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Dyslipidemia and Diabetic Retinopathy: Effect of n6 and n3 Polyunsaturated Fatty Acids (PUFA) on Inflammation in human Retinal Endothelial Cells

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Weiqin Chen

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DYSLIPIDEMIA AND DIABETIC RETINOPATHY: EFFECT OF N6 AND N3 POLY-UNSATURATED FATTY ACIDS (PUFA) ON INFLAMMATION IN HUMAN RETINAL ENDOTHELIAL CELLS

BY

WEIQIN CHEN

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ABSTRACT

DYSLIPIDEMIA AND DIABETIC RETINOPATHY: EFFECT OF N6 AND N3 PUFA ON INFLAMMATION IN HUMAN RETINAL ENDOTHELIAL CELLS

BY

WEIQIN CHEN

Early diabetic retinopathy (DR) has been recognized as a low-grade chronic inflammatory disease. The mechanism(s) leading to inflammatory conditions in the diabetic retina are not well understood, but likely involve diabetic hyperglycemia and dyslipidemia. The effects of hyperglycemia have been studied in detail; while the role of dyslipidemia in the development of DR has received less attention. Diabetes induces a decrease in the major n3-PUFA, docosahexaenoic acid ($DHA_{22:6n3}$) in the plasma and the retina with a shift toward a higher n6/n3 PUFA. **The increase in n6/n3 PUFA ratio can profoundly affect the inflammatory state in the retinal endothelium due to the proinflammatory role of n6-PUFA and the anti-inflammatory effect of n3-PUFA.**

We first demonstrated that n6-PUFAs have a profound proinflammatory effect in human retinal vascular endothelial cells (hRVE) by inducing intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 expression and leukocyte adhesion to hRVE. The induction was through lipoxygenase (LOX) pathway as only LOX, but not COX or MOX inhibition blocked n6-PUFAs response in hRVE cells.

To assess the anti-inflammatory effects of n3-PUFA, the signaling of major inflammatory cytokines upregulated in diabetic eyes, TNFα, IL-1β and $VEGF_{165}$, was first characterized. All three cytokines stimulated CAMs expression in hRVE through NFκB activation. $DHA_{22:6n3}$ dramatically decreased the cytokines induced CAM expression in hRVE cells. A hypothesis that $DHA_{22:6n3}$ exerts the anti-inflammatory

effects through direct activation of nuclear receptors PPARs was first addressed. Activation of PPARα downregulated cytokines induced CAMs expression. However, saturated 16:0, n6-PUFAs and $DHA_{22:6n3}$ could all activate PPARα similarly. Moreover, $DHA_{22:6n3}$ could not only prevent nucleus NFκB binding, but also inhibit IκBα phosphorylation and degradation implying that $DHA_{22:6n3}$ works upstream of IκBα phosphorylation and not at the nuclear receptors level.

The hypothesis that $DHA_{22:6n3}$ treatment results in modification of membrane lipids that affects signal transduction in specific membrane microdomains, caveolae/lipid rafts was next addressed. Biochemical fractionation coupled with mass spectrometry was employed to characterize proteins of caveolae/lipid rafts resulting in an identification of about 70 proteins involved in many crucial endothelial cellular functions. The integrity of caveolae/lipid rafts together with its exclusive residents, the Src-family kinases (SFK) Fyn and c-Yes were required in cytokine induced inflammatory signaling. $DHA_{22:6n3}$ treatment led to a significant displacement of Fyn and c-Yes from caveolae/lipids rafts. The mechanisms of selective displacement of SFKs were further investigated. Analyses of Lipids from caveolae/lipid rafts indicated a significant incorporation of $DHA_{22:6n3}$ into its phospholipids, causing an increase in the unsaturation index and cholesterol depletion from caveolae/lipid rafts. nanoESI-MS/MS further confirmed $DHA_{22:6n3}$ incoporation into the major phospholipids known to localize in the caveolae/lipid rafts.

In summary, our study is the first to show that diabetes induced decrease in $DHA_{22:6n3}$ with a concomitant increase in the n6 to n3 PUFA ratio promotes basal and cytokine induced adhesion molecules expression and leukocyte adhesion in the retina.

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Key to Abbreviations

AA	arachidonic acid (20:4n6)
AGE	advanced glycation end product
BREC	bovine retinal endothelial cells
CAM	cell adhesion molecule
CE	cholesterol ester
COX	cycloxygenase
DAG	diacylglycerol
DHA	docosahexaenoic acid (22:6n3)
DPA	docosapentaenoic acid (22:5n3)
DR	diabetic retinopathy
DRM	detergent resistant membrane
eNOS	endothelial nitric oxide synthase
EC	endothelial cell
EEA	early endosomal antigen
EPA	eicosapentaenoic acid (20:5n3)
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ESI	electrospray ionization
FFA	free fatty acid
Flk-1	Fms like kinase receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDL	high density lipoprotein

HETE	hydroxyeicosatetraenoic acid
HMG-CoA	hydroxymethylglutaryl coenzyme A
HODE	hydroxyoctadecadienoic acid
HPETE	hydroperoxyeicosatetraenoic acid
hRVE	human retinal endothelial cells
HSL	hormone-sensitive lipase
IκBα	inhibitor of NFκB alpha
IKK	IκB kinase
ICAM-1	intercellular cell adhesion molecule -1
IL	interleukin
LA	linoleic acid (18:2ω-6)
LAT	linker for activation of T cell
LC-PUFA	long-chain polyunsaturated fatty acid
LDL	low density lipoprotein
LOX	lipoxygenase
LPL	lipoprotein lipase
LT	leukotriene
MMP	matrix metalloprotease
MCD	methyl-β-cyclodextrin
ME	macular edema
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NDGA	nordihydroguaiaretic acid

NEFA	nonesterified fatty acid
NFκB	nuclear-factor kappa B
PAI	plasminogen activator inhibitor
PC	phosphocholine
PDR	proliferative diabetic retinopathy
PE	phosphatidylethanolamine
PG	prostaglandin
PI	phosphoinositide
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
PS	phosphotidylserine
PUFA	polyunsaturated fatty acid
PVR	proliferative vitreous retinopathy
RAGE	receptor for advanced glycation end product
ROS	reactive oxygen species
RTK	receptor for tyrosine kinase
SFK	Src family kinase
SM	sphingomyelin
SRA	saturated fatty acid
STZ	streptozotocin
TG	triglyceride
TGF	transforming growth factor
TF	tissue factor

TNF	tumor necrosis factor
uPA	urokinase-type plasminogen activator
SMC	smooth muscle cell
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein

I. Literature review

1. Etiology of diabetic retinopathy

Diabetic retinopathy (DR) is a microvascular complication of diabetes and a leading cause of blindness in adults [1, 2]. It occurs when diabetes damages capillaries inside the retina, the light-sensitive tissue at the back of the eye. Diabetic retinopathy, in the way of proliferative diabetic retinopathy (PDR) and macular edema (ME), is the commonest cause of new cases of legal blindness in Europe and in North America in the age group 20 to 70-74 years[3]. Approximately 5.3 million out of 16 million people in the US living with diabetes have some form of DR. About 24,000 people are blinded each year by the disease. Both Type 1 and Type 2 diabetics are at risk of developing diabetic retinopathy. PDR may be present in half of those who have had Type 1 diabetes for 15 years with approximately 10% for those with Type 2 diabetes sustaining the same duration of disease[4].

The earliest stage of DR is diagnosed as mild nonproliferative retinopathy characterized by vascular basement membrane thickening, microaneurysms (out-pouchings of capillaries) with small areas of balloon-like swelling in the retinal capillaries; As the disease progresses, selective loss of intramural pericyte attachment to the capillaries occurs which leads to acellular or nonfunctional capillaries causing the dot and blot hemorrhages (tiny hemorrhages in the retina itself) and exudates (retinal deposits occurring as a result of leaky vessels). These symptoms, often present without any visual compromise, are also called background DR and happen at any time with the onset of diabetes. As more capillaries are blocked, severe nonproliferative retinopathy happens.

The blockage of capillaries compromises the blood circulation, causing downstream ischemia as manifested by an increase in the size and number of intraretinal hemorrhages. The down stream hypoxia thus induces synthesis of an endothelial-cell-specific angiogenic factor, vascular endothelial growth factor (VEGF). The increase of VEGF acts as a local hormone to induce more new blood vessel synthesis (neovascularization), a hallmark of proliferative retinopathy. PDR is the most advanced stage of the disease carrying the greatest risk of visual loss. The proliferating new vessels could develop along the retina and break into the surface of the clear, vitreous gel that fills the inside of the eye and eventually lead to serious retinal detachment. The newly synthesized blood vessels are abnormal and fragile, tending to leak blood into the center of the eye, blurring vision or even causing blindness. Also, fluid can leak into the center of the macula, the part of the eye where sharp, straight-ahead vision occurs. The fluid makes the macula swell thus blurring vision. This is called macular edema. Macular edema can occur at any stage of diabetic retinopathy. About half of the people with proliferative retinopathy also have clinically significant macular edema[5].

Despite the extensive research, only a few therapies are currently available to DR patients. Scatter laser photocoagulation has been recommended for treatment of advanced PDR. The risk of visual loss is reduced by more than 50% for patients with macular edema who undergo focal laser photocoagulation. It was estimated that timely detection and photocoagulation treatment could prevent 95% of severe vision loss in patients with diabetes[4]. More recently, the Diabetes Control and Complications Trial has demonstrated the efficacy and cost effectiveness of glycemic and blood pressure control in reducing the incidence and progression of DR[6]. Phase II and III clinical

studies involving anti-vascular endothelial growth factor, protein kinase C (PKC) inhibitors and antioxidants for the management of DR and diabetic macular edema (DME) are underway (See reviews [5, 7]). The inhibition of these biochemical pathways holds the promise of intervention for DR at earlier non-sight-threatening stages.

Even though careful screening, good control of blood glucose, and laser photocoagulation can help mitigate the effects of DR, patients suffering blindness from diabetes are still arising, with approximately 5,800 new cases reported annually. The identification of risk factors and determinants for early onset of retinopathy becomes crucial for our understanding of disease mechanisms thus developing strategies to prevent the disease at the early stage.

2. Inflammation and diabetic retinopathy

More than 40 years ago, aspirin was shown to decrease the severity of diabetic retinopathy in humans[8], which provides a link between inflammation and DR. Only recently, additional data suggested that early-stage diabetic retinopathy is a low-grade chronic inflammatory condition[9-11]. In experimental diabetes, the earliest event of leukocyte adhesion to the retinal vasculature results in early blood retinal barrier breakdown, capillary non-perfusion and endothelial cell injury and death. Support for this view is provided by the finding that a marked increase in leukocyte density and retinal vascular ICAM-1 and P-selectin immunoreactivity was found in human eyes with diabetic retinopathy[12]. Lymphocyte activation, an increased serum L-selectin level and increased lymphocytes adhesion to the endothelium in DR patients were observed significantly higher than control normal and diabetic (with no DR) patients[13]. In a canine model aspirin prevented certain classic histopathological features of DR, including formation of acellular capillaries; retinal hemorrhage; and an indicator of cell degeneration, capillary sudanophilia[14]. In a rat model of diabetic retinopathy, nonsteroidal anti-inflammatory agents such as aspirin, meloxicam and eternacept prevent early diabetic retinopathy development by decreasing the endogenous level of retinal proinflammatory cytokine TNFα thus suppressing diabetic retinal ICAM-1 expression, leukocyte adhesion and blood–retinal breakdown[10]. Furthermore, in CD18-/- and ICAM-1-/- mice a marked reduction of STZ diabetes-induced blood-retinal barrier breakdown, pericyte and endothelial cell loss and formation of acellular capillaries were observed [15].

Several inflammatory pathways are activated at the early stage of diabetic retinopathy. A pro-inflammatory cytokine TNFα is found in the extracellular matrix, endothelium and vessel walls of eyes of patients with proliferative diabetic retinopathy[16] and is elevated in the vitreous from human eyes with this complication[17-20]. Moreover, inhibition of TNFα signaling with a TNFα receptor/Fc construct reduced leukocyte adhesion and suppressed blood-retinal barrier breakdown in STZ diabetic rats[10].

The VEGF family of growth factors and their receptors have been well studied for their function in regulating endothelial proliferation and migration, vascular permeability and tube formation (reviewed in [21]). They are widely involved in controlling pathological angiogenesis and increased vascular permeability in important diseases such as cancer[22]. VEGF also has been strongly implicated in the pathogenesis of both background and proliferative diabetic retinopathy[23-27]. Increased intraocular VEGF levels as well as VEGF receptor 1 and 2 were detected in rat and human diabetic retina[23-26, 28-32]. In addition to its well known mitogenic and angiogenic activity, VEGF was recently recognized as a proinflammatory cytokine[33, 34]. As such, VEGF induces ICAM-1 expression on endothelial cells[33]. Specific inhibition of VEGF activity inhibited ICAM-1 expression, leukocyte adhesion, blood-retinal barrier breakdown and neovascularization in STZ diabetic rats[33].

Another principal inflammatory cytokine IL-1β recently has also been related to the progress of diabetic retinopathy. The level of IL-1β was increased by more than twofold in retina of rats with two months of diabetes[35]. Injection of IL-1β into vitreous

of rat eyes induced microvascular apoptosis and acellular capillaries[35]. High glucose could increase IL-1β secretion in bovine retinal endothelial cells (BREC), while application of ILRa significantly decreases IL-1β induced EC injuries[36]. These data suggests a possible role of IL-1β and its receptors mediated signaling pathways in inducing EC injury that contributes to the development of diabetic retinopathy.

NFκB is a family of transcription factors prerequisite for many inflammatory genes expression such as adhesion molecules, cycloxygenase 2 (COX2), tissue factors (TF), matrix metalloprotease (MMP) and inflammatory cytokines[37, 38]. It was first described as B-lymphocyte-specific nuclear protein, essential for transcription of immunoglobulin kappa (κ) light chains. There are five NFκB subunits-(RelA (p65), RelB, c-rel, p50 and p52) forming homo- and heterodimers and characterized by the conserved 'rel homology' domain in mammalian cells. In resting cells, NFκB is sequestered in the cytoplasm with members of the inhibitor of NFκB (IκB) family which consist of IκBα, IκBβ, Iκε and Bcl3[39]. Many different stimuli including inflammatory cytokines, LPS, oxidized LDL and microbial agents have the potential to activate the NFκB pathway through different signaling components and cascades depending on the cell type and stimuli. However, all will lead to the phosphorylation and activation of IκB kinases (IKK) complex, a central component to the NFκB cascade. The activation of IKKs triggers the phosphorylation of IκB at N-terminal serines which facilitates its recognition by ubiquitin kinase complex thus degradation by 26s proteosome. This finally leads to the liberation of NFκB from the inactive complex and transport to the nucleus for DNA binding (reviewed in [40], [41]). Both IL-1β and TNFα, and $VEGF_{165}$ could activate the

canonical NFκB pathway as Fig. 1. However, depending on different cell types, different signaling components are probably utilized.

Activation of NFκB (p65 and p50) has been well documented in diabetes especially in the retinal vasculature of diabetic patients and animals[10, 42, 43]. NFκB was shown to be activated in the pericytes of rat and human retina affected by diabetic retinopathy[42]. Also, NFκB was activated in the retinas of advanced glycation end-products (AGEs)-treated rats[44] and in bovine retinal endothelial cells treated with high glucose[43]. The activation of NFκB by inflammatory cytokines in human retinal endothelial cells has not been thoroughly characterized.

While there is a developing consensus on the role of inflammation in retinopathy, individual molecular steps leading to retinal adhesion molecules expression and to diabetic retinopathy are not well resolved. Two major metabolic disorders that are likely to play role in diabetes-induced inflammation in retina are hyperglycemia and dyslipidemia. Considerable progress in understanding of hyperglycemia-induced disease has been made over the past decade, while the role of diabetic dyslipidemia in the development of microvascular complications has received much less attention and the link between diabetic metabolic disorders and retinopathy still eludes us.

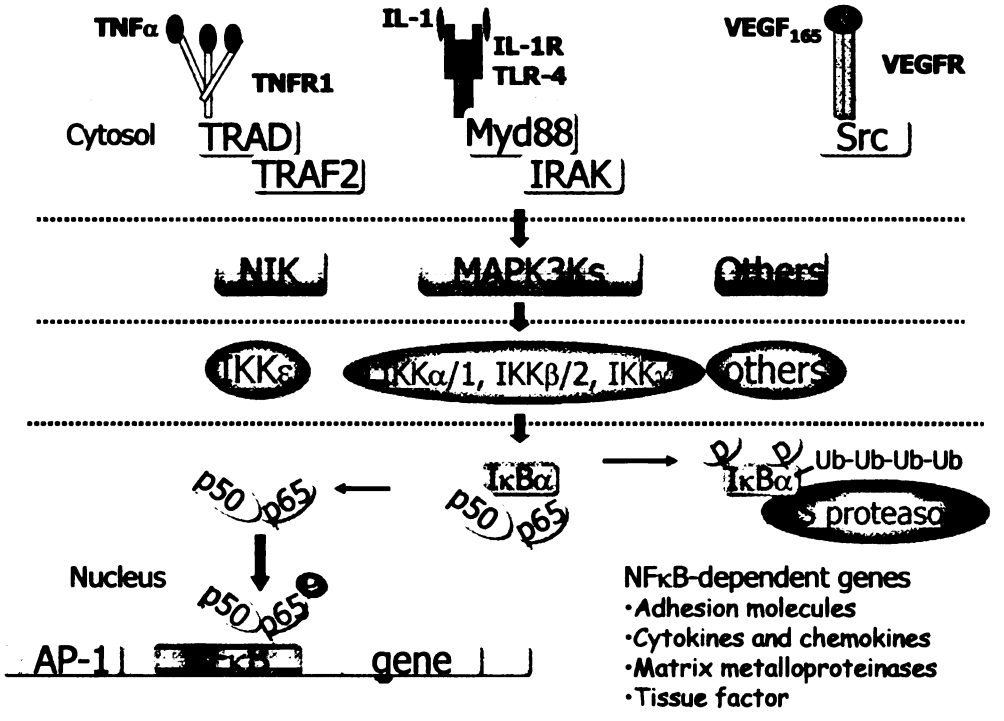


Fig. 1. Canonical pathways of NFκB activation by inflammatory cytokines.

3. Hyperglycemia and diabetic retinopathy

Both Type 1 and Type 2 patients can develop DR. Proliferative DR typically develops with Type 1 diabetes, whereas nonproliferative retinopathy with maculae edema is more common in Type 2 diabetes. However, the microvascular alterations in both conditions have the same pathophysiological basis in that the occurrence and progression of retinopathy has been largely correlated with the degree of hyperglycemia. The molecular mechanisms of hyperglycemia induced retinal endothelial dysfunction have attracted a lot of attention. Several pathways have been implicated, including increased flux of glucose through the polyl pathway; increased advanced glycation end product formation and receptor activation[45]; activation of PKC isoforms[46]; and increased hexoamine pathway flux[47] (Fig. 2).

Hyperglycemia induces increases in polyl pathway flux and acts through aldose reductase that converts glucose to sorbitol. Sorbitol then is further oxidized to fructose by sorbitol dehydrogenase. This process can either produce sorbitol as an osmotic stress activator or change the cellular NADH/NAD^+ or NADPH level that could potentially affect the redox status inside the cell and thus affect cellular functions. However, sorbitol levels in diabetic vessels are far too low to cause osmotic stress. Studies using aldose reductase inhibitors failed to prevent retinopathy and the thickening of the basal membrane of the retina in dogs in vivo[48, 49], although it prevented diabetic nephropathy.

The nonenzymatic glycation and oxidation of proteins produce intracellular and extracellular AGEs (Fig. 2). Other than causing abnormal protein function, cell-matrix or

matrix-matrix interactions, AGEs could affect endothelial cells function mainly through binding to one of specific AGE receptors (RAGE) to produce reactive oxygen species, causing the activation of NFκB[50] and thus pathological changes in gene expression. A wide range of evidence has implicated AGEs, mainly through RAGE, in priming proinflammatory mechanisms in endothelial cells by inducing proinflammatory molecules expression such as adhesion molecules (VCAM-1, ICAM-1 etc) and tissue factor expression[51-53]. Increased amounts of AGEs are found in diabetic retina vessels concomitant with the higher level of RAGE[45, 54]. Furthermore, polymorphisms of RAGE genes have been associated with diabetic retinopathy[55].

Hyperglycemia also induces the *de novo* synthesis of lipid second messenger Diacylglycerol (DAG) which activates PKC isoforms[56]. Activation of PKC has been associated with many vascular abnormalities in retina, renal and cardiovascular diseases[56]. Among PKC isoforms, PKCβ and δ are preferentially activated in the vasculatures of diabetic animals, such as in the retina and glomeruli[56, 57]. Activation of PKC has a number of pathogenic consequences by affecting expression of endothelial nitric oxide synthase (eNOS)[58], VEGF, transforming growth factor (TGF-β)[59] and plasminogen activator inhibitor-1 (PAI-1)[60], and by activating NF-κB[61] and membrane associated NAD(P)H oxidases.

Shunting of excess intracellular glucose into the hexosamine pathway might also cause several manifestations of diabetic complications. The increase in the hexoamine pathway flux causes the modification of important transcription activators such as Sp1 to be modified by n-acetylglucosamine (GlcNAc), a product of hexoamine pathway. This

modification of Sp1 could increase the transcription of tumor growth factor TGF-α, TGF-β1 and PAI-1[62], resulting in many changes in both gene expression and protein function, which together contribute to the pathogenesis of diabetic retinopathy. Other than Sp1, O-glycosylation of NFκB components was also reported to regulate NFκB binding activities thus influence the expression of NFκB dependent genes such as VCAM-1[63]. Blocking the hexoamine pathway could prevent experimental DR[47].

It has been suggested that hyperglycemia activates all these mechanisms by a single underlying process: overproduction of superoxide by the mitochondrial electron transport chain with subsequent inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity (reviewed in[64]). This leads to the diversion of upstream metabolites from glycolysis pathways into pathways of glucose overutilization such as the increased flux of dihydroxyacetone phosphate (DHAP) to DAG, an activator of PKC; and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Also, increased flux of fructose-6-phosphate to UDP-*N*-acetylglucosamine increases modification of proteins by *O*-linked *N*-acetylglucosamine (GlcNAc) and increased glucose flux through the polyol pathway consumes NADPH and depletes glutathione (GSH) (Fig. 2). The central role of reactive oxygen species (ROS) in hyperglycemia mediated pathological effects are confirmed by the evidence that normalization of mitochondrial ROS by an inhibitor of electron transporter chain complex 2, by an uncoupler of oxidative phosphorylation, by uncoupling protein 1 and by manganese superoxide dismutase[65] could block the above pathways of hyperglycemic damage *in vivo*. However, this hypothesis still needs to be further tested.

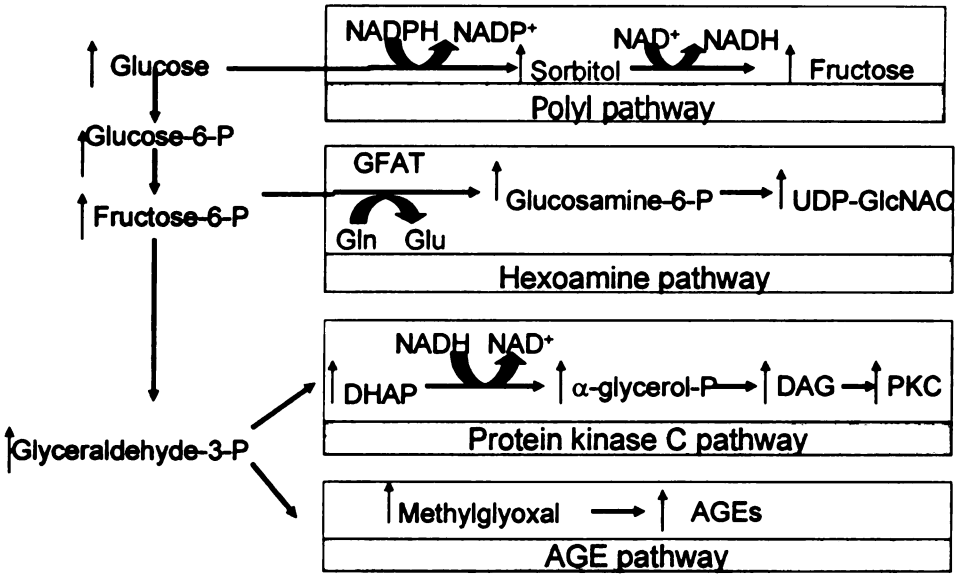


Fig. 2. Potential mechanisms by which hyperglycemia activates four pathways of hyperglycemic damage.

4. Dyslipidemia and diabetic retinopathy

Although a multitude of pathogenic mechanisms have been proposed, the underlying dysfunctional biochemical and molecular pathways that lead to initiation and progression of DR remain largely unresolved. Hyperglycemia is at a very late stage in the sequence of events leading from insulin resistance to frank diabetes; whereas lipoprotein abnormalities are manifested during the largely asymptomatic diabetic prodrome and may contribute substantially to the increased risk of macrovascular and microvascular diseases. The potential role of dyslipidemia in the development of microvascular complications has received much less attention compared to hyperglycemia and will be the focus of this dissertation.

4.1. Diabetic dyslipidemia

Dyslipidemia is a major metabolic syndrome prevalent in both Type 1 and Type 2 diabetes. An imbalance in the coordinated complex regulation of fatty acid uptake, metabolism, release by adipocytes, and clearance from circulation causes changes in serum triglycerides, lipoproteins and free fatty acids profiles. Indeed, Type 2 diabetes is frequently associated with elevation of blood levels of LDL-C, triglycerides and free fatty acids along with decrease in HDL cholesterol[66-72]. In Type 1 diabetes the overall cholesterol, triglycerides and nonesterified fatty acid levels do not significantly differ from the control values[73-75]; however, there is a substantial change in fatty acid profile of these pools. Changes in serum lipids and lipoproteins have been observed in Type 1 diabetes mainly with an increased level of linoleic acid (LA) and alpha linoleic acid (α-LA)[73, 76]. The total n3-polyunsaturated fatty acids (PUFA) especially

docosahexaenoic acid ($DHA_{22:6n3}$) is decreased in both plasma and retina of diabetic children and human diabetic eyes[73, 77].

4.2. Insulin and lipid metabolism

Fatty acids are structurally classified according to the number of carbons, double bonds, and proximity of the first double bond to the methyl terminal of the fatty acyl chain. For example, fatty acids of n6 family contain a double bond at the sixth carbon from the methyl end while n3 family contains a double bond at the third carbon. The nomenclature and the chemical structures of the major classes of fatty acids such as saturated (SRA), mono-unsaturated (MUFA) and PUFA are indicated as Fig. 3. As such, DHA is represented as 22:6n3, indicating carbon chain length of 22 with 6 double bonds and the first unsaturated bond is inserted at carbon 3.

To understand the effects of diabetes on the plasma and tissue fatty acid compositions, two metabolic routes have to be considered: *de novo* lipogenesis and polyunsaturated fatty acids remodeling pathways, or the Sprecher pathway[78] (Fig. 4). Saturated (SRA), mono- (MUFA) and PUFA are synthesized from dietary precursors (glucose, 16:0, 18:1n9, 18:2n6, 18:3n3 & n6, 20:5n3) through a series of desaturation (Δ5-desaturase [Δ5D], Δ6-desaturase [Δ6D] or Δ9-desaturase [Δ9D]) and elongation (Elovl-2, -5 & -6) reactions. The Δ6 and Δ5 desaturations are considered to represent the rate-limiting steps in fatty acid metabolism, and insulin is the most potent activator of the desaturase enzymes, as well as the induction of Δ5, Δ6, and Δ9 desaturases[67, 69, 74-76, 79-83]. Thus, subnormal availability of insulin in the liver will result in reduced activity of the desaturase enzymes and, consequently, will lead to accumulation of the substrates

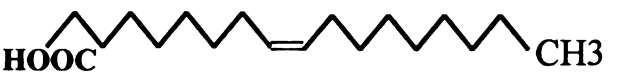
and depletion of the products. The overall effect of such metabolic perturbations would lead to a shift in fatty acid profile towards higher saturation index and shorter fatty acid chains. Indeed, tissue lipid profiles of people with diabetes are characterized by lower than normal concentrations of long chain PUFAs (LCPUFA) and higher than normal concentrations of monounsaturated and saturated fatty acids.

Insulin inhibits hormone-sensitive lipase (HSL) and activates lipoprotein lipase (LPL) [81, 84]. HSL is an intracellular neutral lipase capable of hydrolyzing triacyl, diacyl and monoacyl glycerols and cholesteryl esters as well as other lipid and water soluble substrates in many tissues[85]. Responsive to many hormones such as catecholamines, ACTH and glucagon, HSL is responsible for release of free fatty acids from adipose tissue thus providing the major source of energy for most tissues. Altered expression and activity of HSL in different cell types may be associated with obesity and Type 2 diabetes [85, 86]. Lipoprotein lipase (LPL) is synthesized in adipose and muscle and transported to the surface of endothelial cells where it hydrolyzes the core of triglyceride-rich lipoproteins (chylomicrons and very low density lipoprotein) into free fatty acids and monoacylglycerol, facilitating the removal of triglyceride-rich lipoproteins from the bloodstream. In the liver, activation of LPL by insulin stimulates conversion of fatty acids to triglycerides, followed by secretion as very low-density lipoprotein (VLDL)[87]. Thus, insulin resistance in Type 2 diabetes and low portal insulin levels in Type 1 diabetes resulting in an increased activity of HSL and a decreased activity of LPL, are expected to have a profound effect on plasma lipid levels, lipid profile and fatty acid composition. Indeed, diabetes is also characterized by increased lipolysis and altered lipogenesis that leads to increased concentration of nonesterified fatty acids (NEFA).

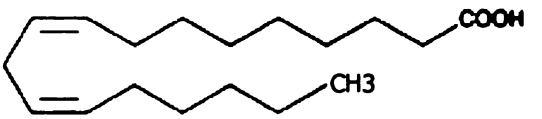
Patients both with Type 1 or Type 2 manifest decreased adipose LPL activity, and this is accompanied by a higher rate of HDL-cholesterol catabolism thus an increase in plasma triglycerides [79, 88].

- **Saturated Fatty Acids (SA)**
  Palmitic Acid (16:0)

- **Mono Unsaturated Fatty Acids (MUFA)**
  Oleic Acid (18:1,n9)



- **Poly Unsaturated Fatty Acids (PUFA)**
  Linoleic Acid (18:2,n6)
  (n6-PUFA)



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

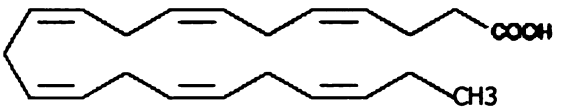


Fig. 3. Classification of fatty acids. Classification is based on the carbon lengths, number of double bonds and the site of the first double bond from the methyl end. Fatty acids can be classified into saturated, monounsaturated and polyunsaturated (n6 and n3) fatty acids.

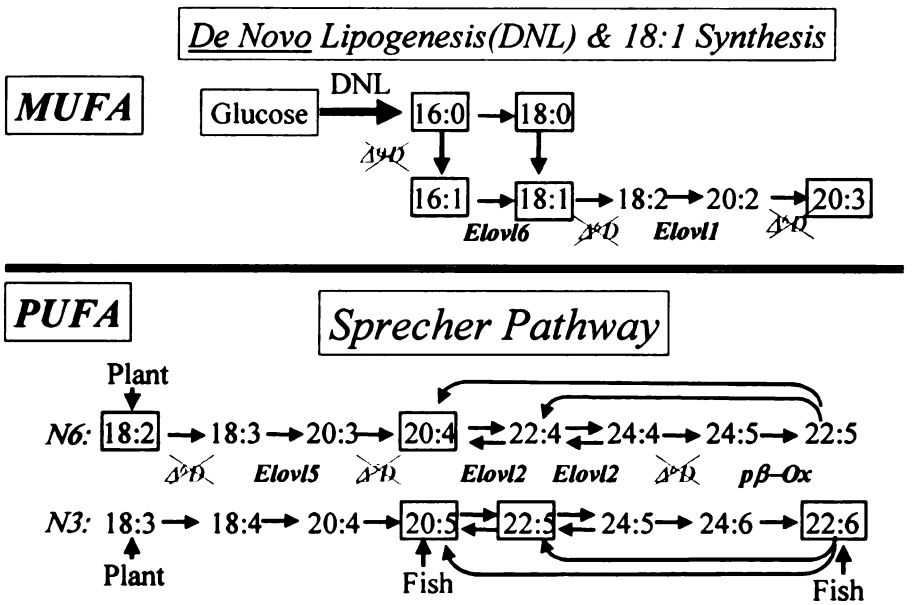


Fig. 4. Effect of insulin on unsaturated fatty acid synthesis. Dietary 16:0, 18:1n9, 18:2n6 and 18:3n3 are converted to long chain unsaturated fatty acids *in vivo* by a series of desaturation (Δ5-desaturase [Δ5D], Δ6-desaturase [Δ6D] or Δ9-desaturase [Δ9D]) and elongation (Elovl-2, -5 & -6) reactions. Fatty acids that accumulate in most animal and human tissues are in solid boxes. Dietary 18:2n6 and 18:3n3 are obtained from plants; 20:5n3 and 22:6n3 are rich in fish meals. There is no interconversion between n3, n6 and n9 fatty acids in the animals. Insulin controls Δ5-, Δ6-, and Δ9-desaturases. The activity and expression of these desaturases is low in Type 1 diabetes, as indicated by Xs.

4.3. Dyslipidemia and diabetic retinopathy

Clinical data show that dyslipidemia could be a critical factor in the development of diabetic retinopathy. Retinal hard exudates are the component of diabetic retinopathy most likely to be related to plasma lipoproteins, because the exudates are lipid rich. The Early Treatment Diabetic Retinopathy Study (ETDRS) has demonstrated that higher levels of total triglycerides, total cholesterol, and LDL cholesterol are strongly associated with an increased risk of development of hard exudates in the macula and visual loss[89-91]. In a recent clinical treatment of diabetic patients with simvastatin, a hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, a significant retardation of the progression of diabetic retinopathy was documented[92, 93]. Another clinical trial with atorvastatin in patients with Type 2 diabetes with dyslipidemia demonstrated a reduction in the severity of hard exudates and subfoveal lipid migration in clinically significant macular edema, suggesting that lipid-lowering therapy could be an important adjunct in the management of clinically significant macular edema[94, 95]. The association of retinopathy with cardiovascular disease and elevated plasma LDL cholesterol[96] was also suggested from another population-based study. The newly conducted Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study (DCCT/EDIC) cohort study revealed new associations between retinopathy status and the subclasses of lipoproteins. A strong inverse association between the severity of DR and average VLDL particle sizes was defined with gender differences in Type 1 diabetes[97].

