was collected and transferred to a separate tube. The pellet was again lysed by the addition of 0.33 ml base buffer with divalent cations and protease inhibitors, followed by sheering 20 times through a 22gx3" needle. After centrifugation at 1,000*g* for 10 minutes, the second postnuclear supernatant was combined with the first. An equal volume (0.66 ml) of base buffer containing 50 % OptiPrep was added to the combined postnuclear supernatants and placed on the bottom of a 4 ml centrifuge tube. A 2.4 ml gradient of 0 % to 20 % OptiPrep in base buffer was poured on top of the lysate. Gradients were centrifuged for 18 hours at 200,000*g* at 4 °C using a swinging bucket rotor and 0.4 ml samples were collected carefully from the top of each fraction. A band confined to fractions one and two was designed as caveolae/lipid raft- enriched membrane domains.

3.8. Isolation of Plasma Membrane Domains

The method has been previously described in chapter II.

3.9. Lipid Extraction

Lipid extraction from whole HREC. 3 x 10^6 HREC were washed twice with cold PBS, collected in 500 µL cold 40% methanol and homogenized in a glass homogenizer on ice. The cell homogenate was transferred to a screw-cap glass tube and lipids were extracted as previously described (44). The samples were dried under a stream of nitrogen and re-extracted twice to remove non-lipid contaminants. The lipid film was dried under nitrogen, then further dried overnight in a speedvac, and resuspended in an appropriate amount of isopropanol : methanol : chloroform (4 : 2 : 1) based on the protein concentration of each sample.

Lipid extraction from HREC caveolae/lipid raft fractions and plasma membranes. For determination of sphingomyelin and ceramide content, plasma membranes and pooled caveolin-1 enriched OptiPrep fractions were subjected to lipid extraction with alkaline hydrolysis of glycerophospholipids (45). Lipids were dried under a stream of nitrogen, and re-extracted twice as outlined above for whole HREC lipid extracts to remove non-lipid contaminants. Lipids were dried and resuspended as described above for whole HREC lipids.

3.10. Mass Spectrometry Analysis

Synthetic lipid standards (Sphingolipid Mix I, Avanti) were added to lipid extracts used in mass spectrometry analysis at 20 nmol/mg protein and 5 nmol/mg protein, respectively. Prior to analysis, lipids were appropriately diluted in isopropanol/methanol/ chloroform (4 : 2 : 1 v /v/ v) containing 20 mM ammonium hydroxide, centrifuged, then loaded into Whatman Multichem 96-well plates (Fisher Scientific, Pittsburgh, PA) and sealed with Teflon Ultra-Thin Sealing Tape (Analytical Sales and Services, Pompton Plains, NJ). Lipids were introduced to a Thermo model TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA) as previously described (44). Ceramide (Cer) and sphingomyelin (SM) molecular species were monitored as their $[M+H]^+$ ions by tandem mass spectrometry (MS/MS) precursor ion scanning for the formation of characteristic product ions at m/z 264.4 (Cer) and m/z 184 (SM) in positive ionization mode. All MS/MS spectra were acquired automatically for 5-10 minutes at a rate of 500 m/z sec^{-1} by methods created using Xcalibur software (Thermo, San Jose, CA). Correction for ^{13}C isotope effects and quantitation of lipid molecular species against appropriate internal standards was performed using the Lipid Mass Spectrum Analysis (LIMSA) software (46) peak model fit algorithm in conjunction with an expanded user-defined database of hypothetical lipid compounds. Ceramide and sphingomyelin molecular species were quantitated ratiometrically by comparison with the peak area of their synthetic lipid internal standard. The LIMSA software peak model fit algorithm was used as previously described for peak finding and isotope correction to arrive at an "absolute" value for each identified lipid species. All identified ceramide and sphingomyelin molecular species were summed in order to quantify the total ceramide

and total sphingomyelin amount in the samples. The final result for each sample was presented as ceramide/sphingomyelin ratio. MS/MS collision energies, CID gas pressure, and Q1 and Q3 settings were optimized using synthetic lipid standards corresponding to the individual lipid classes of interest.

3.11. Immunofluorescence Assay

Cells were cultured on cover slips. The next day, cells on cover slides were removed from the media and rinsed in warm 1xPBS three times. Then cells were fixed and permeabilized in ice-cold methanol for 15 minutes at -20°C, washed with warm 1xPBS three times before blocking in a 2% BSA in 1xPBS solution for one hour at 37°C, and then incubated with primary anti-Caveolin-1, anti-ASMase and anti-LAMP1 antibodies and in a corresponding secondary antibody: Alexa Fluor 488 for Caveolin-1and LAMP1 and Alexa Fluor 495 for ASMase. All the primary and secondary antibodies were used in 1:100 dilutions. The cells were washed three times (five minutes each) with warm 1xPBS before mounting the cover slips on the slides. ASMase and Caveolin-1 colocalization was visualized with a confocal fluorescence microscope and measured with Olympus FluoView 1000 colocalization software. For images shown in Figure 3.2 B cells were fixed in 2% paraformaldehyde in PBS and where indicated, cells were permeabilized by ten minutes incubation in 0.1% TritonX-100.

3.12. Statistical Analysis

Data are expressed as the mean ± SD. Factorial ANOVA with post hoc Tukey test (GraphPad Prism5, GraphPad Software, San Diego, CA) was used for comparing the data obtained from independent samples. T-test was used to compare data obtained from two independent measurements. Significance was established at $P<0.05$.

4. Results

4.1. ASMase Expression and Activity in Human Retinal Cells

ASMase activity and expression level was assessed in three human retinal cell cultures: human retinal endothelial cells (HREC), human adult retinal pigment epithelial cells (HRPE) and human Muller cells (HMC). In these three retinal cell types, we found that HREC have the highest ASMase activity (Figure 3.1 A) and protein levels (Figure 3.1 B,C) when compared to HRPE and HMC.

4.2. ASMase Cellular Distribution in Human Retinal Cells

ASMase is ubiquitously expressed in the lysosomal compartment of all cell types. Lysosomal ASMase plays an important role in sphingomyelin metabolism but is not associated with inflammatory signaling. Recently endothelial cells were shown to secrete copious amounts of ASMase in response to inflammatory cytokines and the enzymatically active ASMase isoform was found in the caveolae membrane microdomain compartment of these endothelial cells (33). Caveolae are highly enriched in sphingomyelin and 70% of total cellular sphingomyelin is found in these membrane microdomains (26, 36-39). In order to hydrolyze plasma membrane sphingomyelin to pro-inflammatory and pro-apoptotic ceramide, ASMase has to be present in the caveolae. Therefore, we investigated the cellular distribution of ASMase in HREC, HRPE and HMC. In HREC, we found that ASMase and caveolin-1, a marker for caveolae microdomains, colocalize both at the membrane level and within the cytoplasm 56.38±5.85 % of ASMase staining colocalizes with caveolin-1 (Figure 3.2 A, upper

panel); however, we also observed a strong punctuate perinuclear staining negative for caveolin-1, which likely represents lysosomal ASMase. To confirm that perinuclear ASMase lies within the lysosomal compartment, lysosomal associated membrane protein 1 (LAMP1) was used as a marker for this cellular compartment. We found that perinuclear ASMase closely associates with LAMP1 (Figure 3.2 A, lower panel) as indicated by the yellow color in the merged panel. Moreover, although ASMase was ubiquitously expressed in the perinuclear intracellular compartment in HREC, HRPE and HMC (Figure 3.2 B,D), HREC were the only cell type with pronounced plasma membrane expression of ASMase (Figure 3.2 B,C)

4.3. DHA Effect on ASMase Activity in Human Retinal Cells

As we have previously shown, DHA-pretreatment decreases basal ASMase activity in HREC. To compare the effect of DHA on ASMase activity on various retinal cell types, we next determined ASMase activity in HRPE and HMC that were grown in control media or media supplemented with DHA. DHA had no effect on ASMase activity in HRPE (Figure 3.3 A) and HMC (Figure 3.3 B) when compared to control cells.

4.4. Downregulation of caveolar ASMase by DHA

Next we assessed ASMase colocalization with caveolin-1 in the caveolae/lipid raft fractions isolated from HREC by sucrose discontinuous gradient ultracentrifugation. Partial caveolae localization of ASMase was confirmed by western blot (Figure 3.4 A,C).

Moreover, DHA pretreatment of HREC prior to isolation of plasma membrane microdomains shows downregulation of ASMase in caveolae microdomains (Figure 3.4 B,C) with no effect on the ASMase expression in the intracellular compartment.

4.5. Sphingomyelin Enrichment in Caveolae/Lipid Rafts by DHA

To address the effect of DHA on sphingolipid composition of caveolae/lipid rafts, these microdomains were isolated by OptiPrep continuous gradient ultracentrifugation then subjected to lipid extraction and lipid analysis by tandem mass spectrometry for sphingomyelin and ceramide molecular species. Confirmation of isolated lipid rafts was achieved by showing enrichment of Caveolin-1 in density gradient fractions one and two. We found a significant decrease in ceramide/sphingomyelin ratio in the caveolae/lipid raft fractions isolated from HREC treated with DHA compared to BSA (vehicle control) or linoleic acid (lipid control) (Figure 3.5A). Moreover, DHA pretreated HREC prevented TNFα-induced increase in ceramide/sphingomyelin ratio (Figure 3.5 C). We found no significant modification of ceramide/sphingomyelin ratio in the plasma membrane domains isolated from control and DHA treated HREC (Figure 3.5 B). These data show that DHA-induced ASMase downregulation in the caveolae compartment results in formation of sphingomyelin-enriched caveolae/lipid rafts membrane microdomains.

4.6. Gene Silencing of ASMase Prevents Proinflammatory Cytokine-Induced HREC Activation

Treatment of HREC with 5ng/mL IL-1β for 6 hours induced dramatic upregulation of ICAM-1 protein expression (Figure 3.6 A,B). DHA significantly reduced cytokine-induced ICAM-1 protein expression in HREC (Figure 3.6 A,B). More importantly, ASMase gene silencing recapitulated the effects of DHA on cytokine-induced adhesion molecules expression in HREC, preventing the IL-1β-induced increase in ICAM-1 protein level in HREC (Figure 3.6 C,D).

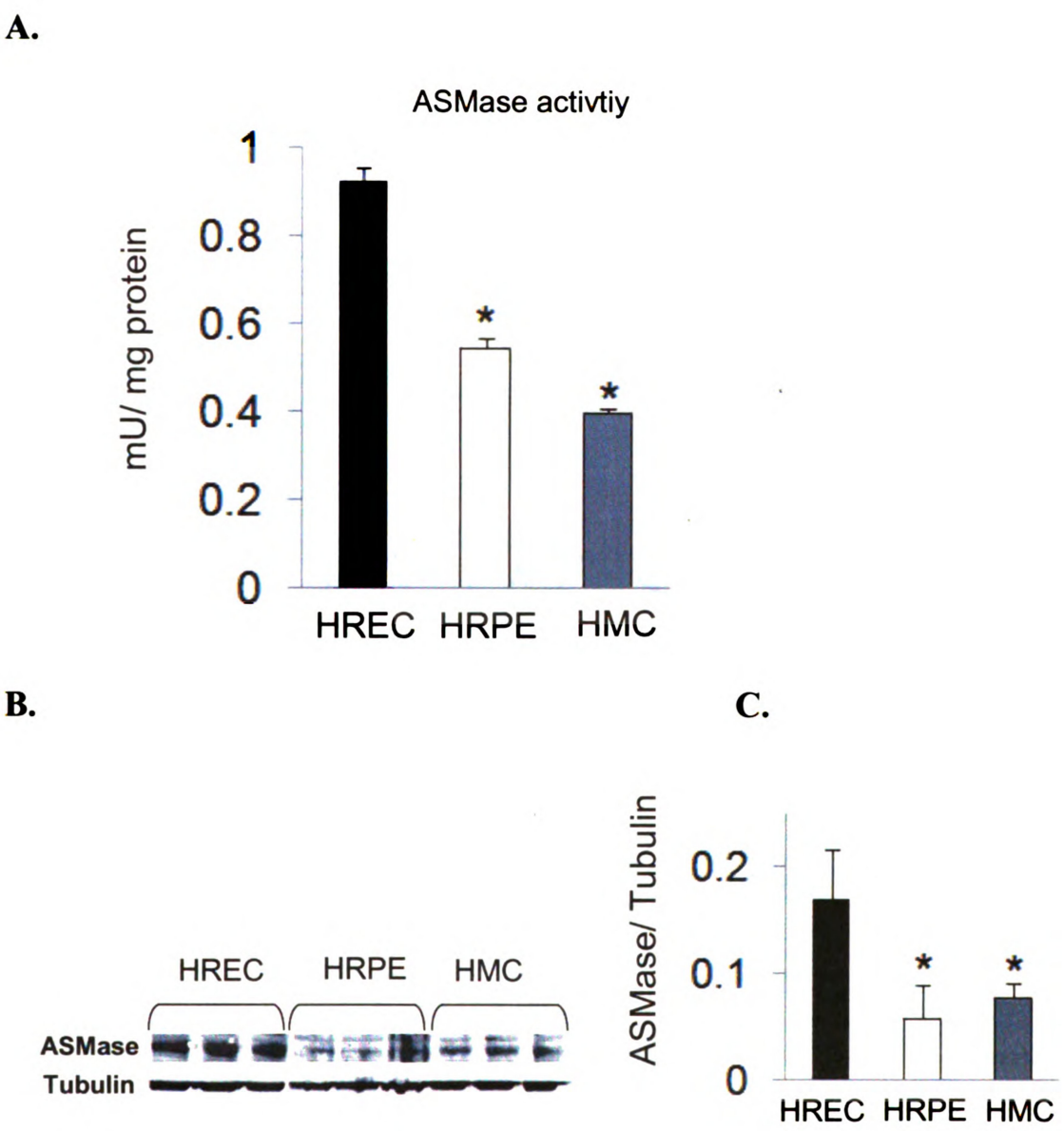


Figure 3.1. ASMase expression in human retinal cells. Three human retinal cell cultures are shown: human retinal endothelial cells (HREC), human retinal pigment epithelial (HRPE) and human Muller (HMC) cells. **(A)** ASMase activity in HREC, HRPE and HMC. **(B)** ASMase expression in HREC, HRPE and HMC was determined by immunoblot. **(C)** Quantitative analysis of the data in panel **(B)** for ASMase normalized to tubulin level. The results are mean±SD of at least 3 independent experiments; *P<0.05 compared to HREC.

A.

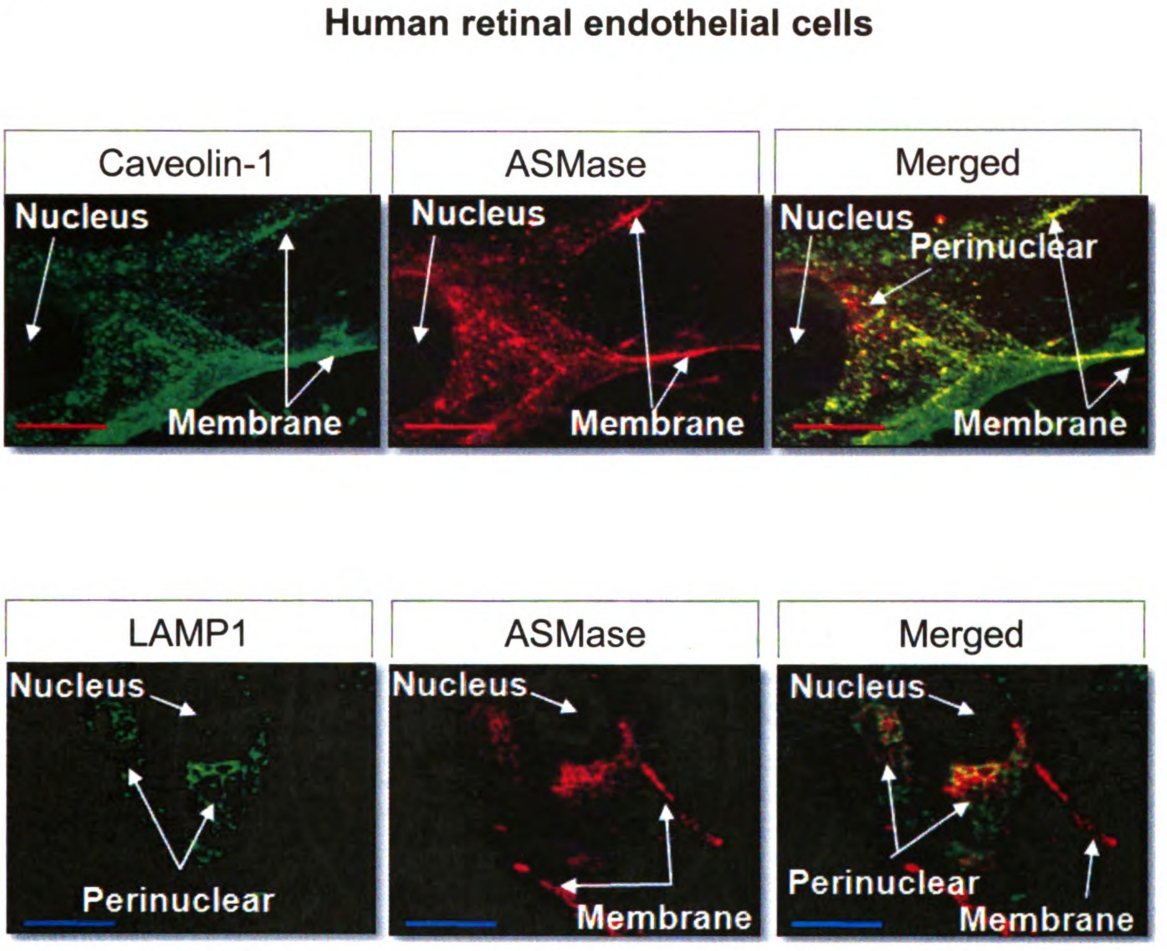


Figure 3.2. ASMase cellular distribution in human retinal cells. Three human retinal cell cultures are shown: human retinal endothelial cells (HREC), human retinal pigment epithelial (HRPE) and human Muller (HMC) cells. (A) ASMase, Caveolin-1 and LAMP1 colocalization in HREC was assessed by immunohistochemistry. There was 56.38±5.85 % colocalization of ASMase and caveolin-1 both at the plasma membrane level and in the cytoplasm (upper panel). However, there was strong punctuate perinuclear staining, negative for caveolin-1 that associates with LAMP1 (lower panel), a marker for the lysosomal compartment (yellow color in the Merged panel).

B.

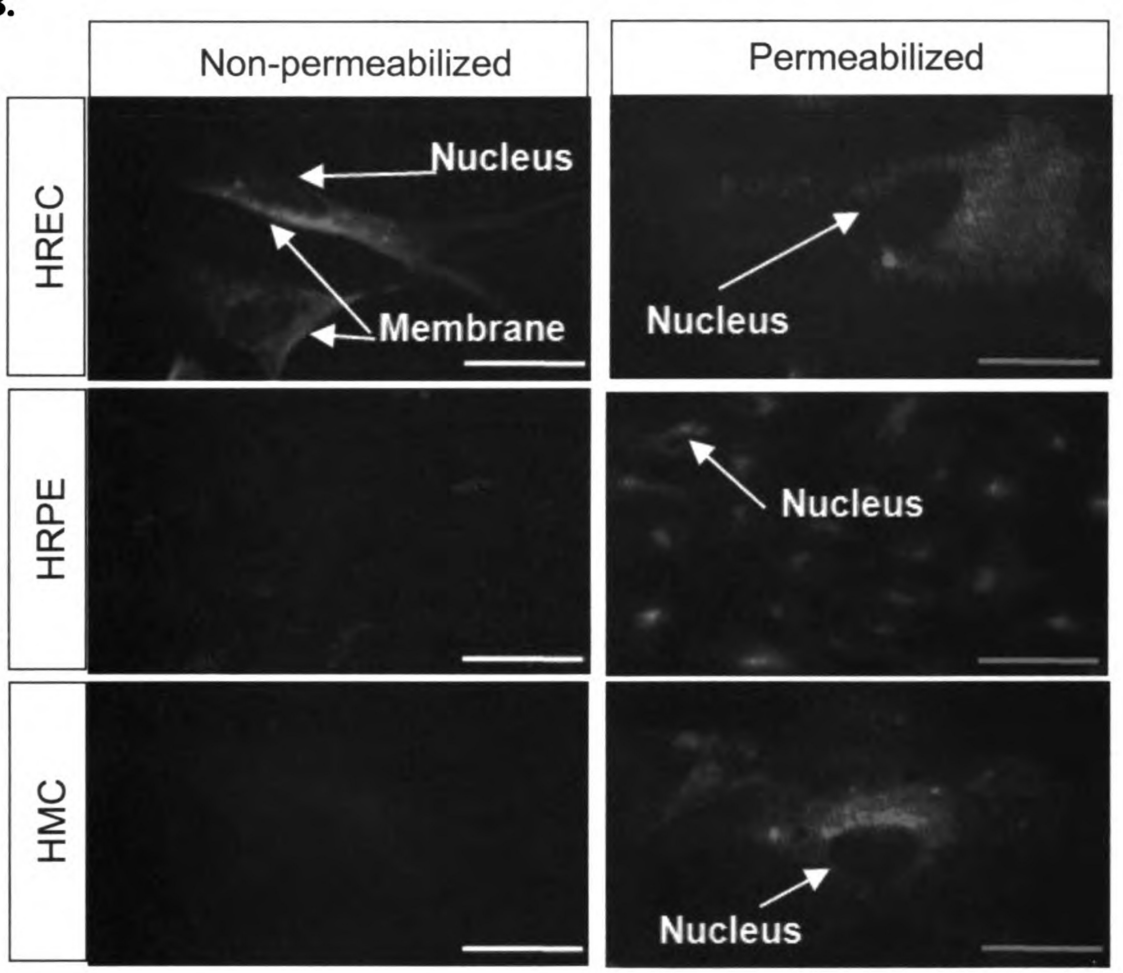
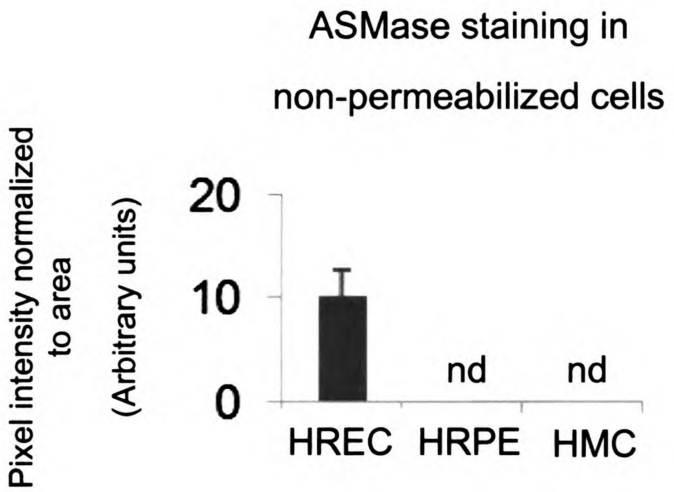


Figure 3.2 (continued). ASMase cellular distribution in human retinal cells. (B) Immunofluorescent assessment of ASMase cellular distribution in non-permeabilized (left) and permeabilized (right) HREC, HRPE and HMC.

C.



D.

