A Lipidomic Screen of Hyperglycemia-Treated Human Retinal Endothelial Cells Links Lipid Metabolites of 12/15-Lipoxygenase to Microvascular Dysfunction during Diabetic Retinopathy via NADPH Oxidase

Ahmed S. Ibrahim1,2,3, Sally Elshafey1, Hassan Sellak4, Khaled A Hussein1,2, Mohamed El-Sherbiny1,5, Mohammed Abdelsaid6, Nasser Rizk7, Selina Beasley1,8, Amany M. Tawfik1,2,8, and Sylvia B. Smith2,8, Mohamed Al-Shabrawey1,2,5,8*

1Oral Biology and Anatomy, College of Dental Medicine, Georgia Regents University (GRU), Augusta, GA, USA; 2Ophthalmology and Culver Vision Discovery Institute, Medical College of Georgia (MCG), GRU; 3Department of Clinical Biochemistry, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt; 4Department of Anesthesiology and Perioperative Medicine, GRU, Augusta, GA, USA; 5Department of Anatomy, Faculty of Medicine, Mansoura University, Mansoura, Egypt; 6Department of Physiology, MCG, GRU, Augusta, GA, USA; 7Biomedical science program, Faculty of science, Qatar University, Doha, Qatar; 8Cellular Biology and Anatomy, MCG, GRU, Augusta, GA, USA.

Short running head: 12/15-Lipoxygenase Derived Metabolites and Diabetic Retinopathy

* Corresponding author:

Mohamed Al-Shabrawey, MD, PhD
Department of Oral Biology, Ophthalmology and Cellular Biology and Anatomy
College of Dental Medicine and Medical College of Georgia
Georgia Regents University, 1120 15th Street, Augusta, GA 30912
CB-2602, Tel: (706) 721-4278
Email: malshabrawey@gru.edu
List of Nonstandard Abbreviations:

DR, diabetic retinopathy; 12/15-LOX, 12/15-Lipoxygenase; NOX, NADPH oxidase; HETEs, hydroxyeicosatetraenoic acids; ELISA, Enzyme-linked immunosorbent assay; ROS, reactive oxygen species; STZ, Streptozotocin; HRECs, human retinal endothelial cells; HDoHE, hydroxydocosahexaenoic acid; HODE, hydroxyoctadecadienoic acid; Liquid chromatography–mass spectrometry, LC/MS; VEGF, vascular endothelial growth factor.
ABSTRACT

Retinal hyperpermeability and subsequent macular edema is a cardinal feature of early diabetic retinopathy (DR). Here, we investigated the role of bioactive lipid metabolites, in particular 12/15-lipoxygenase (12/15-LOX) derived metabolites, in this process. Liquid chromatography coupled to mass spectrometry (LC/MS) lipidomic screen of human retinal endothelial cells (HRECs) demonstrated that 15-HETE was the only significantly increased metabolite (2.4±0.4 fold, p = 0.0004) by high glucose (30mM) treatment. In the presence of arachidonic acid (AA), additional eicosanoids generated by 12/15-LOX, including 12- and 11-HETEs, were significantly increased. Fluorescein angiography (FA) and retinal albumin leakage showed a significant decrease in retinal hyperpermeability in streptozotocin-induced diabetic mice lacking 12/15-LOX compared to diabetic wild type (WT). Our previous studies demonstrated the potential role of NADPH in mediating the permeability effect of 12- and 15-HETEs, therefore we tested the impact of intraocular injection of 12-HETE in mice lacking the catalytic subunit of NADPH oxidase (NOX2). The permeability effect of 12-HETE was significantly reduced in NOX2−/− mice compared to the WT. In vitro experiments also showed that 15-HETE induced HREC migration and tube formation in NADPH oxidase dependent manner. Taken together our data suggest that 12/15-LOX is implicated in DR via NADPH oxidase dependent mechanism.

Key Words: Diabetic Retinopathy, 12/15-Lipoxygenase, 12/15-HETEs, Retinal vascular leakage, NADPH oxidase, and retinal inflammation.
INTRODUCTION

Diabetic retinopathy (DR), the most prevalent microvascular complication of diabetes, is responsible for over 10,000 new cases of blindness every year in the United States alone (1). The greatest risk of vision loss occurs with the development of diabetic macular edema (DME) and/or retinal neovascularization (NV), the former being a direct consequence of blood-retinal barrier (BRB) dysfunction and the latter to widespread retinal ischemia (2).