5. Role of PUFA in inflammation

The immunomodulatory properties of lipids were first reported more than half century ago. Cells of the immune system can use the lipids as both intracellular and extracellular signal mediators. It has been reported that lipid mediators can have proinflammatory properties or anti-inflammatory properties depending on the cell target or lipid derivatives[98] (Fig. 5A, 5B). The fatty acid composition of inflammatory and immune cells is sensitive to change according to the fatty acid composition of the diet. In particular, the proportion of different types of PUFA in these cells is readily changed, and this provides a link between dietary PUFA intake, inflammation, and immunity.

5.1. n6-PUFA and inflammation

Unsaturated fatty acids are substrates for oxygenases such as Cyclooxygenases (COX)[99], Lipoxygenases (LOX or LO)[100], and cytochrome P450 Monoxygenases (MOX)[101, 102], and also for nonenzymatic oxidation. The COX pathways including COX-1 and COX-2 enzymes catalyze the first step in the biosynthesis of prostaglandins (PGs) by converting arachidonic acid (AA) to PGH2. PGH2 is further converted into 2-series of PGs and eicosanoids such as PGE2, PGD2, PGF2α, PGI2 (prostacyclin) and thromboxanes (TXB_2)[98, 103]. COX-2 and its potent inflammatory products like PGE2 and thromboxanes have been implicated in the pathogenesis of several inflammatory diseases including diabetic vascular diseases such as atherosclerosis ([99] and reviewed in [104]). Recent research indicates that high glucose as well as ligands for RAGE could upregulate the expression and activity of COX-2 primarily through NFκB activation under diabetic conditions[105, 106],

LOXs are a diverse family of nonheme ferroproteins that catalyze the hydroperoxidation of polyunsaturated fatty acids both region- and stereospecifically. Thus far, six LOXs have been identified in humans: platelet-type 12-LOX, 12(R)-LOX[107], 15-LOX-1, 15-LOX-2, e-LOX-3[108], and 5-LOX[100, 109]. Lipoxygenases products, such as the hydroperoxyeicosatetraenoic acids (HPETE), hydroxyeicosatetraenoic acids (HETE) and their metabolites the leukotrienes, play roles in many of the steps involved in diabetes, inflammation, arthrosclerosis, especially in modulating cell-cell interactions[98, 110]. Under hyperglycemia conditions, endothelial cells have been shown to increase the monocyte-endothelial interactions through 12-LOX pathway by generating 12 (S)-HETE from arachodonic acid[110]. Products of linoleic acid metabolism by leukocyte-type 12-LOX may also play roles in mediating inflammatory processes. 9- and 13-hydroxy-linoleic acid possess chemotactic activity for bovine and human polymorphonuclear leukocytes[111]. Significant quantities of 12-HETE were produced in db/db mice in vivo which corresponded to the increased monocyte-endothelium adhesion[112]. Disruption of 12/15 LOX mRNA in these animals by a catalytic ribozyme blocked the monocyte adhesion[113]. Furthermore, 12-LOX activity and expression were highly increased in HLHG (hyperlipidemia-hyperglycemic) pigs in a diabetic pig model[92]. Other LOXs and their bioactive lipids also have profound proinflammatory effects. Emerging data implicate 5-LOX and its products, especially the 4 series leukotriences LTB4 and LTE4, as major players in the cardiovascular diseases due to their proinflammatory activities[98, 110]. The role of LOXs and their bioactive lipid mediators in the pathogenesis of diabetic retinopathy is not well characterized. The only evidence is from the epiretinal membrane tissue of

patients with PVR and PDR where significant amount of 15-HETE (15-LOX product of $AA_{20:4n6}$) were detected[114].

The mechanisms of the biological activities ascribed to $AA_{20:4n6}$ and $LA_{18:2n6}$ based COX and LOX metabolites including prostaglandins, individual HETEs and HPETEs (from $AA_{20:4n6}$), HODEs and HPODEs (from $LA_{18:2n6}$) etc are not well addressed. Evidence suggests these products can act as discrete signaling molecules through G-protein mediated signaling pathways. 12-HETE has been shown to activate extracellular-signal-regulated kinase (ERK)[115], PI3K[116], PKC and Src kinases[117]. The activation of PKC is linked to 15(s)-HPETE and 12(s)-HETE induced surface expression of cell adhesion molecules (CAM)s in human umbilical vein endothelial cells (HUVEC) associated with an increased binding activity of the transcription factor, NF-κB[118]. Recently, 12(s)-HETE alone has been shown to be able to direct the translocation and activation of PKCs in lens epithelial cells[119]. The regulation of protein kinase C by mono-HETEs has also been suggested through a specific guanine nucleotide-binding protein linked receptor mediated hydrolysis of inositol phospholipids[120], a receptor mediated signaling pathway similar to other eicosanoid receptors identified for prostaglandin E, prostacyclin, thromboxane A2 and leukotriene D4.

Overall, the oxidized lipids of the lipoxygenase and cycloxygenase pathways of $AA_{20:4n6}$ and $LA_{18:2n6}$ metabolism have potent growth, vasoactive, chemotactic, oxidative and proinflammatory properties in vascular smooth muscle cells, monocytes and endothelial cells (Fig. 5A). Cellular and animal models have shown that these enzymes

were induced in diabetic complications suggesting the role of these pathways and their lipid products in the pathogenesis of diabetic vascular diseases[121]. However, the biological effect of fatty acid oxidation also depends critically on particular fatty acid precursors[122].

5.2. n3 PUFA and inflammation

The anti-inflammatory effects of long-chain omega-3 polyunsaturated fatty acids (n-3 LCPUFAs) from fish oil were amongst the earliest identified biological actions of these fatty acids. Animal studies have shown that dietary fish oil results in altered lymphocyte function and suppressed production of proinflammatory cytokines by macrophages[123].

Supplementation of the diet of healthy human volunteers with fish oil-derived n3-PUFAs results in decreased monocyte and neutrophil chemotaxis and decreased production of proinflammatory cytokines[124]. Fish oil feeding has been shown to ameliorate the symptoms in some animal models of autoimmune disease (reviewed in [125]. Clinical studies have reported that fish oil supplementation has beneficial effects in treating asthma[126], atherosclerosis, cancer, and cardiovascular disorders (for recent reviews, see [127, 128]), supporting the idea that the n3-PUFAs in fish oil is anti-inflammatory and immunomodulatory.

5.2.1. n3-PUFA and oxidized lipids

The first established link between the n3-PUFA and anti-inflammatory role is through inhibition of n6-PUFA derived inflammatory eicosanoids. This could be

achieved through the incorporation of n3-PUFA into membrane phospholipids of immune cells which leads to the displacement of the inflammatory n6-PUFA substrates ($AA_{20:4n6}$) available for catalysis. Also n3-PUFA could inhibit the activation of phospholipase A2 (PLA2), the enzyme necessary to release the substrate for metabolism[124, 129]. Generally, eicosapentaenoic acid ($EPA_{20:5n3}$) and $DHA_{22:6n3}$ are poor substrates for oxygenases. $EPA_{20:5n3}$, or $DHA_{22:6n3}$ (by reconversion to $EPA_{20:5n3}$), can to some extent be metabolized by COX and 5-LOX, but the derived 3-series proglandins and 5-series leukatrienes have an order of magnitude lower biological activity compared to n6-PUFA and mainly play an anti-inflammatory function (Fig. 5B) (reviewed in[122]).

Recent studies have provided a novel mechanism(s) for the therapeutic benefits of n-3 dietary supplementation important in inflammation, neoplasia, and vascular diseases. A novel group of mediators -- termed E-series resolvins -- formed from $EPA_{20:5n3}$ by COX-2 in the presence of aspirin and $DHA_{22:6n3}$-derived mediators termed D-series resolvins, docosatrienes and neuroprotectins, also produced by COX-2, have been identified from resolving inflammatory exudates[130] and $DHA_{22:6n3}$ enriched tissues such as brain and retina[131] (Fig. 5B). Aspirin acetylates COX-2, enabling the synthesis of those bioactive 17R-hydroxy-containing di- and tri-hydroxy-docosanoids/epoxyanoids termed resolvins from $DHA_{22:6n3}$ and $EPA_{20:5n3}$ via epoxide-containing intermediates. They are proved to be potent anti-inflammatory mediators with pico- to nanomolar range efficacy to reduce both leukocytes infiltration in vivo and block cytokine production from glial cells. These results indicate that $DHA_{22:6n3}$ or $EPA_{20:5n3}$ are the precursors to potent protective mediators generated via enzymatic oxygenations to novel docosatrienes and

17S series resolvins that each regulate events of interest in inflammation and resolution[132].

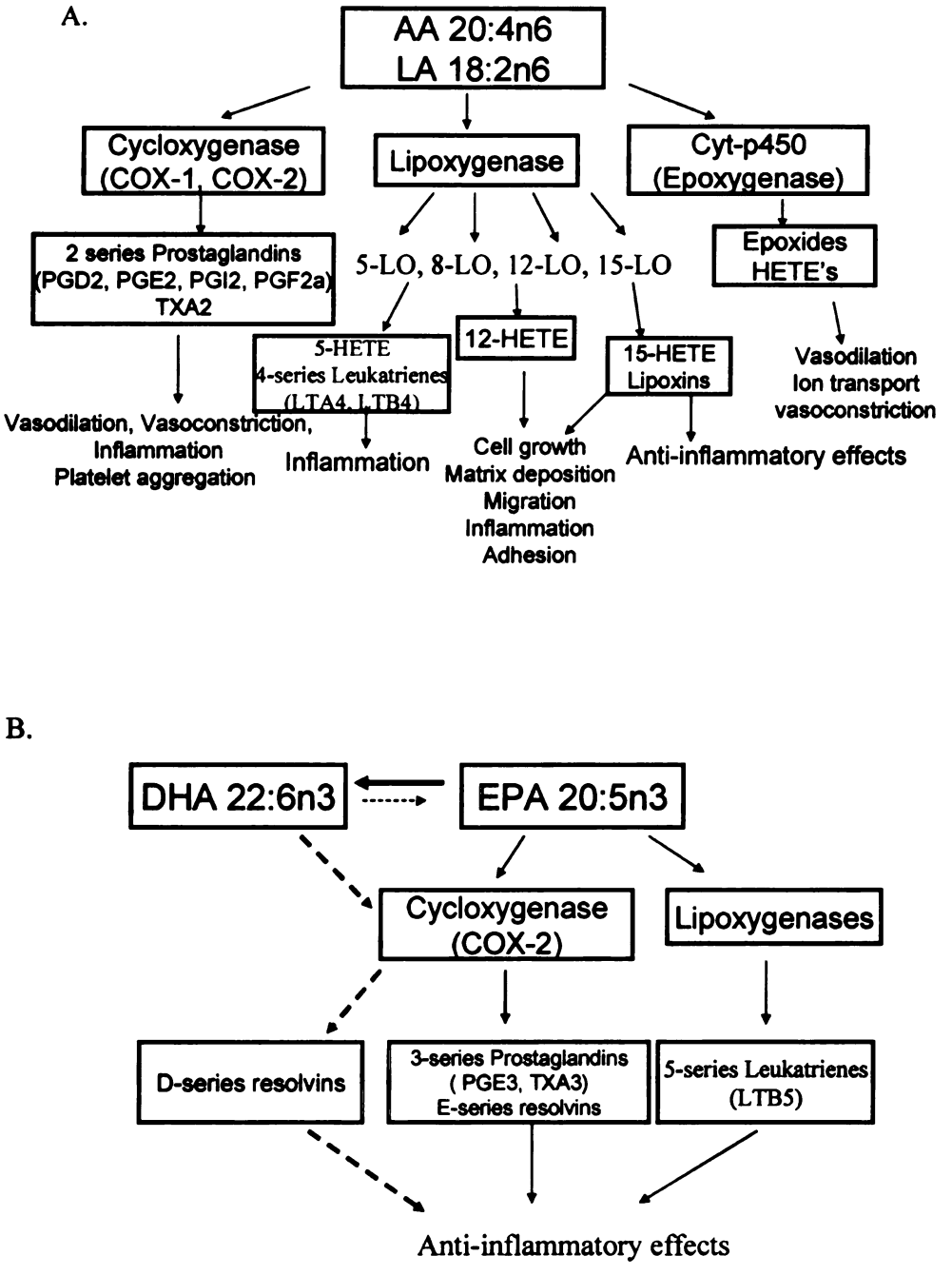


Fig. 5. Syntheses and functions of eicosanoids from PUFAs of n6 (A) and n3 (B). Lipid mediators can have proinflammatory properties or anti-inflmmatory properties depending on the cell target or lipid derivatives.

5.2.2. n3-PUFA and PPARs

Several lipid mediators, mainly PUFA or their derivatives can function as ligands for Peroxisome Proliferator-Activated Receptor (PPAR) family[133, 134]. PPARs are members of nuclear hormone receptor super family transducing environmental, nutritional and inflammatory signals into cell at the level of gene transcription (reviewed in [134]). Three PPAR isoforms – PPARα, PPARβ/δ and PPARγ have been identified with a unique quantitative pattern of tissue distribution and differential functions in regulating glucose and lipid metabolism[134].

The activation of PPARs by various types of fatty acid and their derivatives has some degree of isoform specificity. Endogenous NEFA such as α-$LA_{18:3n3}$, γ-$LA_{18:3n6}$, $AA_{20:4n6}$ and $EPA_{20:5n3}$ are weak activators of PPARγ[135]. PPARα can be activated by similar PUFA but also some medium-chain saturated and mono-unsaturated fatty acids (e.g. palmitic (C16:0) and oleic (C18:1) acids). $DHA_{22:6n3}$ has been shown to be a potent PPARα activator[136]. 13-HODE, 9-HODE, generated from $LA_{18:2n6}$ from endogenous or component of oxidized-LDL, via 12/15 lipoxygenases, are natural ligands for both PPARα and PPARγ[137], while $AA_{20:4n6}$ metabolites from 5 and 8 lipoxygenases such as LTB4 and 8(s)HETE can selectively activate PPARα. The cycloxygenase metabolites of $AA_{20:4n6}$, mainly prostaglandins such as 15dPGJ2 have great potential in activating PPARγ as well as the 15-lipoxygenase metabolite15-HETE[137, 138]. Various types of eicosanoids, including prostaglandin A1 (PGA1), prostaglandin D2 (PGD2) and possible the natural prostacyclins might be the endogenous agonists for PPARβ/δ[139] (Fig. 6).

The composition of the intracellular non-esterified fatty acids (NEFA) pool and their metabolites is an important determinant in the control of PPAR activity.

Several pharmacological compounds have been synthesized to differentially activate PPAR isoforms, as shown in Fig. 6. Fibrates, a class of drugs in the treatment of dyslipidemia, and WY14,643, are synthetic ligands for PPARα, whereas the antidiabetic glitazones are high-affinity ligands for PPARγ. Synthetic ligands for PPARβ/δ have recently also been identified, including the prostacyclin analog carbaprostacyclin and GW501516.

Recent evidence has indicated an important role of PPARs in the control of various types of inflammatory response (reviewed in [140]). The first role of PPARs in inflammation came with the evidence that PPARα-null mice demonstrated a prolonged response to inflammation stimuli[141]. Later on, specific agonists of PPARα such as fenofibrate or WY14,643 have been demonstrated to be able to reduce cytokine induced TF expression in human monocytes and macrophages[142] and IL6 production in human aortic smooth muscle cells[143]; thrombin-induced endothelin-1(ET-1) production[144] and VCAM-1 expression in endothelial cells[145-147] etc. Not only PPARα, but also PPARγ ligands have been reported to suppress the LPS induced T cell active CXC chemokines production in human EC[147]. Modulation of vascular inflammation in HUVEC and in vivo[148, 149] by PPARγ activators were documented with observation of suppressing proinflammatory adhesion molecules expression. However, the anti-inflammatory role of PPARγ is still under debate since the effects are most pronounced with the least selective and active PPARγ agonist, 15d-PGJ2[150]. The role of PPARβ/δ

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in the control of inflammatory responses has not yet been fully investigated due to the lack of isoform-specific agonists. Recently treatment of endothelial cells with new PPARβ/δ agonist GW510156 inhibits the stimulus induced upregulation of VCAM-1 expression[151] and a crucial role of PPARβ/δ in keratinocyte inflammation was also documented[152].

The involvement of PPARs in the control of inflammation and inflammatory gene expression is mainly through the transrepression. Agonist targeted PPARα can effectively antagonize the NFκB and AP-1 signaling pathways by physically interacting with NFκB (p65) through its Rel homology domain and with the amino-terminal c-Jun respectively, thereby resulting in a functional cross-inhibition of both transcriptional activators[144, 153]. Also, ligand-activated PPARα was shown to upregulate the expression of mRNA and protein of the Inhibitor of κB (IκBα), which prevents NFκB translocation into the nucleus[154].

Table 1.

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Table 1. **Natural and synthetic peroxisome proliferator-activated receptor (PPAR) ligands.**

	PPARα	***PPARβ/δ***	***PPARγ***
Naturally occurring FA-derived molecules	NEFA (SRA, MUFA and PUFA eg. LA, AA, EPA, DHA)	NEFA (PUFAs)	NEFA (PUFAs)
	9-HODE	Eicosanoids (PGA1, PGD2)	15-HETE
	13-HODE	prostacyclin	15-dPGJ$_2$
	8S-HETE		9-HODE
	LTB4		13-HODE
Pharmacological compounds	Fibrates (Fenofibrates, benzofibrates) WY14,643	GW501516 Carbaprostacyclin L165041	Glitazones (Rosiglitazone, Pioglitazone, trioglitazone)

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5.2.3. n3 PUFA and Caveolae/lipid rafts

5.2.3.1. Caveolae/lipid rafts

It has long been recognized that incorporation of n3-PUFA into membrane lipids decreases the microviscosity of membranes in general that may affect the mobility and function of membrane proteins. One specialized membrane microdomain whose structure and function could possibly be affected by n3-PUFA is called lipid rafts. Lipid rafts are enriched with cholesterol and sphingolipids, such as sphingomyelin and glycolipids. They also contain polar lipids such as phospholipids that mainly consist of saturated fatty acyl residues. This makes them spontaneously aggregate to form liquid-ordered membrane regions facilitating their isolation as nonionic detergent–resistant membrane domains (DRM).

Caveolae were first identified as 50-500nm flask-shaped invaginations in the plasma membrane nearly fifty years ago[155]. They share some similarities with lipid rafts as caveolae are also DRMs enriched with cholesterol and sphingolipids. Unlike lipid rafts, caveolae has a distinct invaginated form that can be easily detected by electron microscope. This is due to the presence of the scaffolding/regulatory protein caveolin. Caveolae are most numerous in well differentiated cell types such as adipocytes, myocytes, and fibroblasts especially endothelial cells[156]; while some cell types such as lymphocytes and neurons only contain lipid rafts devoid of caveolin. Other structures of caveolae independent of plasma membrane are also present including detached plasmalemmal and tubular-vesicular[156]. The density gradient centrifugation following Triton X-100 extraction at 4°C, the most frequently used method, does not result in pure

caveolae, but also coisolation of lipid rafts from all cellular membranes. Several other methods have been newly devised to study caveolae and lipid rafts separately, including immuno-isolation[157, 158], double-label immunoelectroscopy and photonic force microscopy etc; each with its own advantages and disadvantages (Reviewed in [159]).

Caveolae/lipid rafts consist of dynamic assemblies of cholesterol and sphingolipids in the exoplasmic leaflet of the bilayer. The presence of saturated hydrocarbon chains allows for cholesterol to be tightly intercalated, similar to the biophysical liquid-ordered state in model membranes. The characterization of the inner leaflet lipid composition is still incomplete, but they are probably rich in phospholipids such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC) with saturated fatty acyl chains and cholesterol[160]. The membrane surrounding the caveolae/lipid raft is fluid since it contains more unsaturated phospholipids[160]. The importance of cholesterol to the structure and function of caveolae/lipid rafts was demonstrated by the use of sterol binding agent such as filipin, nystatin or cholesterol depletion agent such as methyl-β-cyclodextrin (MCD) (reviewed in [159]).

A major property of caveolae/lipid rafts is the differential inclusion and exclusion of proteins. Caveolae/lipid rafts are highly enriched with GPI-anchored proteins and doubly acylated proteins such as Src-family kinases (SFK) or the alpha subunits of heterotrimeric G proteins, etc. GPI anchored proteins have affinity with the exoplasmic leaflet of the lipid rafts[157, 161]. The double acylation of some intracellular proteins happens with the irreversible cotranslational modification of myristoylations at the N-terminal together with the s-acylation on cysteines usually with palmitoylation. The high

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packing order of saturated myristol and palmitoyl moieties facilitates the interactions with high-ordered cytoplasmic leaflets of lipid rafts or caveolae membrane subdomains. Some transmembrane proteins are targeted to caveolae/lipid rafts by yet unclear mechanisms with possible involvement of amino acids in the transmembrane domains near the exoplasmic leaflet[162].

5.2.3.2. Functions of caveolae/lipid rafts in endothelial cells

The function of caveolae and lipid rafts in endothelial cells was first suggested to involve transmembrane transport, endocytosis and exocytosis. In endothelial cells, the transcytosis of albumin, insulin and native or modified LDL are reported through receptors localized in caveolae, since this specialized process could be perturbed by sterol binding agent such as filipin or cholesterol depletion such as MCD[163, 164]. The involvement of caveolae in transcytosis suggests a role in regulating vascular permeability. The selective permeability of endothelial cells is especially important in the case of infectious diseases, atherosclerosis, and blood-brain and blood-retinal barriers functioning. Indeed, in the vascular systems of caveolin-1 deficient mice, abnormalities in permeability and contractile functions were observed[165, 166]; and endothelial cells derived from mice lacking caveolae membranes have defects in transcytosis[167].

Caveolae-mediated endocytosis constitutes an alternative endocytic pathway to clathrin-coated pits. The endothelial caveolae have the molecular transport machinery for vesicle budding, docking and fusion including VAMP, NSF, SNAP, annexins and GTPases[168]. Caveolae carry select cargo, distinct from clathrin coated pit mediated endocytosis, and mediate ultimate delivery to the Golgi and endoplasmic reticulum

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(ER)[169, 170] or lysosomes[171], such as the uptake of folate via the folate receptor. Viruses such as SV40 are internalized by caveolae to early endosomal antigen 1 (EEA1) -negative, TfR-negative, fluid-phase marker-negative compartments called caveosomes; followed by subsequent transport to the ER[172]. Recently, an important role for caveolae has been well documented in cholesterol homeostasis. The caveolae structural integral membrane protein caveolin-1 binds to cholesterol directly[171] and participates in shuffling of the free cholesterol through ER, the Golgi complex and the cell surface[173]. The HDL receptor SR-BI[174] and oxidized–LDL receptor CD36 have all been shown to be highly concentrated in caveolae[174, 175] where they mediate cholesterol homeostasis in the cell.

The most important role of caveolae/lipid rafts at the cell surface is their function in signal transduction. It is well established that caveolae and lipid rafts both can act as signaling platforms to recruit and compartmentalize the specific signaling receptors, adaptors, scaffolds and enzymes. Receptor tyrosine kinases such as FcεRI receptor, T cell receptor, B cell receptor are all localized or recruited to the lipid rafts to initiate the signaling upon ligand binding[159, 176, 177]. Several classes of signaling proteins including receptor tyrosine kinases (RTKs), Src famiy kinases, G proteins and GTPases have been identified by biochemical fractionation and immunohistochemistry, mainly from macrovascular endothelial cells or tissues rich in endothelium such as lung[164]. Growth factor receptors such as PDGFR, EGFR, Flk-1/KDR together with receptors for EDG-1, uPAR, and G protein coupled receptors including bradykinin 2 Receptor (B2R) and endothelin receptor (ETA) are all reported to be partially localized in endothelial ceveolae[164]. Caveolin-1, the most characterized caveolin among three caveolin

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isoforms in mammalian cells, interacts with and modulates the functions of many signaling proteins in caveolae, including endothelial isoform of nitric oxide synthase (eNOS), Src family tyrosine kinases and Gα proteins. Caveolae and caveolin-1 coordinately regulate VEGF mediated endothelial proliferation, angiogenesis and permeability that requires NO produced by eNOS. Binding of caveolin-1 to eNOS inhibits eNOS activity as shown in Cav-1-/- mice a constitutive activation of eNOS[166, 178]. Recent report of Cav-1-/- mice showed an absence of caveolae together with uncontrolled endothelial cell proliferation and increased microvascular permeability[165, 166]. Moreover, si-RNA knock down of caveolin-1 in BAEC perturbs the Sphingosine 1 phosphate (S1p) and VEGF mediated Akt and Rac activation[179].

Recent advances in protein identification technology involving proteomics have permitted the identification of tens to thousands of proteins in the lipid microdomains. The protein components in lipid rafts from Jurkat T cells[180], neutrophils[181] and monocytes [182] or in caveolae/lipid rafts from Cos cells[183], Vero cells[184] and human endothelial cells (HUVEC)[185] have all been characterized by mass spectroscopy following the isolation of caveolae/lipid rafts as triton-insoluble, low density membrane fractions. However, the protein components of caveolae/lipid rafts in microvascular endothelial cells from retina have not been addressed yet.

5.2.3.3. n3-PUFA modification of caveolae/lipid rafts

Since n3-PUFA is readily attached to the sn-2 position of membrane phospholipids, incorporation of PUFA into membrane phospholipids will increase the fluidity of the membranes in general, which may influence the localization and function

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of membrane proteins. Inhibitory effects of n3-PUFA on T cells are reported to be primarily due to eicosanoid-independent effects of PUFA in vitro[186]. It has been proposed that n3-PUFA inhibits T cell activation by interfering with lipid raft signaling at the T cell receptor level[187]. Enrichment of T cells with n3-PUFA leads to changes in lipid composition of the lipid rafts which result in the displacement of Src family kinases Lck and adaptor protein LAT from the cytoplasmic leaflet of lipid rafts thus inhibiting T cell activation[188, 189]. N3-PUFA are incorporated into lipids of the cytoplasmic and exoplasmic leaflets of lipid rafts[187]. Moreover, dietary n3-PUFA treatment in vivo leads to a significant decrease in the sphingomyelin (SM) content of mouse T cell lipid rafts which may not only alters the exoplasmic membrane leaflets but also cytoplasmic leaflets[190]. The latest *in vivo* mice feeding study of Fan, YY demonstrated that dietary fish oil or highly purified $DHA_{22:6n3}$ are incorporated into the splenic T cells lipid rafts and soluble membrane phospholipids[190]. This results in a 30% decrease in rafts sphingomyelin content. N3-PUFA feeding attenuates the antibody induced PKCθ recruitment to the TCR complex thus the downstream NFκB, AP-1 activation and IL-2 production[191]. N3-PUFA also has been reported to alter lipid composition and protein localization of caveolae in mouse colon[192]. A 46% decrease in cholesterol level of caveolae was observed in n3-PUFA feeding compared with n6-PUFA. Also the caveolin-1 localization and the subdomain distribution of H-Ras and eNOS in caveolae were negatively modulated by n3-PUFA feeding[193].

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6. Retinal endothelial cell and fatty acid profile

Two tissues, retina and brain, are unique among other peripheral tissues due to a tight barrier that separates them from circulation and a unique fatty acid profile with one of the highest levels of LCPUFA (especially 20:4n6 and 22:6n3) in the body[194-198]. Liver has been shown to be a key site for biosynthesis of LCPUFAs, where they are distributed into plasma lipids and lipoproteins for transport and tissue uptake. LCPUFAs can be transported through choriocapilaries to the retinal-pigmented epithelial cells (RPE) (an outer blood-retinal barrier) to support the needs of the photoreceptors in the retina. Retinal vascular endothelial cells form another inner blood-retinal barrier, a metabolically active interface responsible for fatty acid uptake from blood and delivery to the retina. In addition to uptake and delivery, retinal endothelium can also actively remodel fatty acids through elongation and desaturation providing the retina with long chain highly unsaturated PUFA such as $DHA_{22:6n3}$ ([199] and our preliminary results). Indeed, in cultured primary bovine retinal endothelial cells, $DHA_{22:6n3}$ and $AA_{20:4n6}$ each represents approximately 8% and 10% of total fatty acids and the retroconversion of $DHA_{22:6n3}$ to $EPA_{20:5n3}$ is negligible, indicating a specificity of fatty acid composition and metabolism different from endothelial cells isolated from other vascular tissues ([198] and our unpublished data).

Although healthy retinas are relatively insensitive to changes in blood fatty acid profile (our preliminary data), in diabetic conditions, if hormonal control of retinal desaturases is altered, this could lead to a prominent change in retinal fatty acid profile. The retina selectively accumulates $DHA_{22:6n3}$, but not α-$LA_{18:3n3}$ and $EPA_{20:5n3}$, the

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precursors of $DHA_{22:6n3}$. Thus, in diabetes, a shift in plasma fatty acid profile from products to substrates would favor an increased accumulation of $LA_{18:2n6}$ without accumulation in its n3 counterpart, α-$LA_{18:3n3}$, and a decrease in $AA_{20:4n6}$ and $DHA_{22:6n3}$. Overall fatty acid composition is expected to shift toward more n3 deficient, n6 rich state. The experimental data on the anticipated diabetes-induced changes in retinal fatty acid profiles are very sparse. Retinal fatty acid profiles in diabetic subjects[77] and alloxan diabetic rats maintained for 116 days without insulin[200] were reported. These studies found increased level of $LA_{18:2n6}$ and reduced levels of $AA_{20:4n6}$ and $DHA_{22:6n3}$ in diabetic retina[200]. However, this study was not followed up later on and the time course of profile change and correlation between the fatty acid profile and development of retinopathy is still not known. Overall, n3-PUFA deficiency in the retina was shown to have detrimental effect on the visual acuity[201-203].

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7. Objective of Thesis

The early steps that lead to the inflammatory state in the pathogenesis of diabetic retinopathy are not completely understood. The disturbance of lipid metabolism in diabetes with an increase in $LA_{18:2n6}$ and decrease in long chain $AA_{20:4n6}$ and $DHA_{22:6n3}$ is of particular importance in the retina. The purpose of this dissertation is to investigate the effect of diabetic dyslipidemia especially the n6-PUFA/n3-PUFA ratio change in diabetes on inflammatory signaling using primary microvascular endothelial cells isolated from human retina.

Chapter II describes the proinflammatory aspect of n6-PUFA ($LA_{18:2n6}$ and $AA_{20:4n6}$). Chapter 3 analyzes the anti-inflammatory role of n3-PUFA especially ($DHA_{22:6n3}$) and its possible target PPARs on cytokine induced inflammatory signaling. Chapter IV details the characterization of protein components in a specialized highly lipid-ordered microdomain called caveolae/lipid rafts. The final chapter describes investigations into the anti-inflammatory mechanism of $DHA_{22:6n3}$ by its potential role in the modification of lipid composition in the caveolae/lipid rafts. Results of this work will add to our understanding of the role of dyslipidemia in the development of diabetic retinopathy and facilitate new treatments and preventive strategies.

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II. Dyslipidemia, but not hyperglycemia, induces inflammatory adhesion molecules in human retinal vascular endothelial cells

1. Abstract

Purpose: The initial determinants of retinal microvascular damage in diabetic retinopathy are not well understood, but are likely to be contributed by hyperglycemia and/or dyslipidemia. The purpose of this study was to examine the effect of fatty acids and hyperglycemia on human retinal vascular endothelial cells (hRVE) as means of mimicking diabetic metabolic disorders.

Methods: The expression of adhesion molecules in hRVE and human umbilical vein endothelial cells (HUVEC) was assayed by Western Blotting and confirmed by leukocyte adhesion assay. The mechanisms underlying the induction of adhesion molecules by fatty acids were further investigated by using cyclooxygenase (COX), lipoxygenase (LOX) and P450 monooxygenase (MOX) inhibitors.

Results: Treatment of hRVE cells with n6 polyunsaturated fatty acids (PUFA), 18:2,n6 and 20:4,n6, for up to 24 hrs, resulted in a significant induction of ICAM-1 and VCAM-1 protein levels. In contrast, treatment with high glucose (22 mM) for 24 hrs did not affect CAM expression. Induction of CAM by n6 PUFA was correlated with enhanced leukocyte binding to hRVE cells. The effect of n6 PUFA on ICAM-1 and VCAM-1 was blocked by an inhibitor of LOX, but not COX or MOX inhibitors. In contrast to hRVE cells, n6 PUFA did not induce ICAM-1 or VCAM-1 in HUVEC.

Conclusion: The data obtained in this study demonstrate that acute exposure to linoleic or arachidonic acid, but not hyperglycemia induces inflammatory adhesion molecules expression in a LOX-dependent manner in microvascular hRVE cells, but not in HUVEC. These results are consistent with the emerging hypothesis recognizing early stage diabetic retinopathy as a low-grade chronic inflammatory disease.

2. Introduction

Despite the progress made in the last decade in the understanding of the molecular mechanisms of diabetic retinopathy, the disease is still neither preventable nor curable. Diabetic retinopathy is characterized by capillary occlusions, microaneurysms, selective loss of intramural pericytes, acellular capillaries, hypertrophy of the basement membrane, and finally, angiogenesis and neovascularization. These morphological and pathophysiological changes occur late in the disease. The initial determinants of retinal microvascular damage are not well understood. Recently very early stage diabetic retinopathy was recognized as a low-grade chronic inflammatory condition. Support for this notion is based on the finding that leukocytes (including monocytes, neutrophils and some lymphocytes) attach and transmigrate the endothelium in both experimental and human diabetic retinopathy[1-7]. Adhesion molecules, especially ICAM-1 and VCAM-1 are involved in leukocyte attachment and transmigration[4-8]. ICAM-1 is a member of the immunoglobulin superfamily of adhesion molecules whose ligands include leukocyte β2-integrins CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1). Vascular endothelial ICAM-1 is associated with adhesion and transmigration of leukocytes in the retina[2, 3] and in other vascular systems. Importantly, leukocyte infiltration and expression of retinal vascular ICAM-1 coincide with many of the pathological lesions in diabetic retinopathy[3, 6].

While the mechanisms leading to ICAM-1 induction in diabetic microvessels are not known, carbohydrate and lipid metabolic disorders are likely to be key causative factors in this process. Hyperglycemia and dyslipidemia are two major metabolic

disorders of diabetes mellitus. Despite considerable progress made in understanding of hyperglycemia-induced pathology over the last decade, the link between diabetic metabolic disorders and retinopathy still eludes us. The role of diabetic dyslipidemia in the development of microvascular complications has received much less attention. Insulin controls an array of enzymes and signaling molecules involved in lipogenesis and lipid metabolism. The insulin resistance of Type 2 diabetes is associated with increased plasma FFA levels, triglycerides, LDL cholesterol and a decrease in HDL cholesterol[9-15]. FFA levels parallel the blood glucose level in diabetes and FFAs are often considered an indicator of the severity of the diabetic state[16]. In Type 1 diabetes, low portal insulin levels cause a reduction in delta-6-, delta-5- and delta-9-desaturases in the liver and a corresponding change in FFA profile resulting, for instance, in an increase in linoleic acid and in n6 PUFA/n3 PUFA ratio[10, 17-19].