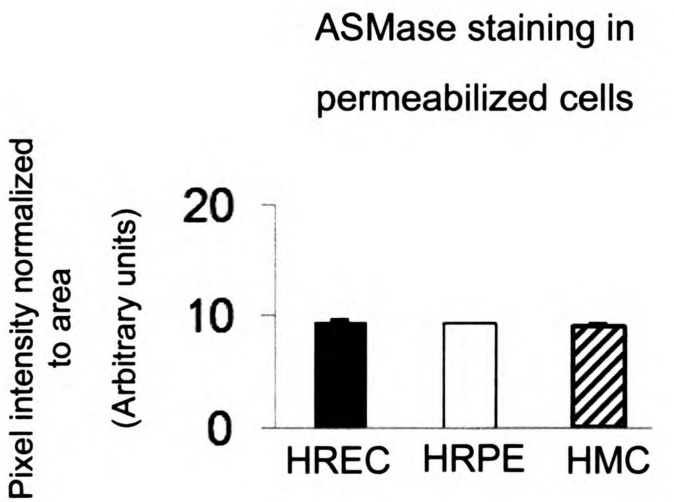
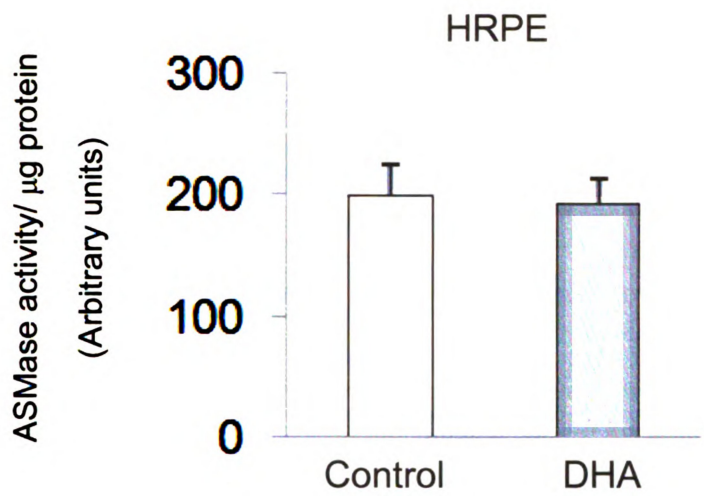


Figure 3.2 (continued). ASMase cellular distribution in human retinal cells. **(C)** Quantification of ASMase staining in non-permeabilized cells corresponding to plasma membrane localization of ASMase in HREC, HRPE and HMC (40 fields per experiment). **(D)** Quantification of ASMase staining in permeabilized cells corresponding to ASMase in the lysosomal compartment in HREC, HRPE and HMC (40 fields per experiment). The results are mean±SD of at least 3 independent experiments; nd= non-determined (red calibration bar = 27.5μm, blue calibration bar = 55μm, white calibration bar = 110μm).

A.



B.

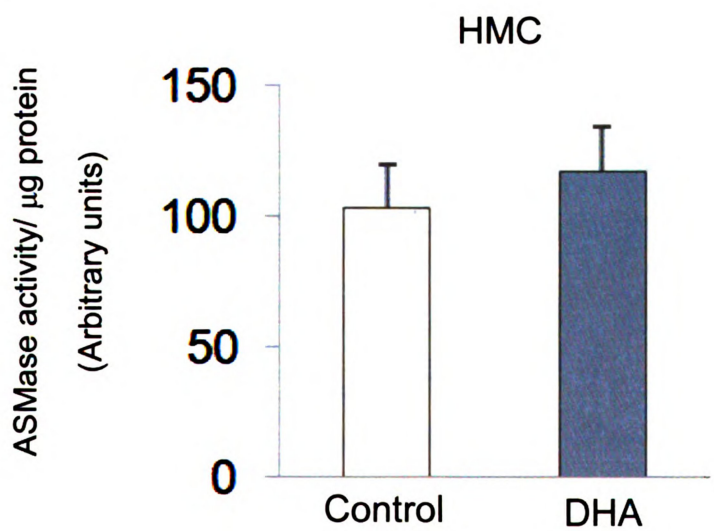


Figure 3.3. The effect of DHA on ASMase activity in HRPE and HMC. ASMase activity in **(A)** HRPE and **(B)** HMC treated for 18 hours with 100 µM DHA compared to control. The results are mean±SD of three independent experiments.

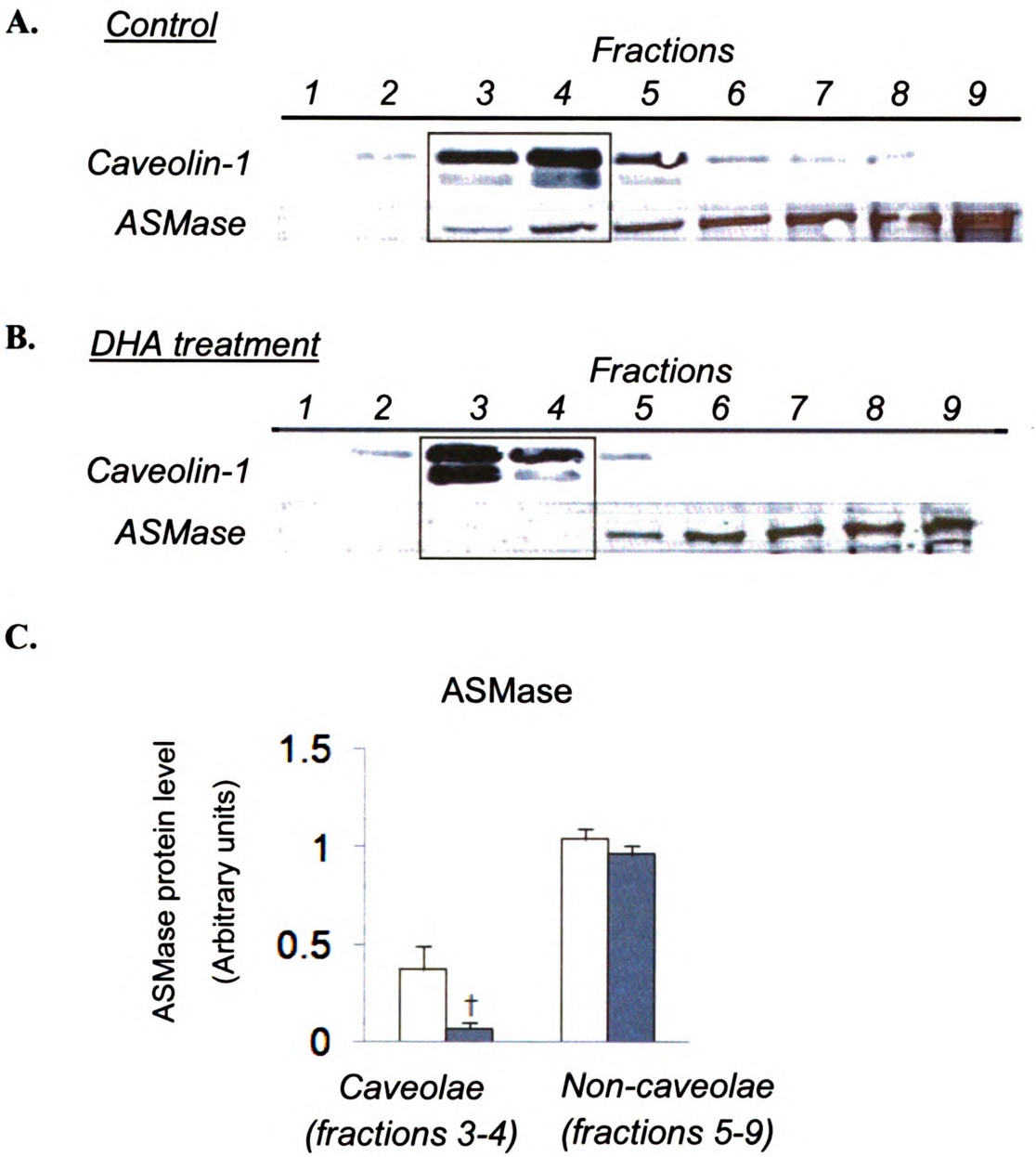
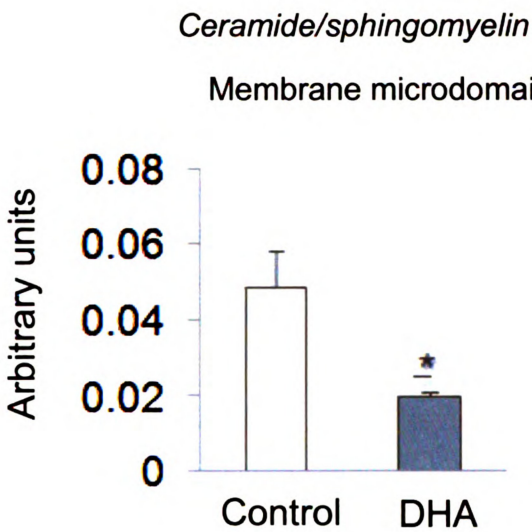


Figure 3.4. Downregulation of caveolar ASMase by DHA. (A-C) ASMase protein expression level in caveolae fractions (fractions 3-4) *versus* non-caveolae fractions (fractions 5-9) in control (**A and C,** *white bar*) and DHA (**B and D**, *gray bar*)-treated HREC. The results are mean±SD of three independent experiments, $^{\dagger}P<0.01$ compared to control.

A.



B.

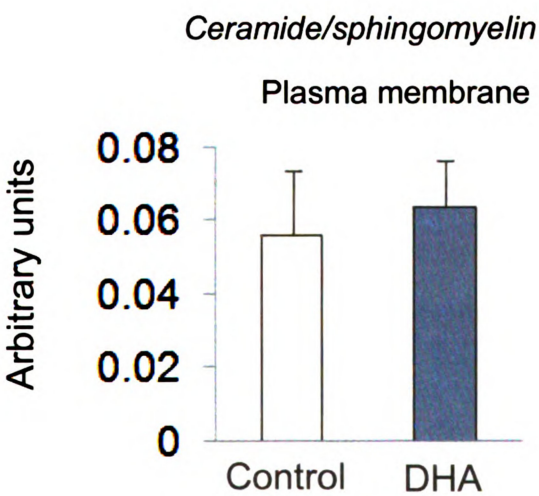
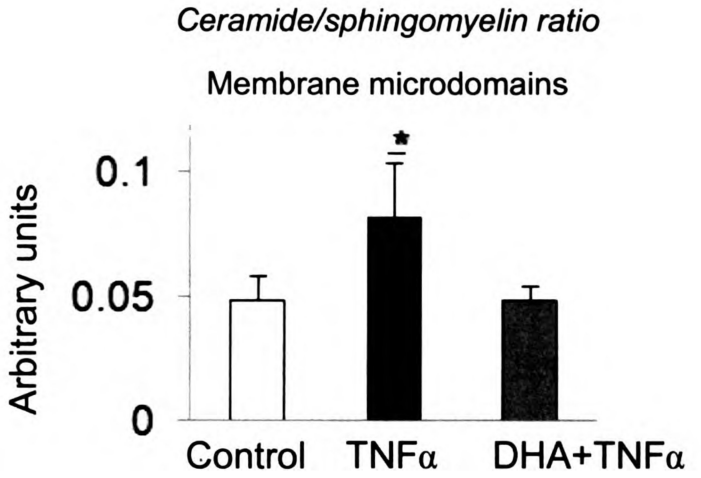


Figure 3.5. The effect of DHA on sphingomyelin and ceramide content of caveolae membrane microdomain in HREC. **(A)** Decrease in ceramide/sphingomyelin ratio in caveolae from DHA treated HREC compared to control. **(B)** No change in ceramide/sphingomyelin ratio in total plasma membrane.

C.



D

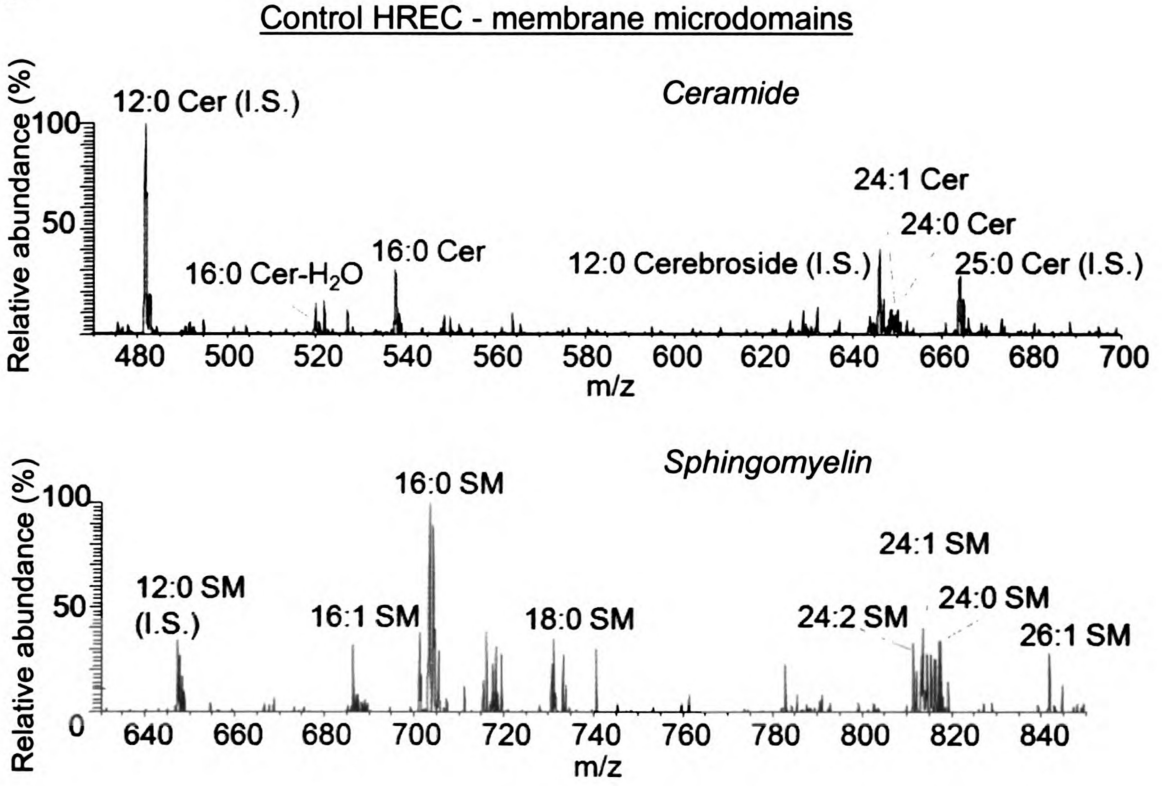


Figure 3.5 (continued). The effect of DHA on sphingomyelin and ceramide content of caveolae membrane microdomain in HREC. (**C**) DHA prevents TNFα-induced increase in ceramide/sphingo-myelin ratio in the caveolae. Tandem mass spectrum of control HREC **(D)**, DHA treated HREC **(E)**, TNFα treated HREC **(F)** and DHA and TNFα treated HREC **(G)** caveolae sphingomyelin species; and control HREC **(H)** and DHA treated HREC **(I)** of plasma membrane sphingomyelin species by positive ion mode PI m/z 184 after alkaline hydrolysis of glycerophospholipds.

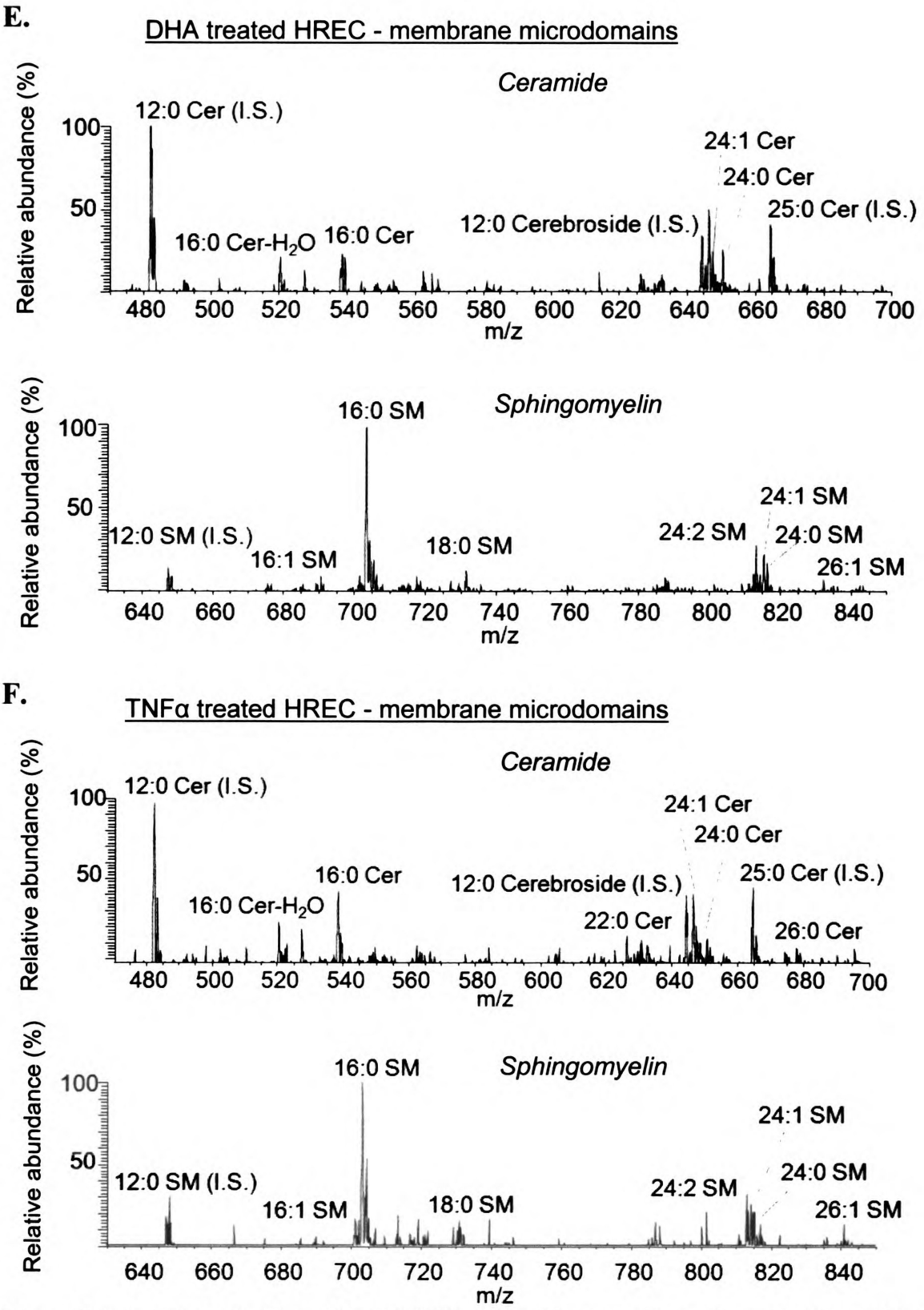


Figure 3.5 (continued). The effect of DHA on sphingomyelin and ceramide content of caveolae membrane microdomain in HREC.

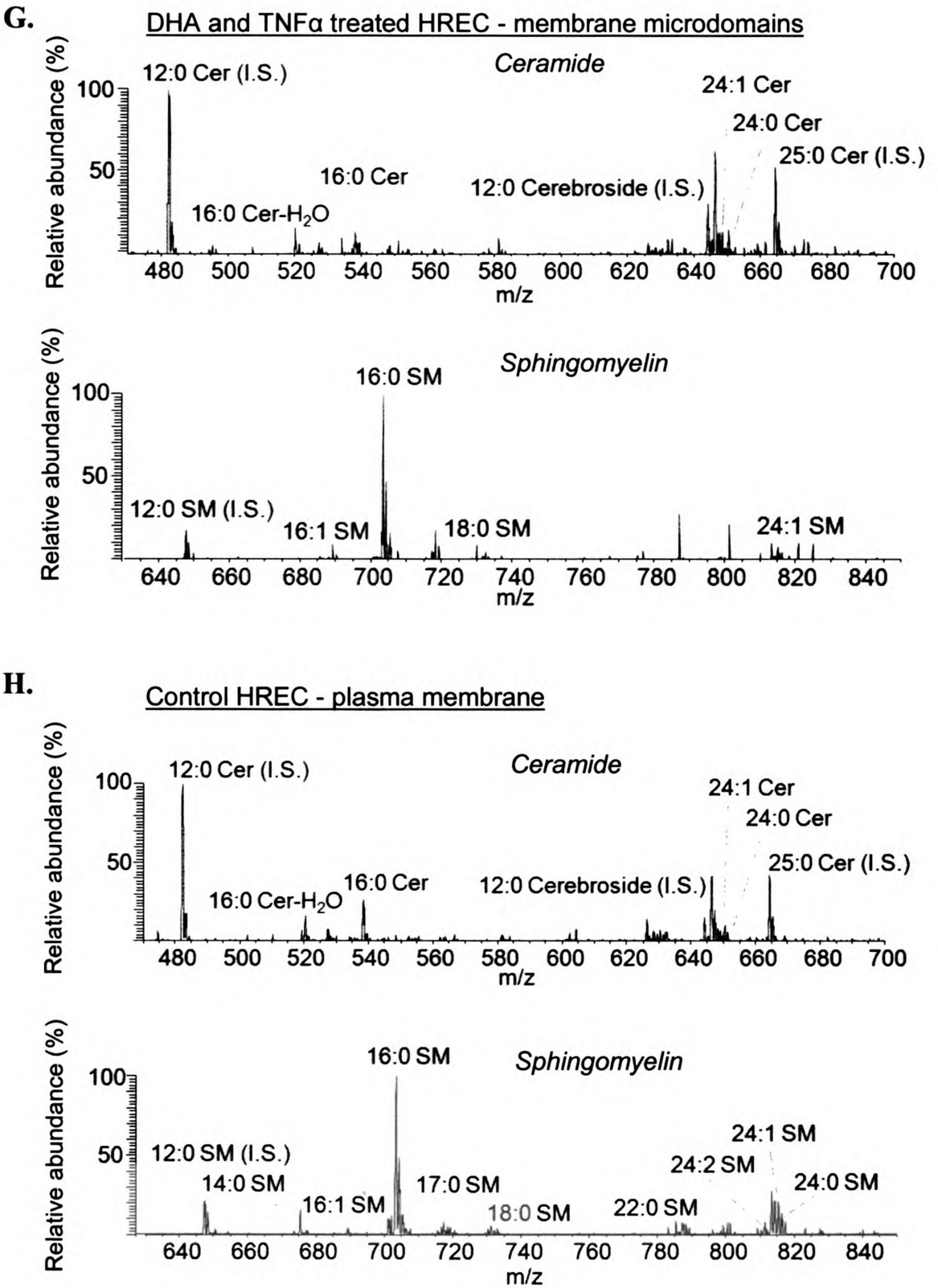


Figure 3.5 (continued). The effect of DHA on sphingomyelin and ceramide content of caveolae membrane microdomain in HREC.