For years, significant effort has been invested in elucidating the mechanisms that underlie destructive preretinal NV in DR (3). Nonetheless, considerably less is known about the molecular events that lead to BRB dysfunction that is characterized by enhanced retinal vascular permeability and recruitment of inflammatory cells. Moreover, existing regimens of treatment carry non-specific adverse effects. These include increased risk of thromboembolic incidence, neuronal toxicity, and geographic atrophy with anti-VEGF (vascular endothelial growth factor) therapies (4, 5). Likewise, cumulative risks of cataract and glaucoma as well as local immunosuppression are frequently associated with corticosteroid intravitreal therapy (6, 7). Even with laser-based photocoagulation, the gold standard treatment for DR, side effects ranging from blurred peripheral vision to scotomas may occur (8). These therapeutic limitations impose the necessity for novel therapeutic interventions via unraveling the pathophysiology of DR.

In recent years, human studies have underscored the strong association between dyslipidemia and the development of DR. Clinically, it has been reported that the severity of retinopathy in Type 1 diabetes was correlated positively with triglyceride level and negatively with the high density lipoprotein (HDL) concentration (9). Additionally, intensive dyslipidemia therapy significantly slowed the progression of DR in Type 2 diabetes over 4 years (10). Diabetic dyslipidemia is characterized by shift in fatty acid profile with an increase in omega 6
polyunsaturated fatty acids (PUFA), in particular arachidonic acid (AA), released from membrane lipids by the activated phospholipase A2 (PLA2) (11, 12). The released AA, in turn, can be converted to inflammatory bioactive lipid mediators such as Hydroxyeicosatetraenoic acids (HETEs), leukotrienes, and prostaglandins via different enzymatic pathways including cyclooxygenase, lipoxygenase (LOX), and cytochrome P450 (13).

Bioactive lipids activate specific signaling pathways that are implicated in cell proliferation, differentiation, and apoptosis. Sustained cellular response to increased bioactive lipids becomes pathological and result in chronic diseases such as cancer and atherosclerosis (14).

While there is irrefutable evidence for the role of dyslipidemia in the progression of DR (9, 15), bioactive lipid metabolites have received limited attention. HETEs are major monohydroxylated arachidonic acid bioactive lipid metabolites that are produced during inflammatory and immunological reactions (16). 12- and 15-HETEs are formed by human 15-LOX and its murine ortholog 12/15-LOX (17) in a variety of mammalian cells such as endothelial, eosinophils, and epithelial cells to exhibit distinct biological activities. These activities include stimulation of endothelial cell mitogenesis, vascular inflammation, and mucus release from human airways(18-20). Recently, we have shown that pharmacological inhibition or deletion of 12/15-LOX reduces retinal NV in the oxygen induced retinopathy (OIR) model (21). Moreover, we have demonstrated in both human patients and animal models that retinal expression of 12/15-LOX is robustly induced during diabetes (21). At the same time, its pharmacological inhibition dampened the levels of inflammatory cytokines, reactive oxygen species (ROS) generation, and pVEGF-R2 expression in retina of diabetic mice (22). However, the cellular source of these lipid metabolites is unknown and could be derived from retinal
tissues including the retinal vascular endothelial, glial, and pigmented epithelial cells as well as from infiltrated inflammatory cells. Moreover, the role of 12- or 15 HETE in mediating the breakdown of barrier function, such as that observed in DR, has not been investigated thoroughly to date.

In addition to dyslipidemia, oxidative stress has attracted a considerable interest as a key factor in mediating retinal vascular injury during DR. Our previous studies highlighted the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase as a major source of ROS generation in retina of experimental diabetic rodents and OIR models as well as in retinal endothelial cells treated with high glucose or hypoxia (23-26). Our recent study showed that inhibiting 12/15-LOX by baicalein reduced diabetes-induced ROS generation and NOX2 expression in mouse retina, suggesting the existence of an inter-connected signaling between LOXs and NADPH oxidase (22). However, the crosstalk between NADPH oxidase and inflammatory bioactive lipids remains ill-defined in DR.