Clinical data support the idea that dyslipidemia could be critical factor for the development of diabetic retinopathy. Thus, a recent clinical trial controlling diabetic dyslipidemia with simvastatin, a HMG-CoA reductase inhibitor resulted in significant retardation of the progression of diabetic retinopathy[20-21]. The data from another population-based study suggested the association of retinopathy with cardiovascular disease and elevated plasma LDL cholesterol[22]. The Early Treatment Diabetic Retinopathy Study demonstrated that higher levels of serum lipids were associated with an increased risk of developing hard exudates in the macula and visual loss[23]. Randomized controlled clinical trials are currently in progress to examine whether lipid lowering agents will reduce the risk of incidence and progression of diabetic retinopathy[22].

Recent progress in lipid research highlighted several pathways through which FFA can exert cellular and functional alterations. These include changes in membrane composition and function as well as in the regulation of gene expression and protein modification[24, 25]. Unsaturated fatty acids are substrates for oxygenases such as COX, LOX and MOX, as well as non-enzymatic oxidation. Fatty acids oxidation leads to generation of a variety of bioactive lipids such as eicosanoids, lipid hydroperoxides and isoprostanes[26-31]. All of these pathways are likely to be relevant to endothelial cells.

The role of fatty acids as the inflammatory agents leading to diabetic retinopathy has not been studied in detail and could represent a missing link between diabetes, dyslipidemia, and microvascular damage. The current study was designed to address this question by analysis of the effect of fatty acids on human retinal vascular endothelial (hRVE) cells. Our data strongly support the hypothesis that elevated plasma fatty acids induce an increase in inflammatory adhesion molecules leading to retinal vascular inflammation involving leukocyte attachment and transmigration.

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); and culture dishes and flasks from Corning. Commonly used chemicals and reagents and chemicals were from Sigma/Aldrich Chemical Co. (St. Louis, MO). TNFα and IL-1β were from R&D Systems (Minneapolis, MN). PMA was from Sigma.

3.2. Cell culture and fatty acid treatments

The present study utilized primary cultures of hRVE cells obtained from 3 separate donors. hRVE cells were prepared as previously described and maintained[32-35] in growth medium consisting of DMEM/F12 (Invitrogen, Carlsbad, CA), 5.5 mM glucose, 10% fetal bovine serum (Invitrogen), endothelial cell growth supplement (Upstate Biotechnologies, Inc., Lake Placid, NY), insulin/transferin/selenium mix (Sigma) and antibiotic/antimycotic solution (Invitrogen). The cells were maintained at 37 °C in 5% CO_2 in a humidified cell culture incubator and passaged at a density of 40,000-100,000 cells/cm^2 in gelatin-coated 75 cm^2 flasks. Passaged cells were plated to yield near-confluent cultures at the end of the experiment. The freshly plated cells were allowed to attach in standard growth medium for at least 72 h. For experimental treatments the cells were transferred to serum-free medium for 18-24 h before addition of the stimulatory agents. Treatment of hRVE cells with fatty acids was performed as follows. Fatty acids stocks were prepared by adding fatty acids (NuCheck Prep. Inc.,

Elysian, MN) to charcoal treated, solvent extracted, fatty acid-free bovine serum albumin (Serologicals Inc., Norcross, GA) in serum-free media to the final concentration of 100 mM fatty acid, 60 μM BSA, stabilized with vitamin E (400 μM) and BHT (0.04%) and neutralized with NaOH, as described previously[36]. The fatty acid stock solutions were diluted into serum-free medium to give fatty acid concentrations of 10-100 μM with corresponding BSA concentrations of 2-20 μM. The fatty acid-to-albumin molar ratio was 5:1[36]. Cells were incubated for the times indicated in the Results. Equivalent amounts of BSA alone were added to control plates. Inhibitors of COX, LOX (Cayman Chemical, Ann Arbor, MI) and MOX (Sigma) were added to the cells at the time of addition of the fatty acids. For hyperglycemia experiments the cells were incubated in normal (5.5 mM) or high (22 mM) glucose for 24 h.

3.3. Electrophoresis and immunoblotting

hRVE cells were grown in 6-cm plates in experimental media for up to 24 hrs. Each plate was rinsed twice with 3 ml of ice-cold phosphate-buffered saline (PBS) containing 130 mM NaCl, 8.2 mM Na_2HPO_4, and 1.8 mM NaH_2PO_4 (pH 7.4). The cells were harvested in 100-300 μl of the lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol) with the freshly added protease and phosphatase inhibitors (1 mM sodium orthovanadate, 0.15 U/ml aprotinin, and 100 μg/ml PMSF). Homogenates were centrifuged at 13,000rpm for 15 min at 4°C. Proteins were fractionated by electrophoresis on SDS-polyacrylamide (10%) mini-gels. The separated proteins were electrophoretically transferred to nitrocellulose (BioRad, Hercules, CA) and blocked for 60 min at room temperature in Tris-Buffered Saline (TBS)

(130 mM NaCl, 100 mM Tris/HCl, pH 7.5) containing 5% powdered milk and 0.1% Tween-20. The membranes were then probed overnight at 4°C in a blocking buffer containing antibody against VCAM-1, ICAM-1 or E-selectin (rabbit polyclonal antibodies, Santa Cruz Biotechnology, Santa Cruz, CA), followed by anti-rabbit horseradish peroxidase conjugate (BioRad, Hercules, CA). Immunoreactive bands were visualized by enhanced chemiluminescence using an ECL kit (Amersham, Piscataway, NJ). Blots were quantitated by scanning densitometry using ImageJ software (version 1.29).

3.4. Leukocyte adhesion assay

U937 cells were labeled with 2 μM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Molecular Probes, Eugene, OR) at 37°C for 30 min, washed twice with fresh RPMI-1640 media with 10% fetal bovine serum and 1 mM L-glutamine. The fluorescent U937 cells were then concentrated to 10^6 cells/ml by centrifugation and resuspended in hRVE culture medium without fetal bovine serum. For the adhesion assay, control or treated hRVE cells in 6 well plates were washed with PBS (23°C) followed by addition of 10^6 fluorescent U937 cells to each well. The cells were then incubated at 23°C on a rotating plate for 2 hrs. The hRVE cells were carefully washed with PBS by decanting and aspirating twice, lightly fixed with 0.5% paraformaldehyde for 15 mins at 23°C and washed with PBS two more times. Adherent florescent U937 cells were directly counted with a fluorescent microscope. For each experiment the total number of fluorescent cells was obtained from 12 to 14 random fields containing a full monolayer of hRVE cells.

3.5. Statistical Analysis

Data were expressed as mean±SEM. Repeated-measures ANOVA was used for comparison of multiple values obtained from the same plate, factorial ANOVA was used for comparing data obtained from 2 independent samples. The Bonferroni procedure was used to control type I error. Significance was established at $P<0.05$.

4. Results

4.1. Polyunsaturated n6 fatty acids induce inflammatory adhesion molecule expression in hRVE cells

We have tested the hypothesis that fatty acids typically found in blood affect the expression of adhesion molecules in hRVE cells. Accordingly, hRVE cells were exposed to 100 μM of BSA bound fatty acids (5 mol fatty acid to 1 mol of BSA) for 12 and 24 hrs followed by immunoblot analysis of ICAM-1 and VCAM-1 expression. Treatment with BSA alone was used as a control. Saturated palmitic (16:0) acid and n3 PUFA docosahexaenoic (22:6,n3) acid treatment had no effect on either VCAM-1 or ICAM-1 expression in hRVE cells (Fig. 1A and B). In contrast, treatment with n6 PUFA linoleic (18:2,n6) or arachidonic (20:4,n6) acid increased VCAM-1 (Fig. 1A and C) and ICAM-1 (Fig. 1A) expression. ICAM-1 and VCAM-1 expression increased after 12 hrs and was maximally induced after 24 h of fatty acid treatment (Fig. 1A). The induction of VCAM-1 was seen with as little as 10 μM linoleic acid and arachidonic acid (Fig. 1C). While ICAM-1 was detectable with no fatty acid treatment, VCAM-1 expression was usually below detectable levels (Fig. 1A, lane 1). The maximal fold induction of VCAM-1 (4-fold) was greater than for ICAM-1 (3-fold) (Fig. 1A, lanes 2, 6 and 8 and B). Fig. 1B shows a quantitation of the results obtained from 3 separate donor cells at the 4 to 6 passage stage.

4.2. Hyperglycemia does not affect CAM expression in hRVE cells

As hyperglycemia is a key metabolic disorder of diabetes, we next examined the effect of high glucose on CAM expression in hRVE cells. In contrast to fatty acid treatment, exposure to high glucose (22 mM) for 24 h did not affect ICAM-1 and VCAM-1 expression (Fig. 2A). As a control IL1-β induced VCAM-1 expression under euglycemic conditions (Fig. 2A).

4.3. Induction of inflammatory adhesion molecules by cytokines and PMA in hRVE cells

Since little is known about CAM expression in hRVE cells, we compared the effects of fatty acids with those obtained following treatment with inflammatory cytokines (TNFα, IL-1β) and the PKC activator, PMA (Fig. 2B). Both cytokines induced strong expression of the ICAM-1 and VCAM-1 molecules in the time range of 6 to 24 h. In contrast, PMA treatment had little effect on CAM expression in the time periods studied. Interestingly, the expression of E-selectin in hRVE cells was not affected by these stimulants (Fig. 2B).

4.4. HUVEC do not respond to fatty acid treatment

HUVEC are a primary cell culture derived from umbilical cord veins widely used as a model for human vascular endothelial cells. Like the hRVE cells, treatment of HUVEC cells with TNFα and IL-1β increased ICAM-1 and VCAM-1 expression (Fig. 3A, lanes 3 and 7). However, in sharp contrast to the hRVE cells, treatment of HUVECs

with fatty acids under the same conditions as used for hRVE cells resulted in no induction of VCAM-1 or ICAM-1 (Fig. 3B). The significant differential sensitivity to fatty acids may represent a fundamental difference between the responses of hRVE cells and other endothelial cells to inflammatory stimuli.

4.5. Inhibition of fatty acid oxidation suppresses fatty acid-induced adhesion molecule expression in hRVE cells

Experiments in progress have shown that radioactive tracer labeled fatty acids taken up from the media by hRVE cells entered a number of metabolic pathways, including esterification and elongation, leading to changes in the intracellular neutral and polar lipid pools. A high percentage of fatty acids remained in NEFA pool providing a possible substrate for oxygenases (data not shown). Since both linoleic and arachidonic acids are precursors of inflammatory mediators such as leukotrienes, thromboxanes and prostaglandins, a possible mechanism for induction of adhesion molecules by fatty acids involves lipid oxidation. This hypothesis was addressed by use of specific inhibitors of the COX, LOX and MOX pathways. Nordihydroguaiaretic acid (NDGA, a general LOX inhibitor; IC_{50} = 3-5 μM[37, 38]) at 5 μM attenuated 18:2,n6 and 20:4,n6 induced ICAM-1 (not shown) and VCAM-1 (Fig. 4A, lane 3 compared to 2) expression by >80%. Higher doses of NDGA completely inhibited ICAM-1 (not shown) and VCAM induction (Fig. 4A, lane 4, 5 and 6). In contrast to NDGA, flurbiprofen, a general COX inhibitor (IC_{50} = 0.04 μM, COX-1 and 0.51 μM, COX-2[39]), at 5 to 10 μM had no effect on fatty acid-induced ICAM-1 (not shown) and VCAM-1 expression (Fig. 4B, lanes 5 and 6 compared to 2). Only at higher, non-specific, concentrations (50-100 μM) did

flurbiprofen inhibit the response (lanes 7 and 8). The specific COX-2 inhibitor NS-398 (IC50=1.77μM)[39] had no effect on the fatty acid-mediated induction of VCAM-1 (Fig. 4B, lanes 3 and 4). We also tested the effect of the MOX P450 inhibitor, 1-ABT, on fatty acid induced adhesion molecule expression. 1-ABT (500 μM) had only a small effect on fatty acid induced ICAM-1 (not shown) and VCAM-1 expression (Fig. 4C). Taken together the data strongly implicate the LOX pathway as a requirement for 18:2,n6 and 20:4,n6-mediated induction of adhesion molecules in hRVE cells.

4.6. Leukocyte adhesion correlates with fatty acid induction of inflammatory CAMs

A leukocyte adhesion assay was used to confirm that fatty acid induced CAMs were functionally expressed (Fig. 5). Human U937 cells with monocytic properties[40] were added to fatty acid, high glucose, or IL-1β treated and untreated monolayers of hRVE cells. Treatment of hRVE cells with 20:4,n6 and 18:2,n6, or with IL-1β resulted in a significant increase of adherent cells as compared to BSA control. In contrast, high glucose or 16:0 treatment did not significantly increase the number of adherent cells. High glucose and 16:0 do not induce CAM (Fig. 1 and 2). The dramatic increase in adhesion correlates well with increased CAM expression observed by immunoblotting.

Fig.1

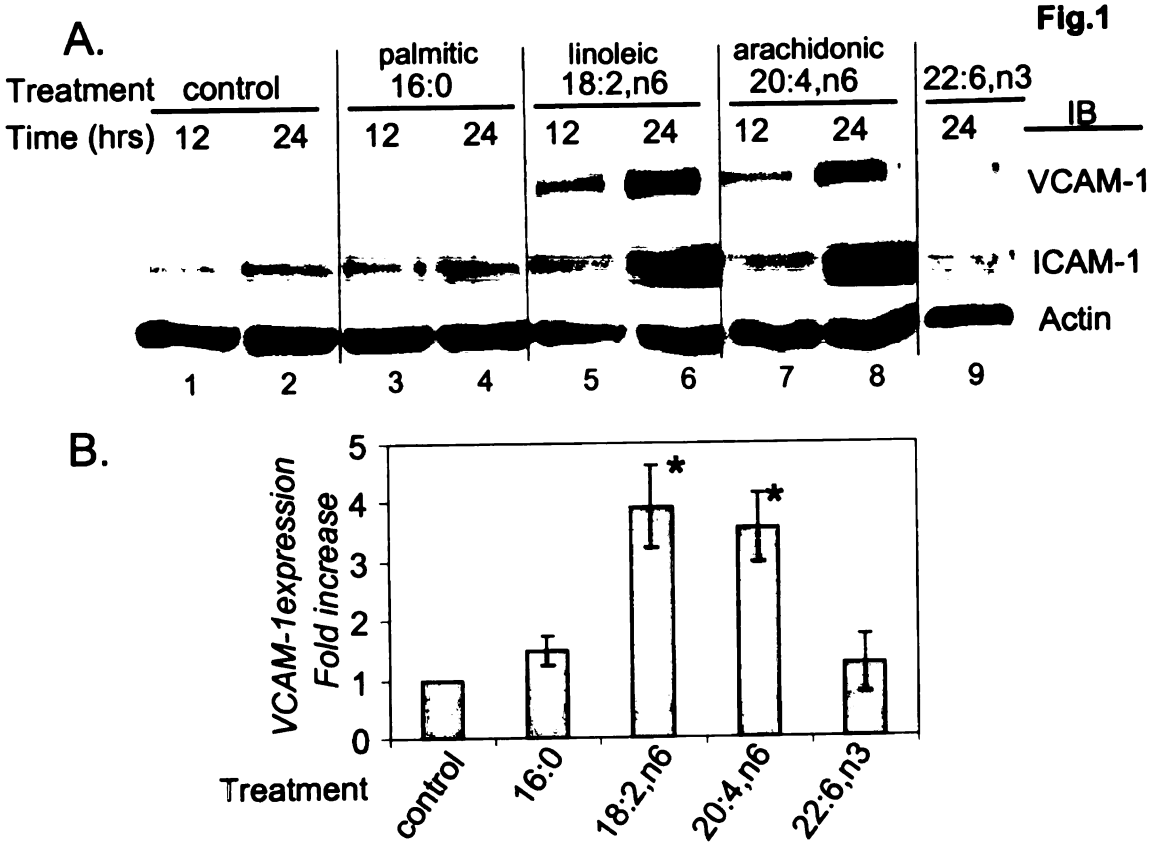

A.
Treatment control
palmitic 16:0
linoleic 18:2,n6
arachidonic 20:4,n6
22:6,n3
Time (hrs) 12 24 12 24 12 24 12 24 24
IB
VCAM-1
ICAM-1
Actin
1 2 3 4 5 6 7 8 9
B.
VCAM-1expression Fold increase
0 1 2 3 4 5
*
*
Treatment
control
16:0
18:2,n6
20:4,n6
22:6,n3
C.
Treatment (μM) 0 10 50 100
18:2,n6
VCAM-1
20:4,n6
VCAM-1

Fig. 1. Induction of endothelial cell adhesion molecules by free fatty acids in hRVE cells. (A) Treatment of hRVE cells with BSA control or with 100 μM of the free fatty acids (16:0, 18:2n6, 20:4n6 and 22:6n3) was performed for the indicated time before analysis of cell lysates for VCAM-1 and ICAM-1 expression by immunoblot (IB). (B) Quantitative compilation of the data on VCAM-1 induction in hRVE cells after 24 hrs of treatment with 100 μM of free fatty acids. The results are obtained from the cells isolated from three independent donors. $*P<0.05$ compared to BSA control. (C) Induction of VCAM-1 in hRVE cells after treatment with different doses of free fatty acids (18:2,n6 and 20:4,n6). A and C are representative results from one donor. Equal amounts of protein were added to each lane and confirmed by probing for actin (representative loading control shown in A). Results are representative samples from greater than three independent experiments.

Fig.2

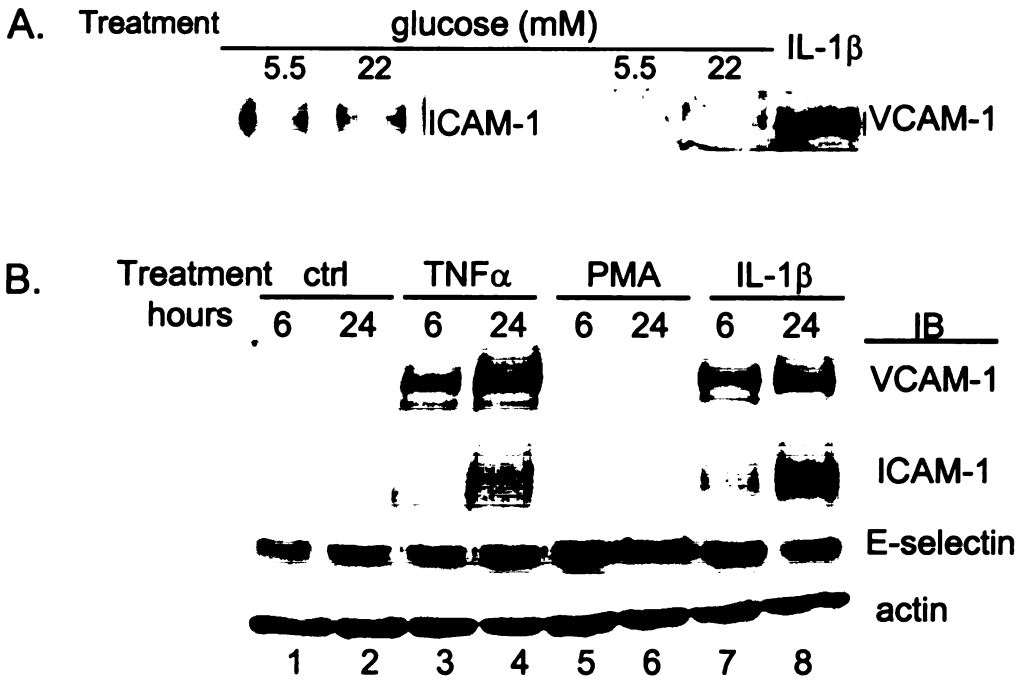


Fig. 2. Evaluation of cell adhesion molecule expression after treatment of hRVE cells with hyperglycemic conditions, cytokines and PMA. The induction of adhesion molecules was assessed by immunoblot analysis (IB) after treatment of cells for the indicated times with each potential stimulant. (A) hRVE cells cells were grown in euglycemic (5.5 mM) or hyperglycemic (22 mM) conditions for 24 hours before analysis. IL1-β (5 ng/ml) was used as a positive control. (B) hRVE cells were stimulated for the indicated times with TNFα (20 ng/ml), PMA (10 ng/ml) or IL-1β (5 ng/ml) followed by immunoblot. For all experiments equal amounts of protein were loaded to each lane and loading was confirmed by probing for actin (shown in B).

Fig.3

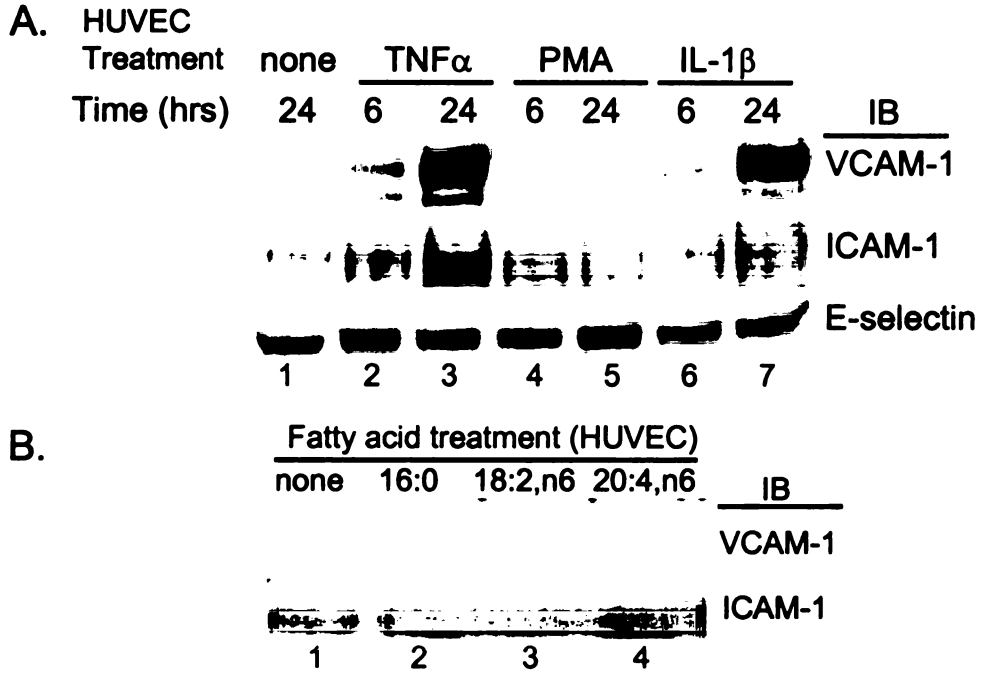


Fig. 3. Fatty acid treatment of HUVEC cells fails to induce endothelial cell adhesion molecule expression. (A) HUVEC cells were treated with TNFα (20 ng/ml), PMA (10 ng/ml) and IL-1β (1 ng/ml) for the indicated times followed by an analysis of VCAM-1, ICAM-1 and E-selectin expression. (B) HUVEC cells were treated with BSA control (none) and with 100 μM fatty acids for 24 h time before analysis of cell lysates for VCAM-1 and ICAM-1 expression. No VCAM-1 was detected and no induction of ICAM-1 was observed in greater than three experiments. Equal amounts of protein were loaded to each lane.

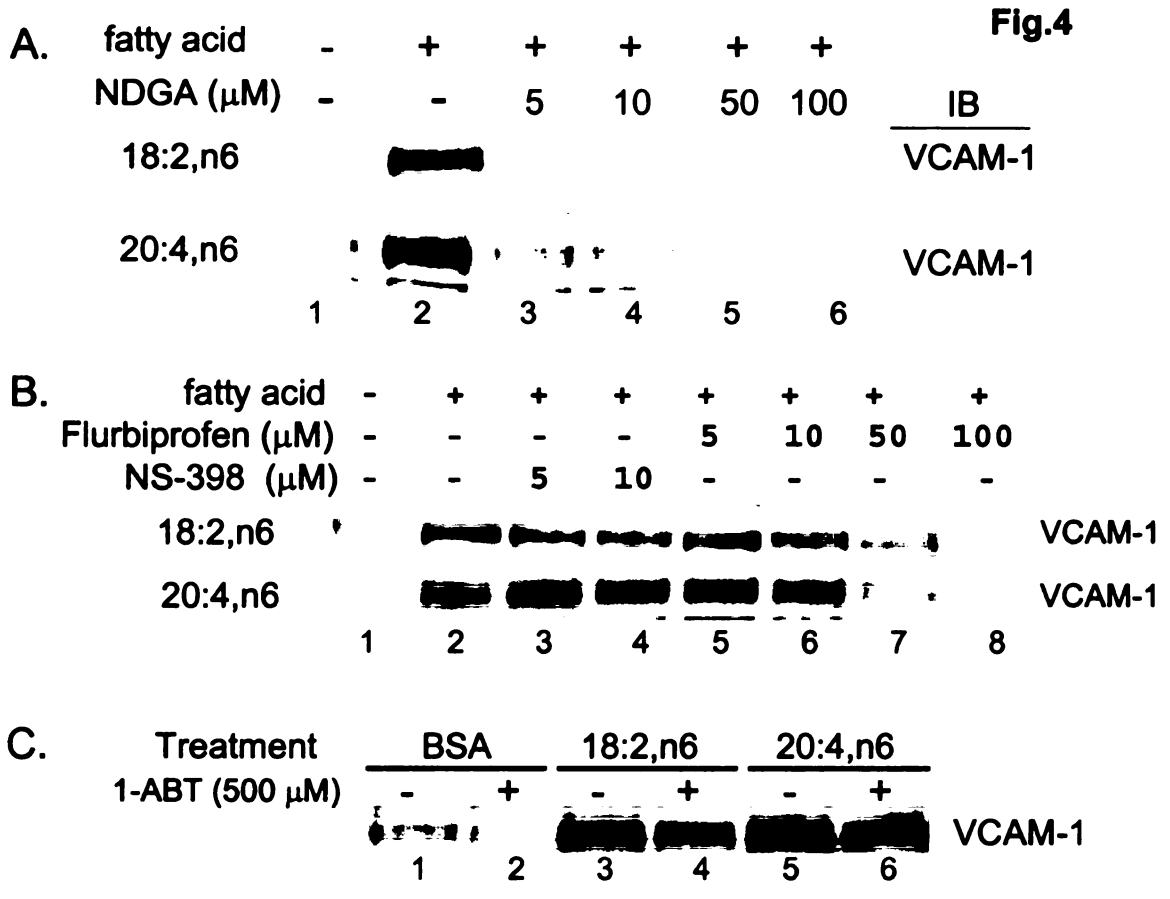
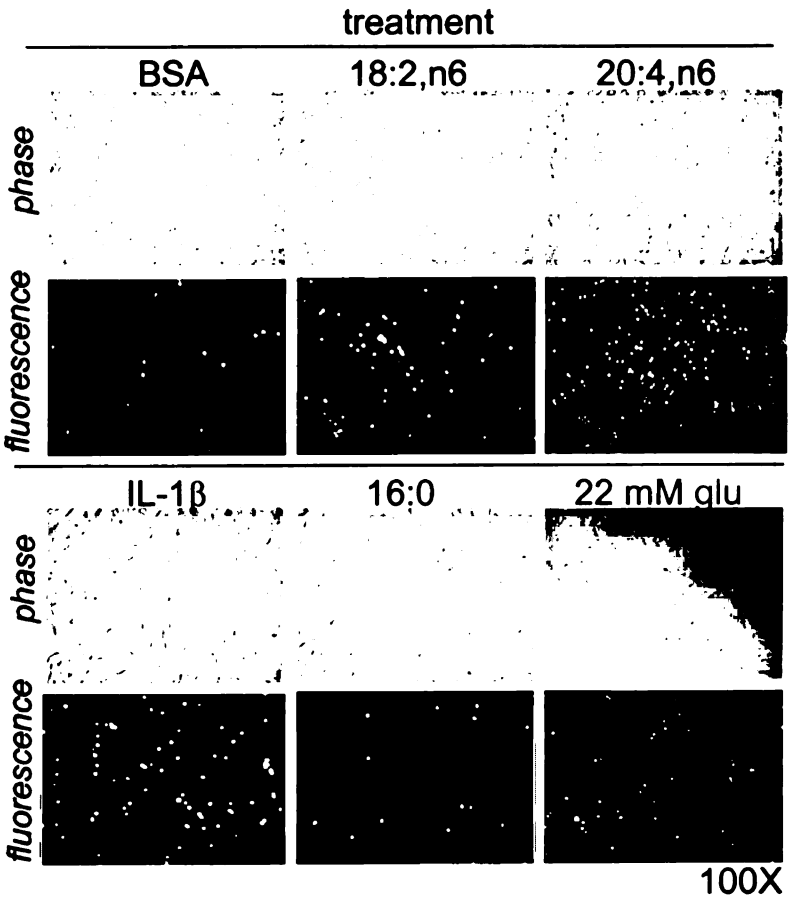


Fig. 4. Induction of cell adhesion molecule expression by fatty acids is inhibited by LOX, but not COX and MOX inhibitors. (A) hRVE cells were treated with 18:2,n6 or 20:4,n6 (100 μM) for 24 h in the presence of increasing amounts of the inhibitor, NDGA, followed by lysis and immunoblot analysis of VCAM-1. (B) hRVE cells were treated with 18:2,n6 or 20:4,n6 for 24h in the presence of increasing amounts of either Flurbiprofen or NS-398, followed by lysis and immunoblot analysis of VCAM-1. (C) hRVE cells were treated with 18:2,n6 or 20:4,n6 for 24 h in the presence of 500 μM 1-ABT followed by lysis and immunoblot analysis of VCAM-1. See methods for details of the treatment. Equal amounts of protein were added to each lane.

Fig.5

A.


treatment
BSA
18:2,n6
20:4,n6
phase
fluorescence
IL-1β
16:0
22 mM glu
phase
fluorescence
100X

B.

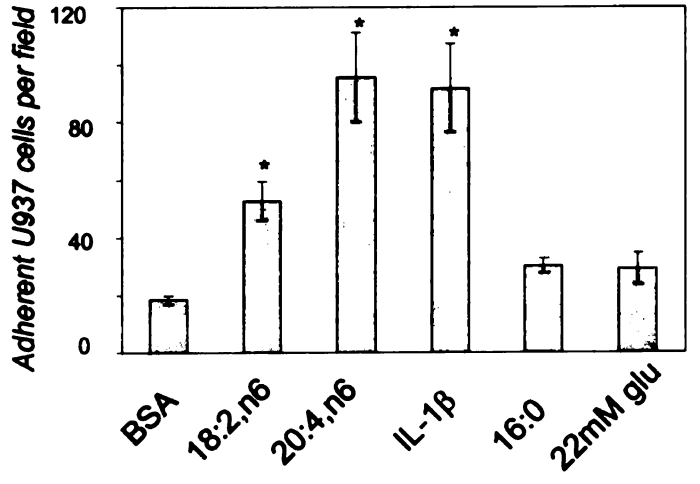

Adherent U937 cells per field
120
80
40
0
BSA
18:2,n6
20:4,n6
IL-1β
16:0
22mM glu

Fig. 5. Increased adhesion of leukocytes after induction of cell adhesion molecule expression by fatty acids. hRVE cells were treated with either BSA; 18:2,n6; 20:4,n6; IL-1β; 16:0; or 22 mM glucose for 24 hours followed by addition of fluorescent-tagged U937 cells (see Methods). **(A)** The number of adherent U937 cells was determined by fluorescent microscopy. A representative fluorescent and phase contrast field is shown for different treatments (all photographs are at 100X). **(B)** The number of fluorescent U937 cells from randomly selected microscope fields exhibiting a monolayer of hRVE cells was determined. The mean and SEM was determined for 12 to 14 fields for each treatment from four independent experiments with hRVE cells from three different donors. $^{*}P<0.05$ compared to BSA control.

5. Discussion

Retinal microvascular damage in early stage diabetic retinopathy has been proposed to be the result of a low-grade chronic inflammatory condition involving endothelial attachment and transmigration of leukocytes[1-3]. Support for this view is provided by the finding that high doses of aspirin are associated with decreased severity of diabetic retinopathy in humans[41] and that a marked increase in leukocyte density and retinal vascular ICAM-1 immunoreactivity was found in human eyes with diabetic retinopathy[6]. In addition, it was recently demonstrated in a dog model that aspirin prevented certain classic histopathological features of diabetic retinopathy, including acellular capillary formation, retinal haemorrhage development, and an indicator of cell degeneratrion, capillary sudanophilia[42]. In rodent models anti-inflammatory agents suppressed diabetic retinal ICAM-1 expression, leukocyte adhesion, and blood-retinal breakdown[5]. However, the molecular steps linking the diabetic state to retinal ICAM-1 expression are not well understood. These causative events could be different in humans compared to animal models. As early inflammatory changes in human eyes do not have any clinical manifestations, human primary cell culture provides an important model to study diabetes-induced low-grade inflammation in human retina.

To investigate the mechanism(s) leading to inflammation in human microvessels we performed experiments with primary human retinal vascular endothelial cells. We first considered hyperglycemia and dyslipidemia, which are two major metabolic disorders of diabetes, as likely contributors to the pathogenesis of retinopathy. We have found no evidence to support a direct causative relationship between hyperglycemia and

inflammatory effects in hRVE cells. Instead, our studies point to dyslipidemia as an important contributor to inflammatory events.

Diabetic dyslipidemia is the result of an imbalance of the complex regulation of fatty acid uptake, metabolism, release by adipocytes and clearance from circulation. Insulin inhibits adipocyte hormone-sensitive lipase and activates lipoprotein lipase[43, 44]. In liver, insulin stimulates conversion of fatty acids to triglycerides followed by secretion as VLDL, as well as the induction of delta-5, delta-6 and delta-9 desaturases[9-12, 14, 17-19, 44-46]. Thus, insulin resistance in Type 2 diabetes and low portal insulin levels in Type 1 diabetes would be predicted to have a profound effect on plasma fatty acid levels and composition. Indeed, Type 2 diabetes is characterized by an elevation of blood levels of cholesterol, esterified and non-esterified fatty acids[12, 14, 15, 45, 47-50], and Type 1 diabetes causes marked changes in FFA profile with an increase in n6 PUFA/n3 PUFA ratio[17]. In our experiments we modeled dyslipidemia by exposure of hRVE cells to n6 PUFA, linoleic and arachidonic acids. Treatment of hRVE cells with either linoleic or arachidonic acid led to a robust increase in VCAM-1 and ICAM-1 expression. The effect was specific for these n6 PUFA, as other fatty acids tested, such as saturated palmitic (16:0), and n3 PUFA docosahexaenoic (22:6, n3) failed to yield a response. Human plasma contains substantial amounts of linoleic (30%) and arachidonic (8%) acid in the triglyceride and FFA pools[51]. As total FFA levels in diabetes are $\geq$ 600 μM[48, 49], the concentrations of FFAs used in this study (100 μM) are comparable to the concentrations expected in diabetic patients.

