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## Inhibition of Cytokine Signaling in Human Retinal Endothelial Cells through Modification of Caveolae/Lipid Rafts by Docosahexaenoic Acid

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### Abstract

**PURPOSE.**—Docosahexaenoic acid (DHA<sub>22:6,n3</sub>) is the principal n3 polyunsaturated fatty acid (PUFA) in the retina. The authors previously demonstrated that DHA<sub>22:6,n3</sub> inhibited cytokine-induced adhesion molecule expression in primary human retinal vascular endothelial (hRVE) cells, the target tissue affected by diabetic retinopathy. Despite the importance of vascular inflammation in diabetic retinopathy, the mechanisms underlying anti-inflammatory effects of DHA<sub>22:6,n3</sub> in vascular endothelial cells are not understood. In this study the authors address the hypothesis that DHA<sub>22:6,n3</sub> acts through modifying lipid composition of caveolae/lipid rafts, thereby changing the outcome of important signaling events in these specialized plasma membrane microdomains.

**METHODS.**—hRVE cells were cultured in the presence or absence of DHA<sub>22:6,n3</sub>. Isolated caveolae/lipid raft-enriched detergent-resistant membrane domains were prepared using sucrose gradient ultracentrifugation. Fatty acid composition and cholesterol content of caveolae/lipid rafts before and after treatment were measured by HPLC. The expression of Src family kinases was assayed by Western blotting and immunohistochemistry.

**RESULTS.**—Disruption of the caveolae/lipid raft structure with a cholesterol-depleting agent, methylcyclodextrin (MCD), diminished cytokine-induced signaling in hRVE cells. Growth of hRVE cells in media enriched in DHA<sub>22:6,n3</sub> resulted in significant incorporation of DHA<sub>22:6,n3</sub> into the major phospholipids of caveolae/lipid rafts, causing an increase in the unsaturation index in the membrane microdomain. DHA<sub>22:6,n3</sub> enrichment in the caveolae/raft was accompanied by a 70% depletion of cholesterol from caveolae/lipid rafts and displacement of the SFK, Fyn, and c-Yes from caveolae/lipid rafts. Adding water-soluble cholesterol to DHA<sub>22:6,n3</sub>-treated cells replenished cholesterol in caveolae/lipid rafts and reversed the effect of DHA<sub>22:6,n3</sub> on cytokine-induced signaling.

**CONCLUSIONS.**—Incorporation of DHA<sub>22:6,n3</sub> into fatty acyl chains of phospholipids in caveolae/lipid rafts, followed by cholesterol depletion and displacement of important signaling molecules, provides a potential mechanism for anti-inflammatory effect of DHA<sub>22:6,n3</sub> in hRVE cells.

Polyunsaturated fatty acids (PUFAs), especially n3-PUFAs such as eicosapentaenoic acid (EPA<sub>20:5n3</sub>) and DHA<sub>22:6,n3</sub>, are well known to have anti-inflammatory properties.<sup>1,2</sup> Vascular inflammation is a process that involves the activation of vascular endothelium and circulating leukocytes. The anti-inflammatory action of n-3 PUFAs has been studied in association with their ability to suppress leukocyte activation and function.<sup>3-11</sup> However, the

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effect of PUFAs on the vascular component of inflammation has received less attention. hRVE cells are the target vascular tissue affected by diabetic retinopathy, a condition that has been recently associated with low-grade inflammation in the retina. We have previously demonstrated that the growth of human retinal vascular endothelial cells in media enriched in DHA<sub>22:6,n3</sub> resulted in the inhibition of IL-1 $\beta$ , TNF- $\alpha$ , and VEGF-induced intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 expression.<sup>12</sup> The mechanism of anti-inflammatory action of DHA<sub>22:6,n3</sub> in hRVE cells is unknown.

Recent studies strongly suggest that the inhibitory effects of n3-PUFA on T-lymphocyte signal transduction are, at least in part, caused by the modification of functional membrane lipid microdomains known as lipid rafts.<sup>3,5,7,9,10,13</sup> Vascular endothelial cells, such as hRVE cells, contain lipid rafts and a second type of specialized micromembrane domain known as caveolae. The lipid composition of caveolae is similar to that of lipid rafts, but caveolae are stabilized by the caveolin family of structural and regulatory proteins, which give them a characteristic flasklike shape.<sup>14-17</sup> Caveolae are important in regulating vascular permeability, vesicle trafficking, cholesterol homeostasis, and, in particular, signal transduction. Despite debate regarding the spatial and temporal aspects of lipid rafts and the initiating factors in lipid raft assembly,<sup>16,18-22</sup> it is well accepted that caveolae and lipid rafts are enriched in cholesterol and sphingolipids and depleted in glycerophospholipids, resulting in a glycerophospholipid/sphingolipid/cholesterol molar ratio of 1:1:1.<sup>23</sup> Furthermore, the glycerophospholipids in lipid rafts are highly enriched in saturated fatty acyl chains. Highly saturated structures in the lipid raft membranes stabilized by cholesterol form lipid-ordered domains that govern lipid-protein interactions.<sup>23-26</sup>

A number of signaling molecules are attracted by the highly ordered raft lipid domain, including glycosylphosphatidylinositol (GPI)-anchored proteins in the exoplasmic domain and dually acylated nonreceptor tyrosine kinases in the cytoplasmic domain.<sup>27-31</sup> Most of the Src family kinases, such as Fyn, Lck, and c-Yes, are myristoylated on the amino-terminal glycine residue and palmitoylated on amino-terminal cysteine<sup>3</sup> residue.<sup>32,33</sup> Dual acylation with saturated fatty acids is proposed to increase the affinity of Src family kinases to caveolae and lipid rafts.

Cytokine signaling in endothelial cells is initiated at the specific receptors localized in the exoplasmic leaflet of the plasma membrane. Many cytokine receptors are GPI-linked proteins that localize in caveolae/lipid rafts, such as TNF-R1<sup>30,31</sup> and VEGF-R2 in endothelial cells.<sup>27,28</sup> Although the direct connection between Src family kinases and cytokine receptors signaling remains to be elucidated, the proximity of Src family kinases localized to the endoplasmic domain of lipid rafts to TNF-R,<sup>34,35</sup> IL-1 $\beta$ R,<sup>36,37</sup> and VEGF-R2<sup>38-41</sup> was documented in several cell types. PUFA treatment was shown to inhibit cytokine signal transduction in T lymphocytes through selective displacement of dually acylated proteins from lipid rafts.<sup>3,5,7,9,42</sup>

In this study we demonstrate that treating human vascular endothelial cells with DHA<sub>22:6,n3</sub> greatly increased the caveolae/lipid raft unsaturation index by enriching fatty acyl chains of phospholipids in caveolae/lipid rafts with this highly unsaturated PUFA. Moreover, DHA<sub>22:6,n3</sub> incorporation into caveolae/lipid rafts caused cholesterol displacement from these specialized membrane microdomains. Changes in lipid composition were associated with decreased affinity of the SFK constitutively expressed in the caveolae/lipid rafts in hRVE cells, Fyn, and c-Yes for the raft fraction. The modification of caveolae/lipid raft lipid composition with PUFA, followed by the displacement of important signaling proteins resident in caveolae/lipid rafts, provides a potential mechanism for the inhibition of cytokine-induced inflammatory signaling by DHA<sub>22:6,n3</sub> in hRVE cells.