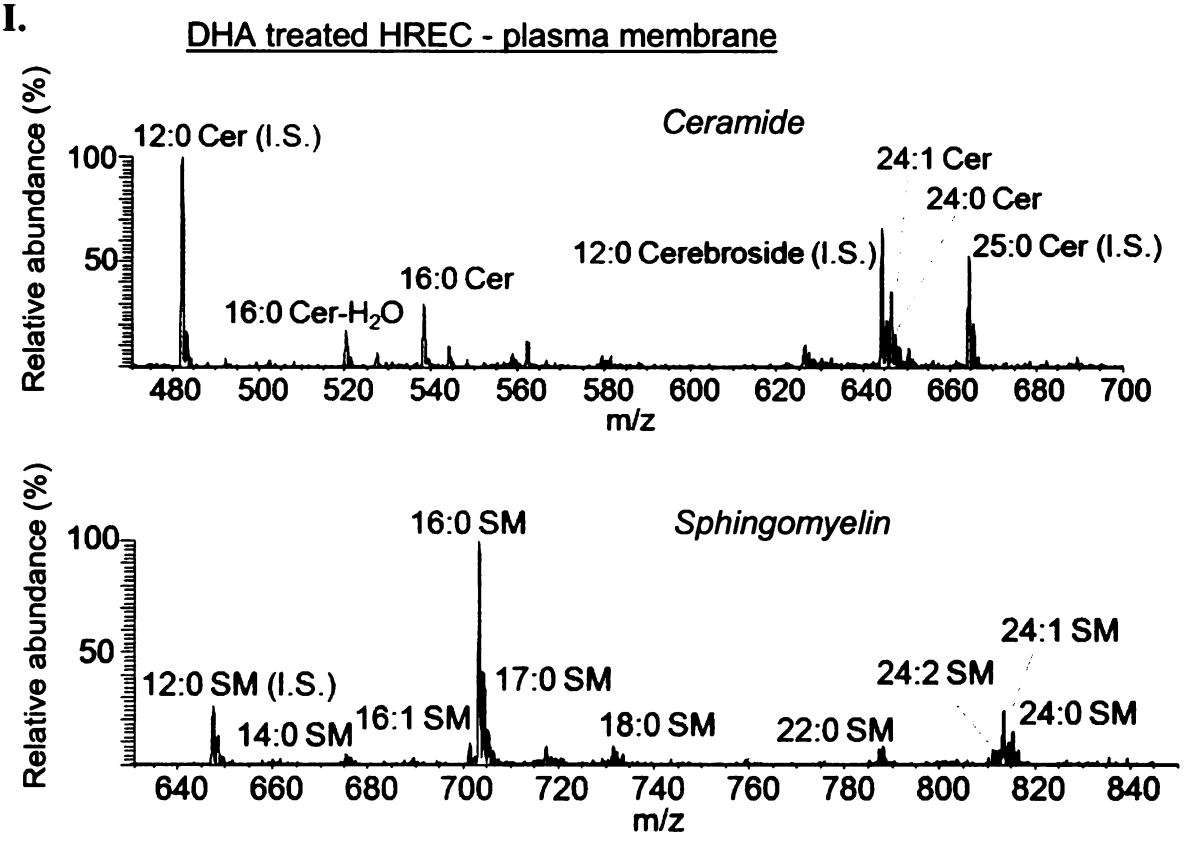
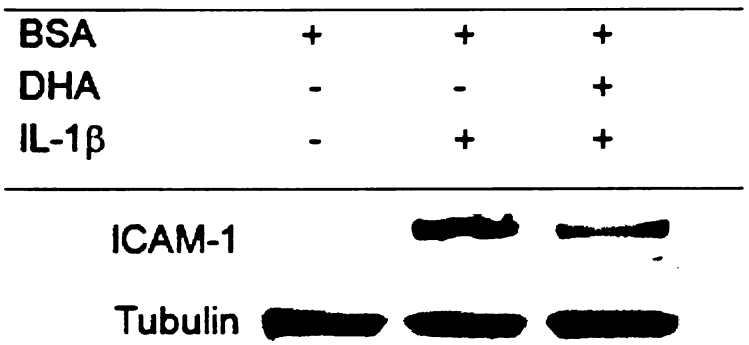


Figure 3.5 (continued). The effect of DHA on sphingomyelin and ceramide content of caveolae membrane microdomain in HREC. Tandem mass spectrum of control HREC **(D)**, DHA treated HREC **(E)**, TNFα treated HREC **(F)** and DHA and TNFα treated HREC **(G)** caveolae ceramide species; and control HREC **(H)** and DHA treated HREC **(I)** of plasma membrane ceramide species by positive ion mode PI m/z 264. The results are mean±SD of three independent experiments performed in triplicates, *P<0.05 compared to control, #P<0.05 compared to control and DHA treated and TNFα stimulated HREC.

A.



B.

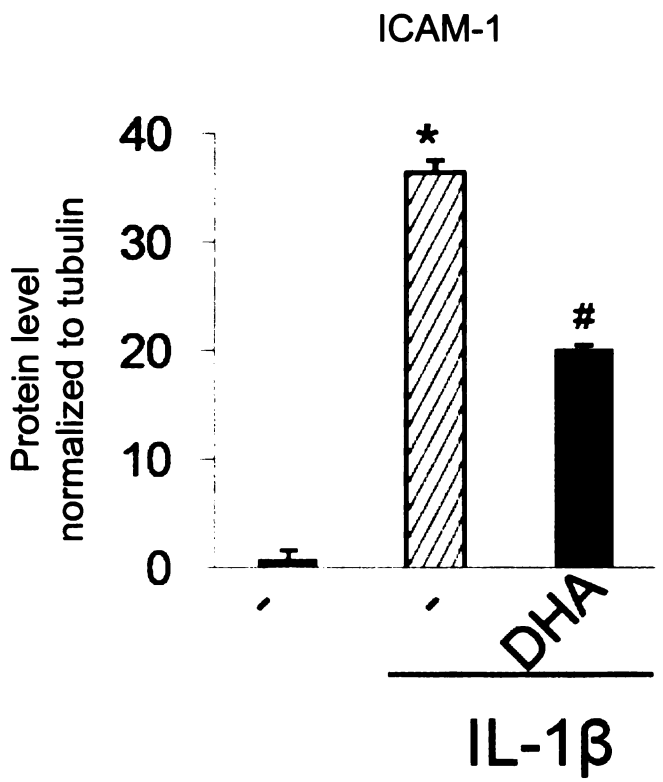
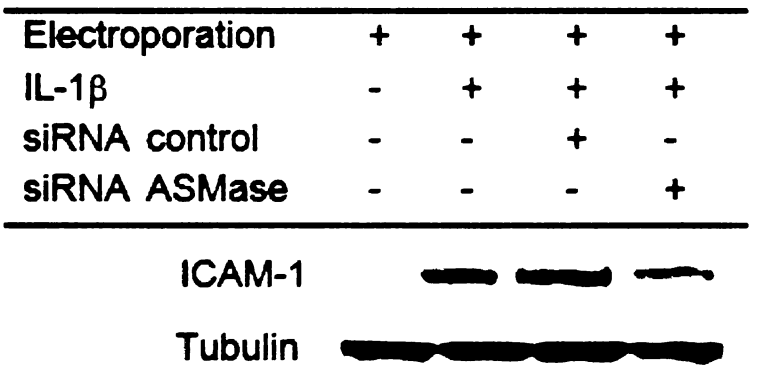


Figure 3.6. The effect of DHA and ASMase gene silencing on IL-1β-induced HREC activation. **(A)** DHA or **(B)** ASMase siRNA treatment prevents IL-1β induced increase in ICAM-1 expression in HREC. Random siRNA was used as control for ASM siRNA experiments. The results are mean±SD of at least three independent experiments performed in triplicates, *P<0.05 compared to control; #P<0.05 compared to IL-1β stimulated HREC, ʃP<0.05 compared to control.

C.



D.

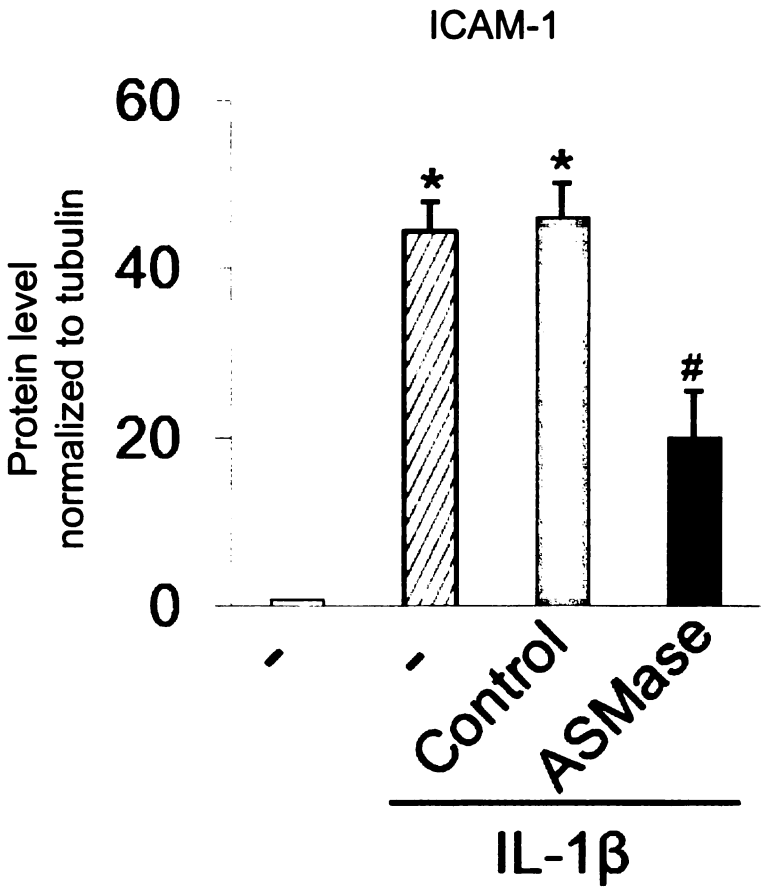


Figure 3.6 (continued). The effect of DHA and ASMase gene silencing on IL-1β-induced HREC activation.

5. Discussion

The fluid mosaic model of the cell membrane structure proposed by Singer and Nicholson in 1972 implied that membranes are in a disordered state with no major selectivity (47). However, recently , an extensive literature has showed the existance of microdomains that exist in a liquid-ordered phase within the membrane structure (48). These membrane microdomains are highly enriched in sphingolipids (sphingomyelin, ceramides) and cholesterol, are static within the membrane, but have the ability to coalesce and rearrange in response to diverse stimuli (49). The generation of ceramide at the plasma membrane level dramatically alters the properties of the cell membrane, since ceramide molecules have the tendency to spontaneously self-associate (50, 51). It is generally accepted that ceramide-rich membrane microdomains could primarily function to reorganize receptors and various signaling molecules at the plasma membrane level in order to facilitate and amplify cellular signaling processes via specific receptors (52).

Sphingomyelinases are sphingolipid hydrolyses that catalyze the breakdown of sphingomyelin to ceramide and phosphorylcholine. Several isoforms of sphingomyelinases have been identified and further distinguished by their catalytic pH optimum, cellular localization, primary structure and co-factor dependence. Alkaline sphingomyelinase activity is confined to the intestinal mucosa, bile and liver and does not participate in signal transduction (36, 53, 54). Neutral sphingomyelinase (NSMase) and ASMase are crucially involved in the pathophysiology of metabolic disorders (55) and play an active role in cellular signaling (56).

ASMase is classically known to localize to the lysosomal compartment. Lysosomal ASMase is ubiquitously expressed in all cell types. However, several studies

also identified a plasma membrane-associated form of ASMase in human fibroblasts (33) and pulmonary vascular endothelial cells (57). Another study brought out evidence of the existence of a small pool of zinc-independent ASMase in the plasma membrane microdomains purified from NIH-3T3 L1 fibroblasts and PC12 cells and more importantly, this pool of ASMase could undergo activation in response to neurotrophins, whereas the lysosomal ASMase did not respond to activation by ligand (58). Various immunohistochemistry studies have shown that cells exposed to different stress stimuli exhibit a change in ASMase location from intracellular compartment to the cell surface (59). Plasma membrane-associated ASMase, rather than intracellular ASMase, is shown to be associated with inflammatory signaling (49), as ceramide generation within the plasma membrane results in coalescence and reorganization of membrane microdomains into large signaling platforms that facilitate receptor clustering and cellular signal transduction (60, 61).

Endothelial cells are well recognized to be an important source of ASMase, expressing as much as 20 times more ASMase as other mammalian cell types (62, 63). Human coronary artery and human umbilical vein endothelial cells, as well as bovine and murine aortic endothelial cells secrete copious amounts of ASMase in response to inflammatory cytokines (63). Our study also shows that HREC exhibit high ASMase activity and expression level when compared to other retinal tissues, including HRPE and HMC cells (Figure 3.1 A,B,C). Moreover, in this study we demonstrate that it is this plasma membrane ASMase that is a key element in the retinal inflammatory response in HREC; HREC was the only cell type to demonstrate positive staining for the plasma membrane form of ASMase, whereas HRPE and HMC presented only the classical,

intracellular form of ASMase. We have found in our study that 56.38±5.85 % of ASMase colocalize with caveolin-1 both at the membrane and intracellular levels and 44 % may represent the lysosomal form of ASMase. In endothelial cells, localization of ASMase to the plasma membrane, and particularly to caveolae microdomains, increases substrate availability for this enzyme.

Omega 3 PUFAs, and especially DHA, have been long accepted to modulate inflammatory processes (64). We have previously shown that DHA pretreatment of HREC decrease ASMase activity and gene expression (65). Our present study demonstrates that DHA downregulates the ASMase protein level in the caveolae microdomain compartment, with no effect on intracellular ASMase protein level. Decreased ASMase expression in caveolae would profoundly impact the ability of HREC to generate ceramide in this membrane microdomain. Although DHA has disparate effects on the caveolar and lysosomal compartments, this differential sensitivity to DHA is likely due to a higher exchange and/or degradation rate of the caveolar ASM, which is dynamically regulated, compared to lysosomal ASM, which is constitutively expressed.

Indeed, regulation of caveolar ASMase expression has a profound impact on membrane microdomains lipid composition, particularly on sphingomyelin and ceramide content. As we have demonstrated in this study, downregulation of caveolar ASMase by DHA induced a significant reduction in ceramide/sphingomyelin ratio and decreased the ability of the proinflammatory cytokine TNFα to induce ceramide production in these specialized membrane microdomains. However, as shown in the previous chapter, DHA also downregulates the activity and cellular expression of NSMase, another critical enzyme capable of sphingomyelin hydrolysis at the membrane level. Therefore, the DHA

effect on microdomain sphingolipid composition may be due to downregulation of both ASMase and NSMase. Yet, inhibition and/or gene silencing of ASMase rather than NSMase had a more pronounced effect on decreasing cytokine-induced adhesion molecules expression in HREC. In addition, several studies have downplayed the role of NSMase in ceramide-enriched platforms formation and transmembrane signal transmission (66, 67). Taken together, our results suggest that ASMase could play a more important role in cytokine-induced formation of ceramide-rich membrane microdomains and amplification of inflammatory signaling in our cell culture system.

In conclusion, we demonstrated that HREC have the highest cellular ASMase expression and protein level when compared to HRPE and HMC. Moreover, the plasma membrane ASMase was expressed only in HREC. DHA downregulated caveolar ASMase expression and promoted formation of sphingomyelin-enriched membrane microdomains. These modified caveolae/lipid rafts have a decreased response to cytokines known to be upregulated in diabetic retina (TNF-α and IL-1β) as confirmed by decreased cellular adhesion molecules expression in HREC. Based on the results obtained in these *in vitro* studies, we addressed in the following chapter the role of sphingomyelinases in retinal vascular inflammation and vessel loss in diabetic retinopathy, using a streptozotocin (STZ)-induced diabetes rat model; we also characterized the effect of DHA supplementation on retinal sphingomyelinase expression, inflammatory status and microangiopathy lesions in this type 1 diabetic rat model.

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Chapter IV

Docosahexaenoic Acid Supplementation Prevents Upregulation of Proinflammatory/Angiogenic Molecules and Vessel Loss in Diabetic Retina Through Downregulation of Acid Sphingomyelinase

1. Abstract

Sphingomyelinases (SMases) are key regulatory enzymes of sphingolipid metabolism, catalyzing hydrolysis of sphingomyelin to the proinflammatory and pro-apoptotic messenger ceramide. In this study, we addressed the role of SMases in retinal vascular inflammation and vessel loss in diabetic retinopathy, using a streptozotocin (STZ)-induced diabetes rat model. Acid (ASMase), but not neutral (NSMase) SMase was upregulated in the retinas with microangiopathy lesions. Proinflammatory/angiogenic molecules were significantly increased in retina of STZ rats at one month and nine month post STZ treatment. However, a docosahexaenoic acid (DHA)-enriched diet restored ASMase in diabetic retina to the control levels and prevented upregulation of inflammatory/angiogenic mediators' gene expression, strongly demonstrating a DHA protective effect against the development of an early inflammatory status in the diabetic retina. More importantly, DHA prevented retinal vessel loss in the retina of STZ treated, diabetic rats. In order to confirm that the protective effect of DHA in the retina is mediated through vascular ASMase, we next used an ischemia/reperfusion (I/R) injury model of retinopathy in rats. Immunohistochemistry analysis of normal rat retina demonstrated that retinal vasculature preferentially express ASMase when compared the

other retinal tissues. Furthermore, rat retinal I/R specifically increased ASMase expression in the vasculature, with little or no effect on ASMase expression in other retinal cells. Notably, DHA supplementation prior to retinal injury prevented ASMase upregulation in the retinal vasculature. Taken together, these *in vivo* data demonstrated ASMase downregulation as a mechanism by which DHA exerts its protective effect against retinal microvascular damage in diabetic retinopathy.

2. Introduction

Visual impairment among people with diabetic retinopathy is a major disability with a profound impact on quality of life (1). Diabetic retinopathy (DR) represents the ocular expression of diabetic microangiopathy. The natural history of DR has been classified into an early, non-proliferative stage (background retinopathy) and a later, proliferative stage. The early stage of DR has distinct vascular lesions that are histologically characterized by the existence of saccular capillary microaneurysms, retinal hemorrhage, venous dilation and beading, retinal lipid exudates, pericyte degeneration, occlusion and degeneration of retinal capillaries (2-3). This early stage diabetic retinopathy represents a persistent low-grade chronic inflammatory disease (4) that involves leukocyte recruitment and adhesion to the retinal vasculature and up-regulation of inflammatory genes. Adhesion molecules, especially intercellular adhesion molecule-1 (ICAM-1), are involved in leukocyte attachment and transmigration into the vascular intima (5-6) that will result in early blood retinal barrier breakdown, capillary non-perfusion, endothelial cell injury and death. Proinflammatory cytokines (TNFα, IL-1β, IL-6 and VEGF) are increased in diabetic eyes and several inflammatory pathways are activated in the very early stages of diabetic retinopathy (7-11).

Proinflammatory cytokines that are upregulated in diabetic eyes, including TNFα and IL-1β have been shown to activate cellular sphingomyelinases in various cell types (12-15), including retinal vascular endothelial cells, as presented in the previous chapters. Sphingomyelinases are enzymes that catalyze the hydrolysis of sphingomyelin (ceramide phosphocholine) to the bioactive lipid ceramide. Caveolae microdomains present on endothelial cells are enriched in sphingomyelin and thus represent an important pool for

ceramide production. Ceramide generation within the outer leaflet of the plasma membrane facilitates receptor clustering and enhances inflammatory signal transduction by several receptors that mediate the pathological changes typically associated with diabetic retinopathy, such as IL-1β and TNFα (16-17). As previously underlined, vascular endothelial cells represent the target tissue affected by diabetic retinopathy and become dysfunctional and degenerate due to diabetic metabolic dysregulation and inflammatory stress. Moreover, vascular endothelial cells are considered to be a very rich source of secreted ASMase and pro-inflammatory cytokines, especially IL-1, increase ASMase secretion in *in vivo* studies (18).

The mechanisms that induce the proinflammatory state in the retina are not well defined, but various studies have shown that hyperglycemia and dyslipidemia play an important role. Hyperglycemia, a major causative factor in the development of diabetic retinopathy, induces cytokine production by several distinct retinal cell types including retinal pigment epithelium, Muller cells, astrocytes, microglia and pericytes, but interestingly not by retinal endothelial cells. We previously demonstrated *in vitro* that retinal endothelial cells increase the production of reactive oxygen species in response to diabetes induced cytokine release by other retinal cells rather than directly to high glucose, suggesting that *in vivo* diabetes-related endothelial injury in the retina is also due to glucose-induced cytokine release and not a direct effect of high glucose on endothelial cells (19). Retinal endothelial cells express TNF-α and IL-1β receptors and stimulation with cytokines activate classic NFκB inflammatory pathways leading to increase expression of inflammatory genes, including adhesion molecules ICAM-1 and VCAM-1.

In addition to hyperglycemia, Diabetes Control and Complications and Epidemiology of Diabetes Interventions and Complications Trials (DCCT/EDIC) demonstrated the strong association between dyslipidemia and development of diabetic retinopathy. We have previously demonstrated that the retina has a unique fatty acid composition exhibiting the highest level of ω3-polyunsaturated fatty acids (PUFA), especially DHA, in the body and that retinal fatty acid metabolism is distinct from the metabolic changes that occur in diabetic plasma or diabetic liver (20).

Omega 3 PUFAs have the ability to modulate various biological processes involved in retinal vascular inflammation, retinal capillary structure and integrity, and retinal angiogenesis (21). We have previously shown that DHA suppresses cytokine induced inflammatory signaling and activation of HREC (22). This pronounced anti-inflammatory effect of DHA was mediated through a reduction in ASMase and NSMase activity and expression (23). Importantly, total ω3-PUFAs, and particularly DHA, are reduced in the retina of human (24) and rat (20) diabetic eyes. Moreover, increasing the retinal levels of ω3-PUFAs by genetic or dietary means has a beneficial effect in reducing pathological retinal angiogenesis (21).

Activation of sphingomyelinases is an important early step in inflammatory cytokine signaling (13-14) particularly in endothelial cells (15, 23). In this study, we tested the hypothesis that downregulation of SMases by DHA may be protective against endothelial activation, injury and death, and thereby preventing retinal vessel loss in a rat model of type 1 diabetes.

3. Materials and Methods

3.1. Reagents and Antibodies

Crude trypsin was obtained from Difco (Detroit, MI)4.. Lyophilized pancreatic elastase was purchased from Calbiochem (San Diego, CA). Streptozotocin (Sigma, St. Louis, MO), Neutral Protamine Hagedorn (NPH) insulin, experimental diets Dyets Inc. (Bethlehem, PA), RT-PCR primers from IDT DNA Technologies (Coralville, IA) (Table 4.4 and Table 4.5). ASMase antibody was a generous gift from Dr. Richard Kolesnick. VEGF antibody was purchased from Novus Biologicals (Littleton, CO). NuPAGE Novex 10% Bis- Tris gels, Platinum SYBR Green qPCR SuperMix-UDG w/ROX, were purchased from Invitrogen (Carlsbad, CA). IRDye infrared secondary antibodies were purchased from Invitrogen, Molecular probes (Eugene, OR).

3.2. Rat Model of Type 1 Diabetic Retinopathy

Male Sprague-Dawley rats weighing 237-283 grams were made diabetic with a single intraperitoneal injection of 65 mg Streptozotocin (STZ) (Sigma, St. Louis, MO) per kg body weight. STZ is a pancreatic ß-cell cytotoxin, similar to glucose, transported into the cell by the glucose transport protein GLUT2. Starting with day 7 after STZ injection, Neutral Protamine Hagedorn (NPH) insulin injections (with a dose of 0-3 units/day) was administrated in order to achieve slow weight gain, but allowing hyperglycemia in the range of 20mM blood glucose. The weight gain and blood glucose measurements from all the groups studied are presented in Table 4.1 and Table 4.2. The retina was harvested and analyzed at several time points (one month and nine months of diabetes).

3.3. The Experimental Diet

In our study, we maintained the recommended 10% caloric intake from lipid and replaced 5% of this value with fish oil in the treatment group. Two dietary experimental groups of animals were established in both control non-diabetic and diabetic animals: a fish oil diet (ω-3-PUFA) and a vegetable oil diet group. The diets were purchased from Dyets Inc. (Bethlehem, PA) and are based on AIN-93M purified rodent diet composition with 10% caloric intake as soybean oil (standard rodent diet ingredient contains 50.8% linoleic acid) or 5% as soybean oil and 5% as Menhaden oil (oil from a small plankton eating fish containing 10.26% DHA and 14.16% EPA). The n6/n3 fatty acid ratio in vegetable oil diet is 8/1, reflecting 15-16.7/1 ratio found in most Western diets (25). The n6/n3 ratio in fish oil diet represents 1.5/1, reflecting the ratios associated with prevention of cardiovascular disease (26) or suppression of inflammation in patients with rheumatoid arthritis (27). The fatty acid composition of the control, standard diet and fish oil (DHA)-enriched diet are presented in Table 4.3.

3.4. Isolation of Retinal Vasculature

Rat retinal vasculatures were isolated by trypsin digestion method as previously described (28-29). The eyes were fixed in formalin for two weeks. The day before trypsin digest, the eyes were opened coronally, cornea and lens were discarded and posterior globe was placed into distilled water. The retina was loosened from sclera with metal micro-spatula and cut loose at optic nerve with microscissors in the way that optic nerve head remained attached to the retina. The retina was placed in a perforated sample holder under gently

running tap water overnight to remove formalin fixative. The next morning the retina was placed in 25 ml glass beaker with 5 ml of 3% crude trypsin in Tris HCl buffer, pH 7.8 with 0.2M NaF and digested for about 90 min. The inner limiting membrane was removed and the digested retina was placed into a Petri dish containing distilled water using a glass pipette as a transfer tool. The retina was gently brushed with cat whisker hair to dislodge neural elements. The vessel network was cleaned three times in pure distilled water, transferred onto mounting slide, and stained with hematoxylin and periodic acid-Schiff.