Both linoleic and arachidonic acids are precursors of inflammatory mediators including leukotrienes, thromboxanes and prostaglandins, as well as other bioactive lipid mediators, such as hydroxyl- and epoxy fatty acids. Our inhibitor studies indicate that the lipoxygenase, but not the cyclooxygenase or P450 monooxygenase pathways may be important for the fatty acid-mediated induction of adhesion molecules in hRVE cells. This conclusion is based on the fact that a LOX inhibitor (NGDA) at specific concentrations was effective at blocking the PUFA-mediated induction of CAM expression. LOXs are a diverse family of nonheme ferroproteins that catalyze the hydroperoxidation of polyunsaturated fatty acids. Thus far, six LOXs have been identified in humans: 12-LOX (platelet type), 12(R)-LOX, 15-LOX-1, 15-LOX-2, e-LOX-3, and 5-LOX[52]. LOX products, such as the hydroperoxyeicosatetraenoic acids (HPETE), hydroxyeicosatetraenoic acids (HETE) and their metabolites the leukotrienes, play a role in inflammation, especially in modulating cell-cell interactions in human aortic endothelial cells[53]. 12-LOX activity and expression were highly increased in a diabetic pig model[20]. With regard to the LOX pathway involving 18:2,n6 and 20:4,n6 mediated induction of CAMs, it is not clear if the exogenous fatty acid, *per se*, is the substrate for this reaction or whether exogenous fatty acids stimulate other mechanisms to generate a substrate for LOX action. Such mechanisms might involve activation of phospholipase A2 or membrane remodeling resulting in release of substrates for LOX action. How LOX products lead to CAM expression is also unknown. Both detailed fatty acid metabolism and signaling studies will be required to define the metabolic pathway involved in PUFA regulation of CAMs. Among the known factors in the transcriptional regulation of VCAM-1 and ICAM-1 by inflammatory cytokines are NFκB, interferon

regulatory factor (IRF)-1, Sp1 and others[54-57]. How these known transcriptional regulators contribute to fatty acid stimulation is currently under investigation.

ICAM-1 and VCAM-1 play an important part in the rolling and attachment of leukocytes to endothelial cells and in normal hemeostatic processes. VCAM-1 is not generally constitutively expressed on endothelial cells and is induced significantly following treatment with inflammatory ligands, which include LPS, TNFα and IL-1β. Induction of VCAM-1 is a critical event in the adhesion and diapedesis of leukocytes resulting in localized inflammation. Our results suggest that fatty acids may also be an important component of the inflammatory process. Although ICAM-1 is constitutively expressed on hRVE cells, n6-PUFA also promoted a greater level of ICAM-1 expression. Vascular endothelial ICAM-1 is associated with adhesion and transmigration of leukocytes in the retina[2, 3] and in other vascular systems. Importantly, leukocyte infiltration and expression of retinal vascular ICAM-1 coincide with many of the pathological lesions in diabetic retinopathy[3, 6]. In our study the physiological relevance of the induction of CAMs was confirmed by performing adhesion assays using hRVE cells and fluorescent–tagged U937 cells. U937 cells were derived from a human histiocytic lymphoma and retain properties of monocytic cells[40]. The adhesion of U937 cells to vascular endothelial cells is primarily dependent on $\alpha_4\beta_1$ integrin interacting with endothelial VCAM-1[58, 59]. The fact that fatty acid treatment induced a large increase in the number of adherent U937 cells strongly supports the notion that 18:2,n6 and 20:4,n6 play an important role in microvascular inflammation.

A significant observation of our study is the finding that while hRVE cells were sensitive to n6-PUFA augmentation of CAM, these same n6-PUFAs did not induce CAM levels in HUVEC cells. However, both hRVE and HUVEC cells were fully capable of producing VCAM-1 and ICAM-1 after treatment with cytokines such as TNFα or IL-1β. HUVEC cells are a primary cell culture derived from umbilical cord veins and represent a macrovascular system. It will be important to determine whether other microvascular and aortic macrovascular cells exhibit higher sensitivity to fatty acids compared to umbilical vein endothelial cells.

In conclusion, our data suggest that diabetic dyslipidemea serves as inflammatory stimulus to initiate and contribute to microvascular complications. This model includes the increase in total lipid in Type 2 diabetes and the shift in fatty acid profile with an increase in n6-PUFA in Type 1 diabetes. N6-PUFAs are known substrates of LOX (and COX) pathways that lead to production of an array of oxidized lipids and bioactive metabolites. Based on our results we propose that chronic exposure of hRVE cells to elevated n6-PUFA associated with the diabetic condition results in longstanding chronic inflammation that gradually progresses to retinopathy.

Note: This part of research has been published at Invest Ophthalmol Vis Sci. 2003 Nov;44(11):5016-22.

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III. Anti-Inflammatory Effect of Docosahexaenoic Acid ($DHA_{22:6n3}$) and Peroxisome Proliferator-activated Receptors (PPARs) on Cytokine Induced Adhesion Molecules Expression in Human Retinal Vascular Endothelial Cells

1. Abstract

Early stage diabetic retinopathy has been recognized as a low-grade chronic inflammatory condition. As such, it is characterized by an increase in inflammatory cytokines including TNFα, IL-1β and $VEGF_{165}$. Diabetes induces changes in lipid metabolism that result in a decrease in the principal n3-polyunsaturated fatty acid (PUFA) in the retina, docosahexaenoic acid ($DHA_{22:6n3}$) in diabetic eyes. $DHA_{22:6n3}$ has been shown to have pronounced anti-inflammatory effect in several inflammatory models. A decrease in $DHA_{22:6n3}$ in the face of increased cytokine production would be expected to further promote inflammatory response in the retinal vascular endothelial cells. The effects of cytokines and $DHA_{22:6n3}$ on inflammatory response in human retinal vascular endothelial cells (hRVE) were not addressed. We report herein that $VEGF_{165}$, TNFα and IL-1β caused significant induction of ICAM-1 and VCAM-1 expression in hRVE cells. Pre-treatment of the cells with 100 μM of BSA-bound $DHA_{22:6n3}$ for 24 hours remarkably inhibited cytokine-induced ICAM-1 and VCAM-1 expression compared to BSA (carrier control) or palmitate (lipid control) treated cells. All three cytokines (IL-1β, TNFα and $VEGF_{165}$) induced NFκB binding to the VCAM-1 promoter in hRVE cells by activating specific NFκB isoforms: p65 and p50. $DHA_{22:6n3}$ pretreatment inhibited cytokines induced NFκB binding to the VCAM-1 promoter by about 25% with IL-1β, and by 40%

with $VEGF_{165}$ respectively. Moreover, $DHA_{22:6n3}$ diminished IL-1β induced IκBα phosphorylation thus preventing IκBα degradation compared to palmitate treated control cells. We further addressed if $DHA_{22:6n3}$ exerted its anti-inflammatory role through activation of the nuclear receptor PPARs. Among three PPAR isoforms (α, β and γ) expressed in hRVE cells, only specific PPARα agonists WY14,643 and fenofibrate downregulated $VEGF_{165}$ and TNFα induced VCAM-1 expression. $DHA_{22:6n3}$ as well as other fatty acids all activated PPARα in hRVE cells to a similar degree. These data suggest that both $DHA_{22:6n3}$ and activation of PPARα can potently suppress proinflammatory cytokine-induced adhesion molecules expression in hRVE cells. Whether activation of PPARα is the principal pathway that $DHA_{22:6n3}$ acts through to inhibit proinflammatory cytokine signaling in hRVE is discussed.

2. Introduction

Diabetic retinopathy (DR) is a leading cause of blindness in adults[1, 2]. The early stage of DR has recently been recognized as a result of chronic inflammatory conditions involving attachment and transmigration of leukocytes to the retinal microvasculature[3-5]. Several inflammatory pathways are activated at the early stage of diabetic retinopathy. The pro-inflammatory cytokines TNFα[6-9] and IL-1β[10] are found to be elevated in the extracellular matrix, endothelium, vessel walls and vitreous of eyes with proliferative diabetic retinopathy and in the retinas of rats with 2 months of diabetes. Inhibition of TNFα and IL-1β signaling with a TNFα receptor/Fc construct[4] or ILRa[11] significantly reduced leukocyte adhesion and endothelial cell (EC) injuries. These data suggests possible roles of TNFα and IL-1β and receptors mediated signaling pathway in inducing EC injury that contributes to the development of diabetic retinopathy.

Another mediator, vascular endothelial cell growth factor (VEGF), has also been strongly implicated in the pathogenesis of both background and proliferative diabetic retinopathy[12-15]. Increased intraocular VEGF levels as well as VEGF receptor 1 and 2 were detected in rat and human diabetic retina[12-20]. In addition to its well known mitogenic and angiogenic activity, VEGF was recently recognized as a proinflammatory cytokine[21, 22]. As such, the induction of adhesion molecues expression such as ICAM-1, VCAM-1 and E-selectin in endothelial cells (HUVEC) and rat retina by VEGF was observed[21][22]. Specific inhibition of VEGF activity inhibited ICAM-1 expression, leukocyte adhesion, blood-retinal barrier breakdown and neovascularization in STZ

diabetic rats[21]. However, the effect of inflammatory cytokines especially VEGF on human retinal endothelial cells is not well studied.

Inflammatory cytokines function through their receptors to initiate a series of signal transduction events that lead to the phosphorylation and degradation of Inhibitor of nuclear factor Kappa B (IκB) followed by the translocation and activation of nuclear factor Kappa B (NFκB) in the nucleus[23]. NFκB is an important transcription factor controlling the expression of an array of inflammatory response genes including adhesion molecules. Activation of NFκB (p65 and p50) has been well documented in diabetes, especially in the retinal vasculature of diabetic patients and in animal models[21, 24]. *In vitro* high glucose has been shown to cause the activation of NFκB in bovine retinal endothelial cells or pericytes. However, the role of NFκB in response to inflammatory cytokines in hRVE cells awaits to be clarified.

Hyperglycemia and dyslipidemia are two major metabolic disorders of diabetes mellitus. Despite considerable progress in understanding of hyperglycemia-induced pathology over the past decade, the link between diabetic metabolic disorders and retinopathy still eludes us. The role of diabetic dyslipidemia in the development of microvascular complications has received much less attention. Dyslipidemia is a major metabolic syndrome prevalent in both Type 1 and Type 2 diabetes. Type 2 diabetes is characterized by the elevation of blood levels of LDL-C, triglycerides and free fatty acids along with decrease in HDL cholesterol[25-31]. Changes in serum lipids and lipoproteins have also been observed in Type 1 diabetes mainly with a reduced level of long chain PUFA such as $DHA_{22:6n3}$ in the plasma of diabetic children[32] and in the

diabetic human eye[33, 34]. Clinical data suggest that dyslipidemia could be a critical factor in the development of diabetic retinopathy[35][36-42].

n3-PUFAs (abundant in marine fish oils) have long been recognized to modulate the inflammatory immune response and are widely applied clinically as an adjuvant immunosuppressant in the treatment of inflammatory disorders (reviewed in [43, 44]). The decrease in the most abundant long chain n3-PUFA in the eye, $DHA_{22:6n3,}$ could expose diabetic eyes to a more proinflammatory environment. The anti-inflammatory role of n3-PUFAs in the regulation of inflammatory cytokines and the induction of adhesion molecules expression in hRVE has not been studied.

PUFAs or their eicosanoid derivatives are natural ligands for the nuclear receptors such as PPARs[45-47]. Recent evidence has indicated that all three PPARs (α, β, γ) are actively involved in regulating inflammatory responses. PPARα, β, γ agonists have been shown to inhibit inflammatory cytokines and adhesion molecules production in a cell type and isoform specific manner[48-53]. It is known that n3-PUFAs are able to activate PPARs effectively[54]. This implies that PPARs and their regulation by n3-PUFAs may play important roles in n3-PUFA mediated anti-inflammatory responses.

Herein, we demonstrate that inflammatory cytokines (TNFα, IL-1β and $VEGF_{165}$) induce VCAM-1 and ICAM-1 expression in hRVE cells through activation of NFκB pathway. This induction was inhibited by treatment with n3-PUFA ($DHA_{22:6n3}$) and specific PPARα agonists. $DHA_{22:6n3}$ might not just target PPARα to exert the anti-inflammatory effect. In fact, $DHA_{22:6n3}$ is acting upstream of IκBα phosphorylation and degradation to suppress cytokine induced NFκB signaling. These results suggest a

beneficial role of $DHA_{22:6n3}$ and PPARα in downregulating inflammatory responses in hRVE.

3. Materials and Methods

3.1. Reagents

DMEM and F12 culture medium, antibiotics, fetal bovine serum, and trypsin were obtained from Invitrogen (Carlsbad, CA). Commonly used chemicals and reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO). TNFα and IL-1ß were from R&D Systems (Minneapolis, MN). $VEGF_{165}$ was purchased from Calbiochem. Trigolitazone (TGZ), Fenofibrate, WY14,643, GW510516 and Alzoyl-PAF were obtained from Caymen chemical (Ann Arbor, MI).

3.2. Cell culture

Primary cultures of hRVE cells obtained from at least three donors were prepared and cultured as previously described[55]. Passages 1-6 were used in the experiments. Primary human umbilical vein endothelial cells (HUVEC, multiple donors) were obtained from Cascade Biologicals (Portland, OR) and cultured in DMEM containing 10% FBS, macrovascular endothelial cell growth supplement (MVGS) and 100 µg/ml penicillin/streptomycin, 100 µg/ml antimycotics in a humidified incubator at 37 °C with 5% CO_2. For experimental treatments, cells were transferred to serum-free medium for 18 to 24 hours before addition of the stimulatory agents. Treatment of cells with fatty acids was performed as follows. Fatty acid stocks were prepared by dissolving fatty acids (NuCheck Prep, Inc., Elysian, MN) in 100% ethanol to a final concentration of 100 mM fatty acid as described previously. The fatty acid stock solutions were diluted in serum-free medium to reach fatty acid concentrations of 100 µM with corresponding

bovine serum albumin (BSA) concentration of 20 μM. Charcoal-treated, solvent-extracted, fatty acid–free BSA was obtained from Serologica Inc., Norcross, GA. The fatty acid-to-albumin molar ratio was maintained at 5:1. Cells were incubated for the times indicated in the Results section. Equivalent amounts of BSA alone were added to control plates.

3.3. SDS-PAGE and 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, 10% glycerol) with freshly added protease inhibitor cocktail (Sigma) and phosphatase inhibitors (1 mM Na_3VO_4, 100 μM glycerophosphate, 10 mM NaF, 1 mM Na_4PP_i). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose, immunoblotted using appropriate antibodies followed by secondary horseradish peroxidase conjugated antibody (Bio-Rad). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ). Blots were quantitated by scanning densitometry using ImageJ software, ver. 1.29 (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

3.4. Electrophoretic mobility gel shift assay

The double-stranded oligonucleotide containing the NFκB binding sequence derived from human VCAM-1 promoter were designed and synthesized as follows: 5' TGCCCTGGGTTTCCCCTTGAAGGGATTTCCCTC3' and

3'GACCCAAAGGGGAACTTCCCTAAAGGGAGGCGG5'. The oligonucleotides were annealed and labeled in the presence of P^{32}dCTP with the Random Primer Kit from Invitrogen used according to manufacturer's protocol. For binding reactions, nuclear extracts (6 μg) were incubated in 25 μL of total reaction volume with ^{32}P-labeled NFκB oligonucleotides for 20 minutes at room temperature. DNA-protein complexes were resolved on a 6% nondenaturing polyacrymide gel and the bands were examined by autoradiography. Incubation of the nuclear extracts with excess cold NF κ B oligonucleotides was used to confirm the specificity of binding activity.

3.5. Real time RT-PCR

Early passage hRVE (p1-p6) from three donors were cultured to 95% confluent and serum starved overnight. RNA was extracted using Trizol reagent. 1 μg RNA was reverse transcribed using oligo d(T18VN) and 1/20 volume of cDNA was used for real time PCR in each reaction. Real-time quantitative RT-PCR primers targeting human PPARα, δ, γ and β-actin were designed by using Primer Express software (Applied BioSystems, Foster City, CA) and sequences are listed as follows: (5' to 3'): PPAR α: Forward: GGAAAGGCCAGTAACAATCC; Reverse: CTGGCAGCAGTGAAAGATG; PPARδ: Forward: GGGCTTCCACTACGGTGTT; Reverse: TTGTTGCGGTTCTTCTTCTG; PPARγ: Forward: AGCCCAAGTTTGAGTTTGCT; Reverse: AATGTCTTCAATGGGCTTCA; β-actin: Forward: CTCTTCCAGCCTTCCTTCCT; Reverse: TGTTGGCGTACAGGTCTTTG. The specificity of each primer to the sequence of choice was checked by National Center for Biotechnology Information (NCBI) Blast module. To assure the specificity of each

primer set, amplicons generated from PCR reactions were analyzed for specific melting point temperatures by using the first derivative primer melting curve software supplied by Applied BioSystems. The SYBR Green I assay and the ABI Prism 7700 sequence detection system (Applied Biosystems) were used for detecting real-time quantitative PCR products. PCR reactions for each sample were done in triplicates for both target gene and β-actin control.

3.6. Transfection of hRVE using lipofectamine 2000

Cells were plated in 6-well plates at $0.08x10^6$/well. The cells were transfected in Opti-MEM using Lipofectamine 2000 (1.5 μl/μg DNA) (Invitrogen) according to the manufacturer's instructions. pM-rPPARα-LBD was a fusion of the Gal4-DNA-binding domain fused to the ligand-binding domain of rPPARα. The TKMH100x4-Luc reporter contains four binding sites for the Gal4-DNA-binding domain.

24h after transfection, cells were changed to serum free media with 100 μM fatty acid (NuChek Prep, Elysian, MN), and bovine serum albumin (BSA to fatty acid ratio was 1:5) or the PPARα agonist, WY14,643. After 24 h of treatment, the cells were harvested for luciferase assays. Each treatment involved triplicate samples, and each study was repeated at least twice. The results were expressed as relative luciferase activity normalized to protein levels.

4. Results

4.1. TNFα, IL-1β and $VEGF_{165}$ induce adhesion molecules expression in hRVE cells

In hRVE cells, both TNFα (5 ng/ml) and IL1-β (1 ng/ml) acutely stimulated the expression of ICAM-1 and VCAM-1 (Fig. 1A). Recombinant $VEGF_{165}$ (20 ng/ml), an important angiogenesis factor in diabetic retinopathy, also induced adhesion molecules expression (both ICAM-1 and VCAM-1) in hRVE cells (Fig.1B). The induction of VCAM-1 and ICAM-1 were time dependent, with VCAM-1 expression peaking at 24 hrs and ICAM-1 expression persisting for up to 48 hrs. Less effect of cytokine stimulation on E-selectin expression was observed in the time points checked. To compare the potency of the principal cytokines such as IL-1β with $VEGF_{165}$, we treated hRVE cells with $VEGF_{165}$ and IL-1β at the doses used above (the commonly recommended doses in cell culture settings for each cytokine) and performed side by side western analyses. As in Fig. 1C, $VEGF_{165}$ has at least one magnitude lower potency than IL-1β, suggesting $VEGF_{165}$ is a weaker proinflammatory cytokine compared to the principal cytokines in hRVE cells.

4.2. $DHA_{22:6n3}$ inhibits TNFα, IL-1β and $VEGF_{165}$ induced CAM expression

n3-PUFAs ($EPA_{20:5n3}$ and $DHA_{22:6n3}$) have been shown to be anti-inflammatory in a number of different cell types. Here we investigated the effect of $DHA_{22:6n3}$ on cytokine induced inflammation in hRVE cells. Pre-treatment of hRVE cells with

$DHA_{22:6n3}$ (100 μM of BSA-bound $DHA_{22:6n3}$ for 24 hours) significantly inhibited IL1-β and TNFα induced VCAM-1 expression by about 40% and 50% respectively (Fig. 2A and B). In contrast, pre-treatment with the saturated palmitic acid (16:0) used as a lipid control did not exhibit a significant effect on cytokine-induced VCAM-1 expression (Fig. 2A, and quantitated in B). Similarly, $DHA_{22:6n3}$ pretreatment inhibited $VEGF_{165}$ induced VCAM-1 and ICAM-1 expression while palmitic acid (16:0) pretreatment had no effect (Fig. 2C). The anti-inflammatory effect of $DHA_{22:6n3}$ was also confirmed in HUVEC cells. $DHA_{22:6n3}$ inhibited $VEGF_{165}$ and IL-1β induced VCAM-1 expression in a dose dependent fashion (Fig. 2D), suggesting a common role of $DHA_{22:6n3}$ functioning as an anti-inflammatory agent in human endothelial cells.

4.3. NFκB is an important transcription factor regulating adhesion molecules expression in hRVE

To investigate the role of NFκB in cytokine induced adhesion molecules expression in hRVE, electrophoretic mobility shift assay (EMSA) was performed. A double stranded DNA probe containing the specific NFκB binding site from human VCAM-1 promoter was used to study the activation and binding of NFκB to the promoters of adhesion molecules. As shown in Fig 3A, all three cytokines induced NFκB binding to the VCAM-1 promoter. $VEGF_{165}$ induced a delayed NFκB activation in the nucleus, with the NFκB induced shifts starting from 1 h and peaking at 2 h (Fig. 3A). Moreover, two specific isoforms of NFκB family members: p65 and p50 accumulated in the nucleus upon stimulation by IL-1β and TNFα (Fig. 3B). Phosphorylation of p65 at Ser 536 required for optimal transactivation of NFκB[56] was

also observed in the nucleus of IL-1β and TNFα stimulated cells (Fig. 3B). Likewise, $VEGF_{165}$ also induced a minor translocation of p50 and p65 into the nucleus (Fig. 3B) with no obvious p65 phosphorylation observed (data not shown).

4.4. $DHA_{22:6n3}$ pretreatment inhibits cytokine induced NFκB binding to the VCAM-1 promoter

To address the molecular mechanism underlining the inhibitory effect of $DHA_{22:6n3}$, we analyzed whether $DHA_{22:6n3}$ is acting through inhibiting NFκB signaling to downregulate CAM expression in hRVE cells. $VEGF_{165}$ induced binding to VCAM-1 promoter at 2 h was decreased about 40% by pre-treatment with $DHA_{22:6n3}$, but not with BSA, palmitic acid (16:0) or linoleic acid (18:2n6) (Fig. 4A). Similarly, $DHA_{22:6n3}$ pretreatment inhibited IL-1β induced NFκB binding to the VCAM-1 promoter by 25% compared with palmitic (16:0) treated controls as shown in Fig. 4B and quantitated in Fig. 4C. This was concomitant with a decrease in the nuclear level of p65 and p50 in $DHA_{22:6n3}$ pretreated cells (Fig. 4D), implying that $DHA_{22:6n3}$ decreases IL-1β induced nuclear translocation of p65 and p50 thus inhibiting their binding to the VCAM-1 promoter.

4.5. $DHA_{22:6n3}$ pretreatment inhibits IκBα phosphorylation and degradation, an immediate event upstream of NFκB nuclear translocation

The specific step that $DHA_{22:6n3}$ acts on to inhibit cytokine induced NFκB activation were further dissected by examining the upstream IκBα phosphorylation and its ubiquitin mediated proteosome degradation. $DHA_{22:6n3}$ pretreatment caused an inhibition of IL-1β induced IκBα phosphorylation compared with palmitic (16:0) treated controls at the time points checked (Fig. 5). The inhibition was correspondent to the prevention of IκBα degradation by $DHA_{22:6n3}$ pretreatment. $VEGF_{165}$, even at the highest dose used, was not as potent an activator of the NFκB pathway as IL-1β and TNFα. Therefore, $VEGF_{165}$ induced IκBα phosphorylation and degradation was below the sensitivity level to be detected by our analyses used in this study.

4.6. Expression pattern of PPAR isoforms in hRVE by RT-PCR

Since PUFAs and the eicosanoids are natural ligands for PPAR isoforms, we first wanted to characterize the expression pattern of PPAR isoforms in hRVE. Reverse transcription followed by real time PCR was utilized to compare the mRNA level of PPAR isoforms in a semi quantitative fashion. The abundance of PPAR mRNA levels was compared to β-actin copies (Fig. 6A). hRVEs express all three isoforms with PPARδ appearing as the most abundant. Western blot analysis was used to verify the protein expression levels in hRVEs isolated from three donors (Fig. 6B).

4.7. PPARα specific agonists partially inhibit cytokine induced CAMs expression

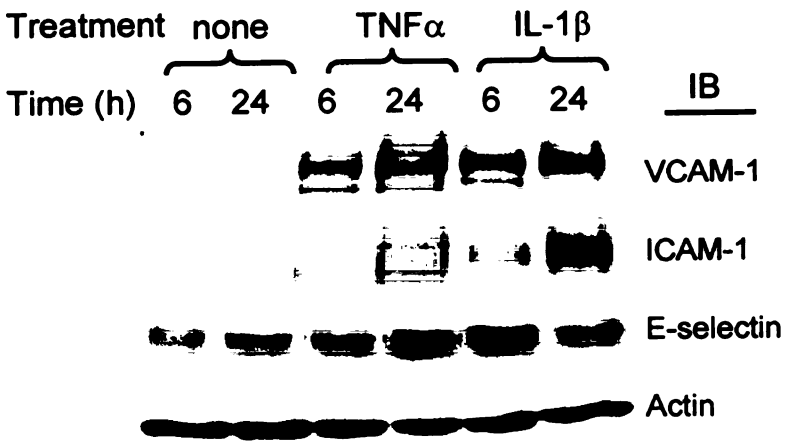
All three isoforms of PPAR have been shown to modulate immune responses[57]. To investigate in hRVE cells which PPAR isoform may play a role in regulating inflammatory cytokine induced immune responses, specific agonists for each isoform were used. Fig. 7 showed that specific PPARα agonist WY14,643 dose dependently attennuated $VEGF_{165}$ induced both VCAM-1 and ICAM-1 expression in hRVE cells, while the PPARγ agonist, TGZ, had no effect. Similar results were obtained with TNFα induced VCAM-1 expression (Fig. 8) which was inhibited by specific PPARα agonists WY14,643 and Fenofibrate, but not by specific PPARδ (GW510516) and PPARγ (TGZ and azoyl-PAF) agonists. These results demonstrate that in hRVE cells, activation of PPARα, but not PPARδ and γ may specifically prevented the cytokine induced adhesion molecules expression.

4.8. $DHA_{22:6n3}$ activates PPARα in hRVE

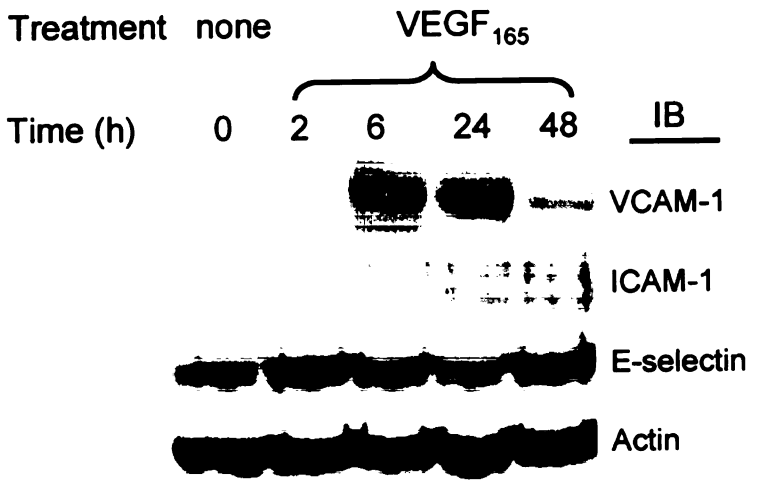
To study whether $DHA_{22:6n3}$ could activate PPARα in hRVE, we used the chimeric receptor Gal4-rPPARα-LBD (which shares high homology to hPPARαLBD) to assess the sensitivity of PPARα to fatty acid regulation. Accordingly, cells were transfected with Gal4-MHTK-luciferase together with the chimeric receptor Gal4-rPPARα-LBD plasmid. PPARα specific agonist WY14,643 induced about a 5 fold increase in Luc activity, suggesting that WY14,643 specifically targeted to PPARα in hRVE cells (Fig. 9). Interestingly, exogenous free fatty acids such as saturated palmitic

acid (16:0), proinflammatory n6-PUFAs ($LA_{18:2n6}$ and $AA_{20:4n6}$), together with anti-inflammatory n3-PUFA ($DHA_{22:6n3}$) all increased Luc activity to a similar level (about 2 fold). Docosapentaenoic acid ($DPA_{22:5n3}$) and $EPA_{20:5n3}$ were less potent activators for PPARα in hRVE. This implies that different free fatty acids have different affinities for PPARα ligand binding domain. However, anti-inflammatory properties of $DHA_{22:6n3}$ might not be only acting through activating PPARα in hRVE.

A.


Treatment none TNFα IL-1β
Time (h) 6 24 6 24 6 24
IB
VCAM-1
ICAM-1
E-selectin
Actin

B.


Treatment none VEGF165
Time (h) 0 2 6 24 48
IB
VCAM-1
ICAM-1
E-selectin
Actin

C.

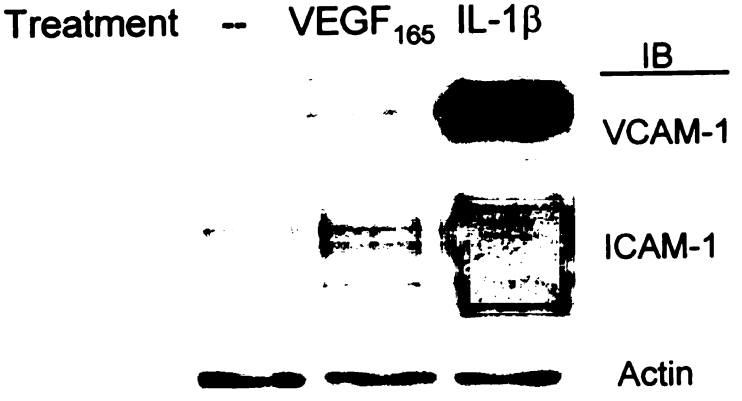
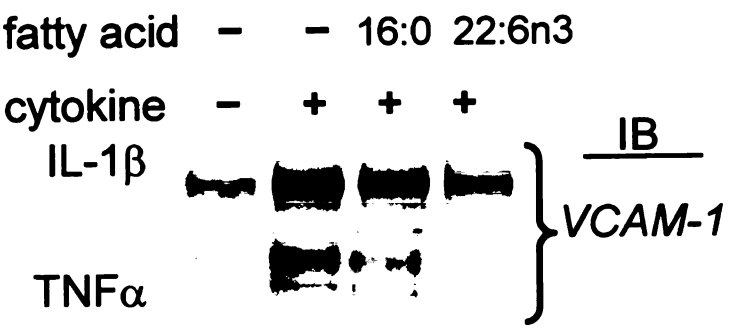
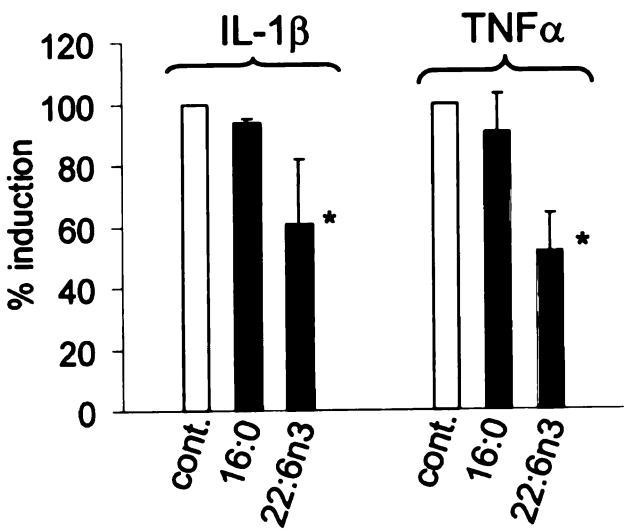

Treatment -- VEGF165 IL-1β
IB
VCAM-1
ICAM-1
Actin

Fig. 1. Induction of cell adhesion molecules after treatment of hRVE cells with cytokine TNFα, IL-1β and VEGF165. hRVE cells were serum starved overnight and stimulated with 5 ng/ml TNFα, 1 ng/ml IL-1β (panel A), 20 ng/ml $VEGF_{165}$ (panel B) for different time periods as indicated. The potency between $VEGF_{165}$ (20 ng/ml) and IL-1β (1 ng/ml) were compared in panel C. The induction of adhesion molecules, VCAM-1, ICAM-1 and E-selectin was assessed by immunoblot analysis. Equal amounts of protein were added to each lane and confirmed by actin blot. Representative results from at least tree independent experiments were presented for each panel.

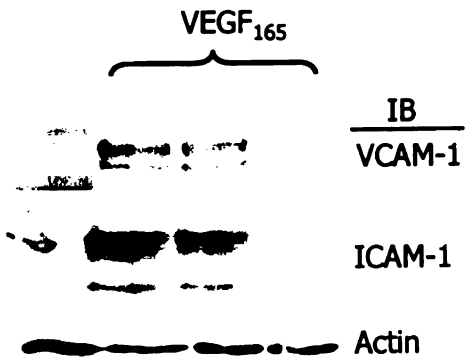
A.


fatty acid - - 16:0 22:6n3
cytokine - + + +
IB
IL-1β
TNFα
VCAM-1

B.


IL-1β
TNFα
% induction
120
100
80
60
40
20
0
*
*
cont.
16:0
22:6n3
cont.
16:0
22:6n3

C.


VEGF165
IB
VCAM-1
ICAM-1
Actin

D.