## MATERIALS AND METHODS

### Reagents and Antibodies

HPLC-grade acetonitrile, acetic acid, methanol, chloroform, methyl-cyclodextrin (MCD), and water-soluble cholesterol were purchased (Sigma, St. Louis, MO), and the antibodies mouse anti-caveolin-1 and flotillin-1 (Upstate Biotechnology, Inc., Lake Placid, NY) and mouse anti-c-Src, Fyn, rabbit antibodies against c-Yes, and CD36 (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

### Cell Culture

Primary cultures of hRVE cells were prepared from tissue (National Disease Research Interchange, Philadelphia, PA) cultured as previously described. Passages 1 to 6 were used in the experiments. For experimental treatment, cells were transferred to serum-free medium for 14 to 24 hours before stimulatory agents were added.

### Fatty Acid Treatment

Fatty acid stocks were prepared by dissolving fatty acids (NuCheck Prep, Inc., Elysian, MN) in ethanol to a final concentration of 100 mM fatty acid, as described previously.<sup>43</sup> The fatty acid stock solutions were diluted to 50 to 100  $\mu$ M in serum-free medium containing 10 to 20  $\mu$ M charcoal-treated, solvent-extracted, fatty acid-free bovine serum albumin (BSA; Serologica Inc., Norcross, GA) as a fatty acid carrier. The fatty acid/albumin molar ratio was maintained at 5:1. The final concentration of ethanol in the media was less than 0.1%. Cells were incubated for the times indicated in Results. Equivalent amounts of BSA and ethanol were added to control plates.

Fatty acids at concentrations higher than 200  $\mu$ M were shown to induce apoptosis in human umbilical vein endothelial cells.<sup>44</sup> Concentrations of fatty acids used in this study were well below the proapoptotic range; however, we routinely verified, according to manufacturer's protocol (Vybrant Apoptosis Assay Kit 4; Invitrogen, Carlsbad, CA), that the rate of apoptosis in fatty acid-treated hRVE cells was not higher than in control cells.

### MCD and Cholesterol Treatment

To deplete cholesterol from the membranes, cells were treated with 8 mM MCD for 30 minutes. To replenish cholesterol, cells were treated with 25  $\mu$ M water-soluble cholesterol (25  $\mu$ M cholesterol complexed with 250  $\mu$ M MCD; Sigma) for 30 minutes. After MCD or water-soluble cholesterol treatment, the cells were washed twice with PBS containing 40  $\mu$ M BSA before stimulation with cytokines for the time indicated in Results.

### 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<sub>2</sub>, 1 mM EGTA, 1% Triton X-100, and 10% glycerol) with freshly added protease inhibitor cocktail (Sigma) and phosphatase inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub>, 100  $\mu$ M glycerophosphate, 10 mM NaF, 1 mM Na<sub>4</sub>PPI). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using appropriate antibodies followed by secondary horseradish peroxidase-conjugated antibody (Bio-Rad, Hercules, CA). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ). Blots were quantitated by scanning densitometry (ImageJ software, version 1.29; available by ftp at [zippy.nimh.nih.gov](http://zippy.nimh.nih.gov) or at <http://rsb.info.nih.gov/nih-image>; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

## Subcellular Fractionation

Postnuclear supernatants were prepared as described previously.<sup>45</sup> Cells were lysed in hypotonic buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM MgCl<sub>2</sub> freshly added with protease inhibitors) for 30 minutes on ice, then homogenized with 20 strokes in a nonstick (Teflon; DuPont, Wilmington, DE)/glass homogenizer and spun down at 500g for 5 minutes. Supernatants were subjected to centrifugation (16,900g, 60 minutes, 4°C), and pellets were collected as plasma membrane-enriched fractions.

Isolation of caveolae/lipid raft-enriched detergent-resistant membrane domains were prepared using a slightly modified sucrose gradient ultracentrifugation protocol.<sup>46</sup> Briefly,  $5 \times 10^6$  hRVE cells were washed twice with cold PBS, lysed in 0.8 mL MNE buffer (25 mM MES, pH 6.5, 0.15 M NaCl, and 5 mM EDTA) containing 1% Triton X-100, fresh protease, and phosphatase inhibitors, and kept on ice for 20 minutes. Lysates were then homogenized with 10 strokes of a tight-fitting Dounce homogenizer and spun down at 4000g at 4°C for 10 minutes. Supernatant (0.8 mL) was mixed with the same volume of 80% sucrose prepared in MNE buffer and was placed at the bottom of an ultracentrifuge tube. Then 1.6 mL of 30% sucrose and 0.8 mL of 5% sucrose were overlaid on top of the sample to form a 5% to 30% discontinuous sucrose gradient. After 16 hours of centrifugation at 200,000g and 4°C in a swinging bucket rotor, 0.4-mL samples were collected carefully from the top of each fraction. A band confined to fractions 2 through 4 was designated as caveolae/lipid raft-enriched membrane domains. The combined 2 to 4 fractions were diluted 3 times in MNE buffer and spun at 200,000g and 4°C for another 2 hours to obtain the caveolae/lipid rafts. Fractions 6 to 10 were also combined and designated as nonraft membranes.

## Fatty Acid Metabolism

hRVE cells were plated at  $0.12 \times 10^6$  cells/6-cm plate and cultured as indicated to 90% confluence. Cells were then serum starved overnight and treated with 100  $\mu$ M DHA<sub>22:6,n3</sub>/20  $\mu$ M BSA containing tracer amounts of <sup>14</sup>C-DHA<sub>22:6,n3</sub> (0.5  $\mu$ Ci in 3 mL media) for 1.5 and 24 hours. <sup>14</sup>C-DHA<sub>22:6,n3</sub> (1.7 Ci/mol) was purchased (PerkinElmer Life Sciences, Wellesley, MA). At harvest, cells were washed once with PBS + 20  $\mu$ M BSA and once by PBS alone. Cells were then resuspended in 500  $\mu$ L 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, Clifton, NJ) in hexane/diethyl ether/acetic acid (90:30:1). Polar lipids were separated in chloroform/methanol/acetic acid (30:20:4). TLC plates were dried, and radioactivity was detected and quantified by phosphor imaging. Location of total lipids was compared with authentic standards for triacylglycerol (TAG), diacylglycerol (DAG), cholesterol esters (CEs), and fatty acids (Sigma). Location of phospholipids was also compared with authentic standards for phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE; Avanti Polar Lipids, Alabaster, AL).

## Fatty Acid and Cholesterol Analysis

Total lipids from caveolae/lipid rafts and 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. Internal standards consisting of 19:1 (1  $\mu$ mol/mg protein) and 14:1/14:1 PC (70 nmol/mg protein) were used. A fraction of the total lipids was further fractionated on an aminopropyl (Alltech, Lexington, KY) column to obtain neutral lipids, neutral phospholipids, acidic phospholipids, and nonesterified fatty acids (NEFAs), as described.<sup>47</sup> Neutral lipids were separated by a 5- $\mu$ M, 250  $\times$  4.6-mm column (YMC-Diol-120NP; Waters, Milford, MA) using evaporative light scatter to detect cholesterol. Pure lipid standards for cholesterol, triacylglycerol, and diacylglycerol (Avanti Polar Lipids) were

used after each experiment to confirm retention times and purity. 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 hour) for fatty acid composition analysis. 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) with a separation technology column (J'sphere ODS-H80; YMC Co., Ltd., Kyoto, Japan) and a sigmoidal gradient starting at 86.5% acetonitrile + acetic acid (0.1%) and ending at 100% acetonitrile + acetic acid (0.1%) over 50 minutes with a flow rate of 1.0 mL/min and a controller (model 600; Waters). Fatty acids were detected using ultraviolet absorbance at 192 nm (model 2487; Waters) and evaporative light scatter (model 2420; Waters). Samples were normalized using recovery standards. Calibration curves were prepared for each fatty acid at the end of the experiment in accordance with fatty acid standards for RP-HPLC (Nu-Chek Prep, Elysian, MN).