Therefore, the current study has been taken up to characterize the effect of hyperglycemia on bioactive-lipid profile in human retinal endothelial cells to determine whether endothelial cells are a potential source of 12/15-LOX derived hydroxyeicosanoids and whether these metabolites play a causative role in the pathogenesis of BRB dysfunction during diabetes. Furthermore, the present work aimed to gain insights into the underlying molecular mechanisms by portraying the causal relationship with NADPH oxidase.
RESEARCH DESIGN AND METHODS

Animal Preparation and Experimental Design

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication, NIH 80-23) and Georgia Regents University guidelines. Six to eight-week-old male 12/15-lipoxygenase knockout (LOX\(^{--}\)), mice lacking the NOX2 subunit of NADPH oxidase (NOX2\(^{--}\)), and corresponding littermate controls wild-type (WT) mice in C57BL/6J background (Jackson Laboratory, Bar Harbor, ME), were matched according to sex, age, and weight. Animals were given intraperitoneal (I.P) injections of freshly prepared Streptozotocin (STZ, 45 mg/kg, Enzo Life Sciences) dissolved in 0.9% NaCl after four hour-fasting for 5 consecutive days. Mice with blood glucose levels >300 mg/dL were considered diabetic. Six weeks after establishment of diabetes, retinal vascular permeability has been evaluated using Fluorescein Angiography. Thereafter retina samples were used for total albumin analysis by Western blot.

For intravitreal injections, the procedure was essentially the same as previously described (27). To avoid uncontrolled intraocular pressure increase, the volume of intravitreal injections was limited to 1 µl. 12-HETE was dissolved in ethanol and a working solution of 10x was prepared by diluting 0.32 µl of stock solution (312 µM) to 100 µl with PBS, assuming the vitreous volume of mouse eye is \(~\)10 µl (28). Then by injecting 1 µl of this working solution, an 0.1 µM vitreal concentration of 12-HETE was obtained. The vitreal concentration of ethanol was 0.032%. The volume of the injected solution apparently did not cause significant pressure-induced retinal damage, because 0.032% ethanol-PBS-injected control eyes showed normal retinal morphology with no apparent apoptosis within 7 days. The dose of 12- or 15-HETE was
chosen according to what detected previously in the vitreous of patients with DR, 50 ng/mL (~0.1 uM) (21).

**Microvascular human retinal endothelial cells (HRECs)**

*In vitro* experiments were performed using cultured HRECs (Cell Systems Cooperation). After the cells were 80-90% confluent, they were serum starved (2% FBS) overnight, then treated with 12/15 HETEs (0.1 uM) with or without apocynin 30 µM or N-acetyl-L-cystein (NAC) 50 µM or high glucose (HG) (D-Glucose, 30 mM) in presence or absence of 20 µM of Arachidonic acid (AA). The osmolarity of control group, in the HG experiments, was adjusted using L-Glucose. Cytotoxicity of tested inhibitors were assessed by MTT assay as previously described (29). Transcellular Electrical Resistance (TER) and cell Migration were done using Electric Cell-substrate Impedance Sensing (ECIS, Model 1600R, Applied BioPhysics) as previously described (30, 31). Experiment was terminated after 24 hours of 12/15-HETE treatment for analysis of leukocyte adhesion and tube formation. Conditioned media were used for multiplex assay of various cytokines as previously described (30). Meanwhile, the HG experiment was terminated 5 days after initiation of treatment and cell lysates were collected and analyzed for 15-Lipoxygenase-1 (15-LOX) and 5-LOX protein expression using Western blot analysis. At the same time, collected cell lysates were assayed for phospholipase A2 (PLA2) activity according to manufacturer’s instructions. For lipidomic analysis, the cells were incubated in indicator-free media for 5-days and then collected with cells together, sonicated, centrifuged to remove cell debris and then freezed for the analysis in the Lipidomics Core Facility (Wayne State University, Detroit, Michigan) as described before (32).
**Immunofluorescence**

Retinal paraffin sections of human subjects with or without DR obtained from Capital Bioscience (Rockville, MD) were fixed in 10% neutral buffered formalin. Following rehydration of the paraffin section and two washes in PBS, sections were treated with Proteinase K for 10 min and washed twice in PBS followed by blocking with 10% normal goat serum (NGS) and then incubated with Phospho-PLA2 antibody overnight in a humidified container at 4 °C. In the next day the sections were incubated in Oregon green-labeled anti-rabbit antibody. Sections were covered using DAPI mounting medium and images were obtained with confocal microscopy (LSM 510; Carl Zeiss).