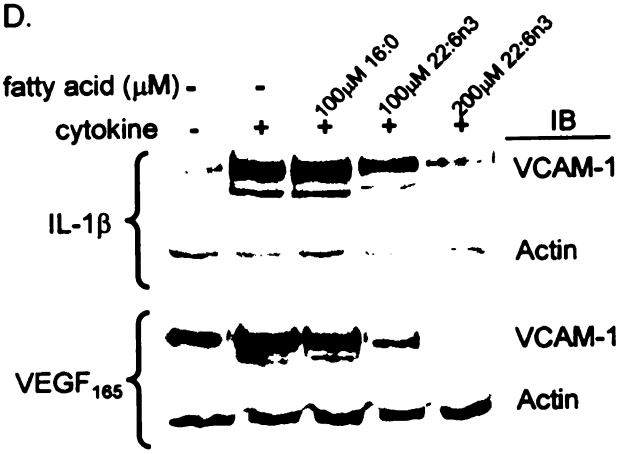
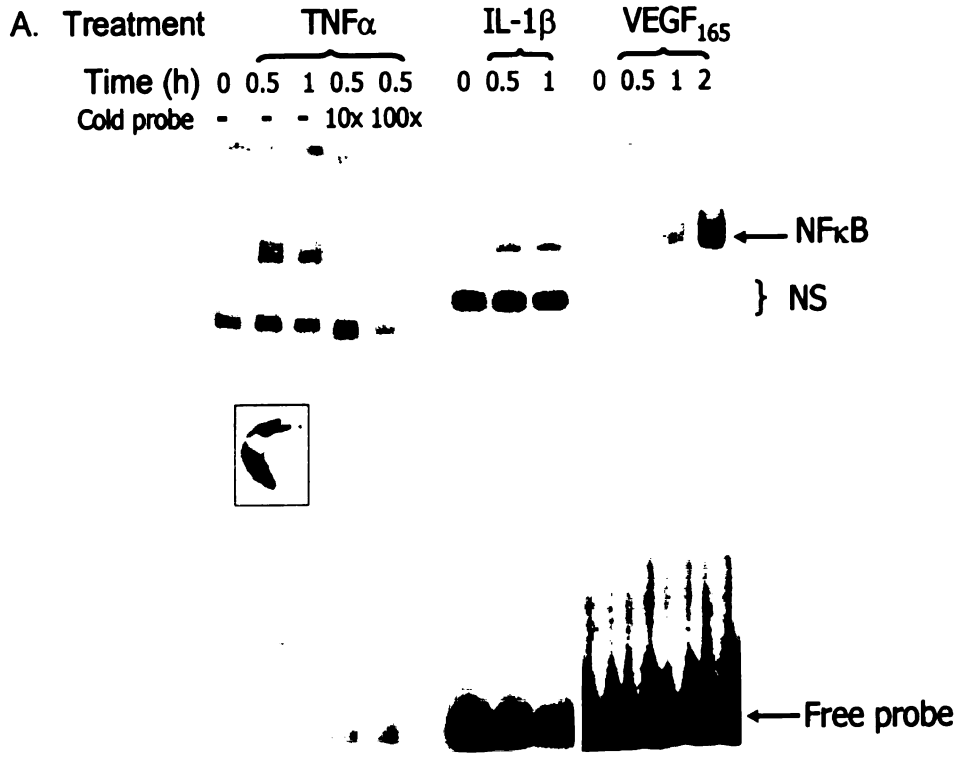

fatty acid (μM) - - 100μM 16:0 100μM 22:6n3 200μM 22:6n3
cytokine - + + + +
IB
IL-1β
VCAM-1
Actin
VEGF165
VCAM-1
Actin

Fig. 2. n3-PUFA pretreatment specifically downregulates the induction of VCAM-1 by proinflammatory cytokines. (A) hRVE cells were serum starved overnight and then cells were either untreated or treated with 100 μM 16:0 and 22:6n3 for 24 hrs. Cells were then stimulated with 1 ng/ml IL-1β, 5 ng/ml TNFα and 20 ng/ml $VEGF_{165}$ for 6 h. Lysates were prepared and same amounts of protein were used for analysis by immunoblot to detect the expression of VCAM-1 and ICAM-1 (panel A and C). Representative data were presented from at least 3 independent experiments using hRVE cells from different donors and quantitated as in panel B for TNFα and IL-1β. *$P<0.05$. (D) HUVEC cells were treated with BSA alone or BSA bound 16:0 and 22:6n3 in the presence of 1% FBS media as indicated for 24 h. Cells were then stimulated with 0.2 ng/ml IL-1β and 20 ng/ml $VEGF_{165}$ for 6 h. Lysates were prepared and analyzed as above and representative data from at least three independent experiments were presented


A. Treatment
TNFα
IL-1β
VEGF165
Time (h) 0 0.5 1 0.5 0.5 0 0.5 1 0 0.5 1 2
Cold probe - - - 10x 100x
NFκB
} NS
Free probe

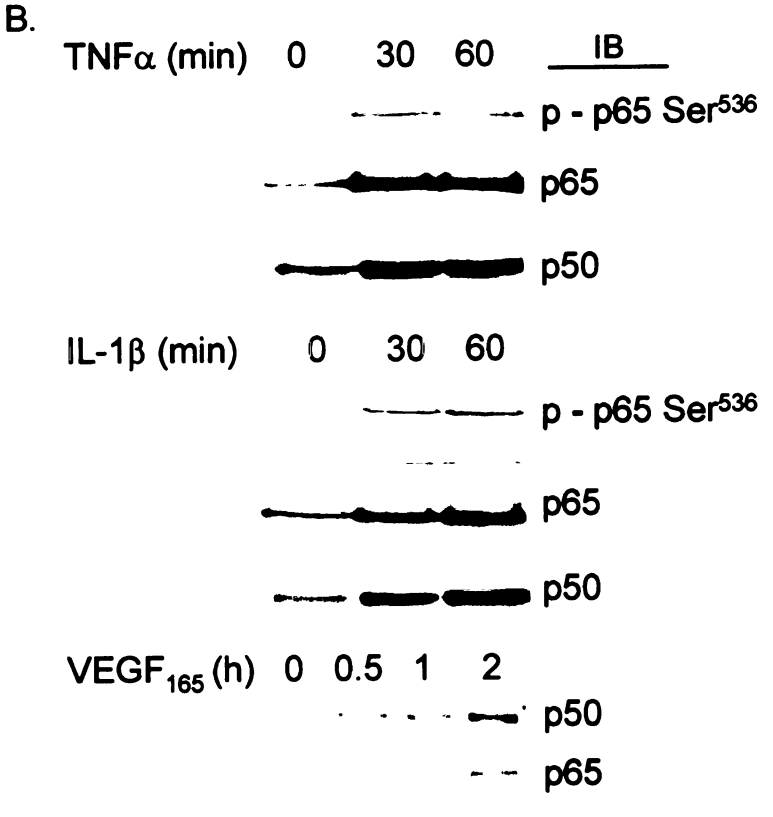

B.
TNFα (min) 0 30 60
IB
p - p65 Ser536
p65
p50
IL-1β (min) 0 30 60
p - p65 Ser536
p65
p50
VEGF165 (h) 0 0.5 1 2
p50
p65

Fig. 3. Inflammatory cytokines activate NFκB signaling to induce adhesion molecules expression in hRVE cells. (A) hRVE cells were serum starved overnight and treated with TNFα (10 ng/ml), IL-1β (1 ng/ml) and $VEGF_{165}$ (20 ng/ml) as indicated. Nuclear extracts were prepared. EMSAs were performed using probes containing specific NFκB binding motif to the human VCAM-1 promoter. Arrows indicate the NFκB induced shift, NS stands for the nonspecific band. The specific NFκB induced shift band was confirmed by adding cold probes to compete away the labeled probe. (B) Equal amounts of nulear extracts were loaded for western blot analyses against p65, p-p65Ser^{536} and p50 for their transport into nucleus. Representative results were presented from at least three independent experiments.

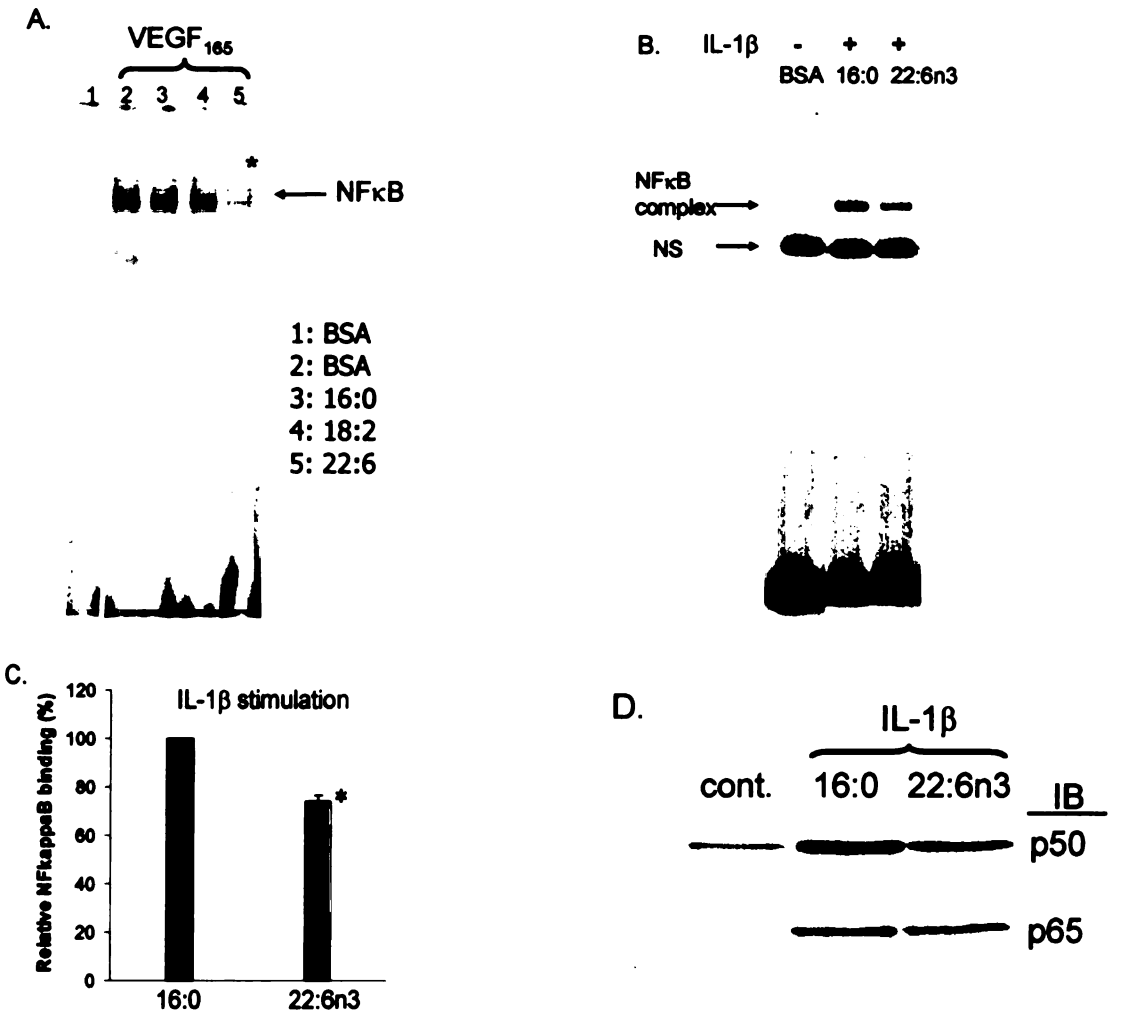


Fig. 4. DHA22:6n3 inhibits VEGF165 and IL-1β induced NFκB signaling. (A) hRVE cells were serum starved overnight and treated with 100 μM BSA bound 16:0, $LA_{18:2n6}$ and $DHA_{22:6n3}$ for 24 h. Cells were then stimulated with 20 ng/ml $VEGF_{165}$ for 2 h and nuclear extracts were prepared. EMSAs were performed as before. (B) hRVE cells were treated with 100 μM BSA bound 16:0 and 22:6n3 for 24 h and then stimulated with 1ng/ml IL-1β for 30 min followed by EMSA. Arrows indicate the NFκB induced shift, NS stands for the nonspecific band. Representative results from three independent experiments were shown and statistic analyses were performed as panel C with $p<0.005$. (D) The same amounts of nuclear extracts from B were analyzed against p65 and p50 by western blot.

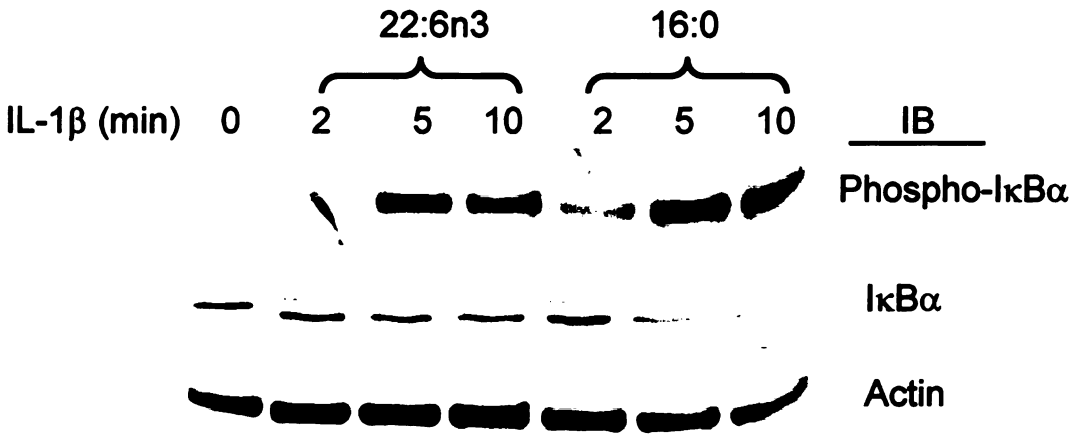
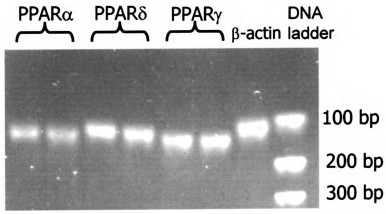
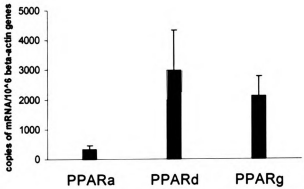


Fig. 5. Inhibition of IL-1β induced IκBα phosphorylation and degradation by DHA22:6n3 pretreatment in hRVE. hRVE cells were serum starved overnight and treated with 100 μM BSA bound 16:0 and $DHA_{22:6n3}$ for 24 h. Cells were then stimulated with 1 ng/ml IL-1β for the indicated time periods and harvested. Western blots were performed to detect the IκBα phosphorylation and degradation. Representative data were presented from at least 3 independent experiments.

A.


PPARα
PPARδ
PPARγ
DNA
β-actin ladder
100 bp
200 bp
300 bp


copies of mRNA/10^6 beta-actin genes
5000
4000
3000
2000
1000
0
PPARa
PPARd
PPARg

B.

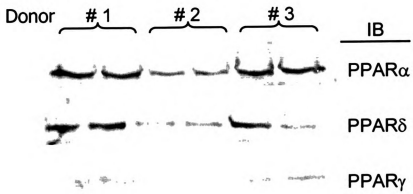

Donor
#1
#2
#3
IB
PPARα
PPARδ
PPARγ

Fig. 6. Relative mRNA abundance and protein expression of PPAR isoforms in hRVE. (A) hRVE cells were serum starved overnight and total RNAs were extracted for reverse transcription and quantitative real time PCR analyses using specific primers against human PPARα, δ and γ. The results were calculated relative to β-actin to compare the mRNA abundance of three major PPAR isoforms averaged from hRVE cells isolated from three different donors and the amplified DNA bands for each gene were presented. (B) Same amounts of whole cell lysates of hRVE cells from three donors were applied for SDS-PAGE and blotted using antibodies against each PPAR isoform. Represent blot was shown.

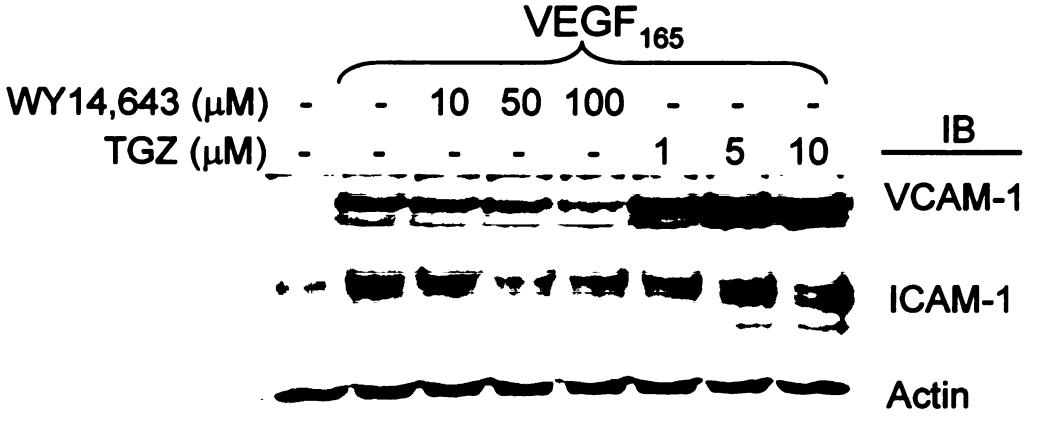


Fig. 7. PPARα agonists inhibit VEGF165 induced CAMs expression. hRVE cells were serum starved overnight and pretreated with PPARα agonist WY14,643 and PPARγ agonist Trigolitazone (TGZ) as indicated for 45 min. Recombinant $VEGF_{165}$ (20 ng/ml) was then added. Cells were harvested after 6 h and total lysates were prepared. Same amounts of protein were submitted for western blot analyses against VCAM-1, ICAM-1 and actin. A representative blot from 3 independent experiments was presented.

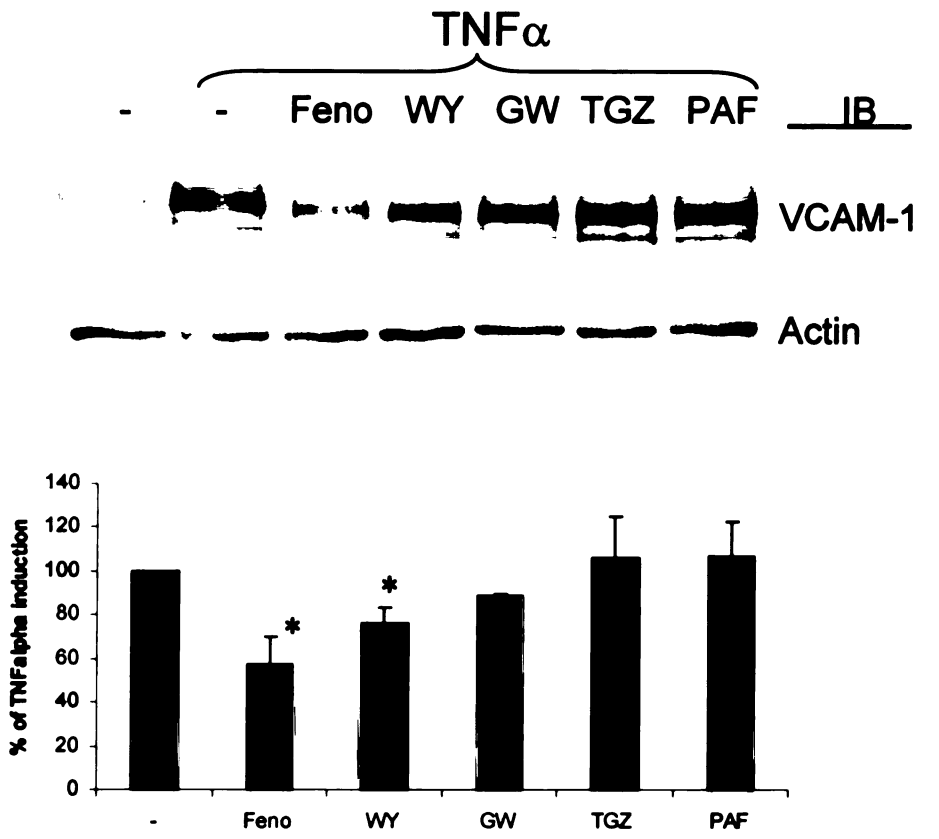


Fig. 8. Effect of PPAR ligands on TNFα induced VCAM-1 expression in hRVE cells. hRVE cells were serum starved overnight and pretreated with PPARα agonist 300 μM Fenofibrate (Feno) and 100 μM WY14,643 (WY), PPARδ agonist 1 μM GW501516 (GW), PPARγ agonists 10 μM Trigolitazone (TGZ) and 1 μM Azoyl-PAF (PAF) for 45 min and stimulated with 5 ng/ml TNFα for another 6 h. Cells were harvested and the same amounts of protein were submitted for western blot analysis against VCAM-1 and actin as a loading control. A representative blot from 3 independent experiments was presented and expressed as the means ± S.D by normalizing to TNFα stimulation alone according to experiments using hRVE derived from two donors ($p<0.05$).

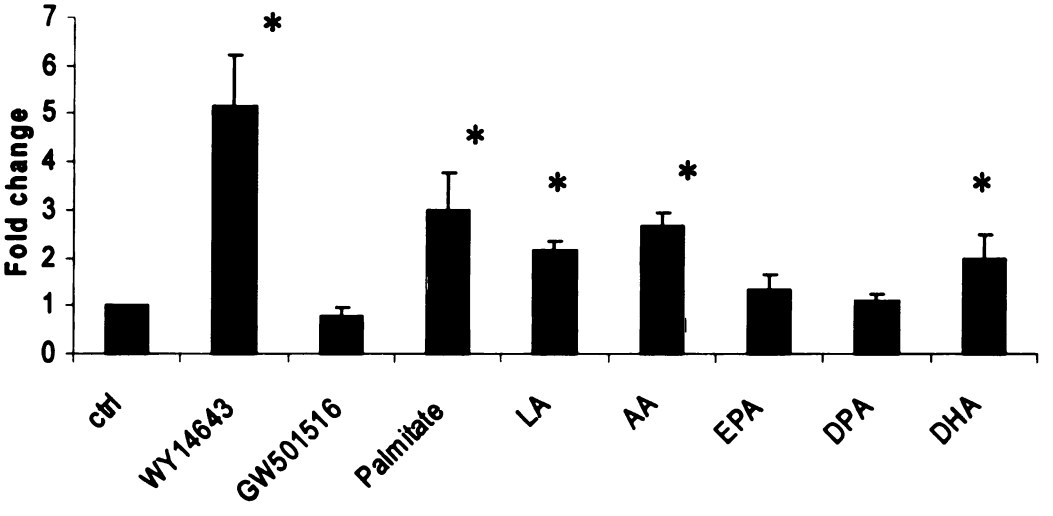


Fig. 9. Activation of PPARα by WY14,643 and exogenous free fatty acids in hRVE cells. hRVE cells were transfected with pMN-MHTK-Luc with pMN-rPPARα-LBD. After an overnight transfection period, the cells were treated with or without WY14,643 (100 μM) or free fatty acids as described in Methods for 24 h. The cells were harvested for protein and luciferase assays. The results were reported as the relative luciferase activity (*RLA*, firefly luciferase activity/μg protein) normalized to the untreated control. The results were expressed as the means ± S.D. of three separate studies with triplicate samples per group. *: $p<0.05$.

5. Discussion

In chronic inflammatory conditions, endothelial cells actively recruit blood borne leukocytes such as monocytes and T lymphocytes to the underlying tissue in response to the activation by cytokines and growth factors. This process is mediated by the increased expression of adhesion molecules on both immune cells (leukocytes and endothelial cells). The early stage of Diabetic retinopathy has been recognized as a chronic inflammatory disease[3-5]. Upregulation of inflammatory cytokines especially TNFα[7-9], IL-1β[10, 11] and VEGF[12-14] along with their correspondent receptors have been well documented in diabetic eyes of human subjects and animal models. However, the effect of these principal cytokines on human retinal endothelial cells adhesion molecules expression, especially VCAM-1, the specific vascular inflammatory marker, has not been tested. In this paper, for the first time we report the effect of cytokines on adhesion molecules expression in human primary retinal endothelial cells isolated from different donors. Also the role of a family of important transcription factors essential to mediate inflammatory response, NFκB, was investigated in regards to their response to different cytokines. Our work further showed the anti-inflammatory properties of the principal n3-PUFA in the retina, $DHA_{22:6n3}$, on cytokine triggered inflammatory signaling. It suggests that the decrease of $DHA_{22:6n3}$ in both plasma and retina in Type 1 diabetes may exacerbate the proinflammatory environment due to the elevated levels of the proinflammatory cytokines in diabetic eyes.

NFκB has been suggested as a potential therapeutic target in atherosclerosis and thrombosis due to its important role in regulation of inflammatory diseases[23, 58].

NFκB is involved in the development of diabetic microvascular complications. Retinal NFκB is activated in diabetes and mediates retinal capillary cell death[21, 24]. Our data suggests that retinal endothelial cells contain the cognate receptors for TNFα, IL-1β and $VEGF_{165}$, and activation of these receptors could lead to the increased binding of the important transcription factor NFκB to the VCAM-1 promoter in the nucleus. The major NFκB isoforms activated by the inflammatory cytokines in hRVE are p65 and p50, which usually form a classical p65/p50 heterodimer to mediate DNA binding. Previous reports demonstrated increased accumulation of only p50, but not the p65 subunit of NFκB in nuclei of retinal endothelial cells from diabetic animals[59]; and p65 was shown to be increased in retinal pericyte nuclei but not in endothelial cells from diabetic retinopathy patients and/or in cells from the STZ diabetic rat model[60]. The apparent differences could come from the different systems used. Our study using cultured human retinal endothelial cells demonstrates that both p65 and p50 are important DNA binding transcription factors that can be induced by proinflammatory cytokines in hRVE cells as a route for activation of VCAM-1 and ICAM-1 expression. Moreover, our data agrees with other reports showing that ICAM-1 is a critical adhesion molecule increased in the retinas and that $VEGF_{165}$ is a proinflammatory cytokine in inducing its expression[21][22]. However, our data also suggests that VCAM-1 is also important in mediating leukastasis of the human retina since $VEGF_{165}$, IL-1β and TNFα were potent inducers of VCAM-1 expression in hRVE cells.

n3-PUFAs have long been recognized to modulate immune response and are widely applied clinically as adjuvant immunosuppressant in the treatment of inflammatory disorders[43, 44]. Numerous studies in various cells have shown that

treatment with n3-PUFAs could inhibit adhesion molecules and cytokine expression induced by inflammatory agents[43]. Our study using primary human retinal endothelial cells contributes to the evidence supporting a common role for $DHA_{22:6n3}$ as an anti-inflammatory agent in ameliorating endothelial cells response to cytokines. The specific mechanisms underlying this inhibitory effect have been intensively sought for decades. Several possible mechanisms have been suggested including the displacement of $AA_{20:4n6}$, the major substrates for the synthesis of proinflammatory eicasanoids due to n3-PUFAs incorporation into membrane phospholipids[61]. Direct activation of the nuclear receptors such as PPARs is also involved. Indeed, activation of PPARα by specific agonists WY14,643 and Fenofibrate can suppress cytokine induced VCAM-1 expression in hRVE cells. However, *in vitro* assay demonstrated that not only anti-inflammatory n3-PUFA ($DHA_{22:6n3}$), but also saturated palmitate and proinflammatory n6-PUFAs ($LA_{18:2n6}$ and $AA_{20:4n6}$) could activate PPARα to a similar degree. This implies that in hRVE cells, activation of PPARα is not the sole target that $DHA_{22:6n3}$ impacts to exert its anti-inflammatory function and that additional mechanisms are possibly involved. Further studies to compare the mechanism(s) of PPARα and $DHA_{22:6n3}$ in suppressing cytokine induced inflammatory signaling in hRVE cells are needed.

Moreover, the fact that $EPA_{20:5n3}$, and its elongated metabolite $DPA_{22:5n3}$, have been shown to downregulate the expression of VEGFR2 in endothelial cells, underscores one of the suggested pathways of suppression of VEGF signaling by n3-PUFAs[62, 63]. However, we did not observe the same effect with $DHA_{22:6n3}$, suggesting that $DHA_{22:6n3}$ inhibition of cytokine induced NFκB activation is through a different mechanism in hRVE.

Recently, $DHA_{22:6n3}$ has been shown to suppress LPS induced activation of NFκB through toll-like receptor 4 in murine macrophages[64]. Biochemical studies demonstrate that the molecular targets of $DHA_{22:6n3}$ are probably at the level of receptor localized plasma membrane, upstream of components MyD88 and Akt[64]. Our data suggest that the $DHA_{22:6n3}$ effect could not only prevent the translocation of specific NFκB isoforms into the nucleus (where it binds to the specific promoter) but also inhibit the phosphorylation of IκBα and prevention of its degradation. This implies that $DHA_{22:6n3}$ acts upstream of IκBα to inhibit inflammatory signaling. The more specific steps in $DHA_{22:6n3}$ action are under further study. An interesting hypothesis will be to test whether $DHA_{22:6n3}$ could modify specific plasma membrane domains, such as lipid rafts or caveolae, as reported for T cells[65, 66]. A number of inflammatory signaling proteins have been found to be localized in caveolae/lipid rafts, such as TNFR1[67, 68] and VEGFR2 (FLK-1) in endothelial cells[69, 70]. Whether $DHA_{22:6n3}$ could modify the lipid composition of plasma membrane or caveolae/lipid rafts in hRVE and thus interfere with the inflammatory signaling awaits to be fully determined and is the focus of future study.

In summary, our data demonstrates that three important inflammatory cytokines upregulated in diabetic eyes, TNFα, IL-1β and $VEGF_{165}$ induce VCAM-1 and ICAM-1 expression through activating NFκB in hRVE cells. n3-PUFA ($DHA_{22:6n3}$) and PPARα contribute to antagonizing the cytokine induced inflammatory response possibly through different molecular mechanisms.

6. References

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IV. Proteomic analyses of detergent resistant caveolae/lipid rafts from cultured human retinal vascular endothelial cells (hRVE)

1. Abstract

Endothelial cells (EC) contain caveolae and lipid rafts, the specialized glycosphingolipid and cholesterol enriched micromembrane subdomains that crucially participate in various essential cellular functions such as signal transduction, vesicular trafficking and cholesterol homeostasis. The protein constituents of caveolae/lipid rafts isolated by buoyant density methods from cultured human primary retinal endothelial cells (hRVE) were determined using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS/MS) analysis. About 70 proteins were subsequently identified in combination with LC-MS/MS after in-gel and in-solution digestions. Proteins present included a large number of known caveolae/lipid raft residents, such as caveolin and flotillin. Overall, together with western blot analysis, known glycosylphosphatidylinositol (GPI)-anchored proteins, transmembrane proteins, cytoskeleton and associated proteins, and cell signaling proteins including G proteins, small GTPases, Src-family kinases were found to be present. This was highlighted with the identification of several important EC functional proteins such as CD44, a hyrarunan receptor; CD36, a scavenge receptors for oxidized lipids and free fatty acids; p63; Bene; CD147 and brain acid soluble protein together with retinoic acid induced 3 protein (RAIG1) and signal proteins induced under serum starvation. These findings suggest crucial involvement of caveolae/lipid rafts in numerous retina vascular functions including permeability, migration, angiogenesis, vesicle trafficking, lipid homeostasis

and inflammation. The possible link to the pathogenesis of important retinal vascular diseases such as diabetic retinopathy is discussed.

2. Introduction

Caveolae were originally described as flask-like membrane invaginations of 50-100nm on the surface of endothelial and epithelial cells by electron microscopy 50 years ago[1]. Characterized by the presence of the principal structural and regulatory protein caveolin[2], caveolae and the so-called lipid rafts (devoid of caveolin) share a similar distinct lipid composition notable for high concentrations of sphingolipids and cholesterol. These glycosphingolipid enriched membrane domains which pack in a liquid-ordered structure and are resistant to solubilization by nonionic detergents at low temperatureare also called detergent-resistant membrane raft (DRM) structures.

Endothelial cells are one of the cell types that contain a large number of caveolae and express a high level of caveolin-1[3, 4]. Caveolae/lipid rafts play important roles in endothelial permeability, vesicle trafficking[5-7], cholesterol homeostasis[8], and endothelial cell signaling[9-11]. Recent *in vivo* data from caveolin-1 (CAV1)-null mice showed a lack of caveolae formation in the microvascular endothelium, which significantly alters microvascular permeability[12-14]. More importantly, caveolae/lipid rafts are identified as sites for the sequestration of diverse membrane-targeted signaling proteins (reviewed in [11]). Various signaling molecules, including endothelial nitric oxide synthase (eNOS), G-protein coupled receptors (GPCRs), VEGF receptor 2, PDGFR and EGFR and GPI-linked proteins are partially segregated into these domains with or without activation or ligand binding, suggesting a critical role of caveolae/lipid rafts in endothelial cells signaling transduction[11]. Given their crucial functions in transport, cholesterol homeostasis and signal transduction, caveolae and lipid rafts are

suspected to play an important role in various diseases such as atherosclerosis[15, 16]. More importantly, increased numbers of caveolae in retinal endothelium and pericytes in hypertensive diabetic rats have been demonstrated to be one of the possible mechanisms in hypertension enhanced diabetic microvascular disease, especially diabetic retinopathy[17].

Retinal microvascular endothelial cells form a tight inner blood-retina barrier to regulate the movement of molecules in and out of the retina. Caveolae have particular significance for the highly specialized, continuous endothelium of the retinal vasculature since these organelles are thought to have a unique role in regulating uptake and transcytosis of proteins across the blood retina barriers. Diabetes causes increased permeability of retina microcirculation which results in water, albumin and lipid leakage, with accumulation of lipid exudates and intraretinal fluid[18]. Considering the importance of caveolae/lipid rafts in regulating endothelial cell permeability and signal transduction, further research is needed to determine the function and character of the protein components in caveolae/lipid rafts from human retinal microvascular endothelial cells.

Recent advances in proteomics and protein identification technology have permitted the identification of numerous proteins contained in subcellular fractions such as lipid rafts. The protein components in lipid rafts from Jurkat T cells[19], neutrophils[20] and monocytes[21] or in caveolae/lipid rafts from Cos cells[22], Vero cells[23] and human endothelial cells (HUVEC)[24] have all been characterized by mass spectroscopy (MS). Here we have isolated caveolae/lipid rafts from human primary

retinal vascular endothelial cells using standard methods based on detergent insolubility in Triton X-100 at 4°C. The protein components associated with these cold detergent resistant vesicles were characterized by proteomics using both in-gel digestion and in-solution digestion followed by LC-MS/MS. About 70 proteins were identified including cytoskeletal proteins, ion-channel proteins, signaling proteins such as GPI-linked proteins, G-proteins, proteins involved in angiogenesis, adhesions, and inflammation and some specific retinal endothelial cell proteins. Western blot analysis was also used to identify several less-abundant proteins which had escaped proteomic analysis such as Src-family kinases, and proteins involved in cholesterol and lipid transport. This is the first report that systematically characterized the protein components of the important lipid microdomains involved in variable functions of primary retinal endothelial cells, suggesting caveolae/lipid rafts play key roles in mediating the blood-retina barrier.

3. Materials and Methods

3.1. Reagents and antibodies

Sequence-grade trypsin was purchased from Promega (Madison, WI, USA). Thiourea, triton X-100 Ultra Pure, MES, ammonium bicarbonate and formic acid were purchased from Sigma (St. Louis, MO, USA). Mouse anti-caveolin-1 and flotillin-1 were obtained from Upstate Biotechnologies (Lake Placid, NY); Rabbit anti-ERK1/2 was from Cell Signaling; mouse anti-PKCα, c-Src; Rabbit antibodies against c-yes, CD36, CD44, Na^+/K^+ ATPase and mouse antibodies against Fyn were purchased from Santa Cruz.

3.2. Cell culture

Primary cultures of hRVE cells were prepared as previously described[25]. Cells were maintained in growth medium consisting of DMEM/F12 (Invitrogen), 5.5 mM glucose, 10% fetal bovine serum (Invitrogen), endothelial cell growth supplement (Upstate Biotechnologies), insulin/transferrin/selenium mix (Sigma-Aldrich) and antibiotic–antimycotic solution (Invitrogen). Passages 1-6 cells were plated to yield near-confluent cultures at the end of the experiment. For experimental treatments, the cells were transferred to serum-free medium for 18 to 24 hours before treatment.