### Statistical Analysis

Data are expressed as the mean  $\pm$  SD. Factorial ANOVA with post hoc Tukey test (calculated using <http://faculty.vassar.edu/lowry/VassarStats.html>) was used for comparing data obtained from independent samples. Significance was established at  $P < 0.05$ .

## RESULTS

### Incorporation of DHA<sub>22:6,n3</sub> into Phospholipids in hRVE Cells

Exogenously supplied [<sup>14</sup>C]-DHA<sub>22:6,n3</sub> was taken up by the cells and rapidly incorporated into different cellular lipids, including DAG, NEFA, triglycerides, and, to a large extent, polar lipids (Fig. 1A). Further separation of polar lipids demonstrated that [<sup>14</sup>C]-DHA<sub>22:6,n3</sub> was incorporated mainly into the phosphatidylcholine (PC) fraction (Fig. 1B).

### Characterization of Caveolae/Lipid Rafts from hRVE Cells

Because phospholipids are important structural components of caveolae/lipid rafts, we examined whether the incorporation of DHA<sub>22:6,n3</sub> affected the signaling environment of caveolae/lipid rafts. Caveolae/lipid raft fractions were isolated by sucrose discontinuous gradient ultracentrifugation based on their insolubility in Triton X-100 at 4°C (Fig. 2). Confirmation of isolated lipid rafts was achieved by demonstrating enrichment of caveolin-1 (a caveolae marker) and flotillin-1 (a lipid raft marker) in density gradient fractions 2 to 4. In contrast, the plasma membrane marker Na<sup>+</sup>/K<sup>+</sup>-ATPase was excluded from the lipid raft fractions. PKC- $\alpha$  and ERK1/2 were found in the nonraft membrane fraction (fractions 6–9) under basal conditions (Fig. 2). These results demonstrate that hRVE cells contain caveolae and lipid raft structures and that a caveolae/lipid raft-enriched fraction can be isolated using methods involving detergent insolubility and density gradient fractionation.

### Incorporation of DHA<sub>22:6,n3</sub> into Phospholipids of Caveolae/Lipid Rafts

To determine the effect of exogenously added fatty acids on total plasma membrane and caveolae/lipid raft lipid composition, the fractions were isolated from control cells or cells treated with 100  $\mu$ M palmitic acid<sub>16:0</sub> (lipid control) or DHA<sub>22:6,n3</sub> for 24 hours. After treatment, total lipids were extracted and separated by an amino-propyl column to enrich for polar lipids. The method yields two phospholipid fractions, neutral phospholipids (containing PC, PE, and SM) and acidic phospholipid (containing PI, PS, and PA).<sup>47</sup> Because most (more than 70% in total plasma membrane and more than 85% in caveolae/lipid raft fraction) phospholipids were recovered in the neutral phospholipid fraction, only this fraction was



saponified and analyzed for the fatty acyl composition of phospholipids by RP-HPLC (Fig. 3). As expected, the phospholipids from caveolae/lipid raft fractions from control and palmitate-treated cells were particularly enriched in saturated palmitic<sub>16:0</sub> and stearic<sub>18:0</sub> acids. Monounsaturated oleic<sub>18:1n9</sub>, polyunsaturated linoleic<sub>18:2n6</sub>, and arachidonic<sub>20:4n6</sub> fatty acids were less abundant in caveolae/lipid raft phospholipids than in total plasma membrane phospholipids. Although treating cells with palmitic acid<sub>16:0</sub> had modest effects on membrane lipid composition, treating cells with DHA<sub>22:6,n3</sub> significantly enriched membrane lipids, both in the total plasma membranes and the caveolae/lipid raft fractions (Figs. 3A, 3B). The unsaturation index—i.e., the number of double bonds per fatty acyl residue—was significantly lower in caveolae/lipid rafts than in total plasma membranes, confirming a high lipid-ordered state in caveolae/lipid rafts. DHA<sub>22:6,n3</sub> treatment caused a significant (35%) increase in the unsaturation index in neutral phospholipids (0.44 vs. 0.29 double bonds per fatty acyl residue) in caveolae/lipid rafts compared with that of control hRVE cells.

In plasma membranes, the effect of DHA<sub>22:6,n3</sub> on the unsaturation index was even more apparent (1.52 vs. 0.61 in DHA<sub>22:6,n3</sub>-treated vs. control cells; Figs. 3A, 3B). Taken together, these data show that DHA<sub>22:6,n3</sub> is effectively incorporated into phospholipids of caveolae/lipid rafts and significantly alters the lipid environment of these specialized membrane microdomains.

### Cholesterol Depletion in Caveolae/Lipid Rafts by DHA<sub>22:6,n3</sub>

Cholesterol content in caveolae/lipid rafts was approximately 10 times higher than in total plasma membranes, confirming that caveolae/lipid rafts are highly enriched in cholesterol (Fig. 4). Introduction of bulky acyl chains such as DHA<sub>22:6,n3</sub> into phospholipids, which normally contain saturated fatty acids, can affect fatty acyl chain-cholesterol interaction in caveolae/lipid rafts.<sup>20</sup> Indeed, DHA<sub>22:6,n3</sub> enrichment was associated with a 70% decrease in the cholesterol level in caveolae/lipid rafts compared with control, whereas palmitic acid<sub>16:0</sub> treatment had no significant impact on cholesterol levels. Moreover, the depletion of cholesterol associated with DHA<sub>22:6,n3</sub> enrichment only occurred in caveolae/lipid rafts, with no significant effect on cholesterol levels in total plasma membranes (Fig. 4). Thus, enrichment of caveolae/lipid raft phospholipid with DHA<sub>22:6,n3</sub> (Fig. 3) results in cholesterol depletion from these structures (Fig. 4). Adding 25  $\mu$ M water-soluble cholesterol for 30 minutes replenished the cholesterol content of caveolae/lipid rafts in DHA<sub>22:6,n3</sub>-treated cells (Fig. 4)

### Inhibition of TNF- $\alpha$ - and IL-1 $\beta$ -Induced CAM Expression by Cholesterol Depletion

The cholesterol-depleting agent MCD, known to disrupt the structure of caveolae/lipid rafts, was used to assess the role of caveolae/lipid rafts in cytokine-induced CAM expression in retinal endothelial cells. MCD pretreatment of hRVE cells downregulated TNF- $\alpha$ -induced phosphorylation of inhibitor of NF-kappa B alpha (I $\kappa$ B $\alpha$ ) (Fig. 5A) and ICAM-1 expression (Fig. 5B). In contrast, TNF- $\alpha$ -triggered ERK phosphorylation was not affected. A similar effect was observed with IL-1 $\beta$ -mediated I $\kappa$ B $\beta$  phosphorylation and ICAM-1 expression in response to MCD treatment (data not shown). These data suggest that the integrity of caveolae/lipid rafts is required for cytokine-induced NF- $\kappa$ B signaling and downstream cell adhesion molecules (CAM) expression.

### Reversal of DHA<sub>22:6,n3</sub> Inhibition of TNF- $\alpha$ -Induced ICAM-1 Expression by Cholesterol Replenishment

As we have previously reported,<sup>48</sup> the pretreatment of hRVE cells with DHA<sub>22:6,n3</sub> significantly inhibits TNF- $\alpha$ -induced ICAM-1 expression compared with TNF- $\alpha$ -induced ICAM-1 expression in control (palmitic<sub>16:0</sub> acid-treated) cells (Fig. 6). Replenishing cholesterol reverses the inhibitory effect of DHA<sub>22:6,n3</sub> on TNF- $\alpha$ -induced ICAM-1 expression (Fig. 6).

