Dyslipidemia, but Not Hyperglycemia, Induces Inflammatory Adhesion Molecules in Human Retinal Vascular Endothelial Cells

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Purpose. The initial determinants of retinal microvascular damage in diabetic retinopathy are not well understood, but are likely to be induced 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 (hRVE) cells as a means of mimicking diabetic metabolic disorders.

Methods. The expression of adhesion molecules in hRVE and human umbilical vein endothelial cells (HUVECs) was assayed by Western blot analysis 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 the n6 polyunsaturated fatty acids (PUFAs) 18:2n6 and 20:4n6 for up to 24 hours resulted in a significant induction of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 protein levels. In contrast, treatment with high glucose (22 mM) for 24 hours did not affect CAM expression. Induction of CAM by n6 PUFA 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 by COX or MOX inhibitors. In contrast to hRVE cells, n6 PUFA did not induce ICAM-1 or VCAM-1 in HUVECs.

Conclusions. The data obtained in this study demonstrate that acute exposure to linoleic or arachidonic acid, but not hyperglycemia, induces inflammatory adhesion molecule expression in the presence of LOX in microvascular hRVE cells, but not in HUVECs. These results are consistent with the emerging hypothesis recognizing early-stage diabetic retinopathy as a low-grade chronic inflammatory disease. (*Invest Ophthalmol Vis Sci.* 2003;44:5016–5022) DOI:10.1167/iovs.03-0418

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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 morphologic 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 through 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. Leukocyte infiltration and expression of retinal vascular ICAM-1 coincide with many of the pathologic lesions in diabetic retinopathy.[3,6]

Although 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 in understanding of hyperglycemia-induced disease 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. 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 free fatty acid (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 Δ^{-6}, Δ^{-5} and Δ^{-9}-desaturases in the liver and a corresponding change in FFA profile, resulting, for instance, in an increase in linoleic acid and in the n6 PUFA-to-n3 PUFA ratio.[10,17–19]

Clinical data support the idea that dyslipidemia could be a critical factor in the development of diabetic retinopathy. Thus, in a recent clinical trial treatment of diabetic dyslipidemia with simvastatin, a hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitor resulted in significant retardation of the progression of diabetic retinopathy.[20,21] The data from another population-based study suggest the association of retinopathy with cardiovascular disease and elevated plasma LDL cholesterol.[22] The Early Treatment Diabetic Retinopathy Study has demonstrated that higher levels of serum lipids are associated with an increased risk of development of hard exu-

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

The role of fatty acids as the inflammatory agent leading to diabetic retinopathy has not been studied in detail and could represent a missing link between diabetes, dyslipidemia, and microvascular damage. The present 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.

METHODS

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 (Corning, NY). Commonly used chemicals and reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO). TNFα and IL-1β were from R&D Systems (Minneapolis, MN). Phorbol myristate acetate (PMA) was from Sigma-Aldrich.

Cell Culture and Fatty Acid Treatments

In the present study, primary cultures of hRVE cells obtained from three donors were used. hRVE cells were prepared as previously described and maintained[32–35] in growth medium consisting of DMEM/F12 (Invitrogen), 5.5 mM glucose, 10% fetal bovine serum (Invitrogen), endothelial cell growth supplement (Upstate Biotechnologies, Inc., Lake Placid, NY), insulin/transferrin/selenium mix (Sigma-Aldrich) 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 to 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 hours. For experimental treatments, the cells were transferred to serum-free medium for 18 to 24 hours before addition of the stimulatory agents. Treatment of hRVE cells with fatty acids was performed as follows. Fatty acid 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 medium to a final concentration of 100 mM fatty acid and 60 μM BSA, stabilized with vitamin E (400 μM) and butylated hydroxytoluene (BHT; 0.04%) and neutralized with NaOH, as described previously.[36] The fatty acid stock solutions were diluted in serum-free medium to reach fatty acid concentrations of 10 to 100 μM with corresponding BSA concentrations of 2 to 20 μM. The fatty acid-to-albumin molar ratio was 5:1.[36] Cells were incubated for the times indicated in the Results section. Equivalent amounts of BSA alone were added to control plates. Inhibitors of COX, LOX, (Cayman Chemical, Ann Arbor, MI), and MOX (Sigma-Aldrich) 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 hours.