3.3. Electrophoresis and immunoblotting

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, 10% glycerol) with freshly added protease inhibitor cocktail (Sigma) and phosphatase inhibitors (1 mM Na_3VO_4, 100 μM

glycerophosphate, 10 mM NaF, 1 mM Na_4PP_i). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose, immunoblotted using appropriate antibodies followed by secondary horseradish peroxidase conjugated antibody (Bio-Rad). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ). Blots were quantitated by scanning densitometry using ImageJ software, ver. 1.29 (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

3.4. Isolation of lipid rafts/caveolin-rich membrane domains

Caveolae/lipid rafts were prepared using a slightly modified sucrose gradient ultracentrifugation protocol [11]. Briefly, $5x10^6$ hRVE cells were washed with cold PBS twice and then 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 and fresh protease and phosphatase inhibitors and kept on ice for 20 min. The homogenization was carried out with 10 strokes of a tight-fitting Dounce homogenizer, and then spun at 4,000xg at 4°C for 10 min. Supernatant (0.8 ml) was then mixed with the same volume of 80% sucrose prepared in MNE buffer, and placed at the bottom of an ultracentrifuge tube. 1.6 ml 30% sucrose and 0.8 ml 5% sucrose were overlaid on top of the sample to form a 5-30% discontinuous sucrose gradient. After 16 h of centrifugation at 200,000xg at 4°C using a swinging bucket rotor, 0.4 ml samples was collected from the top for each fraction. A band confined to fractions 2 to 4 was designated as caveolae/lipid raft-enriched membrane domain. The combined fractions 2 to 4 were further diluted 3 times in MNE buffer and

spun at 200,000xg at 4°C for another 2 h to precipitate the caveolae/lipid rafts and the pellet was designated as the insoluble fraction (I). Fractions 6-10 were also combined and designated as the soluble fraction (S).

3.5. Protein in-gel digestion

The combined caveolae/lipid raft fraction (I) was typically separated by SDS-PAGE on a 4-20% gradient gel (Biorad). Protein bands were visualized by sypro-blue staining (Molecular Probes) and then imaged using a Bio-Rad FX Pro+ laser scanner. The entire 1D SDS sample lane was cut into 30 2 mm sections and dehydrated twice with acetonitrile for 20 min, and dried under vacuum concentrator for about 3 min. The dried gels were then rehydrated in 30 μl of sequencing grade trypsin solution (20 ng/μl) with 50 mM ammonium bicarbonate on ice for 10 min with occasional vortex mixing and digestion was performed overnight at 37 °C.

3.6. Protein in-solution digestion

After pelleting the caveolae/lipid rafts fraction, proteins were dissolved in 15 μl buffer containing ammonium bicarbonate (100 mM, pH 8.0) and 2 M thiourea, 2 mM DTT and incubated at 60°C for 45 min to reduce and denature the proteins. Sequence grade trypsin (100 ng in 85 μL of 50 mM ammonium bicarbonate) was added to digest the protein mixture at 37°C overnight. Tryptic peptides were concentrated by Speed-Vac evaporation.

3.7. LC/MS/MS

The peptides were extracted from each gel section and desalted on a 1 x 0.2 mm Magic C18 Captrap cartridge. The bound peptides were then flushed onto a 15 cm x 75 μm New Objectives Picofrit column packed with Microm Magic C18 AQ packing material and eluted over 60 minutes with Buffer A (0.1% formic acid) and a gradient of 5% to 70% B (95% Acetonitrile 0.1% formic acid, starting at 10 min) into a Thermofinnigan Deca XP+ Ion trap mass spectrometer with a flow rate of 250 nl/min. The top three ions in each survey scan were then subjected to automatic low energy collision induced dissociation (CID) and the resulting uninterpreted MS/MS spectra were searched against the IPI_human database using the Mascot searching algorithm. The Mascot results from every gel section in the lane were combined into one database using an in-house database program. Identifications are usually considered positive if 2 peptides per protein are identified with a significant Mascot score ($p < 0.05$).

4. Results

4.1. Isolation of caveolae/lipid rafts from hRVE

Although a number of studies have demonstrated the importance of caveolae/lipid rafts in regulating endothelial cell function, the protein components of these specialized lipid microdomains in human primary retinal endothelial cells have not been determined. We addressed this issue by characterizing endothelial specific caveolae/lipid rafts in cultured hRVE cells. Caveolae/lipid rafts fractions were isolated by sucrose discontinuous gradient ultra centrifugation (Fig. 1) based on their insolubilization in Triton X-100 at 4 °C. The overall raft isolation was confirmed since caveolin-1 (a caveolae marker) and flotillin-1 (a lipid raft marker) were found highly enriched in density gradient fractions 2to 4). In contrast, the general plasma membrane marker, Na^+/K^+ ATPase, was excluded from the lipid raft fractions. PKCα and ERK1/2 were mainly in the soluble fractions (fractions 6-10) under basal conditions (Fig. 1). These results demonstrate that hRVE contains both caveolae and lipid rafts represented by the enrichment of both caveolin-1 and flotillin-1 in the DRMs isolated using detergent insolubility and density gradient fractionation.

4.2. Characterization of caveolae/lipid rafts components by in gel digestion and LC/MS/MS

With the advance in proteomics, several methods have been employed to characterize proteins from specialized microdomains or organelles. To further characterize the protein components in caveolae/lipid rafts in hRVE, we performed

conventional gel band excision followed by in-gel trypsin digestion and LC/MS/MS. This method was used to primarily identify abundant proteins due to limitations of gel loading. High resolution SDS-PAGE (4-20%) gradient gels were run to first determine the level of complexity of the protein content of caveolae/lipid rafts preparations via syprob blue staining. 20 μg caveolae/lipid rafts protein from about $5x10^6$ cells were loaded on the gel. Fig. 2 shows a number of proteins are present in the caveolae/lipid rafts preparations from serum starved hRVE cells.

A total number of about 70 proteins were identified as detailed in Table I. This include proteins representing a number of functional classes, such as caveolae and lipid rafts structural proteins, well known raft-localized GPI-linked proteins, ion channel proteins, cytoskeleton proteins, and signaling proteins, as well as some putative proteins. Some new proteins were also identified especially proteins involved in signal transduction induced by serum starvation, and in retinoic acid-related signal transduction.

4.3. Identification of caveolae/lipid rafts components by in solution digestion and LC/MS/MS

The in-solution digestion coupled with LC/MS/MS was used to additionally identify proteins that might otherwise not be evident from in-gel trypsin digestion. Table II summarizes the proteins collectively identified from three independent experiments using the caveolae/lipid rafts samples purified from 3 different donors. About 25 proteins were unequivocally identified.

4.4. Identification of less abundant proteins in hRVE by western blot

Due to the limitations of proteomic approaches from both in-gel and in-solution digestion, several less abundant proteins were identified by western blotting. These included the important Src family kinases, such as Fyn and c-yes, which were about 90% enriched in the caveolin/raft fraction. CD36, the scavenger receptor for modified lipoproteins or free fatty acids was also present exclusively in caveolae/lipid rafts fractions from hRVE (Fig. 3).

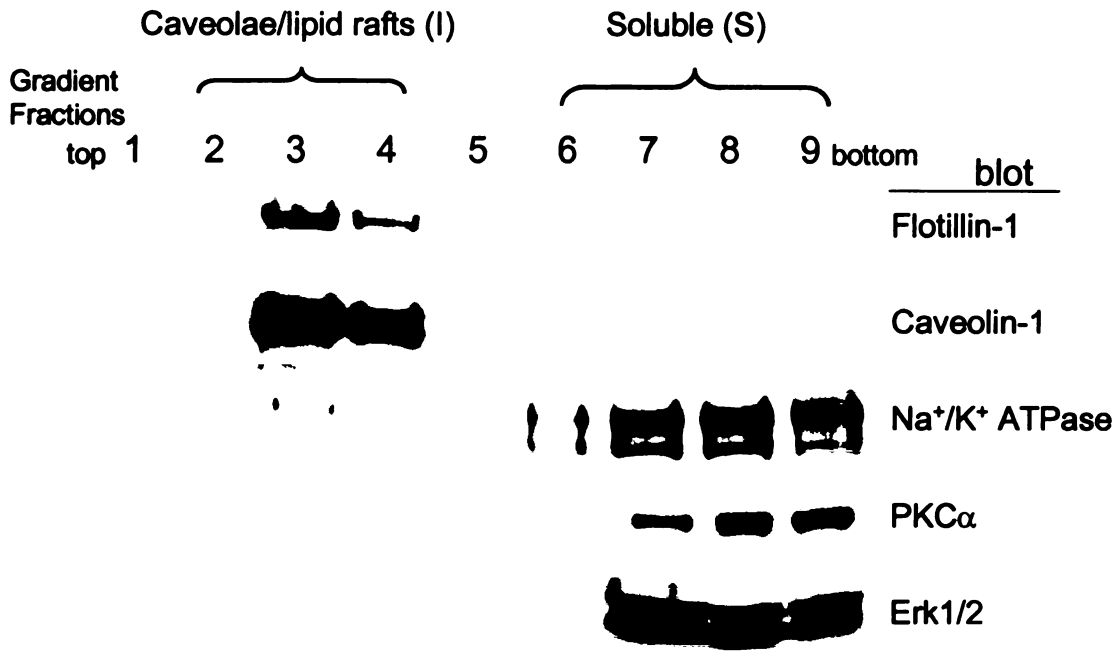

Fig. 1. Characterization of caveolae/lipid rafts in hRVE cells. Caveolae/lipid raft enriched domains were purified as indicated in methods. Gradient fractions were separated by SDS-PAGE. Western blot against the caveolae marker, caveolin-1, and the lipid raft marker, flotillin-1, as well as other proteins such as PKCα and ERK1/2 were performed. The membrane marker, Na^+/K^+ ATPase, was also analyzed to confirm the purity of the purification.

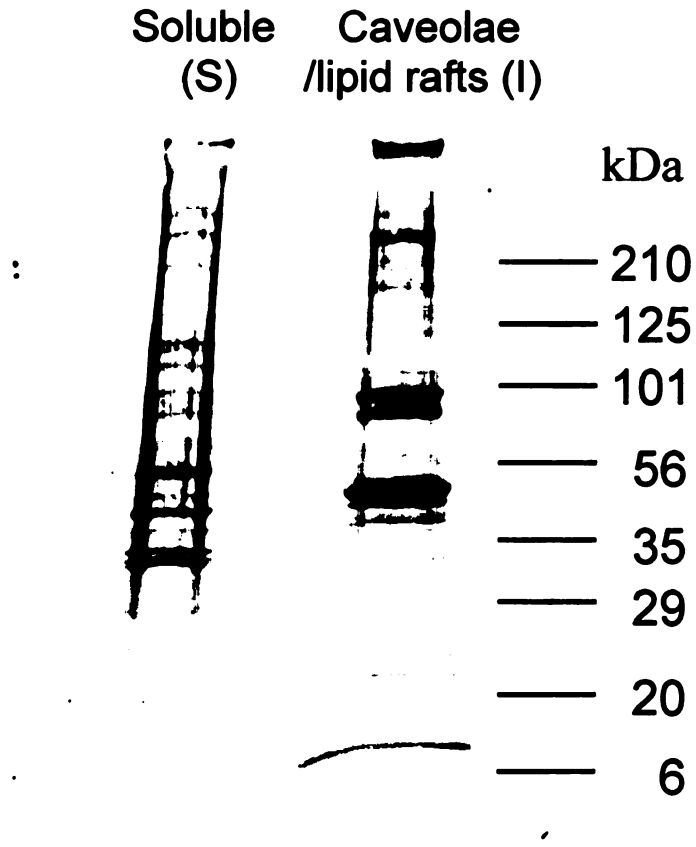

Fig. 2. SyproB Blue staining of caveolae/lipid rafts proteins (I) and soluble (S) proteins isolated from hRVE cells after SDS-PAGE. Caveolae/lipid rafts were obtained from $5x10^6$ hRVE cells as described in Methods. Pooled caveolae/lipid raft fractions (I) and pooled soluble fractions (S) were separated by SDS-PAGE, stained with SyproB Blue and visualized under UV light.

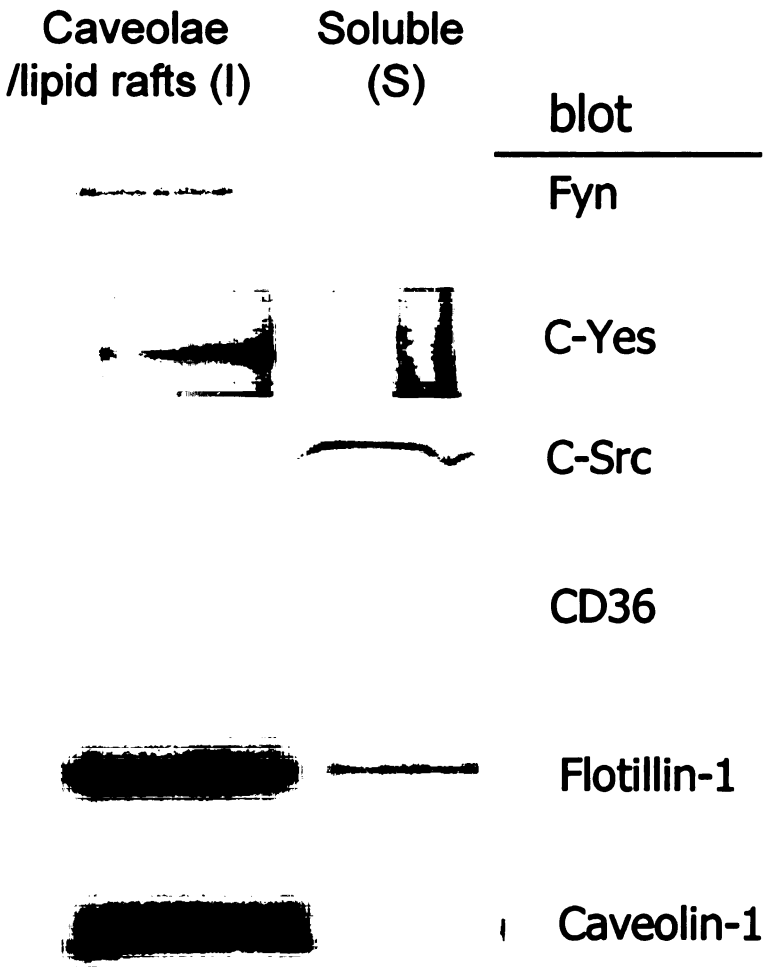

Fig. 3. Localization of Src family kinases in hRVE. Caveolae/lipid rafts from hRVE cells were purified as described in Methods. Pooled caveolae/lipid rafts fractions (I) and pooled soluble fractions (S) were separated on SDS-PAGE and analyzed by western blot.

TABLE I. **Proteins identified by in-gel digestion and LC/MS/MS.**

Identity	Mr(kDa)	Peptides identified	Accession #	Related functions
Marker proteins:				
Flotillin-1	47.3	5	NP_005794	Structural and regulatory proteins of lipid rafts
Flotillin-2	42.6	4	NP_004466	
Caveolin-1	20.458	9	NP_001744	Structural and regulatory proteins of caveolae
Caveolin-2	18.279	3	NP_001224	
Stomatin α	31.71	9	NP_004090	Integral membrane proteins, scaffolding protein of lipid rafts
GPI-linked proteins:				
CD109	161.62	9	NP_598000.1	Alpha2 macroglobulin/complement C3, C4, C5 gene family (AMCOM)
Alkaline phosphatase, tissue non-specific isoform precursor	57.24	2	NP_000469	Exact physiological function unknown
CD59 glycoprotein precursor	14.17	1	P13987	Potent inhibitor of the complement membrane attack complex (mac) action
5'-nucleotidase precursor, CD73	63.327	18	NP_002517.1	Formation of anti-inflammatory and immunosuppressive adenosine from extracellular nucleotides
Cadherin-13 precursor	78.224	5	NP_001248	Calcium dependent cell adhesion molecules
Reversion-inducing cysteine-rich protein with Kazal motifs precursor (hRECK, ST15)	106.38	1	NP_066934	Negatively regulates MMP-9, MMP-2 secretion and activity

Table 1 (cont'd)

Cytoskeleton and related proteins:				
Myosin-IIA HC (non-muscle type A)	226.39	61	NP_002464	Acts as an actin based motor, plays a role in endocytosis and intracellular trafficking
MYO1B protein	131.902	9	NP_036355	
Myosin Ic	117.96	6	NP_203693	
Myosin regulatory light chain (MRCL3)*same peptides as MRCL2	19.78	4	NP_006462 NP_291024	
calmodulin	17.67	1		
Smooth muscle and non-muscle myosin alkali light chain isoform 4	12.93	1	NP_002467	
Tubulin, alpha 6 chain	52.597	3	NP_116093	
Tubulin, alpha 3 chain	52.279	3	NP_006000	
Tubulin beta-1 chain	49.727	5	NP_110400	
Tubulin beta-4 chain	50.4	2	NP_006078	
Actin, cytoplasmic 1 (beta Actin)	41.710	4	NP_001092	
Actin, cytoplasmic 2 (gamma Actin)	41.766	4	P63261	
P63 (CKAP4)	65.98	14	NP_006816	Paramyosin family, integral membrane protein, highly enriched in detergent-insoluble fraction
F-actin capping protein alpha-1 subunit	32.902	2	NP_006126	Mediate actin cytoskeleton organization and biogenesis, cell motility and protein complex assembly
Ion-channel proteins:				
Voltage-dependent anion channel 1	30.639	1	NP_003365	Pore forming proteins also present in plasma membrane especially caveolae other than mitochondria
Transient receptor potential cation channel, subfamily M, member 4 (TRPM4)	136.42	2	NP_060106	A calcium-activated nonselective (CAN) cation channel that mediates membrane depolarization
Dihydropyridine-sensitive L-type, calcium channel alpha-2/delta subunits precursor	123.106	15	NP_000713	Calcium channel protein

Table 1 (cont'd)

Signaling proteins:				
G protein-coupled receptors				
Retinoic acid induced 3 protein (RAIG1 or GPRC5A)	40.225	1	NP_003970	Type 3 G protein-coupling receptor family
G proteins				
G(i)α_2 (GNAI2)	40.27	3	NP_002061	Regulates G protein–coupled receptor activity
Gγ-12 subunit	7.9	1	NP_061329	
GTPases				
R-Ras	23.466	1	NP_006261	A member of the Ras superfamily of small GTPase that has been implicated in promoting cell adhesion and neurite outgrowth
R-Ras2	23.385	1	NP_036382	GTPase
Other signaling proteins				
Serum deprivation response (sdr)	47.14	1	NP_004648	Binds to phosphatidylserine, substrate of PKC
HSRBC	27.625	5	NP_659477	Induced by serum starvation, a binding protein of the protein kinase C, delta (PRKCD)
HSPC121 (butylate induced transcript 1)	44.394	1	NP_057479	GTPase activator activity, involved in Rac mediated JNK activation
Calmodulin	17.5	1	AAB23129	Calcium binding
Chondrocyte-derived ezrin-like domain containing protein (CDEP)	118.55	1	NP_001001715	RhoGEF, crucial for microfilament organization, involved in the adhesion, proliferation, and differentiation

Table 1 (cont'd)

Other proteins:				
CD44	39.41	2	AAA82949	A hyaluronan (HA) receptor
Membrane alanine aminopeptidase precursor (ANPEP)	109.44	8	NP_001141	Member of the zinc-binding metalloprotease superfamily.
Complement C3 precursor	187.1	1	NP_000055	Integral membrane proteins
Complement component C9 precursor	63.133	2	NP_001728	Integral membrane proteins
Protocadherin 10 precursor	118.57	1	NP_065866	Potential calcium-dependent cell-adhesion protein
Heat shock 90kDa protein 1, alpha	84.621	1	NP_005339	Regulation of protein folding and function
Solute carrier family 2, facilitated glucose transporter, member 1	56.9	1	NP_006507	Glucose transporter
Monocarboxylate transporter 4	49.43	1	NP_004687	Proton-linked monocarboxylate transporter.
Lactadherin precursor	43.09	1	NP_005919	Specific ligand for the α5/β3 and α5/β5 receptors, mediate cell adhesion
Basigin long isoform (Basigin 2, CD147)	42.17	1	NP_001719	A regulator of matrix metalloproteinase (MMP) production
Annexin II (lipocortin I)	40.328	3	NP_00100285 8	Calcium-dependent phospholipid-binding protein regulating cellular growth, signal transduction, and exocytosis.
Myeloid-associated differentiation marker	35.25	2	NP_612382	Mal family, help target GPI-linked protein
Vacuolar ATP synthase, catalytic subunit A, ubiquitous isoform	68.26	4	NP_001681	A multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles, necessary for such intracellular processes as protein sorting, zymogen activation, receptor-mediated endocytosis
Vacuolar ATP synthase, subunit B, brain isoform	56.45	4	NP_001684	
Vacuolar ATP synthase, subunit D	40.3	2	NP_057078	
Vacuolar ATP synthase, subunit S1 precursor	52.121	3	NP_001174	
Vacuolar ATP synthase, subunit G 1	13.75	1	NP_004879	
Ubiquitin and ribosomal protein S27a	17.953	1	NP_002945	a fusion protein (ubiquitin and S27a)
Beta-galactosidase binding lectin precursor (galectin-1)	14.70	1	NP_002296	Modulates cell-cell and cell-matrix interactions; may be part of novel anti-inflammatory loop

Table 1 (cont'd)

Possible nonspecific proteins:				
Clathrin heavy chain 1	191.43	2	NP_004850	Main structural protein of the polyhedral lattice surrounding coated pits and coated vesicles, organelles
Hypothetical protein FLJ46846	180.58	6	NP_076965	Moderately similar to Neuroblast differentiation associated protein AHNAK (nucleoprotein)
Desmoglein 2 precursor	122	5	NP_001934	Component of intercellular desmosome junctions mediating cell-cell adhesion
Sodium/potassium-transporting ATPase alpha-3 chain	111.66	2	NP_689509	Sodium pump
Splice isoform SERCA2A of P16615 Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	109.62	2	NP_001672	Intracellular pumps located in the sarcoplasmic or endoplasmic reticula of muscle cells
KIAA0143 protein	103.138	2	NP_055952	Integral membrane protein (potential)
Splice isoform 1 of Q8WXE9 Stonin 2 (stonin 2)	101.102	1	NP_149095	A component of the endocytic machinery that likely regulates vesicle endocytosis
Isoform 3 of Q12906 Interleukin enhancer-binding factor 3 (NFAT90)	82.8	3	NP_703194	Nuclear protein that regulates the activity of protein-arginine methyltransferase I
RPN2 protein (ribophorin II)	73.94	3	NP_002942	Rough endoplasmic reticulum-specific membrane glycoproteins
KIAA0830 protein	59.347	3	XP_290546	ND
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	48.6	1	NP_001924	Mitochondrial, acetyl CoA synthesis

Table 1 (cont'd)

Leucine-zipper protein FKSG13	43.45	5	NP_036364	RNA polymerase I and transcript release factor
α-actin	41.992	3	NP_005150	Smooth muscle actin
Hypothetical protein DKFZp547F237	40.42	1	ND	ND
Glyceraldehyde-3-phosphate dehydrogenase	36.03	2	NP_002037	Dehydrogenase in glucose metabolism
Chromosome 6 open reading frame 188	35.15	2	ND	ND
Muscle-specific DNase I-like precursor	33.8	3	NP_006721	Membrane-bound (potential), belongs to the dnase i family
Hypothetical protein DKFZp564E227 (cytochrome b reductase 1)	31.62	2	NP_079119	Integral membrane, electron transport
Cytochrome c oxidase polypeptide II	25.55	1	NP_653214	Enzyme in electron transport chain
RER1 protein	22.94	1	NP_008964	Golgi integral membrane protein, involved in the retrieval of ER membrane proteins from the early golgi compartment.
Hypothetical protein FLJ46113 (p38IP)	21.114	2	NP_060039	ND

Note: ND: not identified.

TABLE 2. **Proteins identified by in-solution digestion and LC/MS/MS.**

Structural proteins:				
(+) Caveolin-1	20.458	5	NP_001744	Structural and regulatory proteins of caveolae
(+) Stomatin	31.71	9	NP_004090	Integral membrane proteins, scaffolding protein of lipid rafts
GPI-linked proteins:				
(+) CD59 glycoprotein precursor	14.17	1	P13987	Potent inhibitor of the complement membrane attack complex (mac) action
(+) 5'-nucleotidase precursor, CD73	63.327	18	NP_002517.1	Formation of anti-inflammatory and immunosuppressive adenosine from extracellular nucleotides
(+) Cadherin-13 precursor	78.224	5	NP_001248	Calcium dependent cell adhesion molecules
Cytoskeleton and associated proteins:				
(+) Myosin-IIA HC (non-muscle type A)	226.39	15	NP_002464	Acts as an actin based motor, plays a role in endocytosis and intracellular trafficking
(+) MYO1B protein	131.902	2	NP_036355	
(+) Myosin Ic	117.96	2	NP_203693	
(+) calmodulin	17.67	8	AAB_23129	
(+) Actin, cytoplasmic 1 (beta Actin)	41.710	16	NP_001092	
(+) Actin, cytoplasmic 2 (gamma Actin)	41.766	16	P63261	
Ion-channel proteins:				
(+) Dihydropyridine-sensitive L-type, calcium channel alpha-2/delta subunits precursor	123.106	15	NP_000713	Calcium channel protein, integral membrane protein
Signaling proteins:				
(+) G(i)α_2 (GNAI2)	40.27	6	NP_002061	Regulates G protein–coupled receptor activity
G(i)α_3 (GNAI3)	40.506	4	NP_006487	
(+) Gγ-12 subunit	7.9	2	NP_061329	
G(i)/G(s)/G(T) ß subunit 1	37.307	3	NP_002065	
G(i)/G(s)/G(T) ß subunit 4	37.303	3	NP_005264	

Table 2 (cont'd)

Other proteins:				
(+) CD44s	39.417	4	AAA82949	A hyaluronan (HA) receptor
(+) Membrane alanine aminopeptidase precursor (ANPEP)	109.44	6	NP_001141	Member of the zinc-binding metalloprotease superfamily. Type II membrane protein
Annexin A2 (lipocortin II)	38.449	2	NP_001002857	Calcium-dependent phospholipid-binding protein
(+) Myeloid-associated differentiation marker	35.25	2	NP_612382	Integral membrane protein (probable). Mal family, help target GPI-linked protein
Vacuolar ATP synthase, subunit E	31.0	1	NP_001687	A multisubunit enzyme
Thy-1 membrane glycoprotein precursor (CD90)	17.923	4	NP_006279	Activation-associated cell adhesion molecule in EC
Brain acid soluble protein 1 (BSAP1)	22.549	1	NP_006308	Myristoylated protein binds to cholesterol-rich raft-like domains
Bene protein	17.39	1	NP_005425	MAL proteolipid family, an element of the machinery for raft-mediated trafficking in endothelial cells
Nesprin 2	79.585	3	Q8WXH0	Nuclear envelope spectrin repeat protein 2

Note:

(+): indicates the proteins are also identified by in-gel digestion followed by LC/MS/MS.

a: Peptides identified are marked according to the results obtained from in solution digestion.

ND: not identified.

5. Discussion

Here we analyzed the protein composition of caveolae/lipid rafts isolated from cultured human primary retinal endothelial cells using both proteomics and biochemical analyses. As a central organelle of vascular endothelial cells, caveolae/lipid rafts serve as platforms for important functions in mediating endothelial cells signaling, vesicle trafficking, lipid transport and homeostasis. Using Triton X-100 insoluble and low density based sucrose floatation, both caveolae and lipid rafts structural proteins such as caveolin-1, 2 and flotillin-1, 2 as well as the stomatin alpha subunit (which forms oligomers and acts as an important structural protein) were identified by MS and biochemical analyses. Besides its structural role in caveolae, the 22 KDa palmitoylated protein, caveolin-1, also regulates cholesterol efflux due to its high affinity for cholesterol. It also acts as a central scaffolding protein for a variety of signaling complexes in the regulation of EC proliferation, migration and vascular permeability.

Proteins dually acylated by saturated fatty acids are generally believed to specifically target to the caveolae/lipid rafts. This was true in our study in that several proteins known to be dually acylated were all identified in caveolae/lipid rafts from hRVE. This includes G proteins, especially G(i) α2, 3 subunits and G$\beta\gamma$ subunits. The heterotrimeric GTP-binding protein G_i binds to caveolin-1 and plays a fundamental role in the mechanism of caveolae-mediated endocytosis involving Src family kinases[26, 27]. Small GTPases such as R-Ras and R-Ras2 are also present in this fraction. It is worth noting that a new protein called RAIG1 (retinoic acid induced protein 3), which is a type

3 G protein-coupled receptor, was first identified in caveolae/lipid rafts, suggesting a presence of caveolae/lipid rafts mediated retinoic acid activated GPCR signaling.

Our proteomic method failed to identify several well-characterized caveolae/lipid raft localized signaling proteins such as eNOS and members of the Src family kinases. This could be due to their low expression levels in hRVE, since the presence of Fyn and c-Yes were all confirmed by western blot (Fig. 3). We were not able to detect any integral membrane receptors such as PDGFR, EGFR[28] and VEGFR2[29, 30], which were previously reported localized in caveolae in microvascular or cultured endothelial cells by biochemical analyses. This is possible since our caveolae/lipid rafts were isolated from cells under serum starvation that might exclude receptors from caveolae/lipid rafts due to the resting state of the cells. Another possibility is that the expression levels of the receptors in primary cells are not sufficient for detection by both MS and biochemical analyses. Also worthy of mention is that several signaling proteins induced under serum starvation were first identified as being colocalized with caveolae/lipid rafts in hRVE. This includes serum deprivation response (sdr) and HSRBC. The former is a substrate of PKCα which is highly concentrated in caveolae[31]; the latter binds to protein kinase Cδ under serum starvation[32]. This suggests a possible role of caveolae/lipid rafts in PKC mediated serum starvation induced control of cell growth. The exact presence and role of PKC, Sdr and HSRBC in caveolae/lipid rafts need to be further characterized.

GPI-linked surface proteins partition into the exoplasmic membrane leaflets of caveolae/lipid rafts and mediate compartmentalized signaling. They have been claimed

to be mainly present in lipid rafts with a dynamic assembly, with or without ligand binding. A group of GPI-linked glycoproteins were identified including GPI-protein alkaline phosphatase and 5'-nucleotidase precursor (CD73). CD109, a protein belonging to alpha2 macroglobulin/complement C3, C4, C5 gene family (AMCOM), and CD59 glycoprotein precursor, which inhibits the complement membrane complex (mac) action are all identified by in-gel digestion and MS. Interestingly, several complement components such as complement C3, C9 precursors were all found in the caveolae/lipid rafts fraction suggesting that caveolae/lipid rafts may act as platforms for concentrating molecules regulating complement complex recruitment, activation and function.

A connection between caveolae and cytoskeleton has been well described. Our finding that low-density detergent insoluble fraction in hRVE cells contain actin and actin binding membrane skeletal proteins (such as myosin-IIA) is consistent with previous reports in various cells[19, 21, 24]. The identification of all of the known components of the myosin motor (heavy chain, alkali and regulatory light chains) indicates a possible function of caveolae/lipid rafts in the regulation of cytoskeleton reorganization. The presence of tubulin in caveolae/lipid rafts, also reported in Hela cells[24], could have important implications for membrane restructuring since cytosolic tubulin is thought to be added to the plus ends of the microtubules within lipid rafts to maintain the length of the microtubules associating with the plasma membrane. All of this suggests caveolae/lipid rafts are high dynamic microdomains that are associated with cellular microtubule transport machinery to mediate the endocytosis or transcytosis. This notion was further confirmed by the identification of proteins involved in the molecular transport machinery for vesicle budding, docking and fusion including annexins and

GTPase etc. Also worthy of mention is the identification by MS of the type-II transmembrane protein p63 (CKAP4, cytoskeleton-associated protein 4), a member of paramyosin family. The specific role of p63 in microvascular endothelial cells was not characterized with a possible role of binding to tPA to mediate plasmogen activation in vascular smooth muscle cells[33].

Involvement of caveolae in cell interaction with the extracellular matrix is through regulation of matrix degradation, an essential process in mediating endothelial cell migration and angiogenesis. Specific MMPs such as MMP1 and 2 were all reported to colocalize with caveolae in tumor cell lines[34, 35]. In hRVE, membrane alanine aminopeptidase precursor (ANPEP), a membrane zinc-binding MMP superfamily, is concentrated in caveolae/lipid rafts fractions along with several other proteins modulating matrix metalloprotease secretion and activity, such as GPI-anchored protein reversion-inducing cysteine-rich protein with Kazal motifs (RECK), a membrane anchored inhibitor of MMPs involved in inhibiting EC migration[36]. Recent evidence also suggests that CD147, a regulator of MMP production, specifically associates with caveolin-1 which diminishes CD147 MMP-inducing activity[37]. Furthermore, calcium dependent cell adhesion molecules such as cadherin-13 precursor, and protocadherin 10 precursor were all identified in this special compartment. Taken together, matrix-degrading enzymes or their regulators and cell adhesion receptors are enriched in a limited microenvironment (caveolae/lipid rafts) at the cell surface to mediate EC transmigration.

Caveolae/lipid rafts are also shown to be sites for concentration of ion channels that regulate endothelial cell permeability and receptor mediated endocytosis, etc. Voltage-dependent anion-selective channel proteins and vacuolar (H^+)–ATPase subunits are all identified in caveolae/lipid rafts of hRVE. These ion-channel proteins mainly exist in mitochondria, but also in plasma membrane especially caveolae to regulate the intracellular acidity as well as plasminogen activation in EC[38, 39]. The transient receptor potential cation channel, subfamily M, member 4 is also present in caveolae/lipid rafts acting as cation channel regulating ion transport. Two forms of integral membrane calcium channels are identified as indicated in Table I suggesting that caveolae may mediate calcium signaling. The presence of ion-channel proteins in caveolae/lipid rafts implicates specific involvement of caveolae/lipid rafts to regulate cation transport and thus permeability and various other functions.

Surprisingly, glucose transporter 1 (Glut-1) and monocarboxylate transporters are also present in caveolae/lipid rafts. Both Glut-1 and monocarboxylate transporters, a large family of proton-driven transporters possessing 12 membrane spanning domains, were previously detected in the lipid rafts fraction from both Hela[22] and the monocytic cell line THP-1 cells[21]. Like glucose, the monocarboxylates lactate and pyruvate play central roles in retinal cellular metabolism as important energy sources. As stated earlier, CD147, an accessory protein which determines their trafficking, subcellular localization and functional expression was also identified indicating a role of caveolae/lipid rafts in the trafficking and localization of the transporters in endothelial cells and in regulating of energy uptake[40]. This is of particular interest in hRVE cells since hRVE forms an

inner blood retinal barrier to serve as an active interface to provide energy and support retinal functions.