3.5. Histological Assessment of Retinal Capillaries

The mid-retina areas along each major vessel of the vascular bed were systematically investigated (6-8 fields per retina) to quantify the acellular capillaries at 250x magnification (~0.32 mm^2 per field). A capillary was counted as acellular if it had no nuclei from junction to junction and comprises at least 20% of the diameter of surrounding capillaries.

3.6. Simultaneous Extraction of Proteins and RNA

The method was performed as previously described (30). Each retina was homogenized in 1 mL of TRIzol reagent. Then 200 µl Chloroform was added, the sample was covered tightly, shacked vigorously for 15 seconds and let it stay at room temperature for 2-3 minutes; then centrifuge at 13,000 *g* for 15 minutes at 4°C. Phases separation was

obtained: the aqueous phase (RNA), the red organic phase (protein), the interphase (DNA). Any residual DNA was precipitated from the organic phase with 100% ethanol (300 µl), followed by a 2000*g* centrifugation for 5 minutes at 4 °C. The organic phase was resolved in 14 mL of wash buffer (31.25mmol/L Tris-HCl, pH 6.8, 0.1% SDS (sodium dodecyl sulfate)). After centrifugation at 4000g for 5 minutes at 23 °C, the upper phase and wash buffer was transferred to a 5KD MWCO Amicon -15 (Millipore) ultrafiltration tube. The Amicon -15 (Millipore) ultrafiltration tube was centrifuged at 3000*g* for 20 minutes at 23 °C. The filtrate was discarded and the same wash step was performed 3 times with 14 mL of new wash buffer added each time. The protein solution (approx 250 µl retentate) was transferred to an eppendorf tube; 5 µl of protease inhibitor cocktail was added. The sample was stored in -80 °C until analysis.

3.7. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from rat and mice retinas. Specific primers for each gene are listed in Table 1. Primers were designed using IDT DNA PrimerQuest software (Coralville, IA). First strand cDNA was synthesized using the SuperScript II RNase H-Reverse Transcriptase (Invitrogen Carlsbad, CA). Synthesized cDNA was mixed with 2x SYBR Green PCR Master Mix (Invitrogen Carlsbad, CA).and different sets of gene-specific forward and reverse primers and subjected to real-time PCR quantification using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). All reactions were performed in triplicate. The relative amounts of mRNAs were calculated by using the comparative CT method (User Bulletin #2, Applied Biosystems). Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA.

3.8. Western Blot

Proteins were extracted from rat retinas using a simultaneous extraction of proteins and RNA method as previously described. Proteins were resolved by NuPAGE Novex 10% Bis-Tris gels, transferred to nitrocellulose membrane and immunobloted using appropriate primary antibodies followed by secondary IRDye infrared secondary antibodies (Invitrogen, Molecular probes, Eugene, OR). Immunoreactive bands were visualized and quantified by Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

3.9. Immunofluorescence Assay

Rat retinal samples were fixed in Zinc Fixative (BD Biosciences, San Jose, CA) for 48 hours, then washed in PBS. Then the samples were blocked 60 minutes with 3% normal serum in 0.3% Triton-x100 in TBS. The primary antibody incubation was overnight at 4°C on rocker. The next day tissue was rinsed in two changes of TBS+Tween20 (TBST), five min each on rocker at room temperature. Secondary fluorescent antibody incubation was three hours at room temperature on rocker. Then tissue was rinsed in three changes of TBST for 10 min each and mounted on slides and covered with a coverslip.

3.10. Rat Model of Retinal Ischemia-Reperfusion

Male Sprague-Dawley rats weighing 300 grams were used. All procedures involving the animal models adhered to the ARVO statement for the Use of animals in Ophthalmic and

Vision research. Retinal ischemia-reperfusion was created by temporal increase in intraocular pressure to 90 mm Hg as previously described (31). The DHA treated group was on a DHA-enriched diet for 12 days and has also been supplemented via tail vein with 500 nmol/kg DHA one day prior and 250 nmol/kg DHA (32) immediately prior to induction of I/R. The control, no treatment group was on standard rodent diet and subjected to saline solution injection via the tail vein. The retinas were isolated two days after retinal I/R, the time point that corresponds to inflammatory changes similar to the early stage of diabetic retinopathy.

3.11. Slide Preparation and Quantification of the ASMase Staining of the Blood Vessels

Rat retinal samples were fixed in Zinc Fixative (BD Biosciences, San Jose, CA) for 48 hours, followed by routine automated vacuum infiltrating tissue processing to paraffin block. Zinc fixed paraffin blocks were sectioned on a rotary microtome at 4 – 5 microns and placed on 3-aminoalkylethoxysilane coated slides, dried overnight at 56°C. Slides following de-paraffinization and hydration to distilled water were placed in TBST (Scytek Labs – Logan, UT) to allow for pH adjustment to 7.4. All slides were stained on a Dako Autostainer (Dako North America – Carpinteria, CA) with TBST rinses between each staining reagent. Non-specific protein block utilized normal serum from the host of the secondary antibody for 30 minutes (Vector Labs – Burlingame, CA). Endogenous biotin block in Avidin D (Vector) / d-Biotin (Sigma – St. Louis, MO) was used for 15 minutes. Primary antibodies for ASMase and caveolin-1 were applied at optimized

dilutions in normal antibody diluent (Scytek) for 1 hour and then biotinylated anti-Host species IgG H+L diluted to 11μg/ml (Vector) in NAD was applied for 30 minutes. R.T.U. Vectastain® Elite ABC Reagent (Vector) was used next for 30 minutes. The reaction was completed with Vector Nova Red peroxidase chromogen (Vector) for– 15 minutes. Slides were then removed from the auto-stainer; counterstained with Gill 2 Hematoxylin, differentiated, dehydrated, cleared and coverslipped with synthetic mounting medium. The pictures were obtained using a Micropublisher 3.3 Megapixel Color Digital Camera and the amount of staining was quantified using a MetaMorph imaging system (Molecular Devices, Downingtown, PA)

3.12. Statistical Analysis

Data are expressed as the mean ± SEM for gene expression measurements and mean ± SD for western blot quantification and acellular capillary quantification. Factorial ANOVA with post hoc Tukey test (GraphPad Prism5, GraphPad Software, San Diego, CA) was used for comparing the data obtained from multiple independent samples. T-test was used to compare data obtained from two independent measurements. Significance was established at $P<0.05$.

4. Results

4.1. DHA Enriched Diet Prevents the Upregulation of Retinal ASMase in One Month Diabetic Rat Model

The natural history of diabetic retinopathy has been divided into a very early pro-inflammatory stage and a later, proliferative stage. We assessed the very early stage of diabetic retinopathy in rats affected by type 1 diabetes for a one month period of time (Figure 4.1, 4.2 and 4.3). To investigate whether DHA has a beneficial effect on DR, diabetic rats were subjected to either a soybean oil diet (standard rodent diet) or fish oil (DHA) enriched diet for the duration of the study. The control rats were fed a soybean oil diet. As activation of SMases is an early critical step in inflammatory signaling, we first determined the effect of diabetes on SMases. Interestingly, we found that ASMase gene expression (Figure 4.1) and protein level (Figure 4.3 A,B) was dramatically increased in retinas isolated from diabetic rats on soybean oil diet compared to control, healthy rats on the same diet. Moreover, a DHA (fish oil)-enriched diet prevented the upregulation of ASMase gene expression (Figure 4.1) and protein levels (Figure 4.3 A,B) in the diabetic rat retina. We found no effect of diabetes or the DHA enriched diet on NSMase gene expression (Figure 4.1), strongly suggesting that ASMase rather than NSMase might play a more important role in mediating vascular and retinal inflammation in the early stage of diabetic retinopathy.

4.2. DHA Enriched Diet Prevents the Upregulation of Retinal Proinflammatory/Angiogenic Mediators in One Month Diabetic Rat Model

At this initial stage of DR we also found a dramatic upregulation of proinflammatory and pro-angiogenic mediator gene expression, as interleukin 1β (IL-1β), IL-6 and vascular endothelial growth factor (VEGF) gene expression was increased in the retinas of diabetic rats fed a soybean oil diet (Figure 4.2). VEGF protein level (Figure 4.3 A,C) was also increased two fold in the diabetic retina from rats fed a soybean oil diet compared to control retina from rats on the same diet. Intercellular adhesion molecule 1 (ICAM-1) plays a critical role in mediating leukocyte adherence to endothelial walls, leukostasis, and endothelial cell injury and death. ICAM-1 gene expression (Figure 4.2) was increased three fold in the retinas of diabetic rats fed a soybean oil diet when compared to non-diabetic controls. More importantly, rats fed a DHA (fish oil)- enriched diet did not manifest the diabetes induced upregulation of ICAM-1, VEGF, IL-1β and IL-6 gene expression (Figure 4.2), as well as VEGF protein level (Figure 4.3 A,C), strongly demonstrating a DHA protective effect against the development of early inflammation in the diabetic retina.

4.3. DHA Enriched Diet Prevents the Upregulation of Retinal ASMase in Nine Months Diabetic Rat Model

We next investigated the effect of long-term diabetes and a DHA-enriched diet on sphingomyelinase expression in the retina. We found significant upregulation of retinal ASMase gene expression nine months, after induction of diabetes in rats. Interestingly,

there was no change in NSMase gene expression between control rats fed a soybean oil diet and diabetic rats fed either a soybean oil or DHA rich diet (Figure 4.4). These results suggest that ASMase rather than NSMase may play the more important role in mediating retinal inflammation and dysfunction in nine-month old diabetic rats. Moreover, a DHA-enriched diet significantly prevented diabetes-induced upregulation of ASMase gene expression (Figure 4.4) and protein levels (Figure 4.5 A,B).

4.4. DHA Enriched Diet Prevents the Upregulation of Retinal Proinflammatory/Angiogenic Mediators in Nine Months Diabetic Rat Model

Pro-inflammatory and pro-angiogenic molecules, IL-1β and VEGF, were significantly increased in the nine month-old diabetic rat retinas. Feeding diabetic rats a DHA enriched diet prevented upregulation of IL-1β and VEGF gene expression (Figure 4.6). We found no change in ICAM-1 gene expression between the retinas from nine month-old diabetic rats and age matched controls (Figure 4.6). The lack of difference between control and diabetic groups was due to an increase in ICAM-1 gene expression with age in control rats, rather than a decrease in ICAM-1 level in diabetic animals

4.5. DHA Enriched Diet Prevents Retinal Vessel Loss in Nine Month-Old Diabetic Rat Model

To determine whether a DHA enriched diet has a protective effect against the diabetes induced retinal vessel loss, a hallmark for an advanced stage of retinopathy, rat

retina vasculature was isolated and the number of degenerated, acellular capillaries was assessed in the retinas of nine month-old diabetic rats subjected to either a DHA-enriched diet or a standard rodent, control diet. We found that the number of degenerate, acellular capillaries was dramatically increased in diabetic retinas as compared to control retinas isolated from rats fed soybean oil diet (Figure 4.7 A,B). Moreover, retinas from diabetic rats on a DHA-enriched diet showed significantly fewer acellular capillaries then retinas from diabetic rats fed a soybean oil diet (Figure 4.7 A,B). These data suggest that DHA protects the retinal vasculature against vessel loss, a marker of advanced diabetic retinopathy.

4.6. Upregulation of ASMase and Proinflammatory Cytokines TNFα and IL-1β in Human Diabetic Retina

Next we corroborated our findings in diabetic rats with studies in human diabetic retinas and showed that retinas from diabetic donors exhibited increased ASMase, TNFα and IL-1β mRNA levels when compared to control donors (Figure 4.8).

4.7. ASMase Expression in the Rat Retinal Vasculature

Our cell culture data demonstrate that caveolar ASMase expression in endothelial cells rather than other retinal cells plays a key role in cytokine-induced endothelial activation. To determine the distribution pattern of ASMase *in vivo*, we performed immunohistochemistry of retina flatmounts and cross-sections of the retinal layers in rats. ASMase is an important lysosomal enzyme that is ubiquitously expressed in most tissues.

We found basal ASMase expression in all retinal layers (Figure 4.9). Retinal blood vessels, however, exhibited the highest positive staining for ASMase (Figure 4.9) which colocalized with caveolin-1 (Figure 4.9).

4.8. DHA Supplementation Prevents the Upregulation of Vascular ASMase in the Rat Retina Injured by I/R

Next, we investigated the role of ASMase in the activation and degeneration of retinal vessels using a well established model of accelerated ischemia/reperfusion (I/R) injury that closely resembles diabetic retinopathy (31). ASMase expression was examined in the retinal tissue two days following the I/R injury. Immunohistochemistry analysis of retinal cross-sections obtained from uninjured and I/R injured rat eyes, showed that I/R injury specifically increased ASMase expression in the retinal vasculature (Figure 4.10) when compared to uninjured eyes, with little or no effect on ASMase expression in other retinal cells (Figure 4.10). As DHA was an effective ASMase inhibitor in our cell culture experiments, we next determined the effect of this ω3-PUFA on ASMase expression in the rat retinal tissue isolated from uninjured and I/R injured eyes. DHA supplementation prior to retinal injury specifically prevented ASMase upregulation in the retinal vasculature (Figure 4.10).

Table 4.1. Body weight gain and blood glucose of experimental animals with one month of diabetes. Data are mean ± SD.

	Weight gain, g/day	***Blood Glucose (mM)***
Control	8.20±1.38	4.8±0.85
Diabetic control diet	4.13±1.6	25±12.5
Diabetic DHA supplemented diet	4.05±1.79	21.7±13.12

Table 4.2. Body weight, blood glucose and HbA1c of experimental animals with nine month of diabetes. Data are mean ± SD.

	Weight at the end of the study (g)	***Blood Glucose (mM)***	***HbA1c (%)***
Control	733.5 ±74.24	5.04±0.348	5.3±0.6301
Diabetic control diet	547.8±71.87	23.14±4.21	16.228±2.649
Diabetic DHA supplemented diet	586.83±88.27	18.15±5.023	13.05±3.117

Table 4.3. Fatty acid composition of the control diet and DHA (fish oil) - enriched diet

Fatty acid	*Control diet (percent of total)*	*DHA supplemented diet (percent of total)*
20:5n3	0.00	9.49
18:3n3	7.16	6.46
18:3n6	0.00	0.00
22:6n3	0.00	8.90
16:1n7	0.00	6.73
20:4n6	0.00	0.00
22:5n3	0.00	0.00
18:2n6	45.68	21.88
20:3n6	0.00	0.00
16:0	18.98	26.19
18:1n9	22.88	19.40
18:0	5.31	0.95

	n6/n3 ratio
Control diet	6.38
DHA supplemented diet	0.88

Table 4.4. RT-PCR primers for rat

Gene	Sense sequence	Anti-sense sequence
ASMase	caactatgggctgaagaagga	acagctgactggcacacatt
NSMase	ttctcgagctttgtccagggttc	caggaagtgcttctttggctggt
ICAM-1	ccaccatcactgtgtattcgtt	acggagcagcactactgaga
VEGF A	gctctcttgggtgcactgg	caccacttcatgggctttct
IL-1β	caaggagagacaagcaacga	gtttgggatccacactctcc
Cyclophilin	cttcttgctggtcttgccattcct	tggatggcaagcatgtggtctttg

Table 4.5. RT-PCR primers for human

Gene	Sense sequence	Anti-sense sequence
ASMase	caacctcgggctgaagaa	tccaccatgtcatcctcaaaa
TNFα	tccttcagacacctcaacc	aggccccagtttgaattctt
IL-1β	gggcctcaaggaaaagaatc	ttctgcttgagaggtgctga
Cyclophilin	caagactgagtggttggatgg	tggtgatcttcttgctggtct

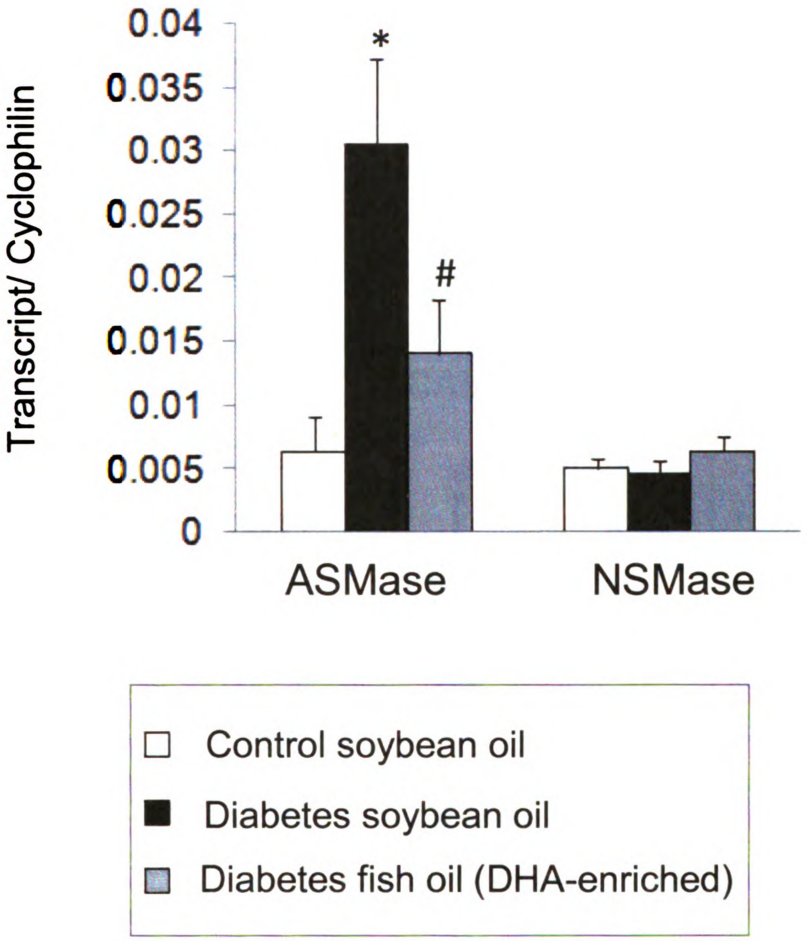


Figure 4.1. The effect of a DHA-enriched diet on ASMase retinal gene expression in rats with one month of diabetes. Retinas from rats fed either a soybean oil (standard rodent) diet or DHA (fish oil)-enriched diet, isolated one month after induction of diabetes were analyzed by RT-PCR for ASMase and NSMase gene expression. Gene expression from retinas isolated from rats subjected to a soybean oil diet (control, *white bar;* diabetic, *black bar)* and a DHA-enriched diet (diabetic, *gray bar*). The relative amounts of mRNAs were calculated by using the comparative CT method. Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. n= 5 to 8 animals; *$P<0.05$ compared to control soybean oil; $^{\#}P<0.05$ compared to diabetes soybean oil.

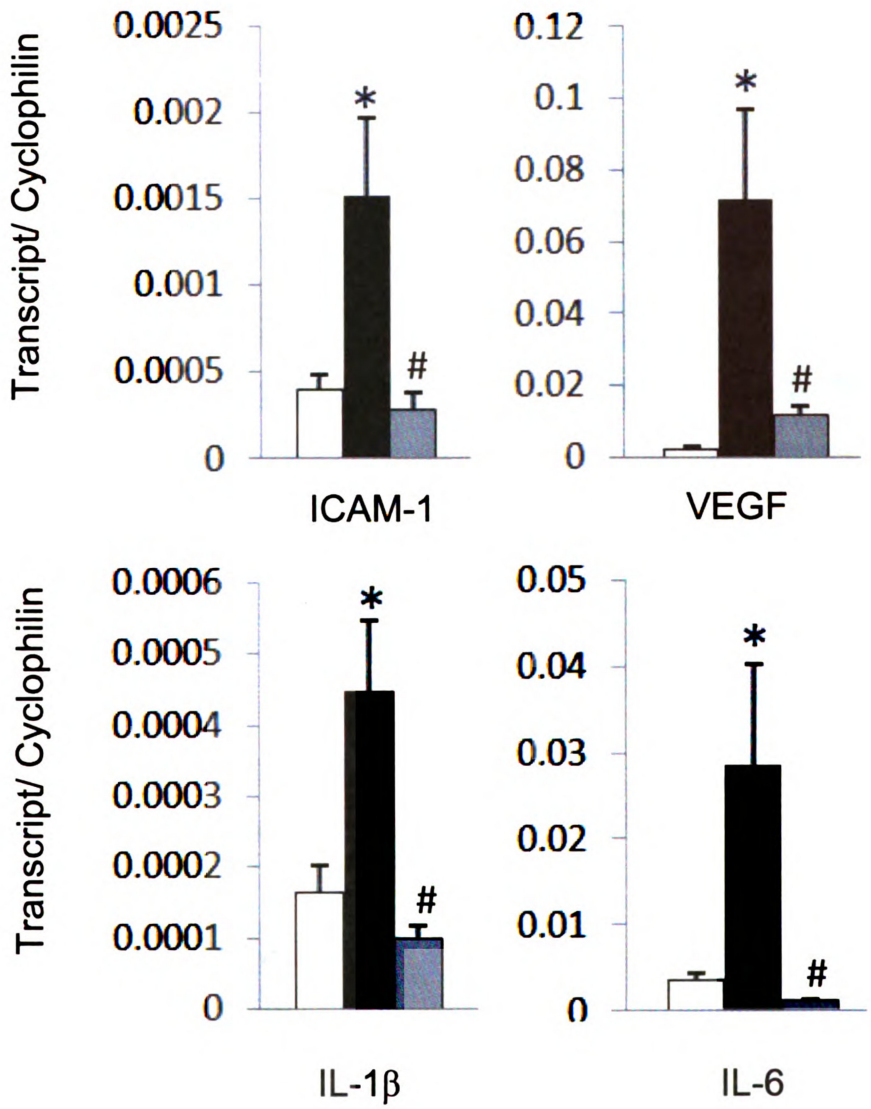


Figure 4.2. The effect of a DHA-enriched diet on retinal inflammatory/angiogenic molecule gene expression in rats with one month of diabetes. Retinas from rats fed either a soybean oil (standard rodent) diet or DHA (fish oil)-enriched diet, isolated one month after induction of diabetes were analyzed by RT-PCR for inflammatory/angiogenic molecule expression. RT-PCR analysis of ICAM-1, VEGF, IL-1β and IL-6 from retinas isolated from rats subjected to a soybean oil diet (control, *white bar;* diabetic, *black bar)* and a DHA-rich diet (diabetic, *gray bar*). The relative amounts of mRNAs were calculated by using the comparative CT method. Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. n= 5 to 8 animals; *P<0.05 compared to control soybean oil; #P<0.05 compared to diabetes soybean oil.