Electrophoresis and Immunoblotting

hRVE cells were grown in 6-cm plates in experimental medium for up to 24 hours. 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 to 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 phenylmethylsulfonyl fluoride [PMSF]). Homogenates were centrifuged at 16,900*g* for 20 minutes at 4°C. Total cell protein (15 μg) was fractionated by electrophoresis on SDS-polyacrylamide (10%) minigels. The separated proteins were electrophoretically transferred to nitrocellulose (Bio-Rad, Hercules, CA) and blocked for 60 minutes 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 (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).

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 minutes, washed twice with PBS. 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 six-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 hours. The hRVE cells were carefully washed with PBS by decanting and aspirating twice, lightly fixed with 0.5% paraformaldehyde for 15 minutes 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.

Statistical Analysis

Data are expressed as the 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 two independent samples. The Bonferroni procedure was used to control type I errors. Significance was established at $P < 0.05$.

RESULTS

Effect of Polyunsaturated n6 Fatty Acids on Inflammatory Adhesion Molecule Expression in hRVE Cells

We 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 BSA-bound fatty acids (5 mole fatty acid to 1 mole of BSA) for 12 and 24 hours, 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 (22:6n3) acid treatment had no effect on either VCAM-1 or ICAM-1 expression in hRVE cells (Figs. 1A, 1B). In contrast, treatment with n6 PUFA linoleic (18:2n6) or arachi-

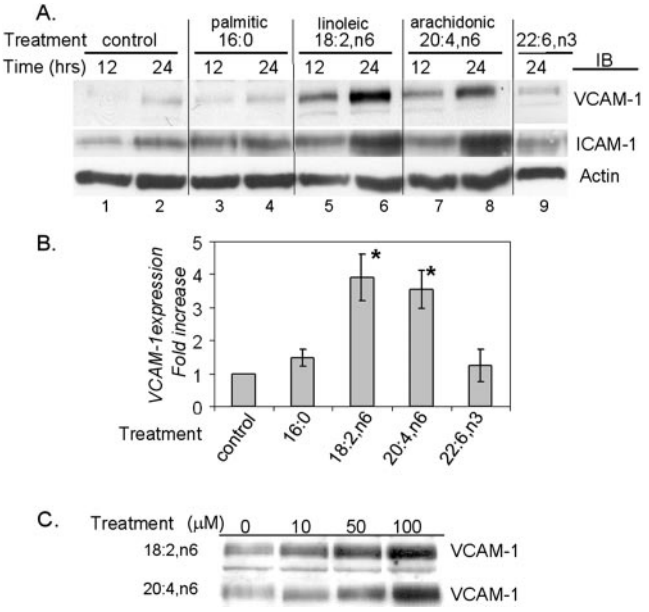

FIGURE 1. Induction of endothelial CAMs by FFAs in hRVE cells. (**A**) hRVE cells were treated with BSA control or with 100 μM of the FFAs 16:0, 18:2n6, 20:4n6, or 22:6n3 for the indicated time before analysis of cell lysates for VCAM-1 and ICAM-1 expression by immunoblot (IB). (**B**) Quantitative compilation of the data on VCAM-1 induction in hRVE cells after 24 hours of treatment with 100 μM FFAs. The results are obtained from the cells isolated from three independent donors. $^*P < 0.05$ compared with BSA control. (**C**) Induction of VCAM-1 in hRVE cells after treatment with different doses of FFAs (18:2n6 and 20:4n6). (**A**, **C**) 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 more than three independent experiments.

donic (20:4n6) acid increased VCAM-1 (Figs. 1A, 1C) and ICAM-1 (Fig. 1A) expression. ICAM-1 and VCAM-1 expression increased after 12 hours and was maximally induced after 24 hours of fatty acid treatment (Fig. 1A). The induction of VCAM-1 occurred with as little as 10 μM linoleic acid and arachidonic acid (Fig. 1C). Whereas ICAM-1 was detectable with no fatty acid treatment, VCAM-1 expression was usually below detectable levels (Fig. 1A, lane 1). The maximum increase in induction of VCAM-1 (fourfold) was greater than for ICAM-1 (threefold; Fig. 1A, lanes 2, 6, 8; 1B). Figure 1B shows a quantitation of the results obtained from cells of three donors at the passages 4 to 6.