**Fluorescein Angiography (FA).**

The anesthetized mouse was placed on the imaging platform of the Phoenix Micron III retinal imaging microscope and Goniovisc 2.5% was applied liberally to keep the eye moist during imaging. Mice were administered 10 to 20 μL 10% fluorescein sodium and rapid acquisition of fluorescent images ensued for ~5 minutes as previously described (33).

**Protein extraction and Western blot analysis**

Cell or retinal lysates were subjected to Western blot analysis using antibodies for 15-LOX1 (Novus biologicals, Littleton, CO, USA), 5-LOX and β-actin (Millipore Corporation, Billerica, MA, USA), VCAM-1 and ICAM-1 (Cell Signaling Technology, Beverly, MA), albumin (Bethyl, TX, USA), NOX2 (BD Biosciences, Franklin Lakes, NJ, USA), and CD45 (Santa Cruz, CA, USA) according to a previous procedure (27).
RNA interference.

HRECs were transfected with NOX2 or control Dicer-substrate RNA (DsiRNA) duplexes (Catalog # HSC.RNAI.N000397.12, Integrated DNA Technologies) using lipofectamine 2000 per the manufacturer’s instructions.

**In-vitro leukocyte adhesion assay**

HRECs were grown to confluence in 12 well plates then treated with or without 15-HETE (0.1 µM) in the presence or absence of apocynin (30 µM) or NAC (50 µM), as well as with positive control, 10 µg/ml of the endotoxin *Escherichia coli* lipopolysaccharide (LPS, Sigma Aldrich, St. Louis, MO) for 24 h. Following the treatment, leukocytes (300,000-400,000 cells/well) purchased from Sanguine Bioscience (Valencia, CA) and labelled with diluted LeukoTracker solution (Cell Biolab) for 60 minutes at 37 °C, according to the manufacturer’s instructions, were added to confluent monolayer HRECs and incubated for 90 min at 37 °C. Immediately before the assay, non-adherent cells were removed by washing three times with RPMI-1640 medium (Life Technology, Grand Island, NY). The adherent labelled leukocytes were counted under the inverted fluorescence microscope at 480 nm/520 nm from three separate fields per well.

**In vitro endothelial tube formation on Matrigel**

Tube formation assay was done as described before (34). Briefly, Vehicle or 15-HETE (0.1 µM) in the presence or absence of different inhibitors, were added to the appropriate wells and the cells were incubated at 37°C overnight. Tube formation was observed under an inverted microscope and the images were captured with a digital camera attached to the microscope. The measurement was done on three randomly chosen microscopic fields per well, and the mean of
the three fields was used as a single observation. Tube formation was further quantified by measuring the total length of tube-like cells using ImageJ software.

Data Analysis

The results are expressed as mean ± SD. Differences among experimental groups were evaluated by using the two-tailed t test or one way analysis of variance (ANOVA). When statistical differences were observed using ANOVA, a post hoc Tukey's test was performed to determine which groups differed.

The Bonferroni correction factor (BCF), which accounts for a large number of comparisons by reducing the alpha level as to decrease the probability of obtaining a type I error, was used when analyzing lipid metabolite profiles among groups. BCF was calculated by dividing the alpha level (0.05) by the number of bioactive lipid metabolites detected. Since 56 individual metabolites were detected, the new alpha value used for individual metabolites was 0.0009 (a = 0.05/56). A new alpha level of 0.0055 (a = 0.05/9) was also calculated for the top 15% metabolites which are used for further analysis by measuring their profile in the presence of exogenous AA as a substrate under hyperglycemic condition.

Differences were considered statistically significant if p < 0.05, except for lipid metabolites, which used the alpha values calculated from the Bonferroni correction.
RESULTS

Hyperglycemia alters bioactive-lipid profile in HRECs

In order to evaluate the potential role of bioactive lipids in mediating the edematous phenotype observed in DR, we first sought to characterize their profile under diabetic milieu. In this regard, the lipidomic profile of HRECs was screened for metabolites whose levels changed dramatically during hyperglycemia. Out of 126 bioactive lipids screened, 70 were not detectable (Table 1), 47 have not changed significantly (t-test, p > 0.05), and 9 metabolites were increased (t-test, p < 0.05) under hyperglycemic conditions (30 mM D-glucose) compared to normo-osmotic control (5mM D-glucose + 25 mM L-glucose), (Figure 1A). However, after the Bonferroni correction, the fold changes in the level of 8 metabolites among the 9-increased were statistically not significant and only 15-HETE has the significant fold increase (2.4±0.4) with a p value of 0.0004 (BCF α = 0.0009).