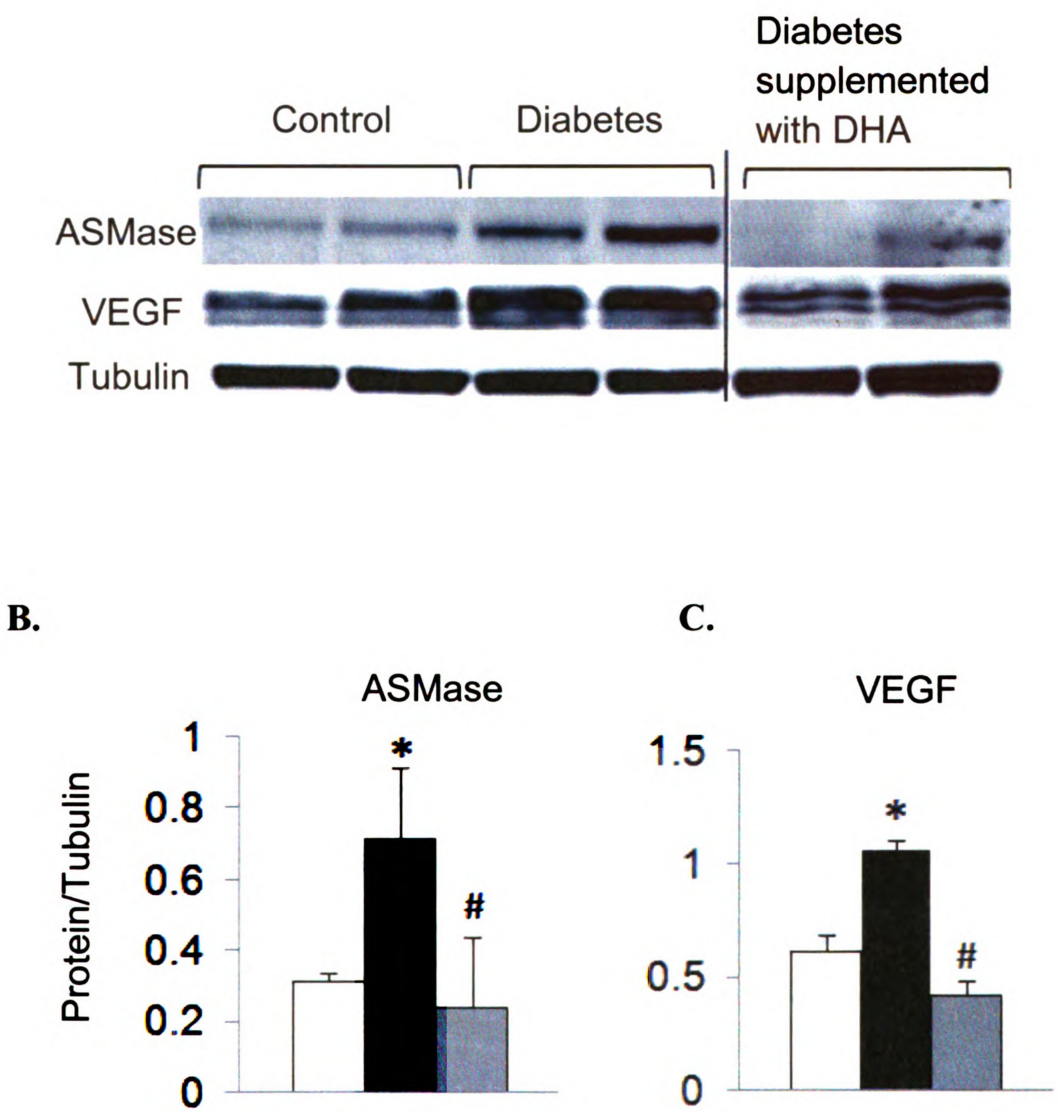


Figure 4.3. The effect of a DHA-enriched diet on retinal ASMase and VEGF protein expression in rats with one month of diabetes. (**A**) Immunoblot of ASMase and VEGF protein levels in retinas isolated from control rats fed a soybean oil diet (*white bar)* and from diabetic rats fed a soybean oil diet (*black bar)* and DHA-enriched diet (*gray bar*). Quantitative analysis of the data presented in panel (**A**) for (**B**) ASMase and (**C**) VEGF expression. The results are mean±SD. (n= 4 rats in each group; $^{*}P<0.05$ compared to control soybean oil; $^{\#}P<0.05$ compared to diabetes soybean oil).

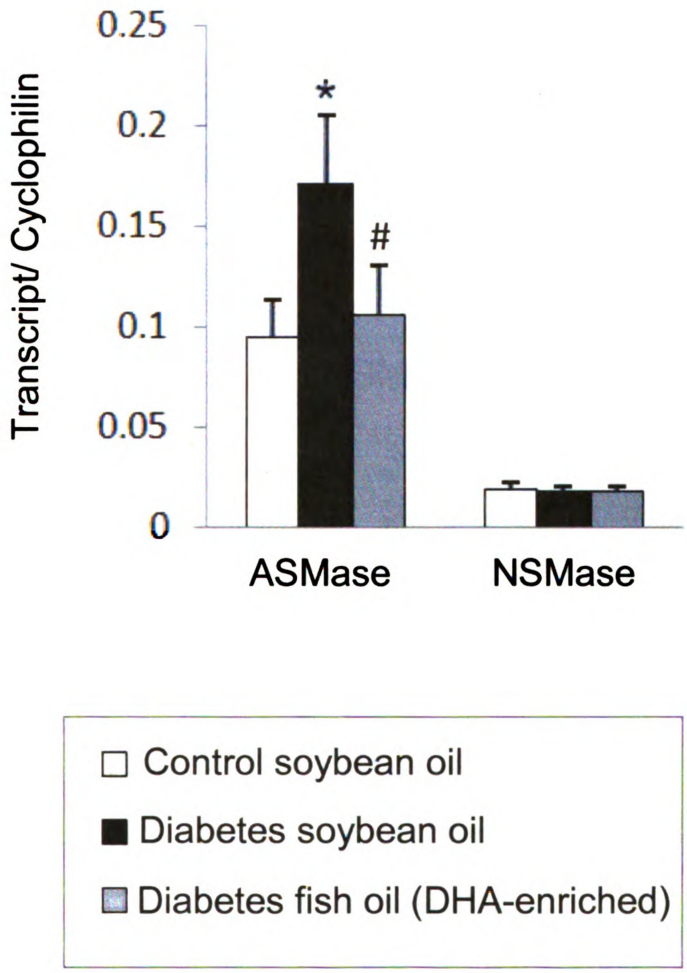
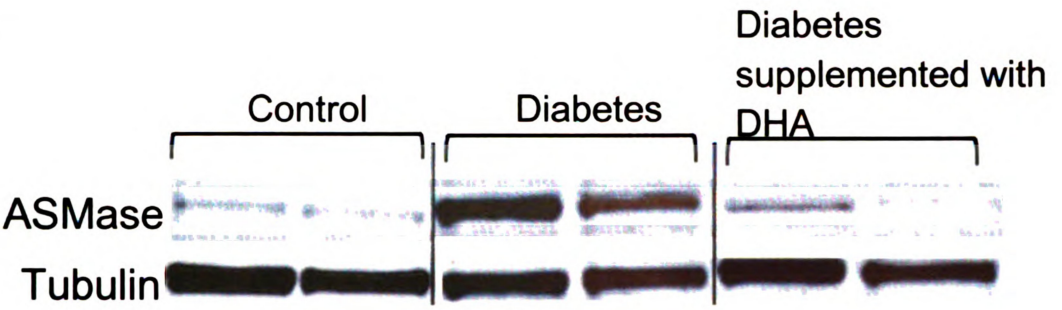


Figure 4.4. The effect of a DHA-enriched diet on retinal ASMase gene expression in rats with nine months of diabetes. Retinas from rats fed either a soybean oil (standard rodent) diet or DHA (fish oil)-enriched diet, isolated nine months after induction of diabetes were analyzed by RT-PCR for ASMase and NSMase gene expression. Gene expression from retinas isolated from rats subjected to a soybean oil diet (control, *white bar;* diabetic, *black bar)* and a DHA-rich diet (diabetic, *gray bar*). The relative amounts of mRNAs were calculated by using the comparative CT method. Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. n= 5 to 8 animals; *$P<0.05$ compared to control soybean oil; $^{\#}P<0.05$ compared to diabetes soybean oil.

A.



B.

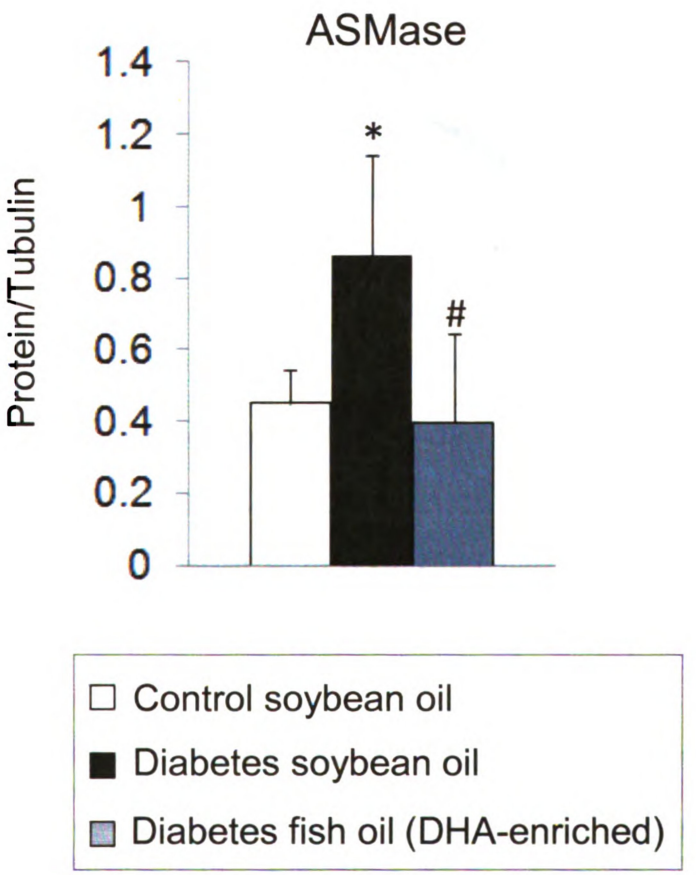


Figure 4.5. The effect of a DHA-enriched diet on retinal ASMase protein level in rats with nine months of diabetes. (**A**) Immunoblot of ASMase protein levels in retinas isolated from control rats on standard rodent diet (soybean oil diet) (*white bar*) and diabetic rats on soybean oil diet (*black bar*) or DHA-enriched diet (*gray bar*). Equal amounts of protein were added to each lane, as confirmed by tubulin levels. **(B)** Quantitative analysis of the data presented in panel **(A)** for ASMase expression. The results are mean±SD. (n=4 rats in each group. *P<0.05 compared to control soybean oil; #P<0.05 compared to diabetes soybean oil).

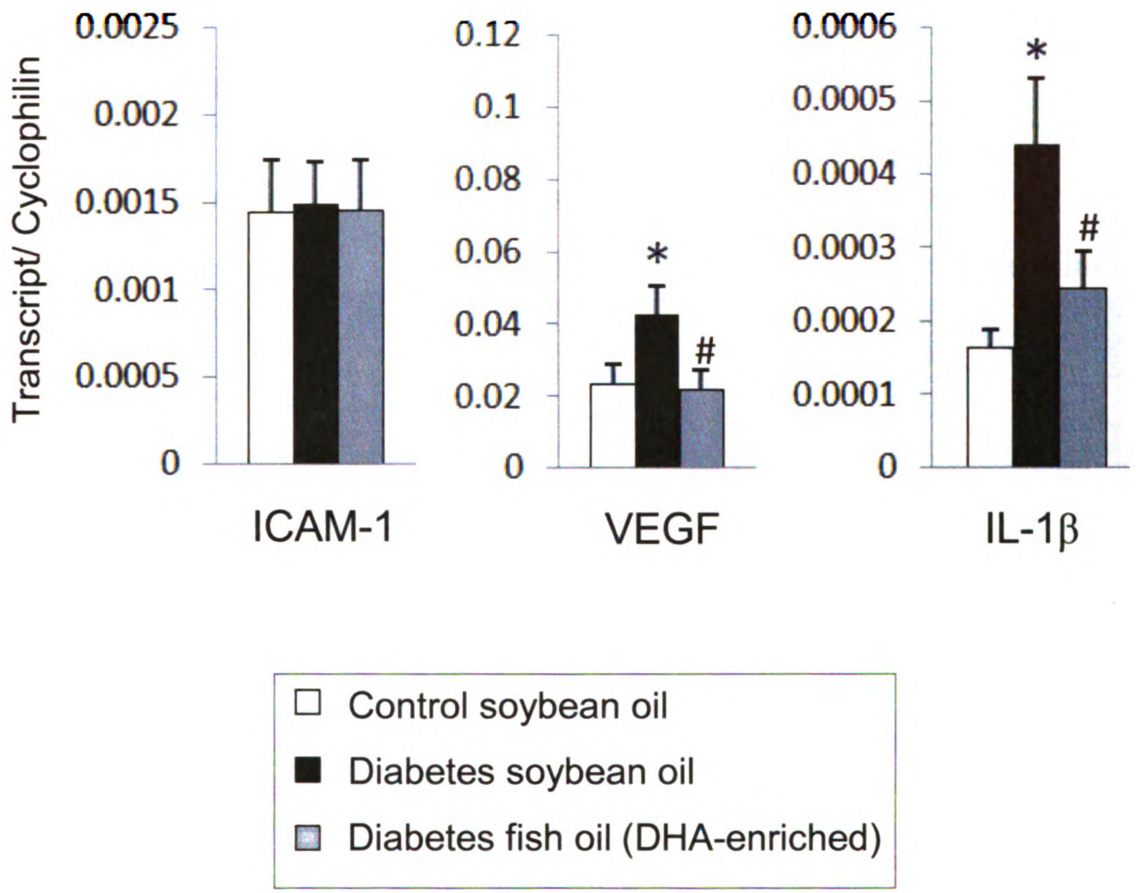


Figure 4.6. The effect of a DHA-enriched diet on retinal inflammatory/angiogenic molecules gene expression in rats with nine months of diabetes. Retinas from rats fed either a soybean oil (standard rodent) diet or DHA (fish oil)-enriched diet, isolated nine months after induction of diabetes were analyzed by RT-PCR for inflammatory/angiogenic molecule expression. RT-PCR analysis of ICAM-1, VEGF and IL-1β from retinas isolated from rats subjected to a soybean oil diet (control, *white bar;* diabetic, *black bar)* and a DHA-rich diet (diabetic, *gray bar*). The relative amounts of mRNAs were calculated by using the comparative CT method. Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. n= 5 to 8 animals; *P<0.05 compared to control; #P<0.05 compared to diabetes soybean oil).

A.

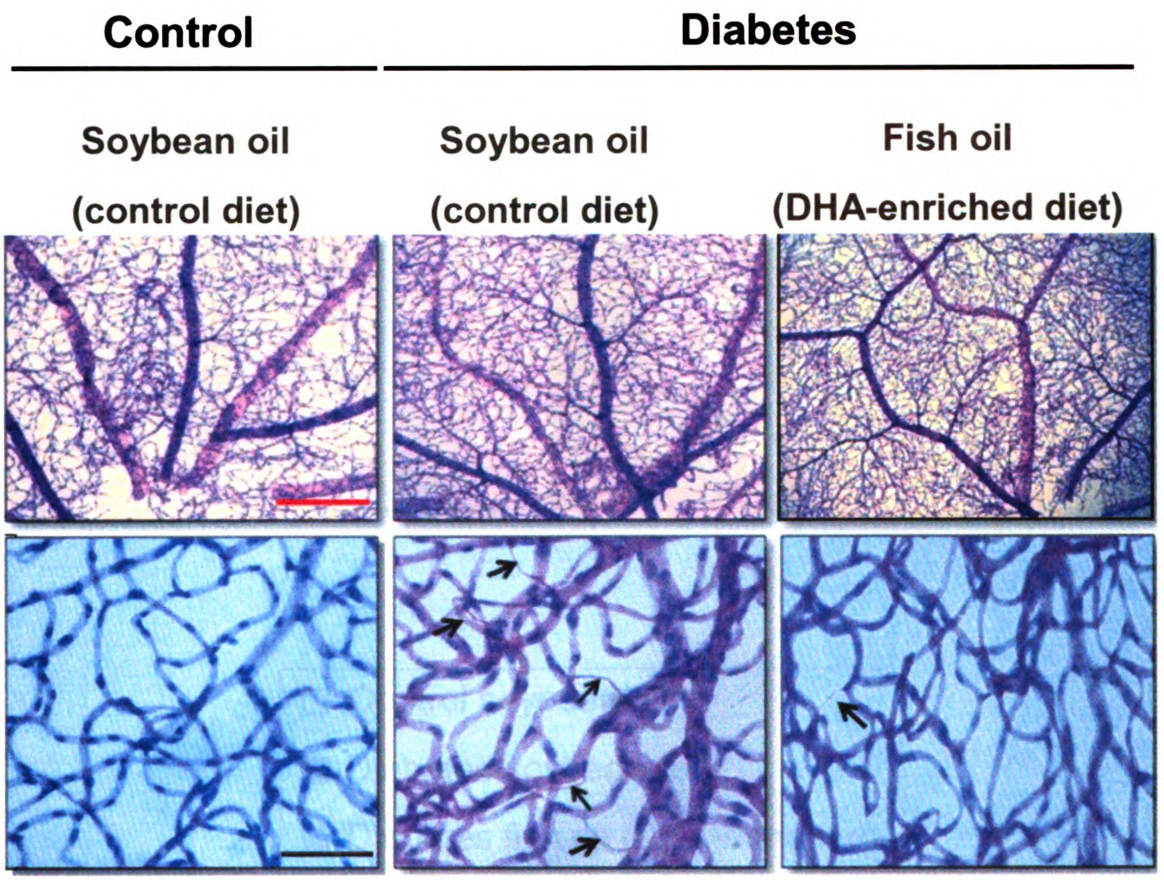


Figure 4.7. The effect of a DHA-enriched diet on retinal vessel loss in rats with nine months of diabetes. Retinas from rats fed either a soybean oil (standard rodent) diet or DHA (fish oil) enriched diet, isolated nine months after induction of diabetes were analyzed by microscopy of mounted retinal vasculature to visualize acellular capillaries. **(A)** Acellular (degenerated) capillary formation (*black arrows*) in the retinal vasculature isolated from control and nine months diabetic rats fed a soybean oil diet (n= 8) or a DHA enriched diet (n= 8).

B.

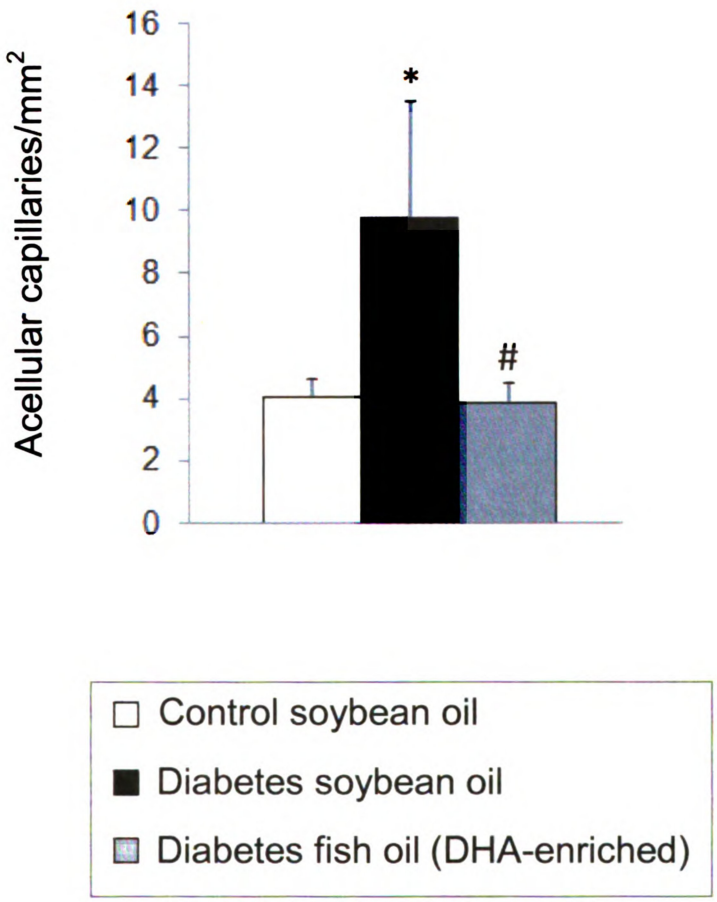


Figure 4.7 (continued). The effect of a DHA-enriched diet on retinal vessel loss in rats with nine months of diabetes. (B) Quantification of the total number of acellular capillaries (average per field, n= 8 animals per group). At least eight fields of retina were counted in duplicates by two independent investigators, *P<0.05 compared to control; #P<0.05 compared to diabetes soybean oil red bar = 550μm; black bar = 110μm.

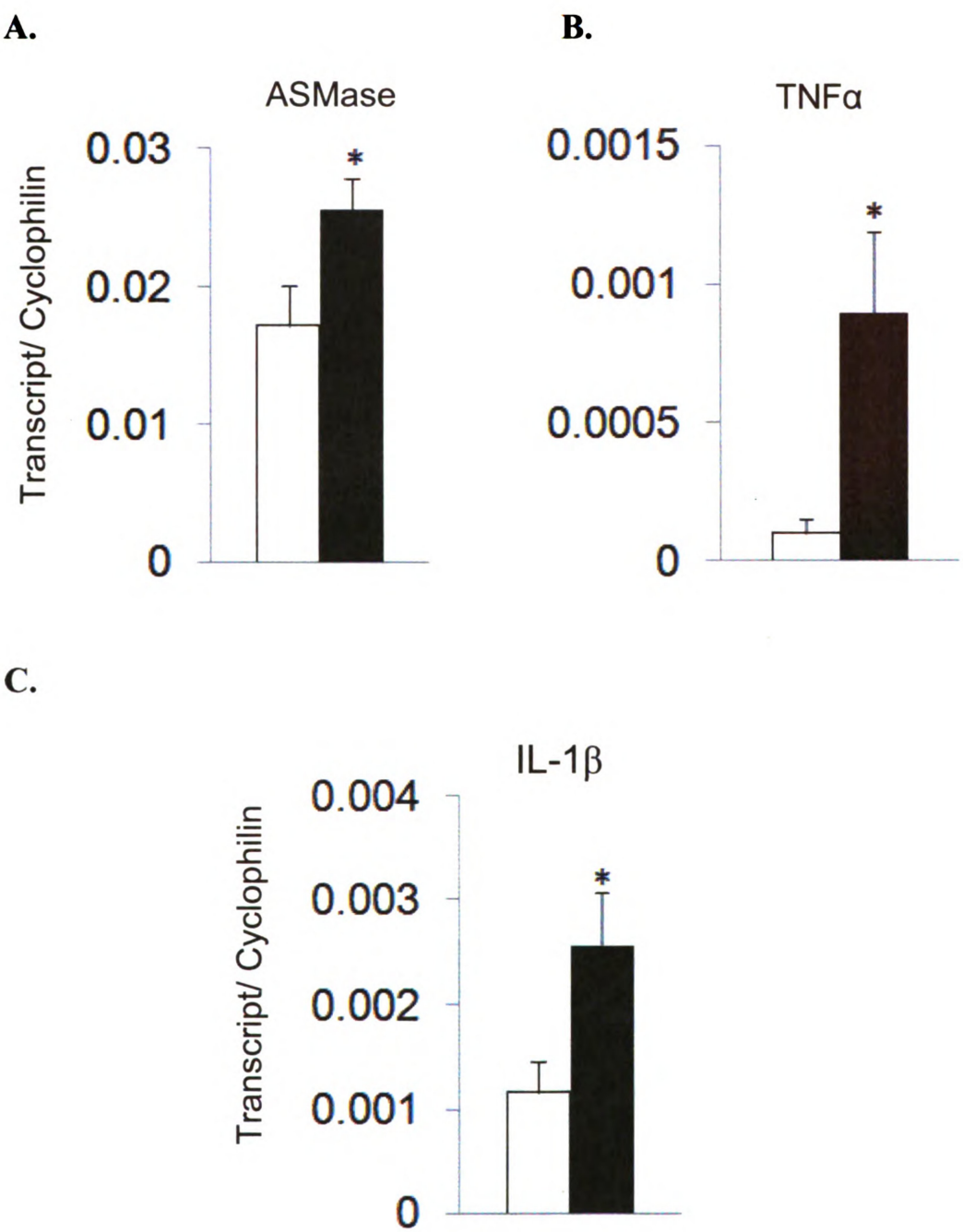


Figure 4.8. Changes in retinal gene expression in control and diabetic human retina. RT-PCR analysis of (**A**) ASMase, (**B**) TNFα and (**C**) IL-1β from retinas isolated from postmortem human control (*white bars*) and diabetic eyes (*black bars*). The relative amounts of mRNAs were calculated by using the comparative CT method. Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. *P<0.05 compared to control.