Effect of Hyperglycemia on CAM Expression in hRVE Cells

Because 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 hours did not affect expression of ICAM-1 and VCAM-1 (Fig. 2A). As a control, IL1-β induced VCAM-1 expression under euglycemic conditions (Fig. 2A).

Induction of Inflammatory Adhesion Molecules by Cytokines and PMA in hRVE Cells

Because little is known about CAM expression in hRVE cells, we compared the effects of fatty acids with those obtained after 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 hours. In contrast, PMA treatment had little effect on CAM expression in the time intervals studied. The expression of E-selectin in hRVE cells was not affected by these stimulants (Fig. 2B).

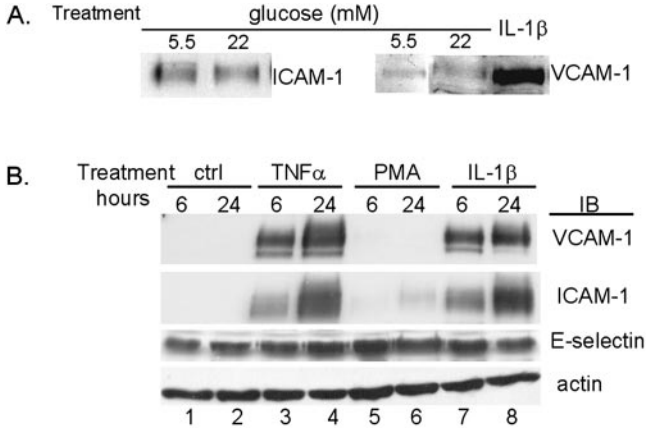

FIGURE 2. Evaluation of CAM expression after treatment of hRVE cells in hyperglycemic conditions and with cytokines and PMA. The induction of adhesion molecules was assessed by immunoblot analysis (IB) after treatment of cells for the indicated times with each potential stimulant. (**A**) hRVE cells were grown in euglycemic (5.5 mM) or hyperglycemic (22 mM) conditions for 24 hours before analysis. IL-1β (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 in each lane, and loading was confirmed by probing for actin (shown in **B**).

Response of HUVECs to Fatty Acid Treatment

HUVECs are primary cells derived from umbilical cord veins and are widely used as a model for human vascular endothelial cells. Similar to the hRVE cells, treatment of HUVECs with TNFα and IL-1β increased expression of ICAM-1 and VCAM-1 (Fig. 3A, lanes 3, 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

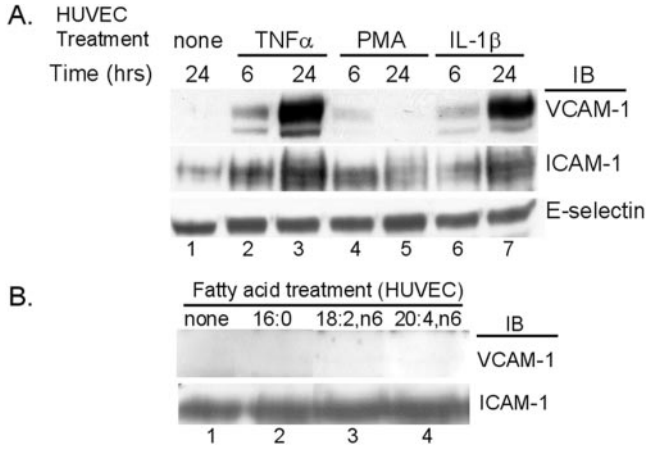

FIGURE 3. Fatty acid treatment of HUVECs failed to induce endothelial CAM expression. (**A**) HUVECs were treated with TNFα (20 ng/mL), PMA (10 ng/mL), and IL-1β (5 ng/mL) for the indicated times, followed by an analysis of expression of VCAM-1, ICAM-1, and E-selectin. (**B**) HUVECs were treated with BSA control (none) and with 100 μM fatty acid for 24 hours before analysis of cell lysates for expression of VCAM-1 and ICAM-1. No VCAM-1 was detected, and no induction of ICAM-1 was observed in more than three experiments. Equal amounts of protein were loaded in each lane.