An interesting finding is that CD44, a phagocytic glycoprotein, is abundantly enriched in caveolae/lipid rafts in hRVE. CD44 is an alpha-helical integral membrane that is recognized as a major cell surface receptor for hyaluronic acid[41]. CD44 is also involved in different physiological and pathophysiological processes including inflammation and tumor metastasis[42, 43]. Western blot using anti-CD44 confirmed its presence (data not shown). The specific functions of CD44 involving caveolae/lipid rafts in hRVE wait to be further investigated.

Due to the high lipid content and existence of hydrophobic membrane proteins in the caveolae/lipid rafts fractions, only a limited number of peptide peaks were detected from in-solution digestion methods. It is believed that this is mainly due to the signal suppression during the ionization and detection processes and also the complexity of the tryptic peptides which were unable to be fully separated in the one dimensional HPLC we applied in our study.

It is not possible to discern how much of this data reflects plasma membrane caveolae/lipid rafts, as the detergent insoluble fraction was isolated from whole cells, without excluding intracellular membranes. Previous biochemical isolations proposed the presence of rafts in other membrane compartments. Clearly, both rafts lipids and proteins are synthesized in the reticulum/Golgi before transport to the plasma membrane. Furthermore, a direct transport route from caveolae to ER exists in endothelial cells[8]. All of this verifies our identification of many synthesis proteins and vesicle trafficking

proteins in the caveolae/lipid rafts fractions isolated from hRVE. Contamination by abundant cytosolic proteins is also a common problem encountered during proteomic analysis of subcellular organelles. A recent analysis using agents that deplete cholesterol to disrupt caveolae/lipid rafts identified about 37% proteins unaffected suggesting that they probably represent non-raft proteins[22].

In summary, of the 70 proteins we identified here, about 25 proteins are possible contaminants from either different organelles or cholesterol nondependent proteins. Some atypical endothelial proteins such as α-actin, a molecular marker for pericytes were also detected. This is explainable, since our preparation of primary hRVE cells can not ensure 100% purity, although above 90% purity is always achieved.

Taken together, our study for the first time systematically characterized the protein components of caveolar/lipid rafts in human retinal endothelial cells using proteomic approaches. The findings of numerous proteins provide compelling evidence for important roles of endothelial caveolae/lipid rafts in regulating the functions of inner blood retinal barrier. The compartmentalization of important signal transducers, effectors and receptors in caveolae/lipid rafts suggest critical roles of caveolae/lipid rafts as functional platforms for the organization and coordination of signaling pathways involved in retinal endothelial cell growth, migration and especially inflammation.

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V. Inhibition of retinal endothelial cell inflammatory response: Modification of caveolae/lipid rafts by Docasahexanoic acid ($DHA_{22:6n3}$) treatment

1. Abstract

An early stage diabetic retinopathy has been recently recognized as a low-grade chronic inflammatory disease involving increased cytokine production, intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) expression and subsequent leukocyte adhesion and blood retinal barrier breakdown. A decrease in a major n3 PUFA, docosahexaenoic acid ($DHA_{22:6n3}$) in the retina of animal models of diabetes was reported. $DHA_{22:6n3}$ inhibits cytokine induced ICAM-1 and VCAM-1 expression in human retinal vascular endothelial cells (hRVE). However, the mechanisms underlying anti-inflammatory effect of $DHA_{22:6n3}$ in hRVE cells remain unresolved. The possibility that $DHA_{22:6n3}$ acts through modifying the lipid composition resulting in change of the affinity of important signaling molecules for a specialized plasma membrane microdomain, the caveolae/lipid rafts, was addressed. We show that the structure of caveolae/lipid rafts as well as its components Src family kinases (SFK) are involved in mediating cytokine induced VCAM-1 expression. Treatment of hRVE cells with $DHA_{22:6n3}$ resulted in significant displacement of the SFKs, Fyn and c-Yes, from caveolae/lipid rafts. Notably, enrichment of $DHA_{22:6n3}$ in endothelial cells resulted in significant incorporation of $DHA_{22:6n3}$ into major phospholipids (PC, PE and PI) that predominantly reside in both exoplasmic and cytoplasmic leaflets of caveolae/lipid rafts which subsequently increased the unsaturation ratio of this specialized high lipid-ordered

membrane microdomain. Moreover, $DHA_{22:6n3}$ enrichment also caused about 70% depletion of cholesterol from caveolae/lipid rafts. $DHA_{22:6n3}$ modification of fatty acyl chains of phospholipids in caveolae/lipid rafts followed by cholesterol depletion could effectively perturb the lipid environment of caveolae/lipid rafts resulting in the displacement of important signaling molecules thus an immunomodulatory effect in endothelial cells.

2. Introduction

Polyunsaturated fatty acids (PUFAs), particularly n3-PUFAs such as eicosapentaenoic acid ($EPA_{20:5n3}$) and docosahexaenoic acid ($DHA_{22:6n3}$) have long been demonstrated to be immunoregulatory by suppressing the activation of immune cells and the production of inflammatory cytokines and adhesion molecules[1, 2]. Numerous clinical studies have shown that dietary supplementation with fish oil abundant in n3-PUFAs has beneficial effects in the treatment of inflammatory disorders such as inflammatory bowel diseases, asthma, arthrosclerosis and cardiovascular diseases and thus are widely applied clinically as adjuvant immunosuppressive agents[3].

Several mechanisms have been linked to the immunosuppressive functions of n3-PUFAs. The replacement of $AA_{20:4n6}$ due to incorporation of n3-PUFAs into membrane phospholipids reduces the amount of $AA_{20:4n6}$ available for oxygenases. This will decrease the production of inflammatory eicosanoids, such as series 1 and 2 thromboxanes and prostaglandins, series 4 leukotrienes, and hydroxy and epoxy fatty acids [4]. Recent discovery of potent anti-inflammatory eicosanoids: E series and D series resolvins derived from $EPA_{20:5n3}$ and $DHA_{22:6n3}$ respectively by COX-2, constitutes a novel mechanism(s) for the therapeutic anti-inflammatory benefits of n-3 dietary supplementation[5-7] and reviewed in [8]. Moreover, n3-PUFAs could also directly bind to and activate nuclear receptors such as peroxisome proliferator-activated receptor (PPAR) that are shown to play an anti-inflammatory role in various cells (reviewed in [9]).

In vitro studies have strongly demonstrated that the inhibitory effects of n3-PUFA on T cell signal transduction are primarily due to eicosanoids independent mechanisms, particularly the modification of functional membrane lipid microdomains called lipid rafts[10]. Lipid rafts are specialized micromembrane domains highly enriched with cholesterol and sphingolipids such as sphingomyelin and glycolipids. Polar lipids in lipid rafts predominantly contain saturated fatty acyl residues that aggregate spontaneously to form liquid-ordered membrane regions insoluble in non-ionic detergents[11, 12]. Lipid rafts are essential in lymphocyte signal transduction[13, 14]. Treatment of cultured T cells with PUFAs selectively displaces acylated proteins such as LCK and LAT from the cytoplasmic leaflet of lipid rafts[15, 16] thus inhibiting the T cell activation and proliferation. This displacement of important signaling molecules is due to the incorporation of n3-PUFAs into the lipids of both exoplasmic and cytoplasmic leaflets of lipid rafts[15]. Dietary n3-PUFA or high purified $DHA_{22:6n3}$ treatment in vivo has also demonstrated a significant displacement of important signaling molecules concomitant with decreases in cholesterol or sphingomyelin contents in mouse colon or splenic T cell lipid rafts due to n3-PUFAs incorporation[17-20]

Vascular endothelium is the interface between blood elements and tissues and plays a vital role in immune responses especially inflammation. Caveolae, a specialized micromembrane domain which shares similar lipid compositions as lipid rafts are highly abundant in endothelial cells[21]. Caveolae are stabilized by a family of structural and regulatory proteins, caveolin, that gives caveolae its characteristic flask-like shape. Caveolae have been shown to be important players in regulating vascular permeability, vesicle trafficking, cholesterol homeostasis and particularly signal transduction[21-25].

A number of cytokine receptors have been found localized in caveolae/lipid rafts, such as TNFR1[26, 27] and VEGFR2 in endothelial cells[28, 29]. Our previous study has demonstrated that n3-PUFAs, particularly $DHA_{22:6n3}$ inhibits cytokine induced inflammatory response in primary human retinal endothelial cells (hRVE). However, the molecular pathways underlying the inhibition are not well resolved in endothelial cells.

In this study we examined the role of caveolae/lipid rafts in inflammatory signaling in hRVE cells. The effect of $DHA_{22:6n3}$ treatment on localization of SFKs and modification of lipid composition of caveolae/lipid rafts were further addressed. Our data provide strong evidence that caveolae/lipid rafts are required for endothelial cell inflammatory responses. Changes in fatty acyl chains of phospholipids and cholesterol displacement from caveolae/lipid rafts induced by $DHA_{22:6n3}$ could represent a principal mechanism for the anti-inflammatory effect of $DHA_{22:6n3}$ in hRVE cells.

3. Materials and Methods

3.1. Reagents and antibodies

HPLC grade acetonitrile, acetic acid, methanol, chloroform and methyl-cyclodextrin (MCD) were purchased from Sigma. 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) was obtained from Calbiochem. The following antibodies were used: Mouse anti-caveolin-1 and flotillin-1 were from Upstate Biotechnology, Inc. (Lake Placid, NY); mouse anti-c-Src, Fyn, rabbit antibodies against c-Yes, and CD36 were purchased from Santa Cruz.

3.2. Cell culture and fatty acid treatment

Primary cultures of hRVE cells obtained from at least three donors were prepared and cultured as previously described[30]. Passages 1-6 were used in the experiments. Primary human umbilical vein endothelial cells (HUVEC, multiple donors) were obtained from Cascade Biologicals (Portland, OR) and cultured in DMEM containing 10% FBS, macrovascular endothelial cell growth supplement (MVGS) and 100 μg/ml antibiotics/antimycotics in a humidified incubator at 37 °C with 5% CO_2. For experimental treatments, cells were transferred to serum-free medium for 18 to 24 hours before addition of the stimulatory agents. Treatment of cells with fatty acids was performed as follows. Fatty acid stocks were prepared by dissolving fatty acids (NuCheck Prep, Inc., Elysian, MN) in 100% ethanol to a final concentration of 100 mM fatty acid as described previously. The fatty acid stock solutions were diluted in serum-free medium to reach fatty acid concentrations of 100 μM with corresponding bovine

serum albumin (BSA) concentration of 20 μM. Charcoal-treated, solvent-extracted, fatty acid–free BSA was obtained from Serologica Inc., Norcross, GA. The fatty acid-to-albumin molar ratio was maintained at 5:1. The final concentration of ethanol in the media is less than 0.1%. Cells were incubated for the times indicated in the Results section. Equivalent amounts of BSA alone were added to control plates.

3.3. SDS-PAGE and western blot

Cells were lysed in the lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1% Triton X-100, 10% glycerol) with freshly added protease inhibitor cocktail (Sigma) and phosphatase inhibitors (1 mM Na3VO4, 100 μM glycerophosphate, 10mM NaF, 1 mM Na4PPi). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose, immunoblotted using appropriate antibodies followed by secondary horseradish peroxidase conjugated antibody (Bio-Rad). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ). Blots were quantitated by scanning densitometry using ImageJ software, ver. 1.29 (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

3.4. Subcellular fractionation

Post-nuclear supernatants were prepared by lysing fatty acid treated cells in hypotonic buffer (10 mM Hepes pH 7.4, 1 mM EDTA, 1 mM $MgCl_2$ freshly added with protease inhibitors) for 30 min on ice, then dounced 20 times in a Teflon vortexer and

spun at 500 X g for 5 min. The supernatants were subjected to centrifugation (16,900 X g, 60 min, 4°C) and the pellets were collected as total plasma membrane enriched fractions (also called bulk membranes).

Isolation of caveolae/lipid raft enriched detergent resistant membrane domains were prepared using a slightly modified sucrose gradient ultracentrifugation protocol [31]. Briefly, $5x10^6$ hRVE cells were washed with cold PBS twice and then 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 and fresh protease and phosphatase inhibitors and kept on ice for 20 min. The homogenization was carried out with 10 strokes of a tight-fitting Dounce homogenizer, and then spun at 4,000xg 4°C for 10 min. Supernatant (0.8 ml) was then mixed with the same volume of 80% sucrose prepared in MNE buffer, and placed at the bottom of an ultracentrifuge tube. 1.6 ml of 30% sucrose and 0.8 ml of 5% sucrose were overlaid on top of the sample to form a 5-30% discontinuous sucrose gradient. After 16 h centrifugation at 200,000xg at 4°C using a swinging bucket rotor, 0.4 ml samples were collected carefully from the top for each fraction. A band confined to fractions 2 through 4 was designated as caveolae/lipid rafts enriched membrane domains. The combined 2-4 fractions were further diluted 3 times in MNE buffer and spun at 200,000xg at 4°C for another 2 h to precipitate the caveolae/lipid rafts and the pellet was designated as insoluble fraction (I). The fractions 6-10 were also combined and designated as soluble fraction (S).

3.5. Fatty Acid Metabolism

hRVE cells were plated at 0.12x106 cells/6 cm plate and cultured as indicated above to about 90% confluence. Cells were then serum starved for 18-24 hours and treated with [^{14}C]22:6n3 in 3 ml of DMEM/F12 containing 100 μM 22:6n3, 0.5 μCi, (1.7 Ci/mol) for 1.5 and 24 h in the presence of 20 μM BSA. ^{14}C-labeled fatty acids were purchased from PerkinElmer Life Sciences. The fatty acid-to-albumin molar ratio was 5:1. At harvest, cells were washed once with phosphate-buffered saline + 20 μM BSA followed by PBS alone once. Cells were then resuspended in 500 μl of 40% methanol and lipids were extracted with chloroform:methanol (2:1); dried under nitrogen and dissolved in chloroform for storage at -80 °C. Total lipids were separated by thin layer chromatography (LK6D Silica G 60A, Whatman) in hexane:diethyl ether:acetic acid (90:30:1). Polar lipids were separated in chloroform:methanol:acetic acid (30:20:4). Location of lipids was compared with authentic standards for triacylglycerol (TAG), diacylglycerol (DAG), cholesterol esters (CE), fatty acids, fatty acid (wax) esters (Sigma), and glycerol- and sphingo-phospholipids (Avanti Polar Lipids).

3.6. Fatty Acid and Cholesterol Analysis

Total lipids of caveolae/lipid rafts and total plasma membranes corresponding to equal amounts of protein (measured by Bradford assay (Biorad)) were extracted with chloroform-methanol [2:1], dried and stored in chloroform at –80°C. A fraction of the total lipids was further fractionated on an amino-propyl (Alltech) column to obtain neutral lipids, neutral phospholipids, acidic phospholipids and nonesterified fatty acids (NEFA) as described[32]. The neutral lipids were separated by Waters YMC-Diol-

120NP 5 μM, 250x4.6 mm column followed by evaporative light scatter detection system to detect cholesterol. Pure lipid standards for cholesterol, triacylglycerol, diacylglycerol obtained from Avanti Polar Lipids were used after each experiment to confirm retention times and purity. In order to quantify the data, calibration curves were prepared for each class of lipids at the end of each experiment.

A fraction of neutral phospholipids and acidic phospholipids were saponified (0.4 N KOH in 80% methanol, 50°C for 1 h) for the fatty acid composition analysis respectively. After saponification, lipids were acidified and extracted with diethyl ether with 0.1% acetic acid, dried under nitrogen and stored in methanol. Saponified lipids were fractionated and quantitated by reverse phase HPLC (RP-HPLC) using a YMC J-Sphere (ODS-H80) column and a sigmoidal gradient starting at 86.5% acetonitrile + acetic acid (0.1%) and ending at 100% acetonitrile + acetic acid (0.1%) over 50 min with a flow rate of 1.0 ml/min using a waters 600 controller. Fatty acids were detected using both UV absorbance at 192 nm (Waters model 2487) and evaporative light scatter (Waters model 2420). Fatty acid standards for RP-HPLC were obtained from Nu-Chek Prep (Eysian, MN). The relative concentration for each fatty acid was obtained by normalizing to the standards.

3.7. Mass spectrometry of phospholipids

Phospholipids analyses by mass spectrometry (MS) were performed using a Thermo LTQ linear quadrupole ion trap mass spectrometer (model LTQ, Thermo-Finnigan, San Jose, CA), equipped with a nanospray ionization source (nanoESI). Total lipid extracts from each sample were dissolved in 50:50:1 methanol/chloroform/28%

ammonia hydroxide prior to introduction to the mass spectrometer by direct infusion through non-coated silica tips with internal diameters of 30 μm (New Objective, Inc. Woburn, MA) at a flow rate of 0.2 μL/min. NanoESI conditions were optimized to maximize the sensitivity and stability of the ions of lipids while minimizing "in-source" fragmentation. Typical nanoESI conditions were: heated capillary temperature 150°C, spray voltage 1.3kV, capillary voltage 20V and tube lens voltage 50V. Quantitative analysis of every sample was based on triplicate MS experiments, each being an average of 50 spectra with 3 micro-scans acquired per spectrum, in both positive and negative ionization modes under the same instrument settings. Intensities of all the ions detected in each sample were first normalized to the internal standards (dimyristoylphosphatidylcholine for positive ionization mode and dimyristoylphosphatidylethenolamine and dimyristoylphosphatidylglycerol for negative ionization mode), and the log of the ratios of normalized intensities between lipids from cells treated with BSA, palmitic acid 16:0 or $DHA_{22:6n3}$ were then plotted to determine those lipids whose abundance were affected most significantly by the different treatments. MS/MS and MS/MS/MS (MS^3) data for each sample were acquired by a data-dependent tandem mass spectrometry experiment, during which an initial MS scan was followed by CID MS/MS (collision energy 30%) of the most abundant precursor ion, followed by MS^3 of the three most abundant product ions obtained from the MS/MS scan. Following the MS/MS scan event, the selected precursor ion was placed on an exclusion list so that subsequent data-dependent acquisitions allowed the analysis of progressively lower-abundance precursor ions. A total of the 40 most intense precursor ions in each sample were sequentially selected for analysis. The structures of the lipids previously

determined to be significantly different between samples from the triplicate MS experiments were identified by interpretation of the spectra from the data-dependent tandem MS/MS experiments.

4. Results

4.1. Caveolae/lipid rafts are involved in $VEGF_{165}$ and TNFα induced CAM expression

In this chapter, we used two different approaches to determine whether caveolae/lipid rafts are involved in cytokine induced CAM expression in endothelial cells. Methyl-cyclodextrin (MCD), a cholesterol-depleting agent was first used to disrupt the structure of caveolae/lipid rafts. As shown in Fig. 1, MCD pretreatment downregulated TNFα induced phosphorylation of IκBα, a critical step of triggering the downstream NFκB activation to mediate cytokine induced adhesion molecules expression. In contrast, the TNFα triggered ERK phosphorylation was generally not affected, although cholesterol depletion had an effect on the basal activation of ERK1/2 (Fig. 1). A similar effect was observed in IL-1β meditated IκBα phosphorylation in response to MCD treatment (data not shown). This suggests that the integrity of caveolae/lipid rafts is required for cytokine induced NFκB signaling and downstream cell adhesion molecule (CAM) expression.

As shown in Chapter III, Src family kinases (SFK) especially Fyn and c-Yes were exclusively localized in caveoae/lipid rafts. Our second approach was to examine whether inhibition of SFKs localized in caveolae/lipid rafts would lead to a decrease of cytokine induced inflammatory response. PP2, a specific inhibitor of SFKs (c-Src, Fyn, and c-Yes) was used to pretreat HUVEC for 30 min before the addition of $VEGF_{165}$ for another 6 h. Fig. 2A demonstrated that PP2 prevented $VEGF_{165}$ induced VCAM-1

expression, suggesting that Src family kinases are involved in $VEGF_{165}$ mediated inflammatory signaling leading to induction of the expression of adhesion molecules. Furthermore, pretreating cells with PP2 also diminished the TNFα induced VCAM-1 expression in hRVE cells (Fig. 2B), indicating that SFKs localized in caveolae/lipid rafts are important in the cytokine induced inflammatory response.

4.2. $DHA_{22:6n3}$ displaces Src family kinase from caveolae / lipid rafts in endothelial cells

The dual acylated Src family kinases are targeted to the cytoplasmic leaflets of caveolae/lipid rafts due to posttranslational modification with fatty acyl moieties. The next experiment was designed to determine whether $DHA_{22:6n3}$ treatment could alter the association of SFKs with caveolae/lipid rafts, thus preventing SFKs recruitment to activated cytokine receptors. Pre-treatment of hRVE cells with 100 μM $DHA_{22:6n3}$ caused about 60% displacement of SFK Fyn from the caveolae/lipid rafts (Fig. 3A and quantitated in Fig. 3B). Displacement of c-Yes from this specialized microdomain was also obvious after $DHA_{22:6n3}$ enrichment compared with BSA alone treated cells (Fig. 3C). $DHA_{22:6n3}$ treatment did not cause the disruption of caveolae/lipid rafts since the major structural proteins of caveolae and lipid rafts: caveolin-1 and flotillin-1 were not affected. Moreover, the localization of CD36, a type II integral membrane scavenger receptor involved in oxidized lipid and fatty acids transport, was also not changed after $DHA_{22:6n3}$ enrichment, suggesting a specific displacement of Src family kinases by $DHA_{22:6n3}$ enrichment. The displacement of Fyn and c-Yes was endothelial cell specific as in

human retinal pigmented epithelial cells (hRPE), treatment with up to 200 μM $DHA_{22:6n3}$ had no effect on SFKs localization in caveolae/lipid rafts (Fig. 3D).

4.3. $DHA_{22:6n3}$ treatment alters fatty acyl compositions of phospholipids residing in caveolae/lipid rafts

The biochemical alterations underlying selective displacement of Src family kinases by $DHA_{22:6n3}$ in endothelial cells are largely unknown. An attractive possibility is that enrichment of caveolae/lipid rafts with $DHA_{22:6n3}$ modifies lipid composition of the rafts resulting in a change of the affinity of acylated proteins, such as SFKs, to the caveolae/lipid rafts. The following approaches were used to determine the changes in lipid composition of caveolae/lipid rafts after $DHA_{22:6n3}$ treatment.

a) $DHA_{22:6n3}$ is metabolized into phospholipids in hRVE

Metabolic labeling of hRVE cells using ^{14}C-$DHA_{22:6n3}$ clearly demonstrated that $DHA_{22:6n3}$ was taken up into the cell and rapidly incorporated into different intracellular lipid complexes such as DAG, NEFA, triglycerides and, to a large proportion, polar lipids (Fig. 4A). Further separation of polar lipid fraction demonstrated the presence of several primary phospholipid subspecies, mainly in the form of phosphotidylcholine (PC), and, to a lesser extent, phosphotidylinositol (PI) (Fig. 4B). Since phospholipids are important structural and signaling lipids present in caveolae/lipid rafts, the possibility that $DHA_{22:6n3}$ could affect the lipid environment of caveolae/lipid rafts to displace acylated proteins is very plausible.

b) $DHA_{22:6n3}$ is incorporated into phospholipids of caveolae/lipid rafts

Next, we isolated caveolae/lipid rafts fractions from cells treated with BSA (carrier control), 100 μM palmitate$_{16:0}$ (Lipid control) or 100 μM DHA$_{22:6n3}$ and assessed the fatty acyl composition of phospholipids from caveolae/lipid rafts in parallel with plasma membranes by RP-HPLC (Table 1). Caveolae/lipid rafts from control BSA and palmitate treated cells were particularly enriched in saturated palmitic (16:0) and stearic (18:0) in neutral phospholipids (mainly PC and PE) and acidic phospholipids (PS, PI and PA). The monounsaturated (18:1n9) and polyunsaturated fatty acids (18:2n6, 20:4n6, and 20:5n3) were less abundant compared with bulk membranes. DHA$_{22:6n3}$ treatment led to its significant incorporation not only in bulk membranes but also in caveolae/lipid rafts (Fig. 5A). The incorporation of DHA$_{22:6n3}$ into neutral phospholipids was much more efficient than acidic phospholipids in both bulk membrane (26.44% vs 2.76%) and caveolae/lipid rafts (3.19% vs 0.67%) (Fig. 5A). This could be due to the fact that PC and PE are the most abundant phospholipids present in plasma membranes. The unsaturation index was significantly lower in caveolae/lipid rafts compared with bulk membranes suggesting a high lipid-ordered state in caveolae/lipid rafts. DHA$_{22:6n3}$ treatment caused a considerable increase (50%) in the unsaturation index in neutral phospholipids (0.36 vs 0.18 double bonds per fatty acyl residue) in caveolae/lipid rafts compared with that of control hRVE cells, although this change is to a lesser content compared with general membranes (1.77 vs 0.55 in DHA treated vs control cells)(Fig. 5B). Palmitate treatment led to an increase of 16:0 levels in both caveolae/lipid rafts and total plasma membranes with a concomitant decrease in both 18:0 and 18:1n9 when compared with control cells. Increased level of 16:1n9, the elongated product of 16:0 was also observed in palmitate treated total plasma membranes (Table 1). The

unsaturation index of total membrane as well as caveolae/lipid rafts lipids was moderately decreased in palmitate treated vs. control cells. Taken together, $DHA_{22:6n3}$ is effectively incorporated into phospholipids of caveolae/lipid rafts and significantly alters the lipid environment of these specialized membrane microdomains.

c) $DHA_{22:6n3}$ alters fatty acyl chains of phospholipids residing in both cytoplasmic and exoplamsic membrane leaflets

Considering RP-HPLC analyses could only provide information about the overall fatty acyl compositions in general phospholipids species, we performed nanoESI/MS and MS/MS to identify the specific phospholipid subspecies into which $DHA_{22:6n3}$ was incorporated. The total lipid extracts of caveolae/lipid rafts and bulk membranes from cells treated with BSA, palmitic (16:0) and $DHA_{22:6n3}$ were analyzed in parallel by nanoESI/MS according to mass to charge (m/z) values. Typical MS profiles were shown for total plasma membranes (Fig. 6A: positive mode; 6B: negative mode). The major phospholipid species identified were listed as in Table 2. Overall, in bulk membranes, substitutions of phospholipids by $DHA_{22:6n3}$ in the fatty acyl moieties were observed mainly with PC and possibly with PI and PS. Accordingly, PC 38:6 (16:0/22:6, m/z 806) was markedly more abundant compared with control (BSA) and palmitate treated bulk membranes suggesting the most probable substitution by $DHA_{22:6n3}$. Palmitate treatment also leads to an increase of PC species of 32:0 (16:0/16:0) and 34:1 (16:1/18:0 or 18:1/16:0). $DHA_{22:6n3}$ treatment did not significantly affect the level of sphingomyelin (SM) present in bulk membranes and no incorporation of $DHA_{22:6n3}$ into sphingomyelin was observed since SM is only present with16:0 at its 2-amino fatty acid group (m/z 703).

Overall, enrichment of $DHA_{22:6n3}$ resulted in considerable incorporation of $DHA_{22:6n3}$ into phospholipids residing in exoplasmic (PC) as well as cytoplasmic (PS and PI) leaflets of membranes. The alterations of phospholipids in the total plasma membranes could unequivocally affect the phospholipids components in caveolae/lipid rafts that is now under intensive study.

4.4. $DHA_{22:6n3}$ enrichment causes cholesterol depletion in caveolae/lipid rafts

The introduction of bulky chains such as $DHA_{22:6n3}$ into fatty acyl chains of phospholipids which normally contain highly packed saturated fatty acids could possibly affect the interaction of fatty acyl moieties with cholesterol in both cytoplasmic and exoplasmic leaflets of caveolae/lipid rafts. As such, incorporation of $DHA_{22:6n3}$ into phospholipids might affect the cholesterol partition in caveolae/lipid rafts. Indeed, Fig. 7 shows that the cholesterol levels in caveolae/lipid rafts are about 10 times higher than total plasma membranes confirming the idea that caveolae/lipid rafts are highly enriched in cholesterol. However, $DHA_{22:6n3}$ enrichment decreased the cholesterol level in caveolae/lipid rafts by about 70% compared with BSA treated control, while palmitic acid 16:0 had no significant impact. Moreover, the depletion of cholesterol induced by $DHA_{22:6n3}$ only occured in caveolae/lipid rafts with no significant effect on total cholesterol levels in bulk membranes.

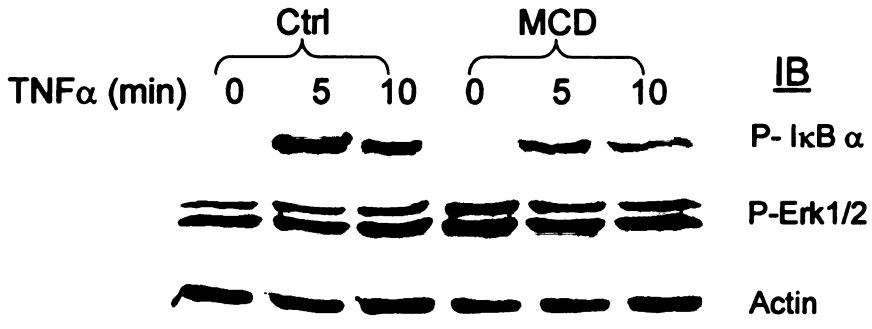


Fig. 1. Methyl-β-cycloxydextrin (MCD) pretreatment disrupts TNFα induced NFκB signaling in hRVE cells. hRVE cells were serum starved overnight then pretreated with 8 mM MCD for 30 min before addition of TNFα (20 ng/ml) for the indicated time. Cells were then harvested and same amounts of protein were loaded for SDS-PAGE and western blot analysis. Representative results were presented from two independent experiments using hRVE cells from two independent donors.

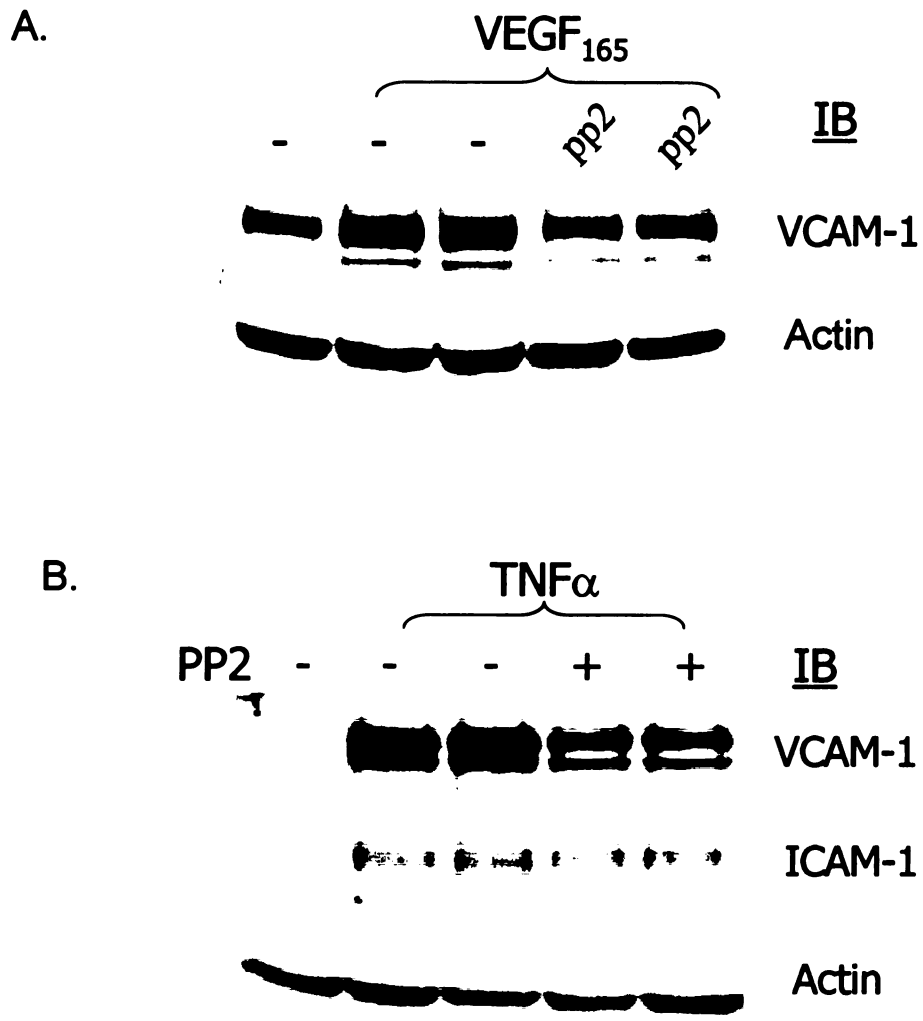
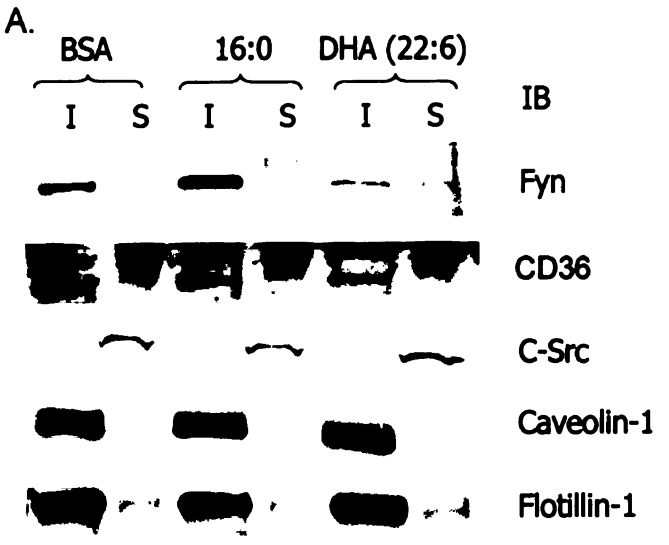
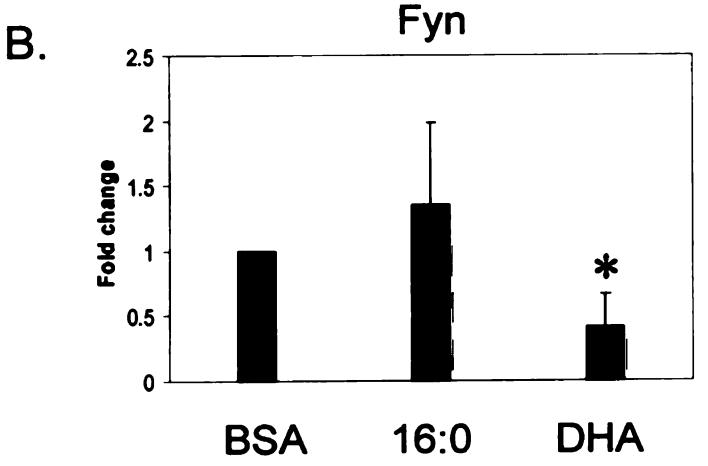
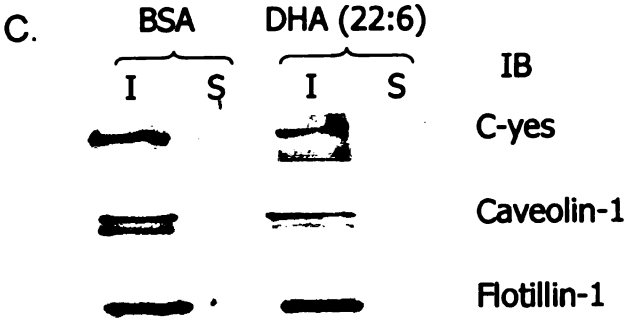


Fig. 2. Src family kinases are involved in VEGF165, TNFα induced VCAM-1 expression. (A) HUVEC cells were pretreated with pp2 (10 μM) for 30 min and stimulated with $VEGF_{165}$ (20 ng/ml) for 6 h. (B) hRVE cells were pretreated with pp2 (10 μM) for 30 min and stimulated with TNFα (5 ng/ml) for 6 h. Cells were then harvested. The same amounts of proteins were loaded for western blot analysis against VCAM-1, ICAM-1 and actin. Representative results from 2 independent experiments were presented.