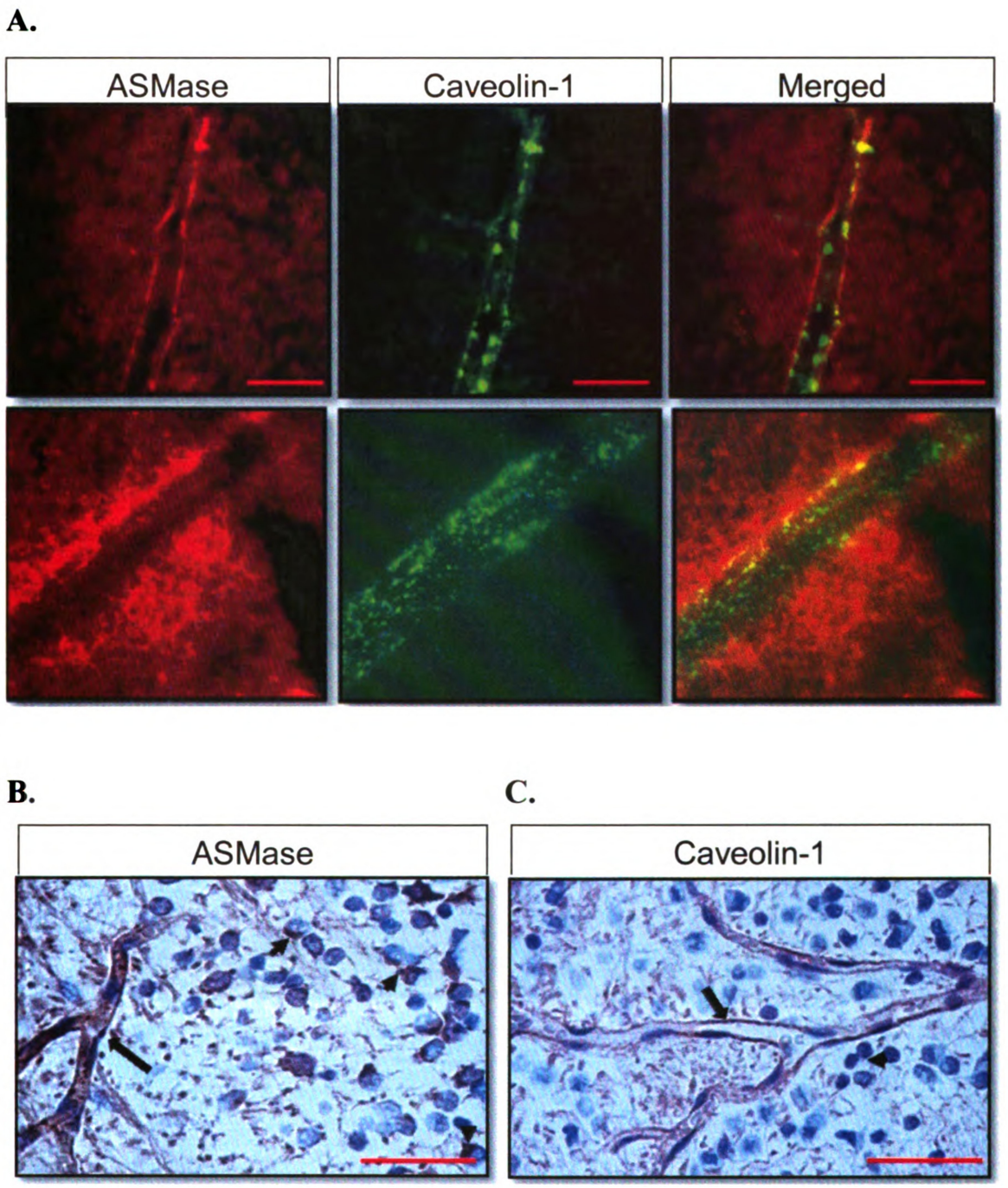


Figure 4.9. Rat retinal ASMase expression. **(A)** Colocalization of ASMase and Caveolin-1 in normal rat retina flat mount. **(B)** ASMase expression in normal rat retina showing ASMase positive staining of retinal vessels (*black arrow*) and perinuclear lysosomal compartment of retinal ganglion cells (*black arrow head*). **(C)** Caveolin-1 expression in normal rat retina showing caveolin-1 positive staining of retinal vessels (*black arrow*); red bar = 55μm.

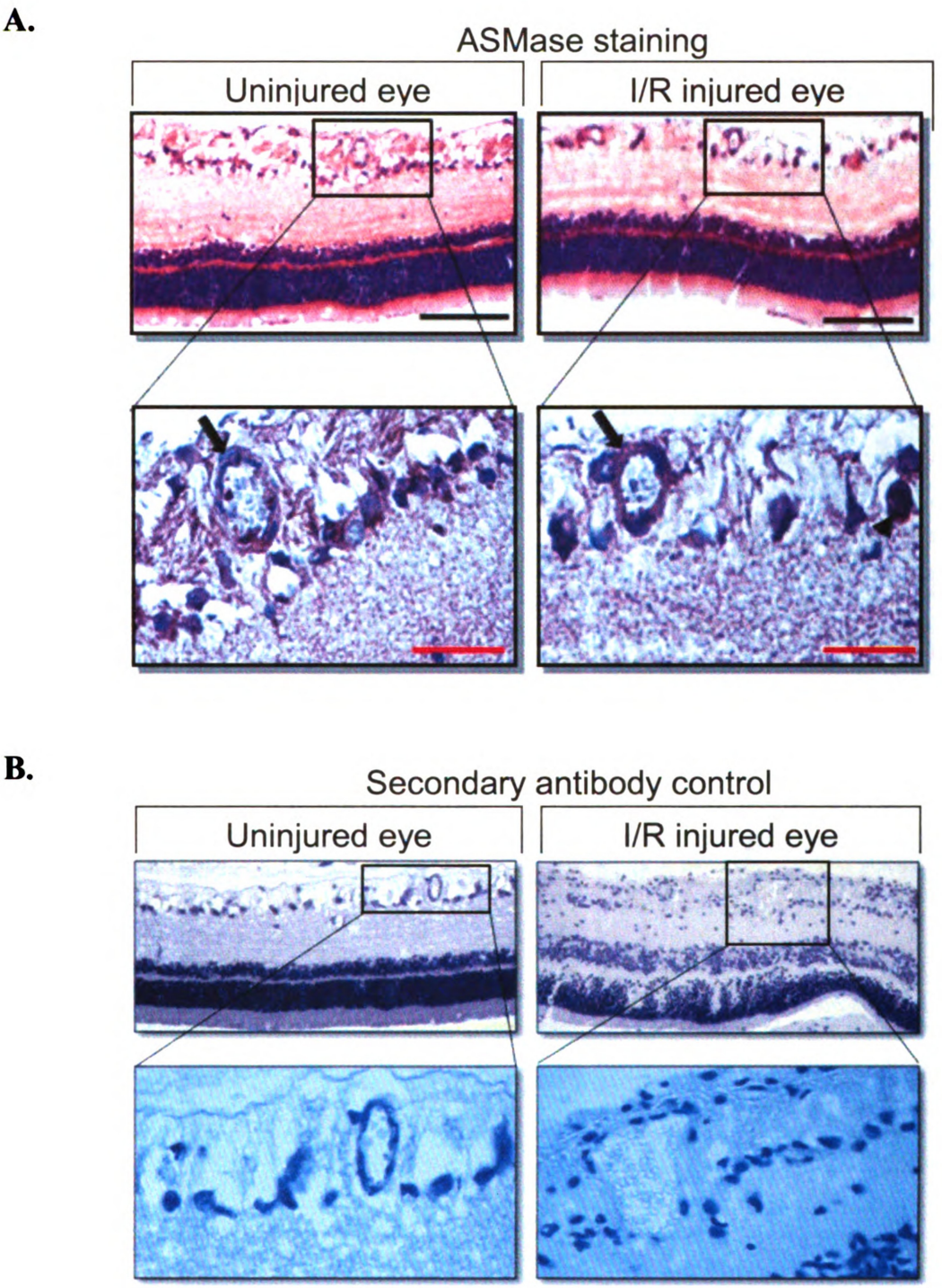


Figure 4.10. Retinal vascular ASMase expression after retinal I/R injury in rats. (A) Specific increase in ASMase expression in the rat retinal vessels (*black arrow*) from I/R injured eye (**A, right**) compared to control (**A, left**). **(B)** Secondary antibody control for **(A).**

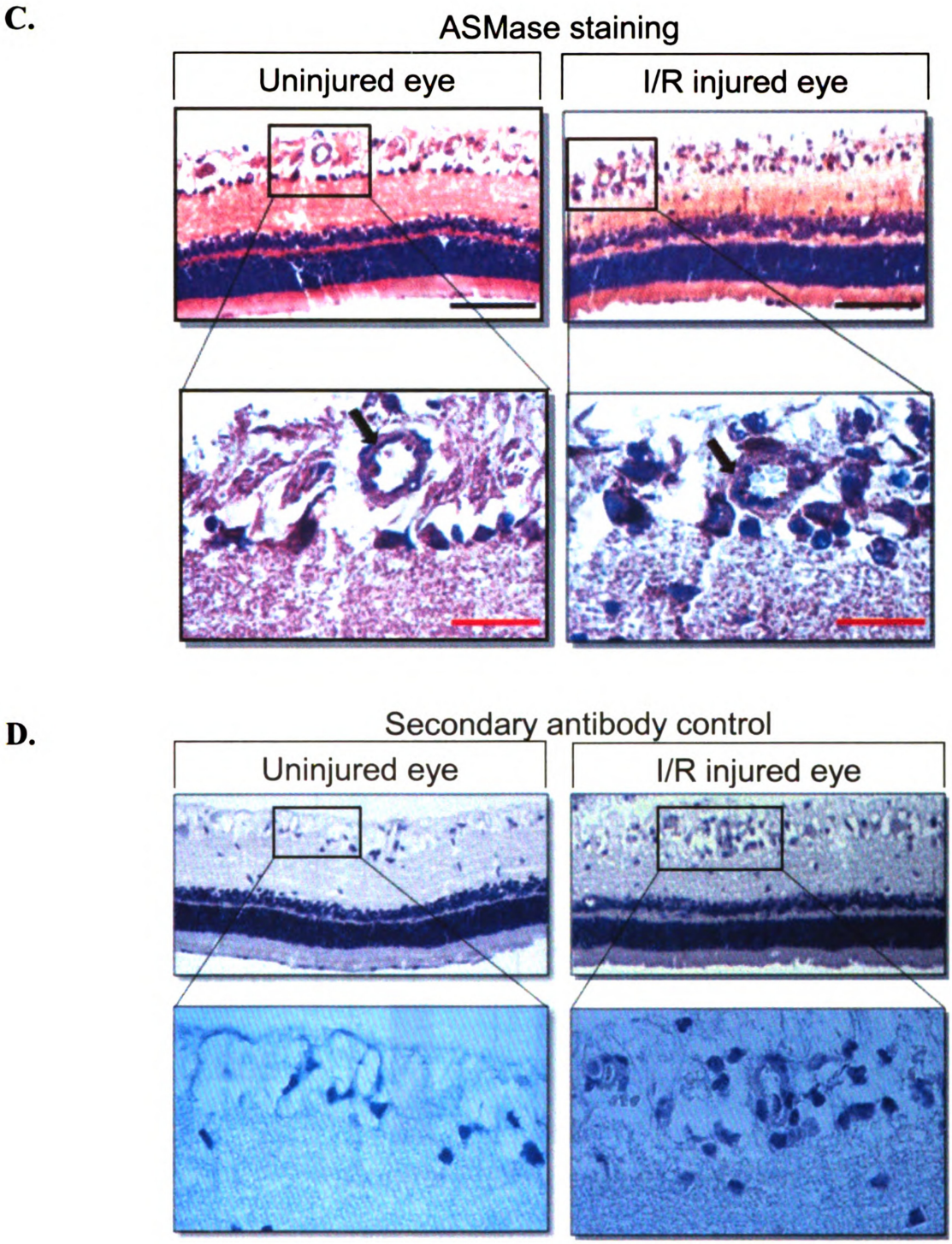
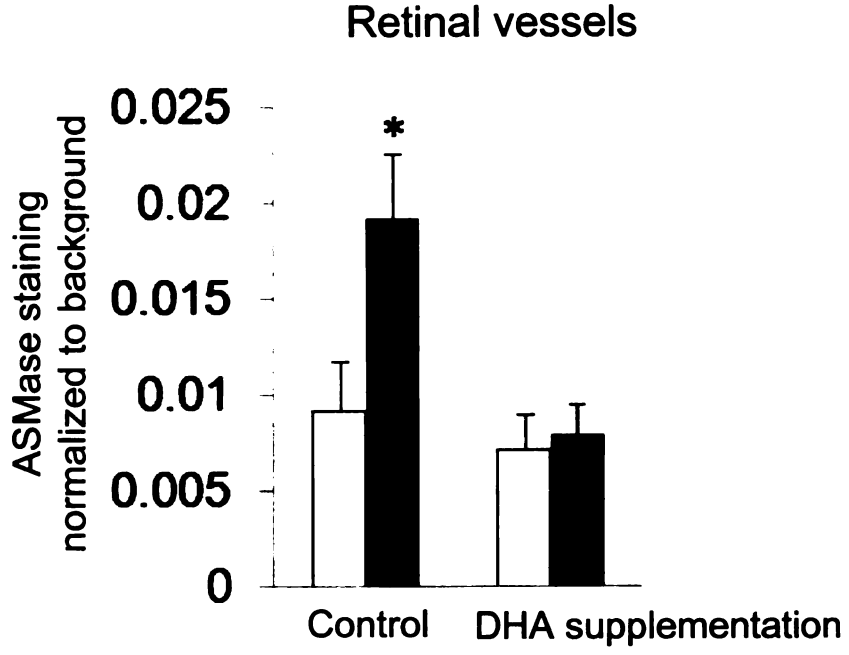


Figure 4.10 (continued). Retinal vascular ASMase expression after retinal I/R injury in rats. DHA supplementation prevents the increase in vascular ASM expression in I/R injured eye (**C, right**). **(D)** Secondary antibody control for **(C).**

E.



F.

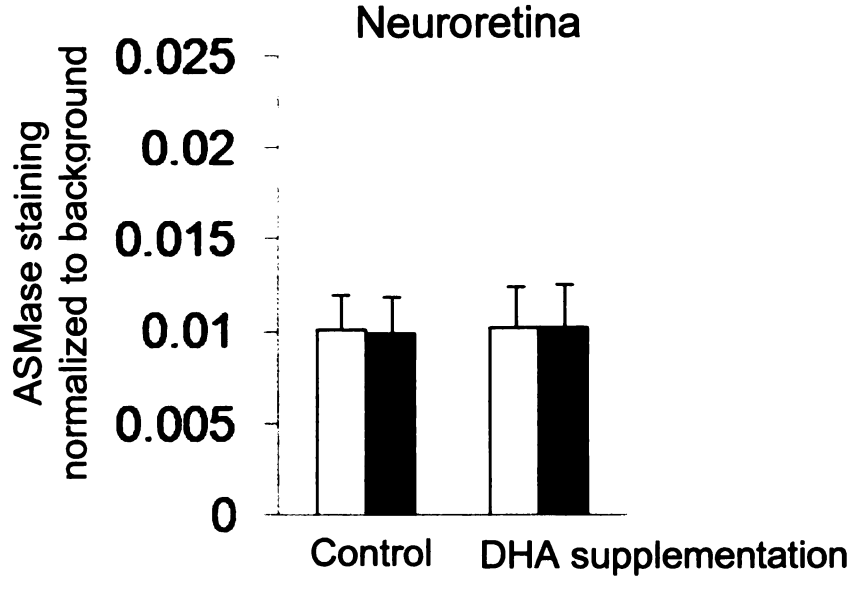


Figure 4.10 (continued). Retinal vascular ASMase expression after retinal I/R injury in rats. Quantification of ASMase staining in retinal vessels (**E**) and neuroretina (**F**). The data is presented from five animals in each group, 11-17 retinal vessels per group; *$P<0.05$ compared to all other groups. Red bar = 55μm, black bar = 220μm.

5. Discussion

Early stage diabetic retinopathy is regarded as a low-grade chronic inflammatory disease. Inflammation is part of the body's normal response to infection and injury; however, when it happens in an uncontrolled or inappropriate manner it can induce excessive damage to the host tissue and disease may ensue. Such uncontrolled or inappropriate inflammatory responses also occur in diabetic retina and are characterized by increased expression of adhesion molecules on the vascular endothelium and leukocyte surfaces, sequestration of leukocytes to sites where they are not commonly found, production of inflammatory mediators and ultimately damage to the host tissue (2). The molecular mechanisms that lead to a persistent inflammation in the diabetic retina are not well defined, but it seems that hyperglycemia and dyslipidemia, two metabolical characteristics of diabetes mellitus, play an important role in the inflammatory process.

Various biochemical pathways have been proposed to represent the link between hyperglycemia and diabetic microangiopathy. Among these pathways, polyol accumulation (33-34), oxidative stress and formation of reactive oxygen species (ROS) (35), increase activity of various PKC isoforms (36-37), and glycation of key proteins that lead to formation of advanced glycation end products (AGEs) (38-40) are metabolic pathways that have received much attention. Numerous studies have also demonstrated that AGEs production(41), PKCδ activation(42), and ROS generation(43), have the ability to activate sphingomyelinases. ASMase and NSMase are considered major candidates for mediating stress-induced cell injury and death; however, these enzymes have individual requirements for their activation and regulation. ASMase functions best

at a pH of 4.5-5.5, whereas optimal NSMase activation occurs at pH 7.4, and requires magnesium or manganese (44). Diabetic hyperglycemia and hypoxemia induced local retinal acidosis along with diabetes-related hypomagnesaemia (45) and this may downplay the role of NSMase in diabetic retinopathy. Indeed, our study demonstrates that ASMase rather than NSMase could represent a key player in mediating vascular damage in diabetic retinopathy.

Dyslipidemia associated with diabetes has also been implicated in promoting the inflammatory status in the retina. Along with DCCT/EDIC findings for a strong association between dyslipidemia and diabetic retinopathy, our recent study has demonstrated a significant decrease in total retinal ω3 PUFAs, and especially DHA, that was tightly coupled with inflammatory status in the diabetic retina (20). At the initial stage of diabetic retinopathy, we also found in this study a dramatic increase in retinal gene expression of the pro-inflammatory and pro-angiogenic mediators, IL-1β and VEGF (Figure 4.2 and Figure 4.3 A,C). The involvement of IL-1β in pathogenesis of diabetic retinopathy has been recently defined in diabetic mice (46). Inhibition of caspase-1, the enzyme involved in production of IL-1β cytokine or inhibition of IL-1β signaling using IL-1β receptor deficient mice protected the mice from development of retinal pathology over seven months duration of diabetes (46). VEGF is a key molecule that plays a well-recognized role in promoting vascular permeability (47) and pathological ocular neovascularization (48). VEGF expression is mainly regulated by hypoxic stimulus; yet there is evidence that it also accumulates in the retina early in diabetes, before any retinal hypoxia is present (49-51) and our results are in accordance with these studies.

Inflammatory mediators can promote the activation and degeneration of capillary endothelial cells with subsequent profound impact on vascular permeability and viability. ICAM-1 plays a critical role in mediating leukocytes adherence to endothelial walls, leukostasis, increased movement of leukocytes from the bloodstream into the surrounding tissue and endothelial cell injury and death. Pro-inflammatory cytokines, including IL-1β and VEGF have a pronounced effect on upregulation of ICAM-1 expression in the retina. We found in our study that ICAM-1 gene expression was increased three fold in the retina from diabetic rats on a standard diet when compared to control (Figure 4.2).

ASMase is regarded as an initial pivotal responder in diverse cellular processes of physiologic and pathologic importance. ASMase induction by various cytokine and stress stimuli has been reported to play an essential role in cytokine-mediated inflammation, apoptosis, cellular differentiation and cellular senescence (52-53). At the initial stage of DR, we show that ASMase gene expression and protein levels were significantly increased in the diabetic retina. The increased expression of many inflammatory proteins is regulated at the gene transcription level through the activation of inflammatory transcription factors, including nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), specificity protein 1 (SP1) and other members of the nuclear receptor family (54-55). Analysis of the ASMase promoter region identified several promoter elements essential for basal and inducible ASMase gene expression; among these, SP-1, AP-1 and AP-2 are mostly characterized and a concerted action of these transcription factors appears to be essential in increased ASMase expression (56-57). Interestingly, the upregulation of ASMase expression in diabetic retina could potentiate the cytokine pleiotropic signaling properties in retinal inflammation. Moreover, I/R injury-mediated upregulation of ASMase expression specifically in the retinal

vasculature may have a profound effect on cytokine induced inflammatory process in the vascular endothelium, which ultimately results in endothelial cells activation, dysfunction and death.

Omega-3 PUFAs, and particularly DHA are regarded as potent anti-inflammatory agents and they may be of therapeutic benefit in a diversity of acute and chronic inflammatory conditions (58). Our findings support the anti-inflammatory effect of DHA and show that DHA supplementation decreases the expression of inflammatory mediators and ICAM-1 in diabetic retina. More importantly, DHA decreases ASMase expression in the diabetic retina and I/R injured vasculature, and this *in vivo* study recapitulates our observations in *in vitro* settings where ASMase downregulation by DHA or ASMase gene silencing had significant inhibitory effects on cytokine-induced upregulation of adhesion molecules expression in retinal endothelium. Furthermore, DHA supplementation conferred significant protection against the development of acellular capillaries, the hallmark of late stage diabetic retinopathy.

In conclusion, we established the protective effect of DHA in *in vivo* models of diabetic retinopathy. DHA downregulates ASMase expression, prevents retinal inflammation and retinal capillary loss in rats with DR. Moreover, DHA modulates vascular ASMase expression in the I/R injured rat retina. These results demonstrate that ASMase may play a pivotal role in retinal inflammation and vascular degeneration in type 1 diabetic rat animal model. Consequently, in the following chapter, we directly address the role of ASMase in retinal vascular microangiopathy using *in vivo* accelerated models of retinopathy, including retinal ischemia-reperfusion and oxygen-induced retinopathy that were induced in an ASMase deficient (ASMase $^{-/-}$) mouse.

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Chapter V

Genetic Deficiency of Acid Sphingomyelinase Prevents Upregulation of Inflammatory/Angiogenic Mediators, Vessel Loss and Neovascularization in the Retina of Mice with Microangiopathy Lesions

1. Abstract

Acid sphingomyelinase (ASMase) is a key candidate for stress-mediated cellular activation and death. We have previously shown that ASMase was upregulated in diabetic rat retina and closely correlated with increased inflammatory/pro-angiogenic molecule levels in the same tissue. More importantly, ASMase expression specifically increased in the rat retinal vasculature injured by ischemia/reperfusion when compared to the non-injured eye. To directly investigate the role of ASMase in endothelial cell activation and death and development of retinal acellular capillaries, we used accelerated models of retinopathy by inducing ischemia/ reperfusion (I/R) injury and oxygen-induced retinopathy (OIR) in wild type ($ASMase^{+/+}$) or $ASMase^{-/-}$ mouse retina. We found that genetic deficiency of ASMase significantly prevented the increase in inflammatory/pro-angiogenic molecules expression in I/R injured retina compared to wild type. Moreover, I/R injured $ASMase^{-/-}$ mouse retinas were protected from both vascular degeneration and neovascularization. These results demonstrate that ASMase deficiency has a protective effect on ischemia-driven endothelial cell dysfunction and death, preventing consequent development of degenerated capillaries, as well as neovessel formation in the injured retinas.

2. Introduction

Sphingomyelin is an important sphingolipid component of all mammalian membranes and consists of a ceramide backbone with a phosphorylcholine moiety. ASMase represents a key regulatory enzyme in sphingolipid metabolism, catalyzing the hydrolysis of sphingomyelin to the bioactive lipid ceramide. ASMase major role in cell signaling is closely linked to its ability to reorganize the plasma membrane where sphingomyelin hydrolysis into ceramide facilitates the formation and coalescence of plasma membrane lipid microdomains. These ceramide enriched microdomains are believed to increase the local protein density, including receptors that often require oligomerization for their activation or facilitate protein-protein interaction (1). Receptor oligomerization and adaptor proteins recruitment appear to be a requirement for TNFα and IL-1 signalosome organization and intracellular inflammatory signaling (2-3).