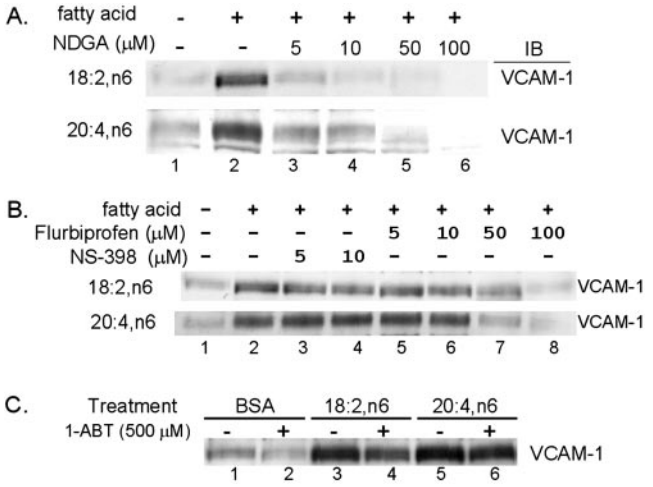

FIGURE 4. Induction of CAM expression by fatty acids was inhibited by LOX, but not COX and MOX, inhibitors. (**A**) hRVE cells were treated with 18:2n6 or 20:4n6 (100 μM) in the presence of increasing amounts of the inhibitor NDGA followed by lysis and immunoblot analysis of VCAM-1. (**B**) hRVE cells were treated with 18:2n6 or 20:4n6 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 18:2n6 or 20:4n6 in the presence of 500 μM 1-ABT followed by lysis and immunoblot analysis of VCAM-1. Equal amounts of protein were loaded in each lane.

VCAM-1 or ICAM-1 (Fig. 3B). The significant difference in sensitivity to fatty acids may represent a fundamental difference between the responses of hRVE cells and other endothelial cells to inflammatory stimuli.

Effect of Inhibition of Fatty Acid Oxidation on Expression of Fatty Acid-Induced Adhesion Molecule in hRVE Cells

Experiments in progress have shown that radioactive-tracer–labeled fatty acids taken up from the medium by hRVE cells enter several 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 the nonesterified fatty acid (NEFA) pool, providing a possible substrate for oxygenases (data not shown). Because 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 18:2n6- and 20:4n6-induced ICAM-1 (not shown) and VCAM-1 (Fig. 4A, compare lane 3 with lane 2) expression by more than 80%. Higher doses of NDGA completely inhibited ICAM-1 (not shown) and VCAM induction (Fig. 4A, lanes 4–6). 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, compare lanes 5, 6 with lane 2). Only at higher, nonspecific, concentrations (50–100 μM) did flurbiprofen inhibit the response (lanes 7, 8). The specific COX-2 inhibitor NS-398 (IC_{50} = 1.77 μM)[39] had no effect on the fatty-acid–mediated induction of VCAM-1 (Fig. 4B, lanes 3, 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 18:2n6- and 20:4n6-mediated induction of adhesion molecules in hRVE cells.

Leukocyte Adhesion and Fatty Acid Induction of Inflammatory CAMs

A leukocyte adhesion assay was used to confirm that fatty-acid–induced CAMs are 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 20:4n6 and 18:2n6 or with IL-1β resulted in a significant increase of adherent cells compared with BSA control. In contrast, high glucose or 16:0 treatment did not significantly increase the number of adherent cells. High glucose and 16:0 did not induce CAM (Fig. 1, 2). The dramatic increase in adhesion correlated well with the increased CAM expression observed by immunoblotting.