## Src Family Kinase Displacement from Caveolae/Lipid Rafts in Endothelial Cells in Response to DHA<sub>22:6,n3</sub>-Induced Change in Lipid Composition

To determine whether DHA-induced changes in lipid composition would lead to the alteration of caveolae/lipid raft signaling components, we first characterized Src family kinase localization in hRVE cells. The dual-acylated Src family kinases Fyn and c-Yes were exclusively localized in caveolae/lipid rafts, whereas only myristoylated c-Src was excluded (Fig. 7). To examine whether the 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, c-Yes) was used (Fig. 8). PP2 diminished TNF- $\alpha$ -induced VCAM-1 expression in hRVE cells (Fig. 8), suggesting that SFKs localized in caveolae/lipid rafts could be important players in the cytokine-induced inflammatory response.

We next determined whether DHA<sub>22:6,n3</sub> had effects on SFK interaction with caveolae/lipid rafts. Treatment of hRVE cells with 100  $\mu$ M DHA<sub>22:6,n3</sub> significantly enriched lipid raft DHA<sub>22:6,n3</sub> content (Fig. 3). DHA<sub>22:6,n3</sub> enrichment in the raft correlated with a 60% displacement of Fyn and a 35% displacement of c-Yes from the caveolae/lipid raft fraction (Fig. 9). DHA<sub>22:6,n3</sub> treatment did not disrupt caveolae/lipid raft structure because the major structural proteins of caveolae and lipid rafts—caveolin-1 and flotillin-1—were not affected by the increased enrichment with DHA<sub>22:6,n3</sub>. Thus, DHA<sub>22:6,n3</sub> enrichment of caveolae/rafts depletes cholesterol and at least two SFKs, Fyn and c-Yes, from these structures.

## DISCUSSION

Ample experimental evidence suggests that early diabetic retinopathy is a low-grade chronic inflammatory disease.<sup>49-56</sup> Under diabetic conditions, retinal endothelial cells were shown to be activated to produce adhesion molecules such as ICAM-1 and VCAM-1. Adhesion molecules interact with their counterpart receptors on activated leukocytes to govern the adherence and transmigration of leukocytes to the endothelium and to mediate inflammation. Although n-3 PUFAs suppress cytokine induced adhesion molecule expression in endothelial cells,<sup>48,57-61</sup> the functional mechanism for this suppression is not well understood. Our results demonstrate that the anti-inflammatory effect of the most abundant n-3 PUFA in the retina, DHA<sub>22:6,n3</sub>, on retinal endothelial cells may be mediated through modification of the important membrane signaling microdomains, caveolae/lipid rafts.

Caveolae/lipid rafts are important in mediating cytokine-induced inflammatory signaling.<sup>31,62-66</sup> Several signaling molecules implicated in the development of diabetic retinopathy, such as VEGFR2,<sup>27,28,67</sup> TNF-R1,<sup>30,31</sup> and IL-2R,<sup>68</sup> have been shown to localize to the caveolae/lipid raft fractions in various cell types, including endothelial cells. Diabetes and hypertension lead to increased caveolin-1 expression in retinal pericytes and endothelial cells.<sup>69</sup> Moreover, hyperglycemia has been shown to induce caveolin-1 expression in a bovine aortic endothelial cell model.<sup>70</sup> We have previously demonstrated that DHA<sub>22:6,n3</sub> inhibits IL-1 $\beta$ -, TNF- $\alpha$ -, and VEGF-induced inflammatory signaling through inhibition of the NF- $\kappa$ B pathway upstream of I $\kappa$ B $\alpha$  phosphorylation and degradation in hRVE cells.<sup>48</sup> The data provided in this study show that the lipid integrity of caveolae/lipid rafts is related to the intactness of cytokine-induced NF- $\kappa$ B activation (Fig. 5). Thus, DHA<sub>22:6,n3</sub> could oppose hyperglycemia-induced proinflammatory changes by modifying caveolae/lipid raft structure and function in the diabetic retina.

The Src family kinase inhibitor PP2 inhibited the cytokine-induced expression of adhesion molecules, implicating the involvement of Src kinases in cytokine-induced inflammatory signaling. In fact, a growing body of literature has demonstrated the requirement of SFKs in TNF receptor-mediated<sup>34,35</sup> and IL-1 $\beta$  receptor-mediated<sup>36,37</sup> inflammatory signaling. The recruitment of specific SFKs to VEGFR2 on VEGF binding was also documented in several

cell types.<sup>38-41</sup> Although the detailed evaluation of the role of Src family kinases in cytokine signaling is beyond the scope of this study, we used Src family kinases as a model to study the effect of DHA<sub>22:6,n3</sub> on signaling components of the caveolae/lipid rafts in hRVE cells.

In agreement with previous studies, dual-acylated SFKs such as Fyn and c-Yes were localized to the caveolae/lipid raft fraction. c-Srk itself, which is only myristoylated because it is missing the cysteine<sup>3</sup> palmitoylation site, was excluded from caveolae/lipid rafts.<sup>32,33</sup> Thus, the highly ordered lipid structure of the caveolae/raft and the posttranslational modification of proteins that direct protein into or out of rafts are likely important regulatory features controlling signal transduction. In this study, we demonstrate that DHA<sub>22:6,n3</sub> enrichment in hRVE cells is associated with displacement of SFKs Fyn and c-Yes from caveolae/lipid rafts.

Altered lipid environment and altered protein acylation are two possible routes by which PUFA can affect protein targeting to lipid rafts. In hRVE cells, DHA<sub>22:6,n3</sub> treatment resulted in its incorporation into phospholipids in the caveolae/lipid rafts. These data are in agreement with reports in T lymphocytes, suggesting that the substitution of fatty acyl chains by PUFA in phospholipids of lipid rafts could change the lipid environment and thus affect the association of proteins dually acylated by saturated fatty acids.<sup>9,10,13</sup> Moreover, in vivo feeding studies demonstrated increased n-3 PUFAs in the lipid rafts from T lymphocytes and colons of mice fed a diet enriched in n-3 PUFAs collectively with displacement of important signaling molecules, such as Ras, caveolin-1, and eNOS.<sup>5,71,72</sup>

DHA<sub>22:6,n3</sub> incorporated primarily into the phosphatidylcholine fraction of phospholipids in hRVE cells. This is different from the reports in T cells showing EPA<sub>20:5,n3</sub> and DHA<sub>22:6,n3</sub> incorporating into phosphatidylcholine and phosphatidylethanolamine,<sup>9,73</sup> underscoring the difference in lipid metabolism between retinal vascular endothelial cells and other cell types. Substitution of phospholipid fatty acyl chains by DHA<sub>22:6,n3</sub> in total plasma membranes was much higher than in caveolae/lipid rafts, suggesting that cells tend to maintain the higher lipid-ordered structure of caveolae/lipid rafts by maintaining a saturated fatty acyl environment. Nevertheless, incorporation of DHA<sub>22:6,n3</sub> resulted in a considerable (35%) increase in unsaturation in acyl chains of neutral phospholipids in caveolae/lipid rafts of DHA<sub>22:6,n3</sub>-treated hRVE cells (Fig. 3B).

The enrichment of DHA<sub>22:6,n3</sub> in raft phospholipids is associated with a decline in arachidonic acid<sub>20:4,n6</sub> (Fig. 3). When released from membranes, arachidonic acid<sub>20:4,n6</sub> serves as a precursor of inflammatory mediators, including leukotrienes, thromboxanes, and prostaglandins, and other bioactive lipid mediators, such as hydroxy and epoxy fatty acids.<sup>74-76</sup> Our previous studies indicated that treatment of hRVE cells with arachidonic acid<sub>20:4,n6</sub> leads to a lipoxygenase-dependent increase in ICAM/VCAM expression.<sup>43</sup> This effect of arachidonic acid<sub>20:4,n6</sub> was observed after 12-hour treatment of hRVE cells. The fact that the effects of cholesterol depletion/replenishment on NF-κB signaling and adhesion molecule expression are obvious in 30 minutes argues against a decline in arachidonic acid<sub>20:4,n6</sub> as a primary mechanism for the inhibitory effect of arachidonic acid<sub>20:4,n6</sub> on caveolae/lipid raft signaling. However, a reduction of arachidonic acid<sub>20:4,n6</sub> phospholipid content by DHA<sub>22:6,n3</sub> could represent an additional mechanism of anti-inflammatory effect of DHA<sub>22:6,n3</sub> in hRVE cells.