A.
BSA
16:0
DHA (22:6)
I S I S I S
IB
Fyn
CD36
C-Src
Caveolin-1
Flotillin-1


B.
Fyn
Fold change
2.5
2
1.5
1
0.5
0
*
BSA
16:0
DHA


C.
BSA
DHA (22:6)
I S I S
IB
C-yes
Caveolin-1
Flotillin-1

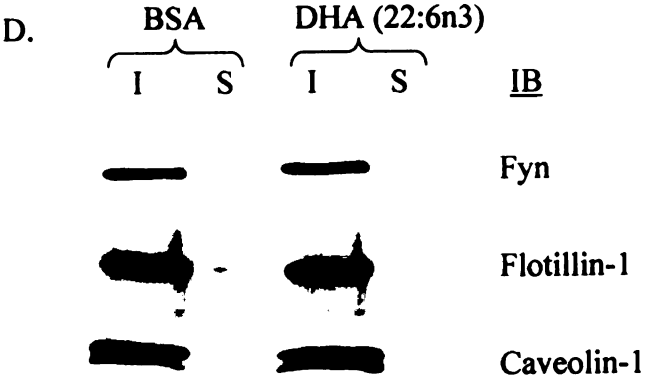

D.
BSA
DHA (22:6n3)
I S I S
IB
Fyn
Flotillin-1
Caveolin-1

Fig. 3. Specific displacement of Src family kinase Fyn and c-yes from caveolae/lipid rafts by DHA treatment in hRVE. (A) hRVE cells were serum starved overnight and treated with BSA (control), 100 μM BSA bound 16:0 or 22:6n3 for 24 h. Caveolae/lipid rafts were isolated and analyzed by western blot. The amounts of Fyn localized in caveolae/lipid rafts from 4 independent experiments were quantitated and normalized to the levels in BSA treated samples as in B. *P<0.005. (C) hRVE cells were treated with BSA (control) and 100 μM BSA bound 22:6n3 as above. The displacement of c-Yes was analyzed by western blot and a representative result was presented from 2 independent experiments. (D) hRPE cells were treated as BSA (control) and 200 μM BSA bound 22:6n3 as above. The displacement of Fyn was analyzed by western blot and a representative result was presented from 3 independent experiments.

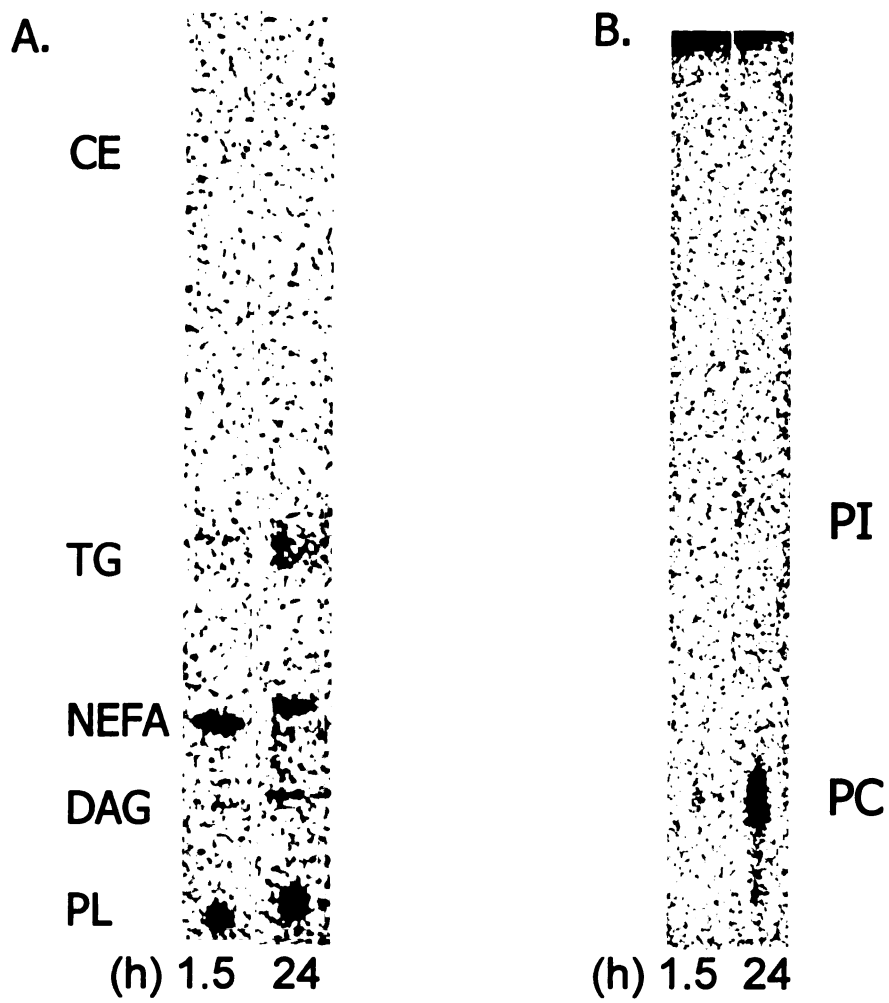


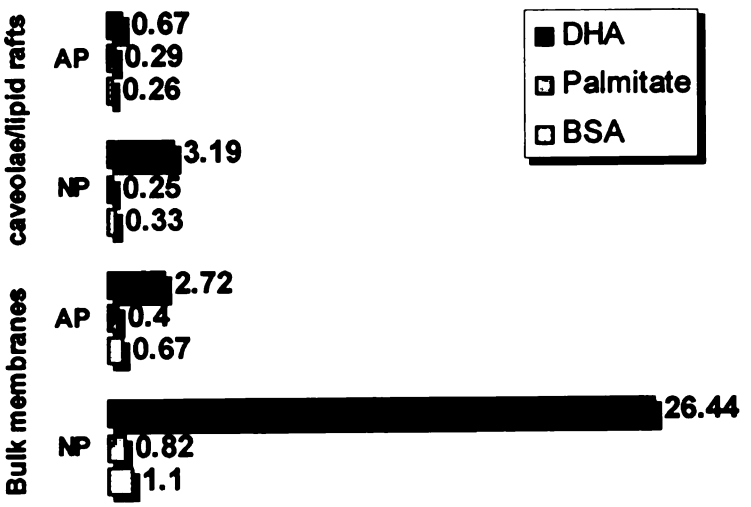
Fig. 4. Incorporation of 14C-22:6n3 into different lipid complexes in hRVE cells. (A). Serum starved hRVE cells were treated with ^{14}C-22:6n3 and labeled for the time periods as described in methods. Total lipids were extracted and submitted for TLC analyses. (B) The polar lipids were extracted from TLC plates and subjected for another TLC to separate different phospholipids subspecies.

TABLE 1. **Fatty acid composition of phospholipids from caveolae/lipid rafts and general plasma membranes of hRVE cells treated with control (BSA), lipid control (16:0) and n3-PUFA (22:6n3)**

Fatty acids	BSA		16:0		22:6n3	
	Rafts	Membranes	Rafts	Membranes	Rafts	Membranes
	Neutral phospholipids (% of total lipids)					
20:5n3	0.20	0.29	0.07	0.14	0.49	0.15
18:3n3	0.04	0.08	0.03	0.02	0.15	0.01
18:3n6	0.03	0.06	0.01	0.02	0.00	0.01
22:6n3	0.33	1.09	0.25	0.81	3.19	26.37
20:4n6	0.73	4.80	0.50	3.37	0.67	1.41
22:5n3	0.10	0.39	0.07	0.33	0.17	0.15
18:2n6	0.31	1.05	0.08	0.85	0.46	0.61
20:3n6	0.33	1.08	0.18	0.72	0.28	0.40
20:3n9	0.14	0.23	0.06	0.14	0.00	0.09
16:1n9	0.00	0.85	0.00	1.39	0.00	0.26
16:0	76.54	46.82	85.06	66.18	67.10	46.34
18:1n9	10.07	19.56	6.95	10.49	8.80	8.79
18:0	11.44	23.69	6.85	15.58	19.33	15.40
	Acidic phospholipids (% of total lipids)					
20:5n3	0	0.59	0	0.14	0	0.80
18:3n3	0.27	0.22	0.07	0.09	0.11	0.99
18:3n6	0.00	0.07	0.02	0.02	0.02	0.10
22:6n3	0.26	0.67	0.29	0.40	0.67	2.72
20:4n6	0.60	1.56	0.62	1.39	0.06	0.30
22:5n3	0.11	0.19	0.15	0.15	0.025	0.14
18:2n6	0.43	0.42	0.34	0.49	0.10	0.41
20:3n6	0.46	0.68	0.37	0.77	0.09	0.46
20:3n9	0.06	0.18	0.12	0.00	0.01	0.00
16:1n9	0	0	0	0	0	0
16:0	39.71	36.25	46.23	57.83	37.77	33.54
18:1n9	3.51	6.83	2.93	5.82	0.61	7.20
18:0	53.62	52.33	48.08	32.89	59.80	53.33

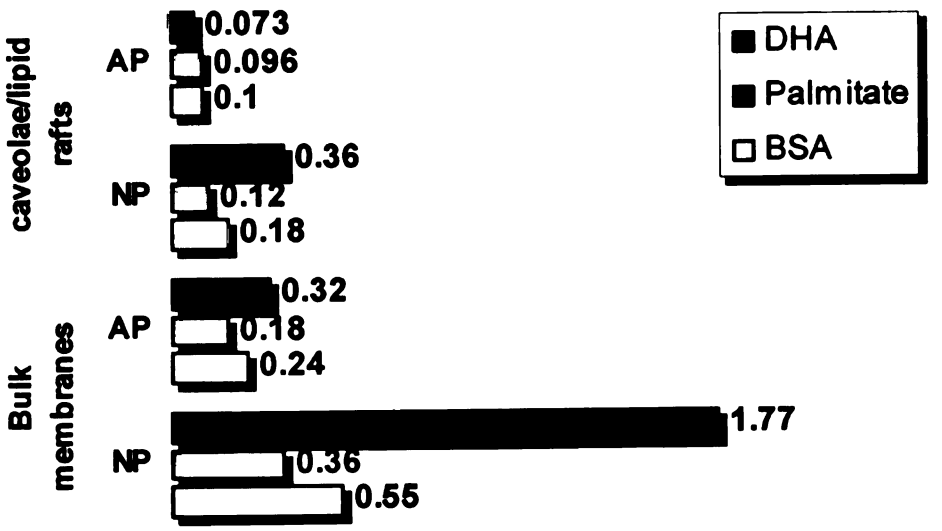
hRVE cells from 3 different donors were treated with vehicle control (20 μM BSA), lipid control (100 μM 16:0) or DHA (100 μM 22:6n3). Total plasma membranes and caveolae/lipid rafts were prepared. Samples corresponding to equivalent amounts of protein were extracted for total lipids followed by separation of neutral phospholipids and acidic phospholipids on amino-propyl column in parallel. The fatty acids composition and amounts after saponification were analyzed by RP-HPLC as described in Methods. The representative fatty acid composition from one donor was presented and expressed in % of total lipids in each phospholipid fraction.

A.



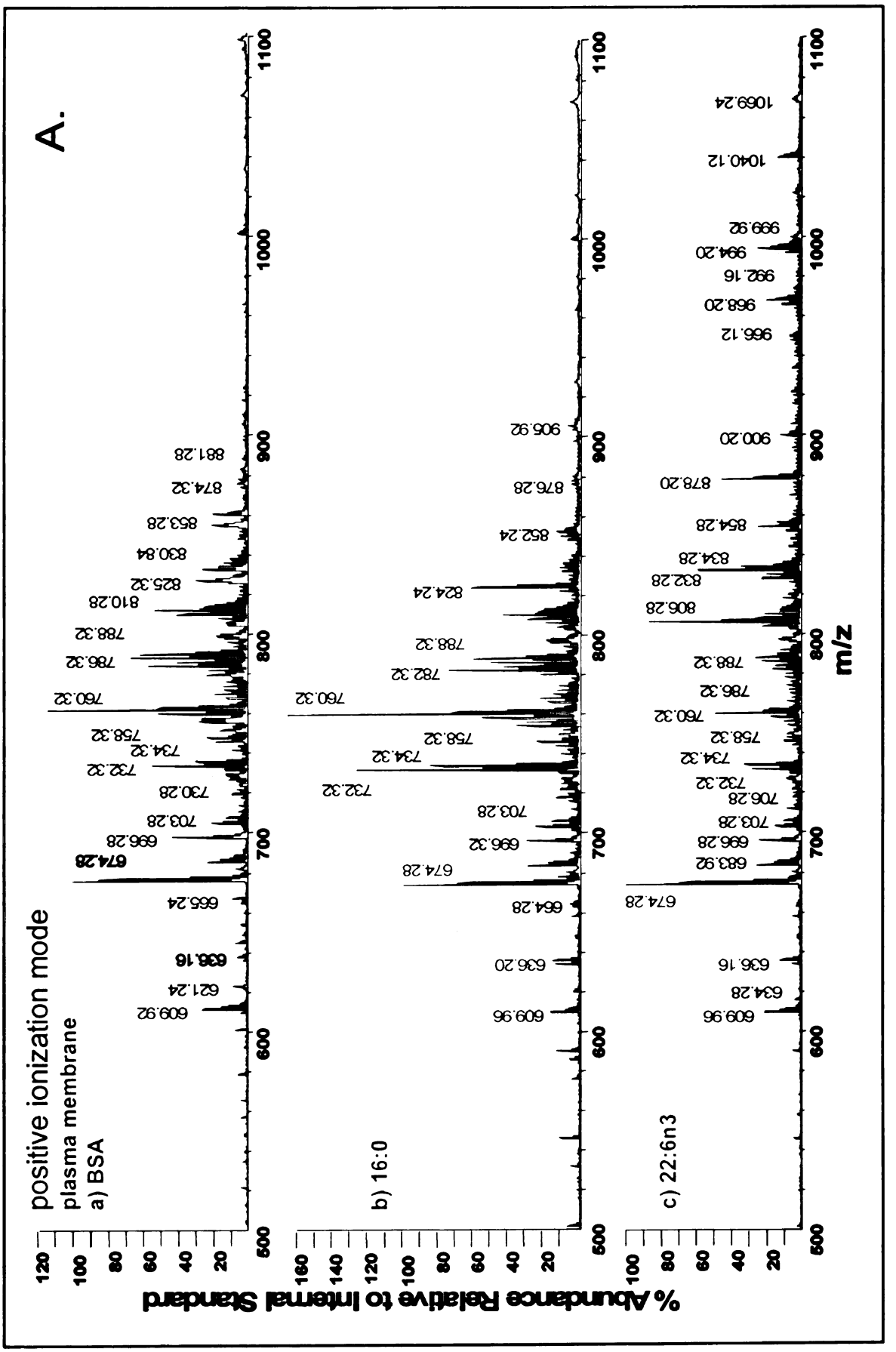
DHA% of total fatty acids

B.



Unsaturation index

Fig. 5. DHA22:6n3 alters fatty acyl compositions of phospholipids in caveolae/lipid rafts and bulk membranes. hRVE cells were serum starved overnight and treated with BSA (control), 100 μM BSA bound 16:0 or 22:6n3 for 24 h. Cells were lysed and same amounts of protein were loaded to isolate caveolae/lipid rafts. 2-4 fractions collected from the gradient were combined and submitted to total lipids extraction and amino-propyl column fractionation. Neutral phospholipids and acidic phospholipids were saponified followed by RP-HPLC analyses. The relative concentration for each fatty acid was obtained by normalizing to the standards and the percentage was calculated according to the total amounts in each fraction. The unsaturation ratio was calculated by the average number of double bonds per fatty acyl residue. A representative set of data from one donor was presented from three independent experiments using cells derived from three different donors. Panel A: the DHA% of total fatty acids in each fraction; panel B: the unsaturation index of phospholipids.


A.
positive ionization mode
plasma membrane
a) BSA
b) 16:0
c) 22:6n3
% Abundance Relative to Internal Standard
m/z

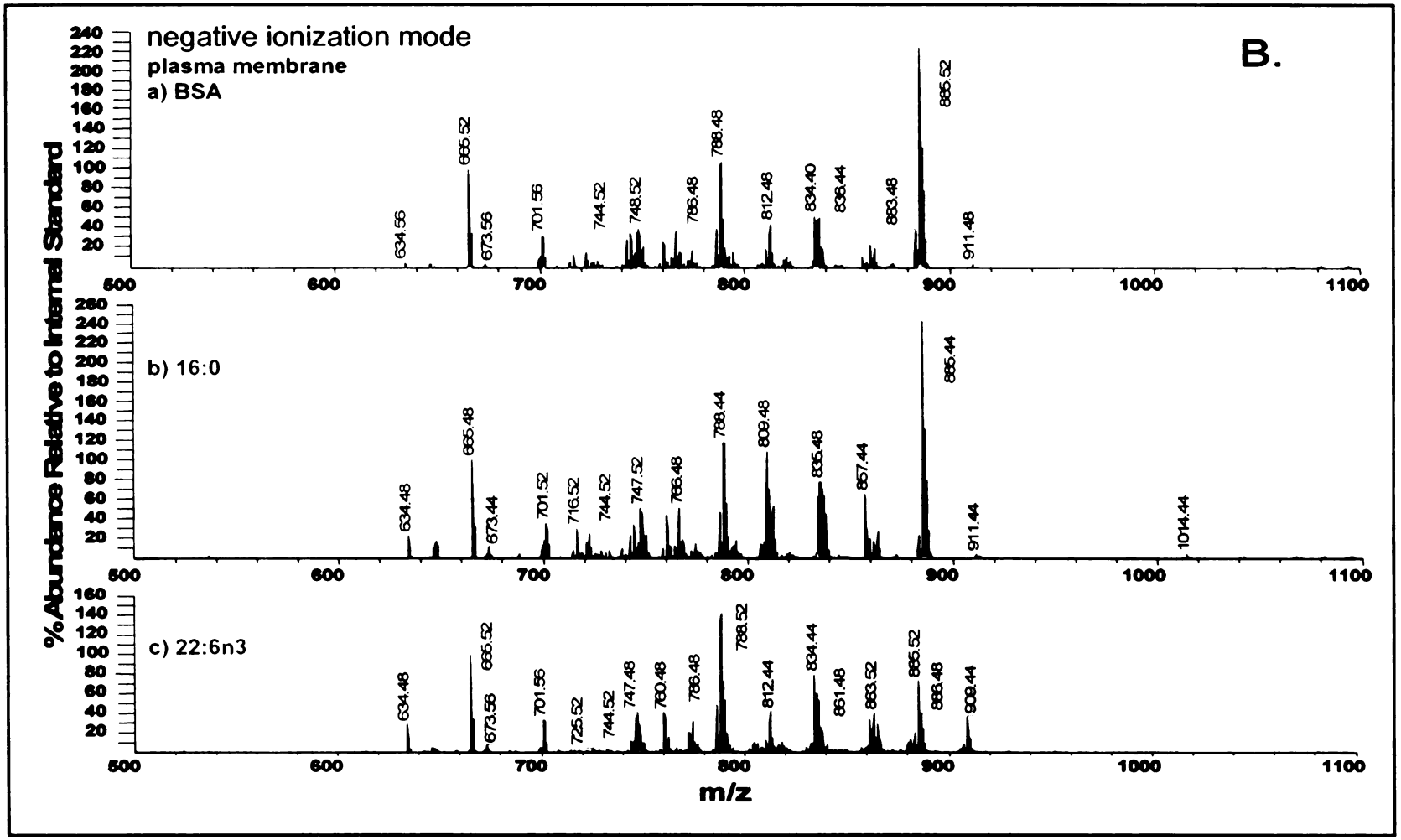


Fig. 6. nano-ESI-MS analyses of total plasma membranes phospholipids from hRVE treated with different fatty acids. Total lipid extracts of general membranes from hRVE treated with BSA, 16:0 and DHA22:6n3 were prepared and applied for nano-ESI-MS analyses under positive mode (panel A) and negative mode (panel B). Representative spectra for each treatment from 3 triplicates runs were presented and normalized to the internal standards (positive mode: PC14:1/14:1, m/z 674; negative mode: PG14:0/14:0, m/z 655).

Table 2. **Identification of the most abundant signals in the ESI mass spectra of total membrane lipid extracts of hRVE cells treated with BSA, palmitate 16:0 and $DHA_{22:6n3}$, as given in Fig. 6 A and B.**

Class of phospholipids	Ion	Total fatty acid carbon no. : no. of double bonds														
		28:0	28:2	32:0	32:1	34:1	34:2	36:1	36:2	36:4	38:3	38:4	38:6	40:5	40:6	16:0
PC	[M+H]+		674[c]	734	732	760	758	788	786	782		810	806[d]		834	
PE	[M+H]+	636[c]														
SM	[M+H]+															703
PE	[M-H]-	634[c]						744				766[b,p]				
PS	[M-H]-						760	788	786		812	810		836	834	
PI	[M-H]-			809[p]		835[p]		863[d]	861[d]	857[p]	883	885		911	909[d]	
PG	[M-H]-	665[c]					747[d, p]									

[M+H]+: positive ionization mode; [M-H]-: negative ionization mode

b: signals highly increased in BSA treated samples; p: signals highly increased in 16:0 treated samples; d: signals highly increased in $DHA_{22:6n3}$ treated samples; c: internal standards added for normalization of ion intensities and quantitation

All PC species have been verified by MS/MS. m/z at 806 was verified as PC38:6 (16:0/22:6n3). The other phospholipids species were only identified by their m/z values under each ion mode and further verification by MS/MS is underway.

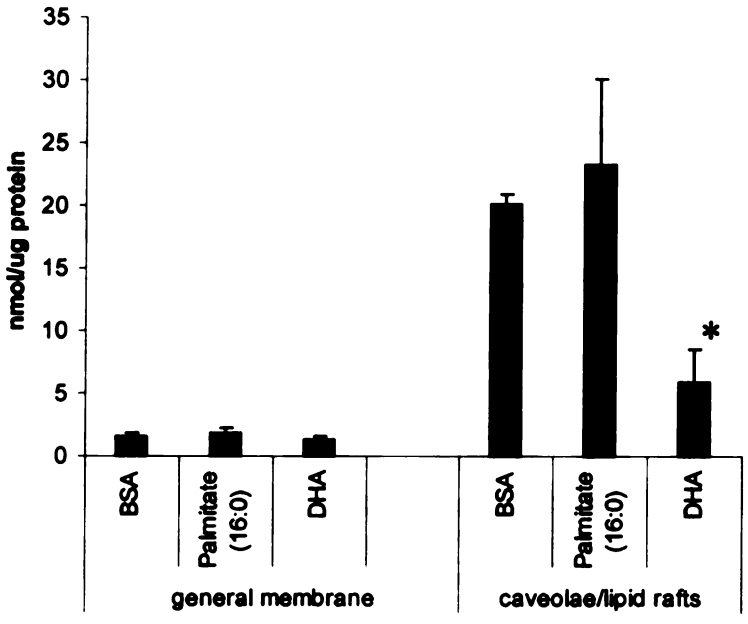


Fig. 7. DHA22:6n3 enrichment causes cholesterol depletion in caveolae/lipid rafts. hRVE cells were treated with fatty acids and isolation of caveolae/lipid rafts as well as the extraction of total lipids and amino-propyl column fractionation were performed as described in Methods. Neutral lipids fractions were submitted for HPLC analyses as indicated in methods. The amount of cholesterol was presented as nmol/μg protein after normalizing to the internal standards in phospholipids fractions analyzed by nano-ESI-MS. The data presented is from 3 independent experiments from hRVE derived from same donor. *$p<0.05$.

5. Discussion

Endothelial cells actively participate in the process of inflammation. Under chronic inflammation, endothelial cells are activated by cytokines to produce adhesion molecules such as VCAM-1 and ICAM-1. Adhesion molecules interact with their counterpart receptors on activated leukocytes to govern the adherence and transmigration of leukocytes to the endothelium and mediate inflammation. The immunosuppressive effects of n3-PUFAs have been well documented in both leukocytes and endothelial cells. Great effort has been made in the past decade to understand the anti-inflammatory role of n3-PUFAs in leukocytes, while little work was done to dissect their functional mechanisms in endothelial cells. In this paper, we provide compelling evidence that caveolae/lipid rafts are required for cytokine induced inflammatory signaling in hRVE cells. In addition, Src family kinases, Fyn and c-Yes, specifically targeted to the caveolae/lipid rafts are important signaling proteins mediating inflammatory signaling. As in T cells, PUFA ($DHA_{22:6n3}$) enrichment in hRVE cells displaces Fyn and c-Yes from caveolae/lipid rafts. This displacement could be due to the modification of the fatty acyl compositions of phospholipids and depletion of cholesterol residing in caveolae/lipid rafts thus altering the lipid environment for the target of dually acylated Src family kinases. Our results for the first time demonstrate that the anti-inflammatory effect of n3-PUFA ($DHA_{22:6n3)}$ in endothelial cells could be through modification of the important membrane signaling microdomains, caveolae/lipid rafts, similar to the mechanism demonstrated in T cells.

Ample evidence points to the idea that caveolae/lipids rafts are important in mediating cytokine induced inflammatory signaling. Biochemical analyses have demonstrated that caveolae/lipids rafts act as platforms to concentrate signaling molecules involving VEGFR2[28, 29, 33], TNFR1[26, 27] and IL-2R[34] mediated signaling pathways in various cell types including endothelial cells. Although the localization of specific cytokine receptors was not analyzed in this study, we did have indirect evidence showing that the integrity of caveolae/lipid rafts is required to maintain the intactness of TNFα induced NFκB activation (Fig. 1). Also, Src family kinase inhibitor PP2 was able to inhibit the cytokine induced expression of adhesion molecules; implicating the involvement of SFK in the cytokine induced inflammatory signaling. In fact, a growing body of literature has demonstrated the requirement of Src family kinases in TNFR[35, 36] and IL-1β receptor[37, 38] mediated inflammatory signaling. The recruitment of specific SFKs to VEGFR2 upon VEGF binding was also documented in several cell types[39-42]. The redundancy within the SFK family has confounded attributing a specific signaling process exclusively to one SFK[43], although c-Src is the major SFK that has been implicated in numerous receptors mediated signaling events[35][39, 42]. In hRVE, only Fyn and c-Yes are more than 90% localized in caveolae/lipid rafts while c-Src is not detectable. The affinity of c-Src for caveolae/lipid rafts is possibly lower since c-Src is only myristoylated as compared to the dually acylated Fyn and c-Yes, which enter caveolae/lipid rafts with high affinity[44, 45]. This can be further complicated by the low abundance of c-Src in primary hRVE since about 5-20% of c-Src could be detected in caveolae/lipid rafts in HUVEC which has much higher c-Src expression level (data not shown). Thus, whether localization of c-Src, like

Fyn and c-Yes, are modified by $DHA_{22:6n3}$ enrichment and which specific SFK is involved in each cytokine induced inflammatory signaling in hRVE needs to be further explored.

Recent evidence suggests that displacement of the Src family kinase Lck and adaptor protein LAT, two important signaling molecules essential for TCR mediated T cell activation, from lipid rafts is the major pathway through which n3-PUFA ($EPA_{20:5n3}$) acts through to suppress T cell activation and proliferation[10, 15, 16]. In endothelial cells, $DHA_{22:6n3}$ enrichment could also displace acylated SFKs localized in caveolae/lipid rafts, suggesting a similar mechanism n3-PUFAs act to mitigate inflammatory response through modifying the affinity of important signaling molecules to this specialized micromembrane domain.

The biochemical mechanisms underlying the selective displacement of acylated proteins from lipid rafts by PUFA treatment are still under debate. An altered lipid environment or changes in protein acylation constitute two possible ways PUFA can affect protein targeting to the lipid rafts. As reported in Cos-1 cells, Fyn can be acylated by fatty acids other than myristate and palmitate[46]. Incorporation of unsaturated fatty acids other than palmitate could displace Fyn from membrane rafts thus preventing it's interaction with other signaling molecules and resulting in the inhibition of signal transduction[46]. Although PUFA ($EPA_{20:5n3}$) was not incorporated into Fyn in T cells[15], whether Fyn can be acylated by PUFA ($DHA_{22:6n3}$) and whether this modification contributes to selective displacement of Fyn and c-Yes from caveolae/lipid

rafts by $DHA_{22:6n3}$ in endothelial cells is beyond the scope of this study and will require further work to address.

In T cells, substitution of fatty acyl chains by PUFA in phospholipids residing in both exoplasmic and cytoplasmic leaflets of caveolae/lipid rafts was suggested to change the lipid environment thus affecting the association of proteins dually acylated by saturated fatty acids[10, 15, 16]. This was further corroborated by the *in vivo* feeding studies demonstrating increased n3-PUFAs in the lipid rafts from T cells and colons of mice fed a diet enriched in n3-PUFAs, collectively with displacement of important signaling molecules such as Ras, caveolin-1 and eNOS[17, 18, 20]. Decreases in SM and cholesterol contents were also observed to cause perturbation of the overall lipid environment and possibly the integrity of caveolae/lipid rafts by n3-PUFAs[17, 18, 20].

Despite the growing number of caveolae/lipid rafts studies, there are relatively few quantitative analyses of caveolae/lipid raft lipids. Besides the work in mast cells[47], epidermal carcinoma cells[48] and neuronal cells[49], the characterization of the lipid composition of caveolae/lipid rafts from human lens[50] and photoreceptor rod outer segment membranes[51] were also reported recently. Our study constitutes the first molecular characterization of caveolae/lipid raft lipid compositions from human retinal endothelial cells and compares favorably with other published results in that caveolae/lipid rafts have higher degrees of saturation relative to the total plasma membrane. The phospholipids in caveolae/lipid rafts contain both neutral phospholipids (PC and PE) and acidic phospholipids (PS, PI and PG) and are mainly esterified with saturated acyl chains compared with total plasma membranes. A significant amount of

$AA_{20:4n6}$ and $DHA_{22:6n3}$ were present in caveolae/lipid raft neutral phospholipids (PC and PE) which is attributable to the relatively high level of $AA_{20:4n6}$ and $DHA_{22:6n3}$ in retinal endothelial cells compared with other sources of cells[15, 48]. Furthermore, the amount of cholesterol was about 10 fold higher in caveolae/lipid rafts relative to total plasma membranes confirming the idea that caveolae/lipid rafts are highly enriched in cholesterol to maintain a high lipid-ordered structure.

$DHA_{22:6n3}$ treatment results in incorporation into the phospholipids, especially PC (mainly localized in exoplasmic) and PI (cytoplasmic leaflets)[52] in the caveolae/lipid rafts in hRVE cells. Substitution of phospholipids fatty acyl chains by $DHA_{22:6n3}$ in total plasma membranes was much higher than caveolae/lipid rafts suggesting that cells tend to maintain the higher lipid-ordered structure of caveolae/lipid rafts by providing a saturated fatty acyl environment through an unresolved compensatory mechanism. Nevertheless, incorporation of $DHA_{22:6n3}$ results in a considerable (50%) increase in unsaturation in acyl chains of neutral phospholipids in caveolae/lipid rafts of $DHA_{22:6n3}$ treated hRVE cells (Fig. 5A). Such modification of phospholipids with $DHA_{22:6n3}$ leads to dramatic changes in the lipid environment in caveolae/lipid rafts. Thus, $DHA_{22:6n3}$ enrichment decreased the cholesterol level by more than 70% in caveolae/lipid rafts in contrast to no significant changes in the overall amount of cholesterol in total plasma membranes (Fig. 7). Cholesterol is the major structural lipid in caveolae/lipid rafts that are required to maintain the ordered state of the rafts membrane. Particularly, interaction of phospholipid saturated acyl chains with cholesterol is required to maintain organization of the cytoplasmic leaflet of caveolae/lipid rafts that lacks sphingolipids in a liquid-ordered phase[11, 53]. It was noted that cholesterol interacts differentially with different

membrane lipids, with a particularly strong association with saturated phospho- and sphingolipids and a particularly weak association with highly unsaturated lipid species (reviewed in [54]). Thus, substitutions of acyl chains mainly at sn-2 position of the cytoplasmic phospholipids such as PE and PI by $DHA_{22:6n3}$ could dramatically affect cholesterol interaction and thus cause a decreased liquid order in the cytoplasmic leaflets of caveolae/lipid rafts. The exoplasmic leaflets of caveolae/lipid rafts may be less prone to be affected by fatty acyl unsaturation due to the presence of sphingolipids. However, the depletion of cholesterol may be detrimental to the interaction of sphingolipids with cholesterol for the formation of a liquid-ordered state[12, 55]. The exact mechanism of specific cholesterol depletion in caveolae/lipid rafts induced by $DHA_{22:6n3}$ enrichment is still not clear. It possibly involves a spontaneous redistribution between membranes due to the changes of lipid environment in caveolae/lipid rafts[54]. Unlike in T cells[15], we did not observe significant incorporation of n3-PUFA ($DHA_{22:6n3}$) into SM even in total plasma membranes which agrees with the notion that mammalian sphingolipids generally have a 16- to 24-carbon saturated chains[56]. Taken together, considerable fatty acyl modifications of the phospholipids localized in caveolae/lipid rafts together with cholesterol depletion are likely to underlie the selective displacement of dual acylated SFKs (Fyn and c-Yes) from caveolae/lipid rafts in $DHA_{22:6n3}$ treated endothelial cells.

In conclusion, for the first time we have characterized the involvement of caveolae/lipid rafts in mediating cytokine ($VEGF_{165}$ and TNFα) induced proinflammatory signaling in endothelial cells. The modification of phospholipids residing in the caveolae/lipid rafts along with the specific cholesterol depletion could provide strong basis for the molecular mechanism of $DHA_{22:6n3}$ induced displacement of

Src family kinases in EC and thus the modulation of the inflammatory signaling in endothelial cells.

6. References

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