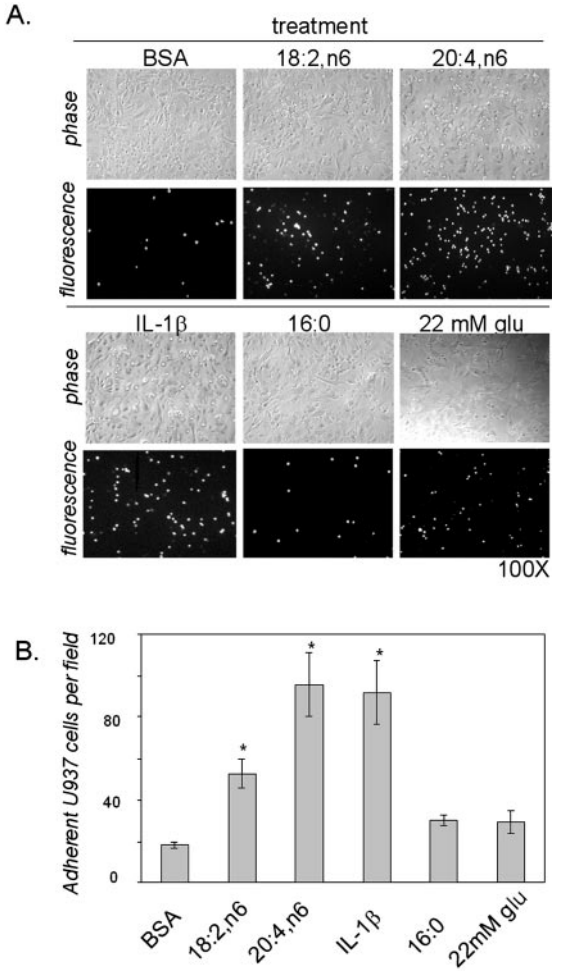

FIGURE 5. Increased adhesion of leukocytes after induction of CAM expression by fatty acids. hRVE cells were treated with BSA; 18:2n6, 20:4n6, IL-1β, or 16:0; or 22 mM glucose for 24 hours, followed by addition of fluorescence-tagged U937 cells. (**A**) The number of adherent U937 cells was determined by fluorescence microscopy. A representative fluorescence and phase-contrast field is shown for the different treatments (all micrographs are at 100×). (**B**) The number of fluorescence-labeled 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 with BSA control.

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 canine model that aspirin prevents certain classic histopathological features of diabetic retinopathy, including formation of acellular capillaries; retinal hemorrhage; and an indicator of cell degeneration, 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 with animal models. As early inflammatory changes in human eyes do not have any clinical manifestations, human primary cell culture provides an important model for the study of 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 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 in 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 the liver, insulin stimulates conversion of fatty acids to triglycerides, followed by secretion as VLDL, as well as the induction of Δ^{-5}, Δ^{-6}, and Δ^{-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 and the presence of esterified and nonesterified fatty acids,[12,14,15,45,47–50] and type 1 diabetes causes marked changes in the FFA profile, with an increase in n6 PUFA-to-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 linoleic or arachidonic acid led to a robust increase in expression of VCAM-1 and ICAM-1. The effect was specific for these n6 PUFA, because other fatty acids tested, such as saturated palmitic (16:0) and n3 PUFA docosahexaenoic (22:6n3), 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 600 μM or more,[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 hydroxy and epoxy fatty acids. Our inhibitor studies indicate that the LOX, but not the COX or P450 MOX pathways, may be essential 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 in 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 18:2n6- and 20:4n6-mediated induction of CAMs, it is not clear whether 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 may involve activation of phospholipase A2 or membrane remodeling resulting in release of substrates for LOX action. How LOX products induce CAM expression is also unknown. Both detailed fatty acid metabolism and signaling studies are needed 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 NF-κB, 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 hemostatic processes. VCAM-1 is not generally constitutively expressed on endothelial cells and is induced significantly after 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. Leukocyte infiltration and expression of retinal vascular ICAM-1 coincide with many of the pathologic 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 fluorescence-tagged U937 cells. U937 cells were derived from a human histiocytic lymphoma and retain properties of monocytes.[40] The adhesion of U937 cells to vascular endothelial cells is primarily dependent on $\alpha 4\beta 1$-integrin's interacting with endothelial VCAM-1.[58,59] That fatty acid treatment induced a large increase in the number of adherent U937 cells strongly supports the notion that 18:2n6 and 20:4n6 play an important role in microvascular inflammation.

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

In conclusion, our data suggest that diabetic dyslipidemia serves as an inflammatory stimulus that initiates and contributes 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 PUFAs associated with the diabetic condition results in longstanding chronic inflammation that gradually progresses to retinopathy.

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