Enrichment of phospholipids with DHA<sub>22:6,n3</sub> dramatically altered the lipid environment in caveolae/lipid rafts. This is particularly evident in the 70% decrease in caveolae/lipid raft-associated cholesterol. The effect of DHA<sub>22:6,n3</sub> on raft cholesterol is specific because significant changes in the overall plasma membrane cholesterol content were not detected (Fig. 4). Cholesterol depletion using MCD mimicked the inhibitory effects on DHA<sub>22:6,n3</sub> on cytokine-induced IκBα phosphorylation and ICAM-1 expression. Moreover, cholesterol



replenishment reversed the inhibitory effect of DHA<sub>22:6,n3</sub> on cytokine signaling in hRVE cells.

Cholesterol is the major structural lipid in caveolae/lipid rafts that is required to maintain the ordered state of the raft membrane. Cholesterol sterol rings tightly bind the ceramide moiety of sphingomyelin in the exoplasmic leaflet of caveolae/lipid rafts, promoting caveolae/lipid raft lateral separation.<sup>77-79</sup> In the cytoplasmic leaflet that lacks sphingolipids, the interaction of phospholipid-saturated acyl chains with cholesterol is required to maintain organization of caveolae/lipid rafts in a liquid-ordered phase.<sup>77,80</sup> Cholesterol interacts differentially with different membrane lipids and has a particularly strong association with saturated phospholipids and sphingolipids and a weak association with highly unsaturated lipid species (for a review, see Silvius<sup>21</sup>). Thus, substitutions of acyl chains of the phospholipids by DHA<sub>22:6,n3</sub> could dramatically affect cholesterol interaction and cause a decreased lipid order in the caveolae/lipid rafts. The mechanism of specific cholesterol depletion in caveolae/lipid rafts induced by DHA<sub>22:6,n3</sub> enrichment is unclear. It may involve a spontaneous redistribution between membranes because of changes of lipid environment in caveolae/lipid rafts.<sup>21</sup>

Decreases in the sphingomyelin content have also been reported in lipid rafts isolated from T lymphocytes and the colons of mice fed a diet enriched in n3-PUFA.<sup>5,71,72</sup> Treating cells with sphingomyelinase, which cleaves the phosphorylcholine head group of sphingomyelin, leading to ceramide production, causes a significant displacement of cholesterol from lipid raft membranes and a modification of lipid raft signaling.<sup>81-83</sup> Because DHA<sub>22:6,n3</sub> was shown to activate neutral sphingomyelinase in cultured MDA-MB-231 breast cancer cells,<sup>84</sup> the activation of sphingomyelinase followed by ceramide production could represent a potential mechanism for the DHA<sub>22:6,n3</sub>-induced cholesterol depletion from caveolae/lipid raft reported here. Sphingomyelinase activity and sphingomyelin and ceramide content were not analyzed in this article and will be the focus of future study.

As reported in Cos-1 cells, Fyn can be acylated by fatty acids other than myristate and palmitate.<sup>7</sup> Acylation of Fyn by unsaturated fatty acids caused the displacement of Fyn from membrane rafts in Cos-1 cells.<sup>7</sup> Determining whether SFKs can be acylated by DHA<sub>22:6,n3</sub>, which would provide an additional mechanism for selective displacement of Fyn and c-Yes from caveolae/lipid rafts in endothelial cells, is beyond the scope of this study.

In conclusion, we have characterized the involvement of caveolae/lipid rafts in mediating cytokine-induced proinflammatory signaling in primary cultures of retinal vascular endothelial cells. The modification of phospholipids residing in the caveolae/lipid rafts and the depletion of caveolae/lipid raft-specific cholesterol provide a compelling model to explain the molecular mechanism of DHA<sub>22:6,n3</sub>-induced displacement of Src family kinases and thus the immunomodulatory effect of DHA<sub>22:6,n3</sub> on endothelial cells.

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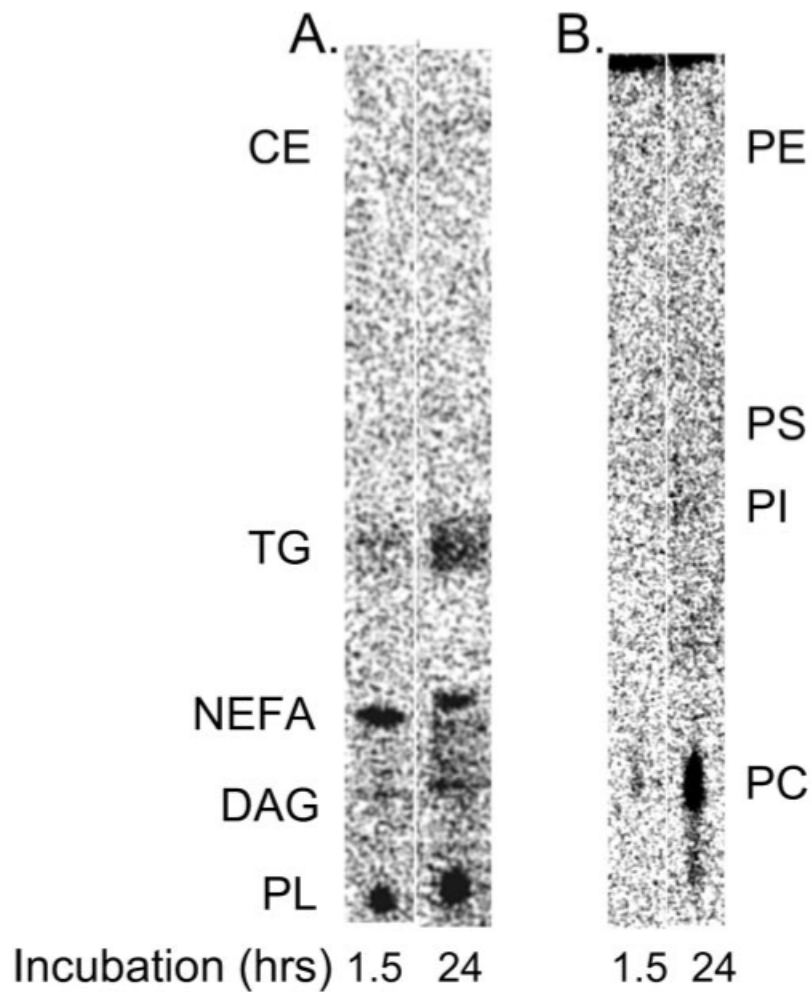
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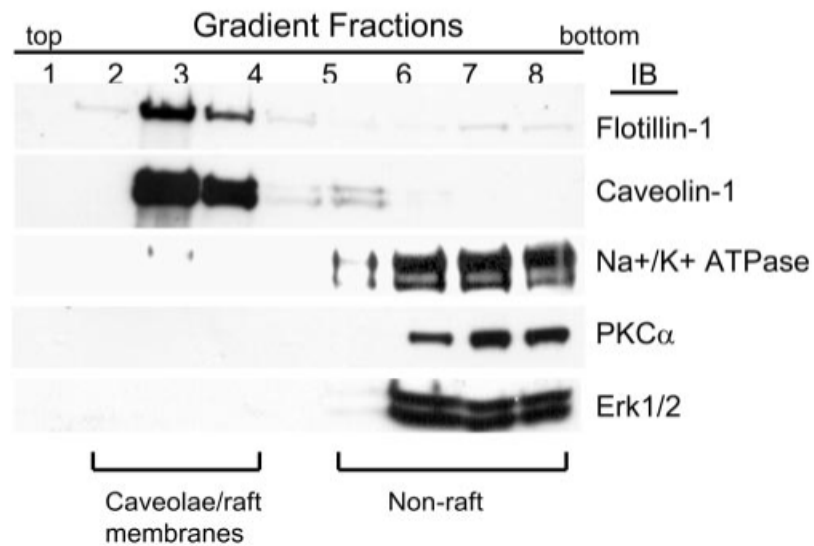
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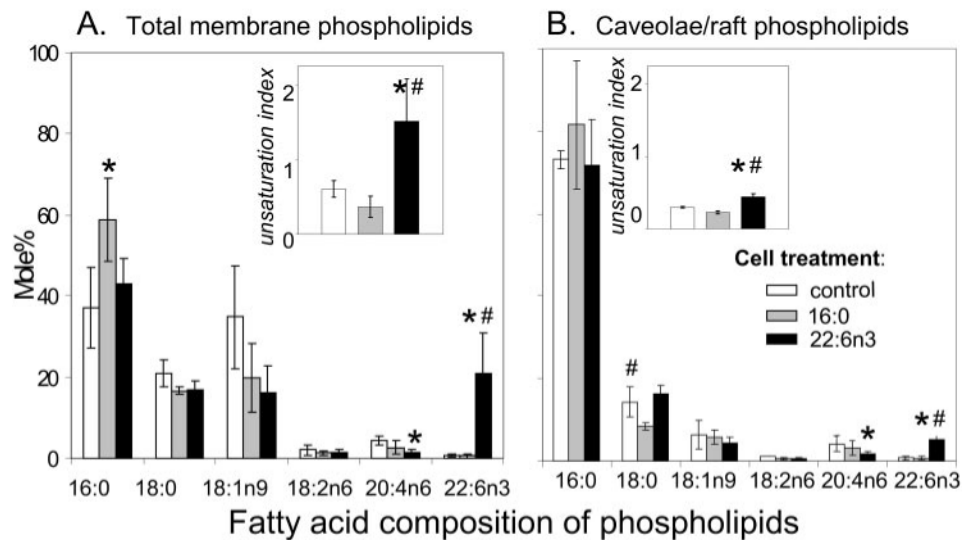