We then narrowed our focus and confirming these experiments, considering the top 15% upregulated metabolites detected in this screen. By measuring their profile in the presence of exogenous AA as a substrate under hyperglycemic condition, we found a significant increase in AA-derived eicosanoids generated by the 12/15-LOX pathway including; 15-HETE (BCF α = 0.0055, Tukey's post hoc, p = 0.001), 11-HETE (BCF α = 0.0055, Tukey's post hoc, p = 0.0001), and 12-HETE (BCF α = 0.0055, Tukey's post hoc, p = 0.001) in which 15-HETE again has the highest (5.48±1.12) fold increase. On the other hand, the levels of other non AA-derived metabolites (8-, 13-, 16-, and 17-HDoHE, and 13-HODE) did not change significantly in the presence of exogenous AA with the exception of 13-HODE, Figure 1B.

Western blot analysis of lipoxygenases from HRECs further corroborated data obtained by LC/MS metabolite measurement. Results revealed that 15-LOX protein expression was
significantly increased by 2.5 fold after treating HRECs with high glucose compared to control. Meanwhile, the protein expression level of 5-LOX did not change significantly under the same treatment, Figure 2A. Given the prominent increase in levels of 15-HETE under the hyperglycemia, we proceeded to investigate the propensity of 15-HETE to disrupt vascular barrier function in HREC monolayer using real-time analysis of transendothelial electric resistance (TER), an indicator of monolayer integrity. We tested the concentration of 15-HETE that has been detected in the vitreous of patients with DR, ~55 ng/mL (0.1- 0.3 uM) (21) and shown by us to elicit transcellular leakage of fluorescein isothiocyanate (FITC)-dextran in bovine retinal endothelial cells (22). Changes in TER was first observed after 26 hours of 15-HETE treatment and continued to decrease throughout the experiment period, Figure 2B.

These data presumably reflect a PLA2-dependent pathway in releasing endogenous free AA as the mediator's precursor followed by the activation of metabolizing lipoxygenases. To this end, we determined the in vitro endothelial PLA2 activity in response to diabetic conditions. As depicted in Figure 2C, treatment of HRECs with hyperglycemia for 5 days stimulated the activity of PLA2 by 2-fold compared to control. This finding of increased PLA2 activity has been substantiated by the detection of phosphorylated PLA2 (active form) in diabetic human retinal sections Figure 2D. Phosphorylated PLA2 was mostly localized in the endothelial layer of the retinal vessels, perivascular retinal glial cells as well as in the outer segment of the photoreceptors.

**Evolving role of 12/15-LOX pathway in compromising retinal barrier function during diabetes**

The aforementioned data from lipidomic analysis provide a rationale to explore the role of 12/15-LOX pathway in diabetes-induced BRB breakdown. We used two complementary
approaches for this investigation. The first approach was aimed to assess whether knocking out retinal 12/15-lipoxygenase, an ortholog of human 15-LOX-1 (17), would efficiently reduce pathological vascular permeability seen in DR. To achieve this goal, we used the well-defined 12/15-LOX (Alox15) KO mice and matched congenic controls. Firstly, these mice were re-genotyped before the study to ensure that they had deletion of 12/15-LOX in the retina according to the Jackson Laboratory’s protocol (data not shown). Next, STZ was administered to ~6-week-old mice and blood glucose was monitored. Mice were considered diabetic if their non-fasted glucose level was higher than 300 mg/dl. After 6 weeks, the magnitude of retinal vascular leakage was assessed via a clinical diagnostic technique, fluorescein angiography (FA). At this time point, FA from WT diabetic mice exhibited a significant elevation almost 2.5-fold in fluorescein hyper-fluorescence compared with the non-diabetic littermates. In contrast, the increase in fluorescein hyper-fluorescence was significantly dropped to 1.9-fold by knocking out 12/15-LOX, Figure 3 A. To further confirm the role of 12/15-LOX pathway in mediating diabetes induced vascular permeability, Western blot analysis of total retinal albumin was performed. As shown in Figure 3B, the total retinal albumin content in diabetic WT mice was 4-fold higher than that in non-diabetic WT mice. However, knocking out 12/15-LOX resulted in 59% reduction of total albumin in the retinas of diabetic mice.