Until the middle of the last decade, interest in ASMase biological role was restricted primarily to researchers studying Niemann-Pick disease, a recessively inherited lysosomal storage disease due to ASMase deficiency. At the same time, ASMase knockout mice were generated and become available for research use (4). The use of these ASMase knockout mice opened new avenues of research since these animals were initially found to be resistant to radiation (5). Subsequently, there has been an advancing crescendo of studies that have begun to shed significant light on our understanding on ASMase involvement in cell survival and pathophysiology of cancer, diabetes, sepsis, cardiovascular, pulmonary and neurological diseases (6). However, the role of ASMase in

the pathology of diabetic retinal microangiopathy has not been address so far and represents the purpose of this study.

Vascular endothelium represents a very rich source of ASMase (5, 7) and, as presented in the previous chapter, retinal vasculature exhibits significant positive staining for ASMase when compared to other retinal tissues. Elevated ASMase concentration in the vasculature makes it an excellent candidate for locally mediation of inflammatory and cell death, signaling pathways that would ultimately result in vascular degeneration.

In this study, we directly tested the hypothesis that ASMase genetic deficiency may be protective against the endothelial activation, injury and death, preventing the retinal vessels loss and subsequent pathological angiogenesis. We have modeled retinopathy in an ASMase knockout (ASMase $^{-/-}$) mouse model of Niemann-Pick disease by using retinal ischemia/reperfusion (I/R) and oxygen-induced retinopathy (OIR), both representing accelerated models of retinopathy; while the first model permits us to investigate the formation of acelullar capillaries, the last one allows us the assessment of neovessels formation.

The ischemia-reperfusion model induces retinal capillary degeneration, is well established and has close similarities to diabetes induced retinal vascular microangiopathy (8). Ischemia and reperfusion lead to individual effects on tissues related to metabolically disregulation induced by both hypoxia and reoxygenation. The arrest of blood supply in the retina results in cell death via both necrosis and apoptosis. Hypoxia leads to disruption of cellular energy metabolism and initiates a harmful cascade of events (9); induction of cytokine production, generation of reactive oxygen species (ROS), degradation of the anti-oxidant system and extracellular buildup of glutamate are

the main changes secondary to ischemia (9-14). The reoxygenation process, although effective in decreasing the ischemic damage, promotes additional cell injury and apoptosis (15) and the reoxygenation-induced cell damage is largely due to generation of reactive oxygen species (ROS) (16), increased levels of extracellular neurotransmitters and waste products that damage previously unharmed cells when being reoxidized (9, 17-18).

Ocular angiogenesis in response to tissue ischemia is commonly seen in various clinical conditions, including diabetic retinopathy, retinopathy of prematurity and retinal vein occlusion (19-20). The oxygen-induced retinopathy model uses a basic premise by which exposure to hyperoxia during early development of retinal vasculature results in the reduction of normal retinal vascular development and the degeneration of newly developed vessels. Removal of the hyperoxic stimulus renders a relative ischemic state in the retina, since the retina is no longer immersed in the high oxygen setting and lacks its normal vasculature. This ischemic situation sets off a rapid pathologic angiogenic process with formation of neovascular tufts which protrude out of the normal retinal vascular plexuses into the vitreal cavity (21).

In the previous chapter, we demonstrated that ASMase gene expression and protein level are significantly upregulated in diabetic rat retinas that exhibited an inflammatory status and microangiopathy lesions; importantly, ASMase was significantly increased in the vascular rat retina with I/R injury. ASMase is considered to be a first responder in mediating signaling processes via a specific receptor and genetic ASMase knockout mice exhibit protection from stress-induced cell death in various organs and systems (6, 22-23). Therefore, in this study we hypothesized that the ASMase genetic

deficiency will render protection against upregulation of proinflammatory/angiogenic mediators, vessel loss and pathological angiogenesis in the retina injured by ischemia-reperfusion and hyperoxic stimulus, respectively.

3. Materials and Methods

3.1. Reagents

NuPAGE Novex 10% Bis- Tris gels, Platinum SYBR Green qPCR SuperMix-UDG w/ROX were purchased from Invitrogen (Carlsbad, CA). Lyophilized pancreatic elastase was purchased from Calbiochem (San Diego, CA). RT-PCR primers from IDT DNA Technologies (Coralville, IA) (Table 1) were used. ASM antibody was a generous gift from Dr. Richard Kolesnick. High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) was used for DNA isolation. DNase, RNase free water, dNTPs, MgCl, Taq DNA Polymerase, Trackit Cyan/Yellow Buffer, TrackIt 1kb Plus DNA ladder were purchase from Invitrogen, (Carlsbad, CA).

3.2 Genotyping Transgenic Mice by PCR and Southernblot

3.2.1. DNA isolation from mouse tail. 0.2 to 0.5 cm of the mouse tail was used for DNA isolation using a High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany). The mouse tail was added to a1.5 mL nuclease-free microcentrifuge tube, along with 200 µL Tissue Lysis Buffer and 20 µL Proteinase K (reconstituted). The mixture was incubated overnight at 55 °C until the tissue was completely digested. Using a 1 mL disposable syringe without needle, the tail sample was lysed by shearing process. Then 200 µL of Binding Buffer and 100 µL of isopropanol were added to the tube, mixed well and subjected to centrifugation for 5 minutes at 13,000 x *g*. Then the liquid sample was pipetted into the upper buffer reservoir of the High Filter Tube that was previously inserted into one Collection tube. The entire High Filter Tube assembly was inserted into a standard table-top centrifuge and centrifuged 1

minute at 8,000 x *g*. After centrifugation, the Filter Tube was removed from the Collection Tube; the flowthrough liquid and the Collection Tube were discarded. The Filter Tube was combined with a new Collection Tube; 500 µL Inhibition Removal Buffer was added to the upper reservoir of the Filter Tube and centrifuged 1 min at 8,000 x *g*. After centrifugation, the Filter Tube was again removed from the Collection Tube; the flowthrough liquid and the Collection Tube were discarded. The Filter Tube was combined with a new Collection Tube; 500 µL Wash Buffer was added to the upper reservoir of the Filter Tube and centrifuged 1 min at 8,000 x *g*. This final step was repeated twice. To elute the DNA, the Filter Tube was inserted into a clean, sterile 1.5 mL microcentrifuge tube; 200 µL prewarmed Elution Buffer was added to the upper reservoir of the filter tube and centrifuged 1 min at 8,000 x *g*. The eluted DNA was stored at -20 °C for further analysis.

3.2.2. PCR amplification of the DNA sample. The following reagents were added to an RNase, DNase free microcentrifuge tube (a total volume of 25 µL): DNase, RNase free water 16 µL, 10x buffer 2.5 µL, dNTPs (10mM) 1 µL, MgCl (150mM) 1.5 µL, Primer Mix for ASMase (0.1 mM) 1.5 µL, Taq DNA Polymerase (Invitrogen, (Carlsbad, CA)) 0.5 µL, DNA 2 µL. The following primers for ASMase have been used: GGCTACCCGT GATATTGC, AGCCGTGTCCTCTTCCTTAC, CGAGACTGTTGCCAGACATC. The PCR mix underwent the following cycling conditions: 94 °C for 3 minutes, followed by a sequence (94 °C for 15 seconds, 64 °C for 30 seconds and 68 °C for 90 seconds) that was repeated 35 times and then 72 °C for 7 minutes.

3.2.3. Southernblot identification of ASMase mutant allele. The PCR product (25 µL) was mixed to 2.5 µL of loading buffer (Trackit Cyan/Yellow Buffer, Invitrogen,

Carlsbad, CA). 1.5% Agarose in 50 mL TBE buffer, mixed with 5 μL of 10,000x SYBR safe stain was used to cast the gel. The TrackIt 1kb Plus DNA ladder was added to the first well. 10 μL of samples to analyze was added to the remaining wells. The ASMase mutant allele was identified at 523 bp, whereas the wild-type allele was localized at 269 bp.

3.3. Mice. All procedures involving the animal models adhered to the ARVO statement for the Use of animals in Ophthalmic and Vision research. **Mouse model of retinal ischemia-reperfusion.** Two months old male C57BL/6J wild type ($ASM^{+/+}$) and C57BL/6J ASMase knock-out ($ASM^{-/-}$) mice were anesthetized. The anterior chamber of one eye was cannulated with a 30 gauge needle attached to a line infusing sterile normal saline solution. The intraocular pressure (IOP) was maintained at 80-90 mmHg; this pressure was determined by the height and the volume of the perfusion bag. This resulted in retinal ischemia, as confirmed by whitening of the iris and loss of the red reflex. The other eye of the same animal was used as a control. The duration of ischemia was 90 minutes. After ischemia, the needle was withdrawn, followed by IOP normalization and reperfusion of the retinal vasculature was documented visually. The mice were sacrificed at different times after reperfusion (two days for assessment of inflammatory mediators and seven days for assessment of acellular, degenerated capillaries). **Mouse model of oxygen-induced retinopathy (OIR).** In the neonatal mouse model of oxygen-induced retinopathy, 7-day-old (P7) C57BL/6J wild type and C57BL/6J acid sphingomyelinase deficient ($ASMase^{-/-}$) mice were placed with their nursing dams in a 75% oxygen atmosphere for 5 days (P7 to P12). Upon return to normal air, these mice develop retinal

neovascularization, with peak development occurring 5 days after their return to normoxia. Retinal neovascularization was assessed at P17 as previously described (21, 24).

3.4. Isolation of Retinal Vasculature

Mouse retinal vascular beds were isolated by elastase digestion method as previously described (25). The fixed mouse retinas were isolated and rinsed in deionized water as described above. The next day retina was incubated for 15 minutes in an agitating water bath preheated to 37°, in 40 units/ml elastase in 100 mmol/1 sodium phosphate buffer with 150 mmol/1 sodium chloride and 5.0 mmol/1 ethylenediamenetetraacetic acid (EDTA) at pH 6.5. The tissue was washed overnight in 100 mmol/1 Tris-HCl (pH 8.5) at room temperature and then transferred to deionized water for removal of the now-loosened vitreous and digested neural elements by gentle agitation using the sides of forceps and the sides and ends of very fine sable brushes as needed. Then the retinas were mounted by flotation in HPLC-grade water over slides. Once air-dried in a dust-free environment, the mounts of the retinal vasculature were stained using the periodic-acid-Schiff reaction and hematoxylin counterstaining.

3.5. Histological Assessment of Retinal Capillaries

The mid-retina areas along each major vessel of the vascular bed were systematically investigated (6-8 fields per retina) to quantify the acellular capillaries at 250x magnification (~0.32 mm^2 per field). A capillary was counted as acellular if it had no

nuclei from junction to junction and comprises at least 20% of the diameter of surrounding capillaries.

3.6. Simultaneous Extraction of Proteins and RNA

The method was performed as described in reference (26). The method has been described in detail in chapter IV.

3.7. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from rat and mice retinas. Specific primers for each gene are listed in Table 1. The method has been previously described in chaper II.

3.8. Statistical analysis

Data are expressed as the mean ± SEM for gene expression measurements and mean ± SD for western blot quantification and acellular capillary quantification. Factorial ANOVA with post hoc Tukey test (GraphPad Prism5, GraphPad Software, San Diego, CA) was used for comparing the data obtained from multiple independent samples. T-test was used to compare data obtained from two independent measurements. Significance was established at $P<0.05$.

4. Results

4.1. Retinal I/R Induces Upregulation of ASMase Gene Expression in the ASMase$^{+/+}$ Retina

Retinas from control and ischemia/reperfusion (I/R) injured eye were isolated from wild type (ASMase$^{+/+}$) and ASMase genetic deficient (ASMase$^{-/-}$) mice 2 days after induction of the retinal damage. Real time qPCR analysis of ASMase gene expression showed a significant upregulation of ASMase mRNA in wild type retinas injured by I/R (Figure 5.1). Conversely, NSMase gene expression was not significantly increased in the wild type retinas with I/R lesions, but was dramatically elevated in the I/R injured retinas isolated from the ASMase$^{-/-}$ mice (Figure 5.2). These results confirmed the role of ASMase as a major candidate in mediating stress-induced cell death.

4.2. ASMase Genetic Deficiency Prevents Upregulation of Cellular Adhesion Molecules in the I/R Injured Retina

Two days after ischemia-reperfusion, a time point that corresponds to an early stage of diabetic retinopathy, the gene expression of intercellular adhesion molecule 1 (ICAM-1) (Figure 5.3 A) and vascular cell adhesion molecule 1 (VCAM-1) (Figure 5.3 B) was significantly increased in the wild type retinas injured by I/R. Conversely, ICAM-1 and VCAM-1 gene expression was significantly decreased in injured retinas isolated form ASMase$^{-/-}$ mice when compared to retinas isolated from ASMase$^{+/+}$ I/R injured eye.

4.3. ASMase Genetic Deficiency Prevents Upregulation of Inflammatory/ Angiogenic Mediators in the I/R Injured Retina

Similarly to adhesion molecules expression, the gene expression of IL-1β (Figure 5.4 A), TNFα (Figure 5.4 B) and VEGF (Figure 5.4 C) was significantly increased in the wild type retinas injured by I/R two days after ischemia-reperfusion. More importantly, ASMase$^{-/-}$ mice exhibit IL-1β, TNFα and VEGF gene expression (Figure 5.4 A,B,C) at a normal level in injured retinas compared to the wild type. Therefore, ASMase genetic deficiency emerges as greatly protective against the development of an inflammatory state in the I/R injured retina.

4.4. ASMase Genetic Deficiency Prevents the Development of Acellular Capillaries in the I/R Injured Retina

Examination of mouse retinal vasculature in wild-type mice seven days after I/R injury, when capillary degeneration response is greatest (8), showed a dramatic increase in the number of acellular capillaries as compared to control retinas (Figure 5.5 A, C). Furthermore, I/R injured ASMase$^{-/-}$ mouse retinas exhibited significantly fewer acellular capillaries compared to the wild type (Figure 5.5 B, C). These results clearly demonstrate that ASMase deficiency has a protective effect on ischemia-driven endothelial cell dysfunction and death, preventing consequent formation of acellular capillaries.

4.5. ASMase Genetic Deficiency Prevents Ocular Neovascularization in OIR Mouse Model

The experiments conducted so far investigated the protective role of ASMase deficiency on the development of vascular retinal nonproliferative changes similar to those that develop in diabetic retinopathy. Owing to the lack of an animal model, that properly reproduces the development of proliferative diabetic retinopathy; we next focused our study on an experimental model of ischemic neovascularization involving oxygen-induced retinopathy (OIR). The OIR model allows the assessment of abnormal retinal angiogenesis, which is similar to aberrant new vessel formation in late proliferative stages of diabetic retinopathy. The degree of neovascularization was quantified by counting the number of endothelial cell nuclei present on the vitreal side of the inner limiting membrane (ILM). We found the average number of nuclei extending past the ILM per 6 μm cross-section to be 12.1±3.4 SD in hyperoxia-treated ASMase$^{-/-}$ mouse eyes, compared to 49.5±12.5 SD in hyperoxia-treated wild type mouse eyes (Figure 5.6). These findings demonstrate that ASMase deficiency prevents the development of pathological angiogenesis in the OIR retina.

Table 5.1. RT-PCR mouse primers

Gene	Sense sequence	Anti-sense sequence
ASMase	aaccctggctaccgagtttaccaa	tggcctgggtcagattcaagatgt
NSMase	atgcacactacttcagaagcggga	aggcattgagcaccagtccactta
ICAM-1	acactatgtggactggcagtggtt	tgaggctcgattgttcagctgcta
VEGF A	tcaccaaagccagcacataggag	ttacacgtctgcggatcttggaca
IL-1β	aagggctgcttccaaacctttgac	atactgcctgcctgaagctcttgt
VCAM-1	cccaggtggaggtctactca	ccagatggtcaaagggataca
TNFα	tctcatgcaccaccatcaagg	accactctccctttgcagaac
Cyclophilin	attcatgtgccagggtggtga	ccgtttgtgggtccagca

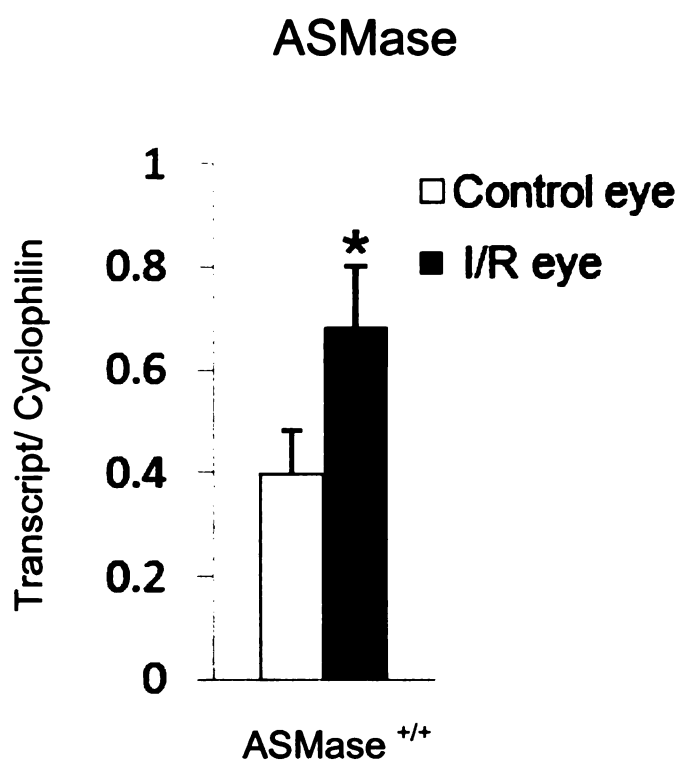


Figure 5.1. Retinal gene expression of ASMase in the I/R retinopathy model. RT-PCR analysis of ASMase in I/R injured retinas isolated from $ASM^{+/+}$ mice 2 days following retinal ischemia-reperfusion injury. The relative amounts of mRNAs were calculated by using the comparative CT method. Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. (n= 7 for $ASMase^{+/+}$ group. $^{*}P < 0.05$ compared to $ASMase^{+/+}$ control eye).

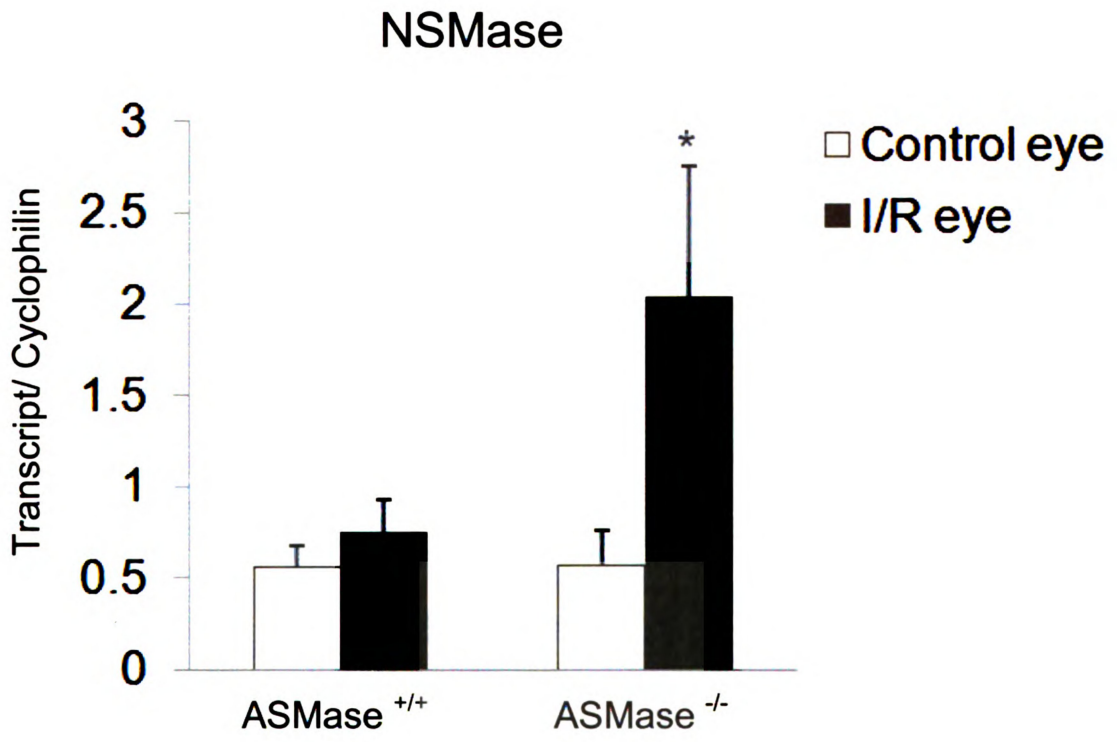
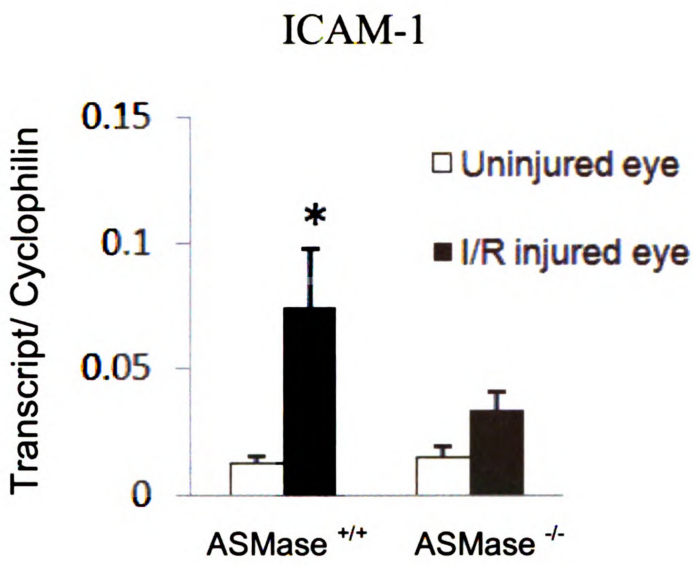


Figure 5.2. Retinal gene expression of NSMase in the I/R retinopathy model. RT-PCR analysis of NSMase in I/R injured retinas isolated from ASMase$^{+/+}$ and ASMase$^{-/-}$ mice 2 days following retinal ischemia-reperfusion injury. The relative amounts of mRNAs were calculated by using the comparative CT method. Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. (n= 7 for ASMase$^{+/+}$ group, n= 3 for ASMase$^{-/-}$ group. $^{*}P < 0.05$ compared to ASMase$^{+/+}$ control eye, ASMase$^{+/+}$ I/R eye and ASMase$^{-/-}$ control eye).

A.



B.

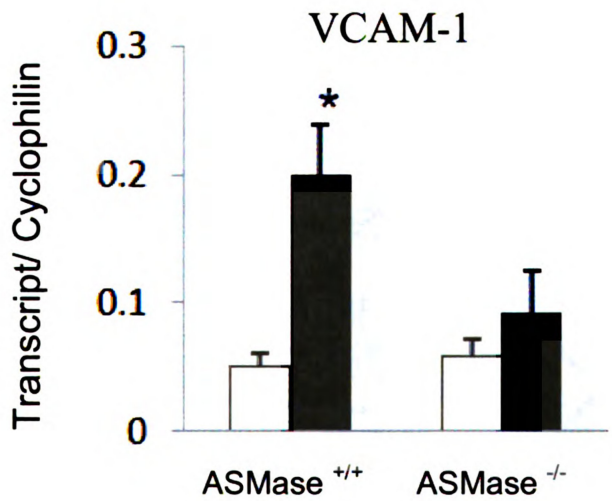


Figure 5.3. Retinal gene expression of cellular adhesion molecules in the I/R retinopathy model. RT-PCR analysis of ICAM-1**(A)** and VCAM-1 **(B)** in I/R injured retinas (*black bars*) isolated from ASMase$^{+/+}$ and ASMase$^{-/-}$ mice 2 days following retinal ischemia-reperfusion injury. The relative amounts of mRNAs were calculated by using the comparative CT method. Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. (n= 7-9 for ASMase$^{+/+}$ group, n= 5 for ASMase$^{-/-}$ group. $^{*}P < 0.05$ compared to ASMase$^{+/+}$ control eye and ASMase$^{-/-}$ control and I/R eye).