**FIGURE 1.** Incorporation of  $^{14}\text{C}$ -DHA<sub>22:6,n3</sub> into hRVE cellular lipids. hRVE cells were treated with  $^{14}\text{C}$ -DHA<sub>22:6,n3</sub> for the time periods indicated. Total lipids were extracted and subjected to thin-layer chromatography (TLC) analysis. Radioactivity was detected and quantified by phosphorimaging. A phosphorimage of total lipid TLC analysis is presented (**A**), as is phospholipid TLC analysis (**B**). Location of the authentic standards for polar lipid (PL), diacylglycerol (DAG), nonesterified fatty acid (NEFA), triacylglycerol (TG), and cholesterol ester (CE) is shown on the total lipid TLC (**A**). Location of the authentic standards for phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE) are shown on the phospholipid TLC (**B**).

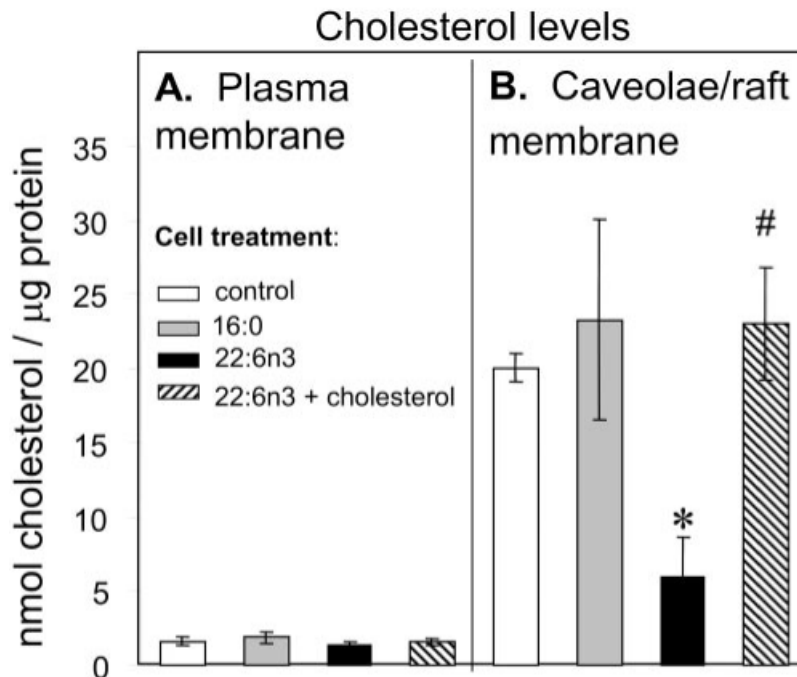


**FIGURE 2.**

Characterization of caveolae/lipid raft protein components in hRVE cells. Membrane lipids were fractionated to enrich for caveolae/lipid rafts and nonraft components. Each membrane fraction was separated on SDS-PAGE. Western blot analysis for the caveolae marker caveolin-1, the lipid raft marker flotillin-1, the plasma membrane marker Na<sup>+</sup>/K<sup>+</sup>ATPase, PKC- $\alpha$ , and Erk1/2 was performed to analyze protein distribution in each fraction. Fractions designated caveolae/raft and nonraft membrane are indicated.

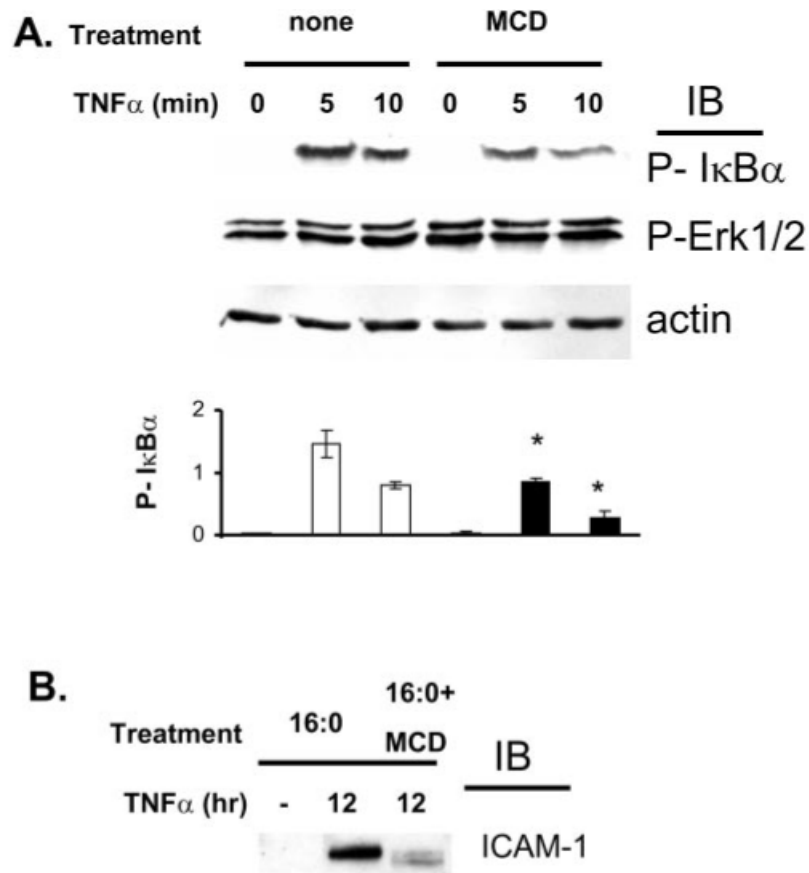
**FIGURE 3.**

Modification of phospholipid fatty acyl composition of caveolae/lipid raft and total plasma membrane of hRVE cells by incubation with palmitic acid<sub>16:0</sub> or DHA<sub>22:6,n3</sub>. hRVE cells were treated with 100  $\mu$ M palmitic acid<sub>16:0</sub> (gray bars) or DHA<sub>22:6,n3</sub> (black bars) for 24 hours. Vehicle (20  $\mu$ M BSA)-treated cells (white bars) were used as a control. Caveolae/lipid raft-enriched and nonenriched fractions were purified as described in Experimental Procedures. Lipids were extracted, and phospholipids and sphingolipids were enriched by aminopropyl column fractionation. Neutral phospholipids (PC, PE, SM) and acidic phospholipids (PI, PS, PA) were saponified and analyzed by RP-HPLC. The concentration for each fatty acid was obtained by normalizing to standards and was presented as the mole percent of total fatty acids in each fraction. The unsaturation index was calculated as the average number of double bonds per fatty acyl residue. Given that most phospholipids were in the neutral phospholipid fraction, only the data for neutral phospholipid fraction are presented. Data are the mean  $\pm$  SD of results in three experiments. \* $P < 0.05$  compared with control. # $P < 0.05$  compared with palmitic acid<sub>16:0</sub>-treated cells.

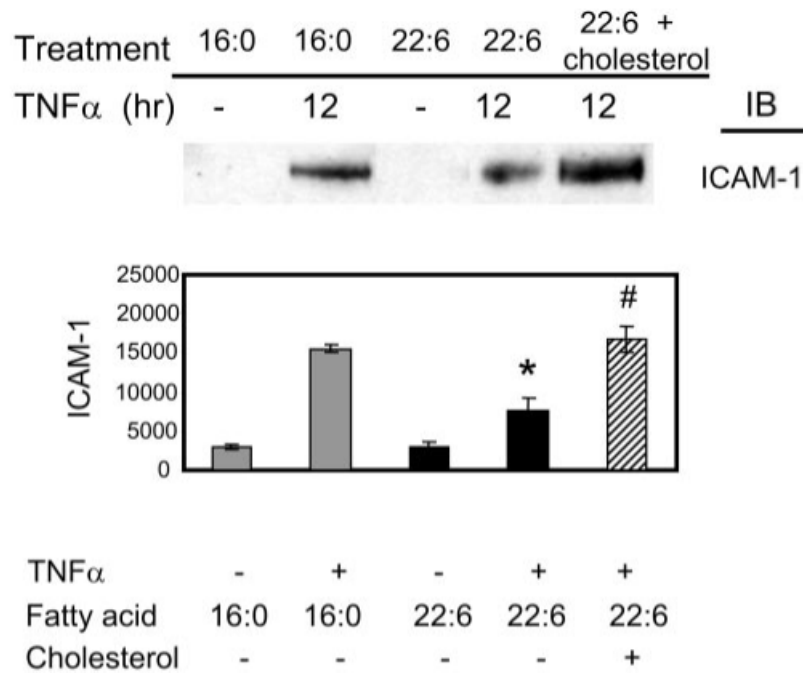
**FIGURE 4.**

DHA<sub>22:6,n3</sub> treatment depletes cholesterol in caveolae/lipid rafts. hRVE cells were treated with 100  $\mu$ M palmitic acid<sub>16:0</sub> (gray bars) or DHA<sub>22:6,n3</sub> (black bars) for 24 hours. Vehicle (20  $\mu$ M BSA)-treated cells were used as a control (white bars). Water-soluble cholesterol (25  $\mu$ M cholesterol complexed with 250  $\mu$ M MCD) was added to DHA<sub>22:6,n3</sub>-treated cells for 30 minutes (striped bars). Caveolae/lipid raft-enriched domains were purified as described in Experimental Procedures and submitted to total lipid extraction and amino-propyl column fractionation. Neutral lipids were fractionated by normal-phase HPLC analyses. The amount of cholesterol was presented as nmol/ $\mu$ g protein. Data are the mean  $\pm$  SD of results in three experiments. \* $P$  < 0.05 compared with control. # $P$  < 0.05 compared with DHA<sub>22:6,n3</sub>-treated cells without cholesterol.