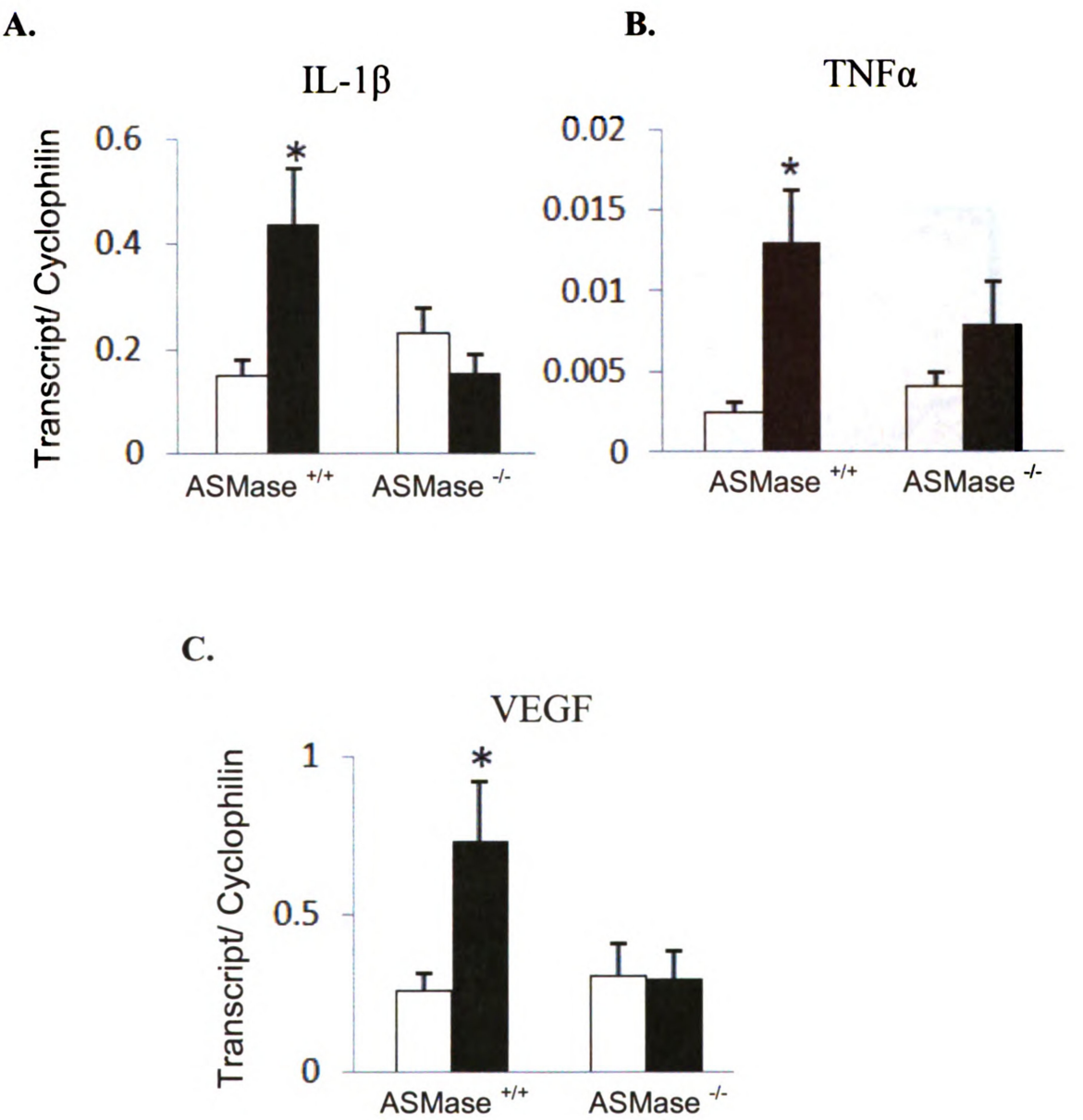


Figure 5.4. Retinal gene expression of inflammatory/angiogenic molecules in the I/R retinopathy model. RT-PCR analysis of IL-1β **(A),** TNFα **(B)** and VEGF **(C)** in I/R injured retinas isolated from ASMase$^{+/+}$ and ASMase$^{-/-}$ mice 2 days following retinal ischemia-reperfusion injury. The relative amounts of mRNAs were calculated by using the comparative CT method. Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. (n= 6-9 for ASMase$^{+/+}$ group, n= 5 for ASMase$^{-/-}$ group. $^{*}P < 0.05$ compared to ASMase$^{+/+}$ control eye and ASMase$^{-/-}$ control and I/R eye).

A.

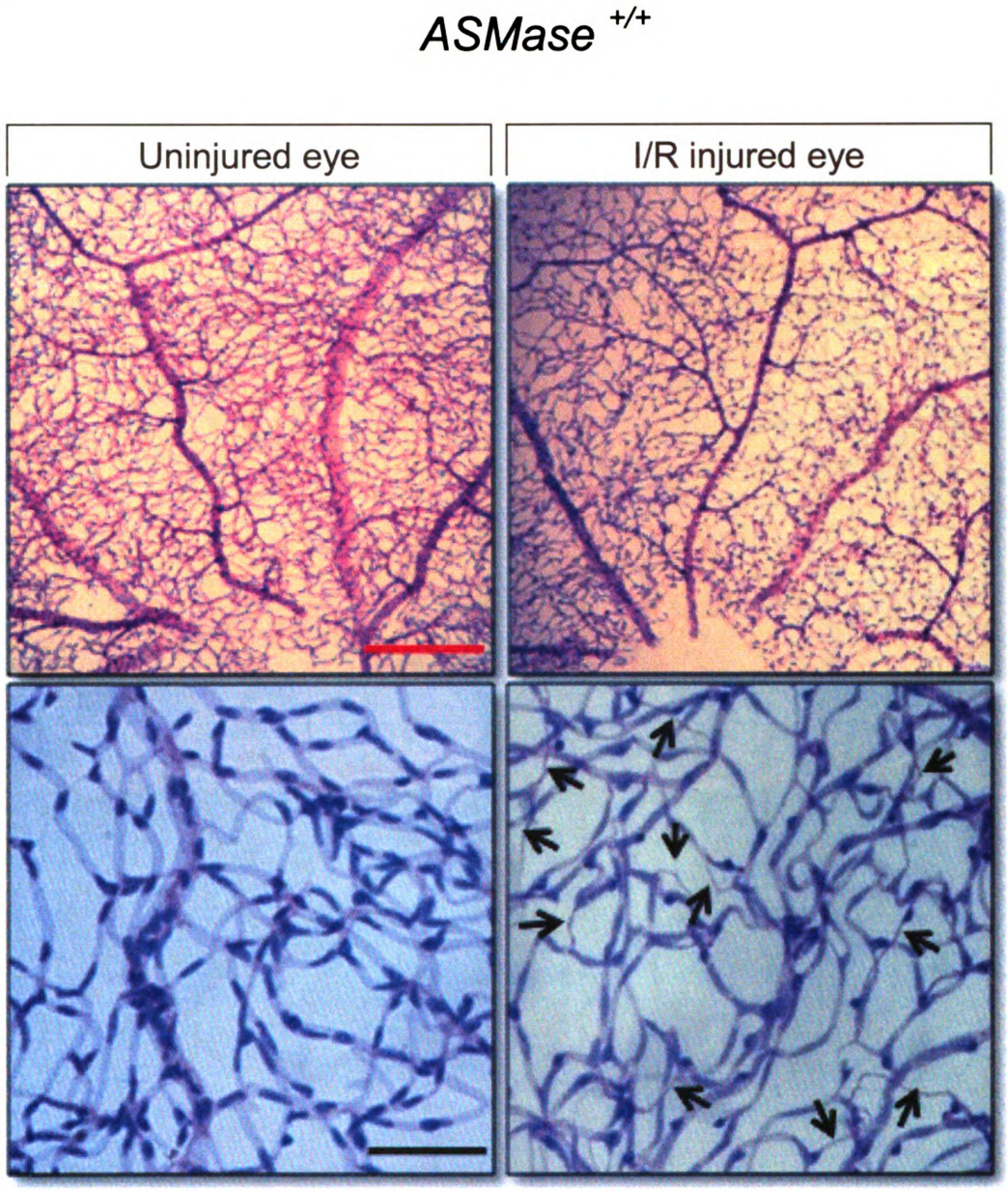


Figure 5.5. Retinal capillary degeneration in retinal ischemia/reperfusion in I/R retinopathy mouse model. Acellular capillary occurrence (*black arrows*) in the retina isolated from (**A**) ASMase$^{+/+}$ and (**B**) ASMase$^{-/-}$ mice 7 days following retinal I/R injury.

B.

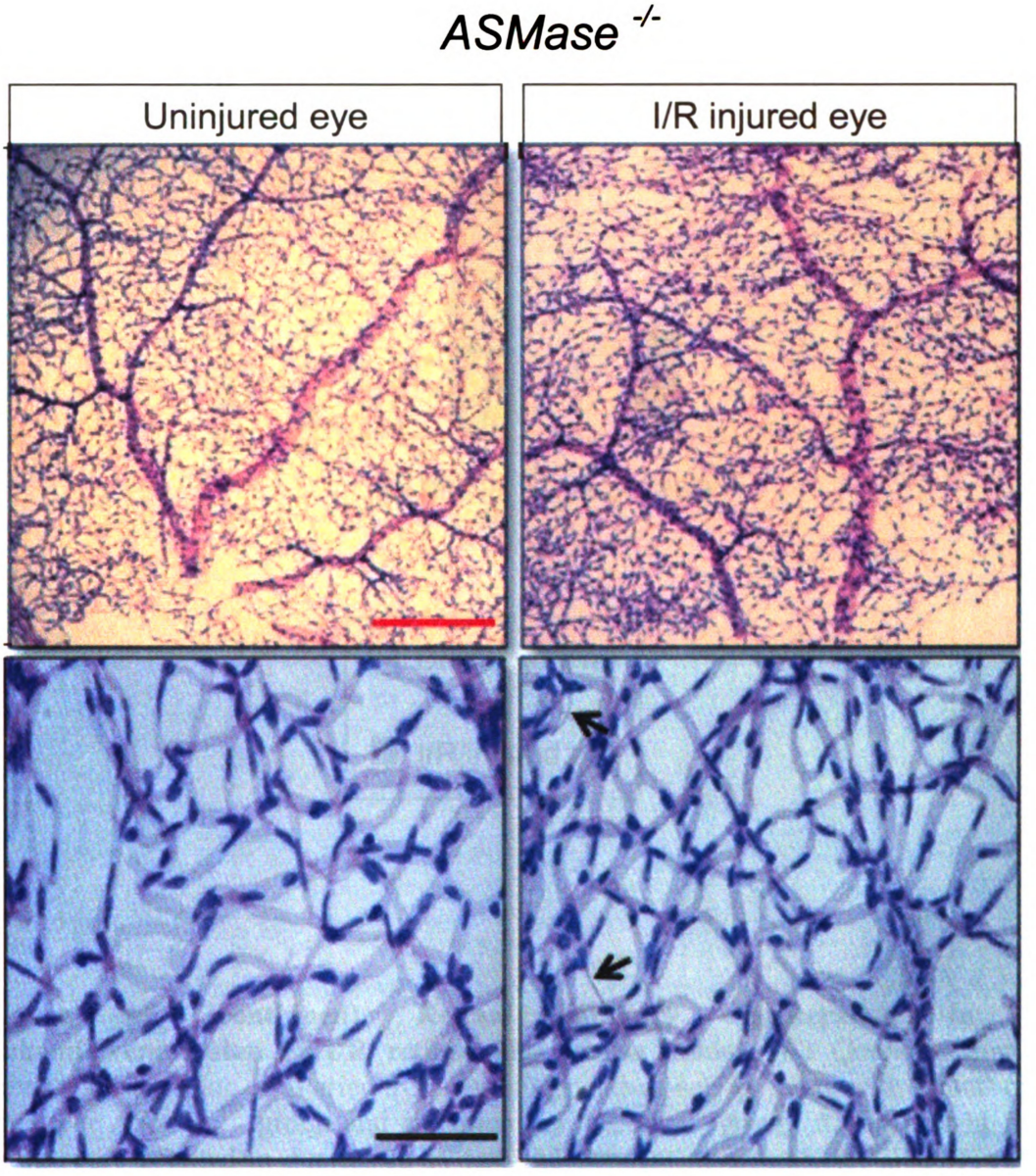


Figure 5.5 (continued). Retinal capillary degeneration in retinal ischemia/reperfusion in I/R retinopathy mouse model.

C.

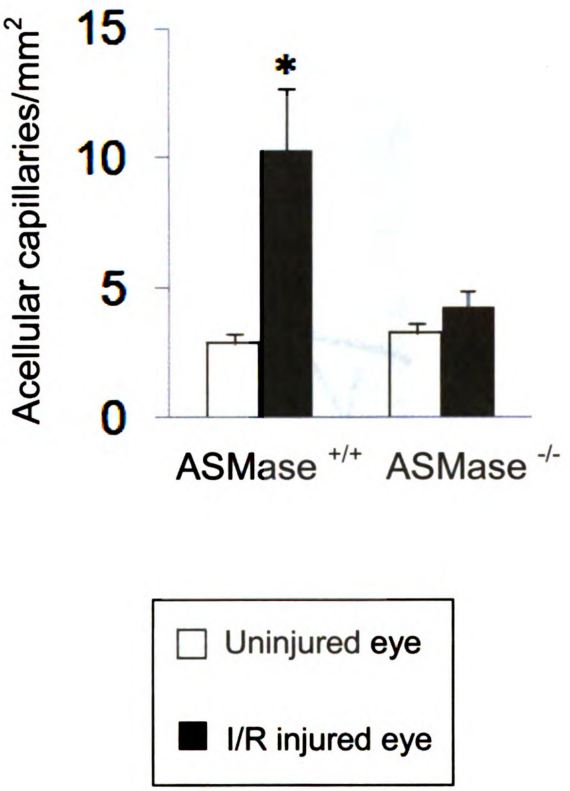


Figure 5.5 (continued). Retinal capillary degeneration in retinal ischemia/reperfusion in I/R retinopathy mouse model. (C) Quantification of the number of acellular capillaries from five independent sets of mice, with a total of seven mice per ASMase$^{+/+}$ group and six mice per ASMase$^{-/-}$ group. At least eight fields of retina were counted in duplicates by two independent investigators; *P<0.05; red bar = 550μm, black bar = 110μm.

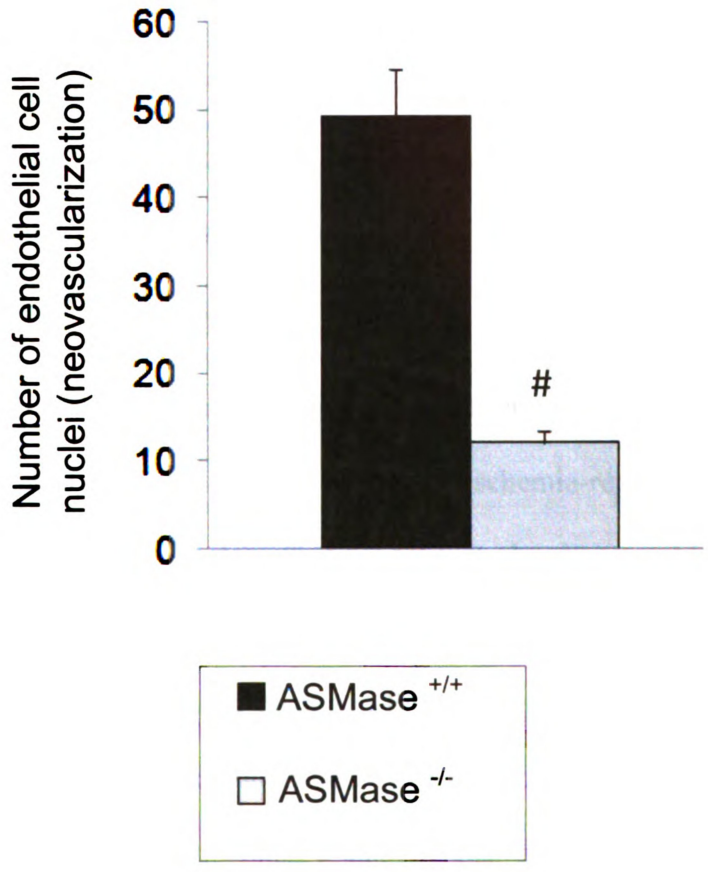


Figure 5.6. Retinal neovascularization in oxygen-induced retinopathy in mice. Quantification of the endothelial cell nuclei representing abnormal retinal neovascularization in the ASMase$^{+/+}$ and ASMase$^{-/-}$ mouse retina. (n= 8 per ASMase$^{+/+}$ group and n= 6 per ASMase$^{-/-}$ group; $^{\#}P<0.05$ compared to number of endothelial cell nuclei in ASMase$^{+/+}$ mouse).

5. Discussion

A large body of literature supports the role of ASMase activation in response to various stress stimuli, including pro-inflammatory cytokine stimulation (TNFα and IL-1β), oxidative damage (27), advanced glycation end products (AGEs) (28) or protein kinase Cδ (PKCδ) activation (29). These biochemical pathways are activated in the diabetic retina and can damage the retinal vasculature, resulting in formation of diabetic retinal microangiopathy lesions (30).

To corroborate the role of ASMase in retinal inflammation and microangiopathy *in vivo*, we used a mouse model of retinal ischemia-reperfusion. Retinal ischemia-reperfusion (I/R) is a well established model that closely resembles diabetic retinopathy (8). We addressed the function of ASMase in retinal microangiopathy by making use of an ASMase$^{-/-}$ mouse model of Niemann-Pick disease (NPD). As the ASMase$^{-/-}$ mouse model of NPD develops neurodegenerative complications early in life and has a short lifespan, we are unable to maintain a diabetic ASMase$^{-/-}$ mouse model for a duration of time sufficient to ensure the development of diabetic retinal complications. Therefore, in the ASMase$^{-/-}$ mouse retinopathy was modeled by retinal I/R injury, which permits us to examine the extent of retinal inflammation and capillary degeneration two days and seven days after injury, respectively (8).

A possible mechanism for capillary degeneration seen in the retinal I/R model could be activation and adhesion of leukocytes to the vasculature, inducing cell death signaling in capillary endothelial cells. Indeed, our study shows that retinal I/R injury

induces significant upregulation of ICAM-1 and VCAM-1 expression in the wild type retina (Figure 5.3 A,B).

Another possible mechanism for capillary degeneration is pro-inflammatory cytokine secretion by various retinal tissues, such as glial cells or retinal pigment epithelial cells, in the injured retina. We have found significant upregulation of IL-1β, TNFα and VEGF gene expression (Figure 5.4 A,B,C) in wild type retinas isolated from I/R injured eyes. These inflammatory mediators can promote the activation and degeneration of capillary endothelial cells with subsequent profound impact on vascular permeability and viability. Indeed, in the present study, retinas with I/R lesions exhibited a significant number of acellular, degenerated capillaries seven days after initiation of retinal injury; this time-point corresponds with late stage development of diabetic retinopathy. More importantly, ASMase genetic deficiency decreased pro-inflammatory/angiogenic mediator's gene expression and thus prevented the development of acellular capillaries in the I/R injured retinas. Therefore, ASMase emerges as a critical initial responder in pro-inflammatory/angiogenic molecules-induced retinal microvascular degeneration in a mouse model of retinal I/R injury.

Interestingly, we observed an increase in NSMase gene expression in the I/R injured retinas from ASMase$^{-/-}$ mice. Both ASMase and NSMase are considered key mediators of stress-induce ceramide generation and cellular injury and death; yet, these enzymes have individual requirements for their activation and regulation. ASMase functions optimally at a pH of 4.5-5.5, whereas optimal NSMase activation occurs at pH of 7.4 (31). NSMase upregulation in the ASMase$^{-/-}$ retinas with I/R lesions might try to compensate the absence of ASMase and mediate the pathological processes that result in

cellular apoptosis. However, the acidic milieu generated in the retina by the ischemia-reperfusion injury may preclude fully activation of NSMase and thus downplay the role of NSMase in promoting the microvascular lesions in the I/R model of retinopathy. Importantly, the retinas isolated from ASMase$^{-/-}$ mice with retinal I/R lesions were significantly protected against the inflammatory status and development of acellular capillaries, suggesting that ASMase rather than NSMase could represent a key player in mediating vascular damage in the I/R model of retinopathy.

In a recent study, Connor *et al.* demonstrated that docosahexaenoic acid (DHA) supplementation by dietary or genetic means confer significant protection against development of retinal pathological neovascularization in an OIR mouse model of retinopathy. As shown in previous chapters, ASMase is increased in diabetic retina and DHA returns ASMase to the basal levels in diabetic retina. Now we demonstrate that ASMase$^{-/-}$ mouse retinas are also significantly protected against pathological angiogenesis in the OIR model of retinopathy.

In conclusion, ASMase genetic deficiency renders protection against retinal capillary degeneration and ocular neovascularization in accelerated retinopathy mouse models.

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Conclusion

This study demonstrates the role of sphingomyelinases, key regulatory enzymes of sphingolipid metabolism, in modulating the inflammatory process in human retinal endothelial cells, the target vasculature affected by diabetic retinopathy.

We showed that proinflammatory cytokines activate sphingomyelinases and sphingomyelinase downregulation (by pharmacologic inhibitors, DHA or gene silencing) prevents cytokines-induced adhesion molecules expression in human retinal endothelial cells. In suport of sphingomyelinases downregulation and more importantly, downregulation of caveolar ASMase by DHA, we found a decreased ceramide/sphingomyelin ratio in caveolae lipid domains; moreover, DHA pretreatment of HREC prevented cytokine-induced increase in ceramide/sphingomyelin ratio in these specialized lipid domains.

Using *in vivo* studies we confirmed that ASMase, but not NSMase expression was significantly upregulated in diabetic rat retinas and closely correlated with the inflammatory status and vessel loss in diabetic retinal tissue. Importantly, ASMase increased specifically in the retinal vasculature from I/R injured eyes.

DHA supplementation significantly prevented increased ASMase expression, inflammatory status and vessel loss in the diabetic retina and prevented ASMase upregulation in the retinal blood vessels. ASMase genetic deficiency prevented upregulation of inflammatory/angiogenic molecules, vessel loss and pathological neovascularization in mouse retinas with accelerated models of retinopathy, and thus recapitulated the protective effects of DHA on diabetic retinas.

Therefore, our data suggests that ASMase, the key regulatory enzyme of sphingolipid metabolism, is a novel mediator and a promising therapeutic target for the prevention of retinal vascular inflammation and diabetic microvascular complications. Inhibition of ASMase by genetic means, pharmacologic inhibitors or DHA supplementation could be beneficial in preventing the development of microangiopathy lesions in diabetic retinopathy. This finding is particularly attractive since currently available approaches for the treatment of diabetic retinopathy do not fully prevent or reverse ocular pathology.

However, further studies are needed to elucidate and confirm the role of ASMase in the development of diabetic retinal inflammation and microangiopathy. One of the studies would be conducted in type 1 and type 2 diabetic rats, and ASMase inhibition will be achieved at the retinal level with inhibitors (i.e. desipramine, imipramine) that can be administered by intravitreal injection or slow-release lipid-based inhibitors implanted preretinal. To directly test the role of vascular ASMase in mediating retinal vessel loss, another study can be performed using vascular ASMase deficient mice that will be made diabetic by streptozotocin injection and retinal vessel damage would be assessed after more then 6 months after induction of diabetes. All these studies may provide new clues and let us understand more comprehensively about the role of ASMase, and particularly its vascular localization, in the development of retinal vascular damage in diabetes.