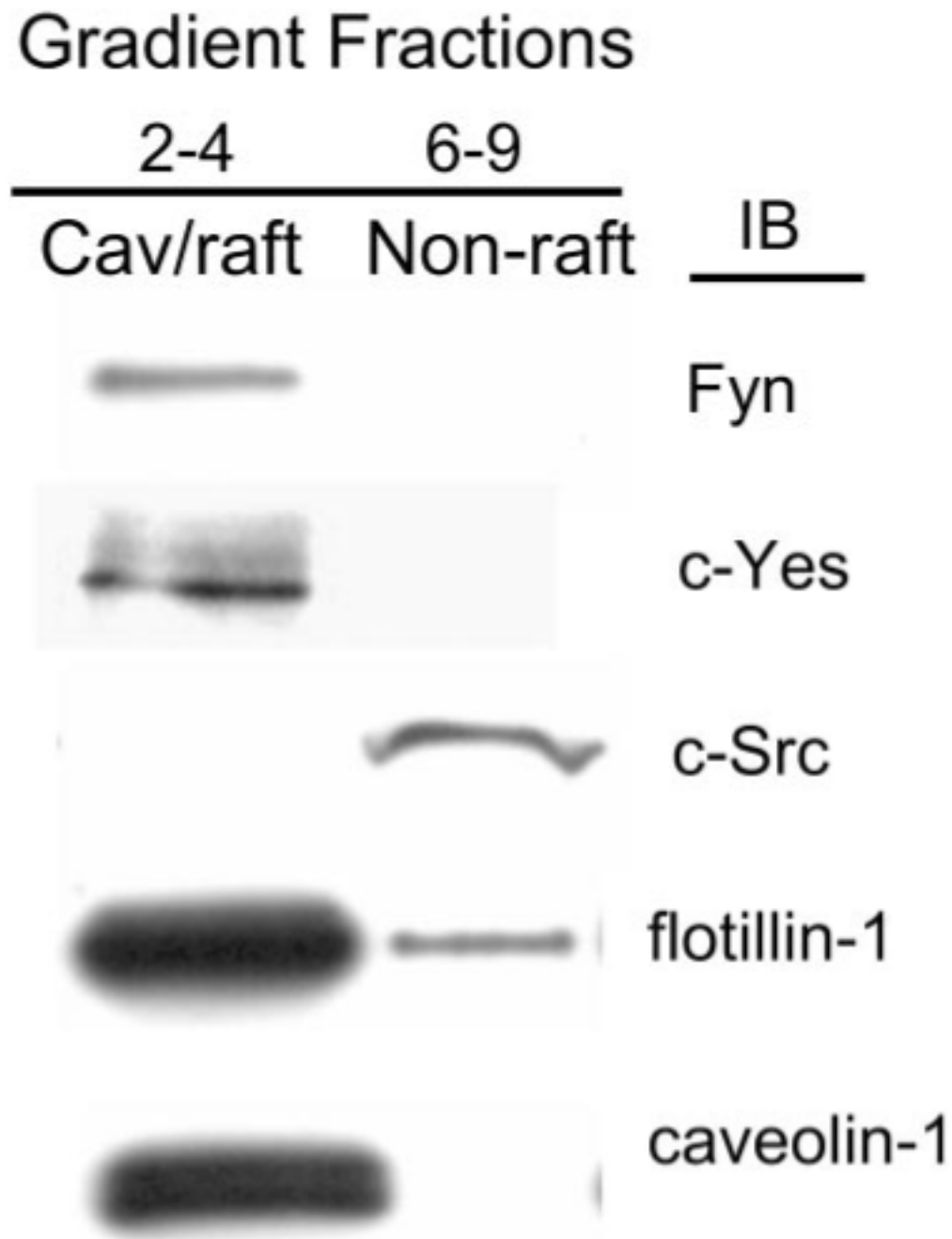




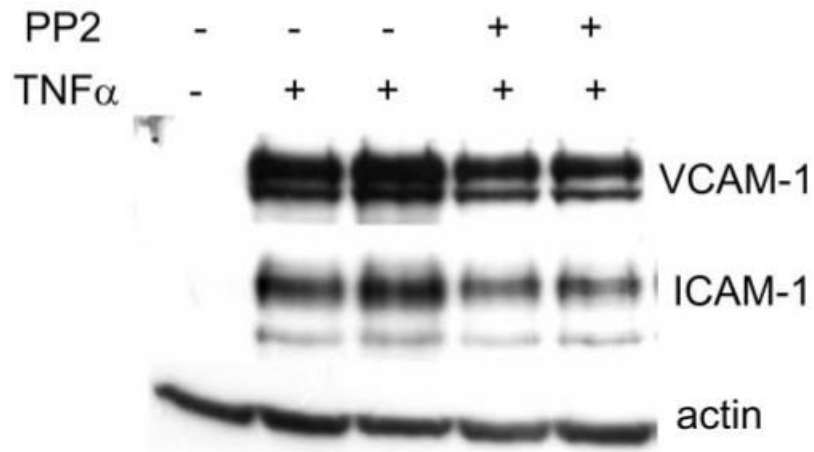
**FIGURE 5.** Inhibition of TNF- $\alpha$ -induced NF- $\kappa$ B signaling by (MCD) pretreatment of hRVE cells. hRVE cells were pretreated with 8 mM MCD for 30 minutes before stimulation with TNF- $\alpha$  (20 ng/mL) for the indicated time. I $\kappa$ B $\alpha$  and ERK phosphorylation levels (**A**) and ICAM-1 expression levels (**B**) were assessed by immunoblot analyses. Equal amounts of protein were added to each lane, as confirmed by actin levels. Data are the mean  $\pm$  SD of results in three experiments. \* $P < 0.05$ .

**FIGURE 6.**

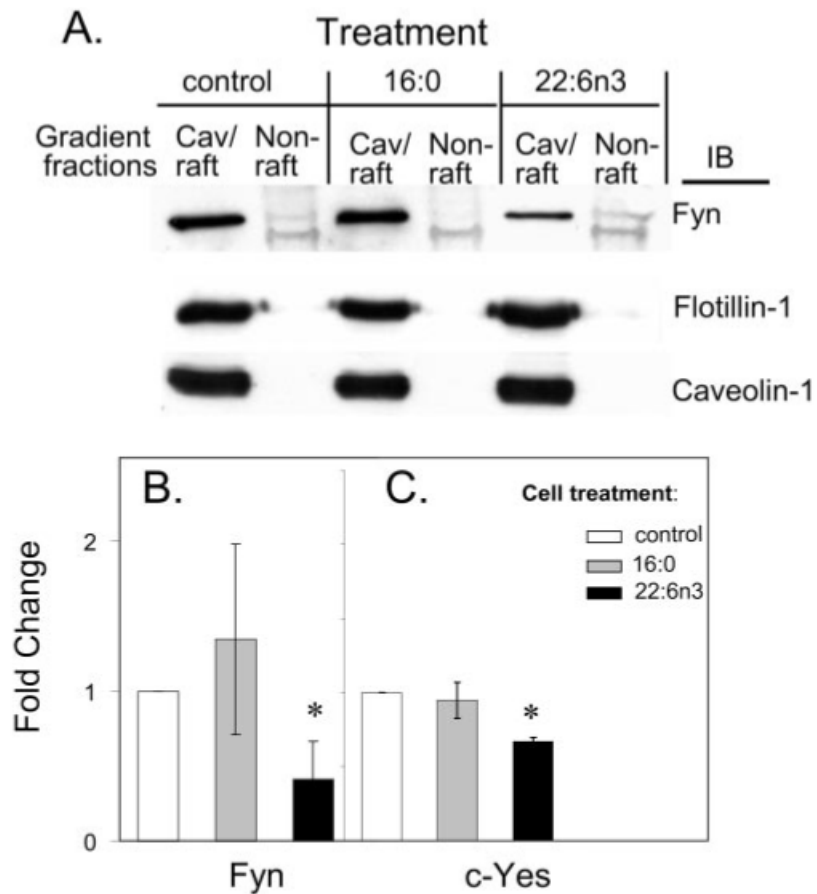
Cholesterol replenishment in caveolae/lipid rafts reverses DHA<sub>22:6,n3</sub> inhibition of TNF- $\alpha$  induced ICAM-1 expression. hRVE cells treated with 100  $\mu$ M DHA<sub>22:6,n3</sub> (black bars) for 12 hours followed by TNF- $\alpha$  stimulation for 12 hours. Cells treated with palmitic acid<sub>16:0</sub> were used as a control (gray bars). Water-soluble cholesterol (25  $\mu$ M cholesterol complexed with 250  $\mu$ M MCD) was added to DHA<sub>22:6,n3</sub>-treated cells for 30 minutes before TNF- $\alpha$  stimulation (striped bar). ICAM-1 expression levels were assessed by immunoblot analyses. Equal amounts of protein were added to each lane, as confirmed by actin levels. Data are the mean  $\pm$  SD of results in three experiments, \* $P$  < 0.05 compared with palmitic acid<sub>16:0</sub> treated cells. # $P$  < 0.05 compared with DHA<sub>22:6,n3</sub>-treated cells without cholesterol.

**FIGURE 7.**

Src family kinase enrichment in hRVE caveolae/lipid rafts. Caveolae/lipid raft fractions were purified, as described in Materials and Methods. Fractions 2 to 4 were combined and designated the caveolae/lipid raft (cav/raft) fractions, and fractions 5 to 9 were combined and designated the nonraft membrane fraction. Fractions were separated on SDS-PAGE and analyzed by Western blot. Results are representative of seven experiments.

**FIGURE 8.**

Effect of SFK inhibition on TNF- $\alpha$ -induced VCAM-1 expression. hRVE cells were pretreated with PP2 (10  $\mu$ M) for 30 minutes and stimulated with TNF- $\alpha$  (5 ng/mL) for 6 hours. VCAM-1 and ICAM-1 expression levels were assessed by immunoblot analyses. Equal amounts of protein were added to each lane, as confirmed by actin levels. Results are representative of three experiments.

**FIGURE 9.**

DHA<sub>22:6,n3</sub> displaces Fyn and c-Yes from hRVE caveolae/lipid rafts. (A) hRVE cells were treated with 100  $\mu$ M palmitic acid<sub>16:0</sub> or DHA<sub>22:6,n3</sub> for 24 hours. Vehicle (20  $\mu$ M BSA)-treated cells were used as a control. Caveolae/lipid raft fractions were isolated and analyzed by Western blot. Amounts of Fyn (B) and c-Yes (C), localized in caveolae/lipid rafts isolated from fatty acid-treated cells, were quantified and presented as a fold change. Data are the mean  $\pm$  SD of results in three experiments. \* $P < 0.05$ .