Further evidence for the ability of 12/15-LOX to compromise endothelial barrier function was obtained from our second approach. In this approach, an in vivo experimental eye model has been used in which normal mice were injected intravitreally with 12/15-LOX derived predominant murine metabolite, 12-HETE, into the right eye and vehicle into the left eye. Thereafter, the putative biological effect of 12-HETE per se on retinal permeability was photographically seen via FA. As shown in Figure 4A, a marked increase in fluorescein leakage
has been observed in eyes receiving 12-HETE compared with the vehicle-injected contralateral eyes. Concordantly, Western blot analysis for total retinal albumin was performed to further corroborate 12-HETE’s ability to induce vascular permeability. As shown in Figure 4B, 12-HETE injection induced a significant 2.8-fold increase in total retinal albumin compared to vehicle-injected controls.

The effect of 12-HETE on retinal vasculature was characterized by a marked increase in the inflammatory response

We next sought to determine whether the deleterious effect of 12-HETE on retinal vasculature is associated with a pro-inflammatory phenotype (increased adhesion molecule expression and leukocyte adhesion, Figure 4C). To address this point, we determined the effect of 12-HETE injection on retinal levels of ICAM-1 and VCAM-1, well established markers of endothelial dysfunction in inflammatory conditions (35). Western blot analysis of ICAM-1 showed 2.9-fold increase in 12-HETE-injected eyes compared with vehicle-injected contralateral eyes. Likewise, retinal VCAM-1 level was higher (2.5-fold, p< 0.05) in 12-HETE injected eyes than in control. Furthermore the inflammatory response to the intravitreally injected 12-HETE was determined by leukocyte infiltration as assessed by CD45, a common inflammatory leukocyte antigen. A significant increase in CD45 immunoreactivity (1.4-fold) in retinas of 12-HETE-injected eyes was observed compared to control.

Intravitreal injection of 12-HETE increased retinal expression of NOX2, the catalytic subunit of NADPH oxidase

Previous studies including from our lab have pointed out that NADPH oxidase-derived reactive oxygen species (ROS) have a central role in both endothelial and leukocyte activation,
keys of retinal inflammatory paradigm (25, 36). Therefore, causal relationship between 12-HETE and NADPH oxidase in mediating endothelial dysfunction has been investigated. To accomplish this, we first assessed the question of whether the retinal expression of NOX2 is enhanced by intravitreal injection of 12-HETE or not. As shown in Figure 4C, the NOX2 protein level was increased 1.7-fold in retinas of 12-HETE injected eyes compared with contra-lateral controls, implying an existing causal relationship.

**12/15-LOX lipid metabolites induce human retinal endothelial cell activation/dysfunction in a NADPH oxidase dependent manner**

Guided by aforementioned results, the role of NADPH oxidase-dependent pathway in 15-HETEs-mediated endothelial dysfunction has been tested in depth using a cell culture model of human retinal endothelial cells. With this model, incubation with either 12-HETE or 15-HETE significantly activates endothelial cells to release multiple of pro-inflammatory cytokines and chemokines. Among these, IL-6, IL-8, IL-17, and MCP-1 appear to dominate (Figure 5A). To ensure that 12 or 15-HETE-activated endothelial cells exhibit features of microvascular dysfunction characterized by augmentation of polymorphonuclear leukocytes (PMNs) adherence, we performed leukocyte adhesion assay. Exposure of HRECs to 15-HETE for 24 hours significantly augmented the number of PMNs adhering to HRECs (5.9 fold increase) compared to control. Next we tested the role of NADPH oxidase-derived ROS in 15-HETE-induced leukostasis by using apocynin, a NADPH oxidase inhibitor, to inhibit ROS generation as well as N-acetyl cysteine (NAC, an ROS scavenger) to eliminate ROS as they are generated. When, apocynin or NAC, was added before 15-HETE, the number of adherent leukocyte induced by 15-HETE dropped back to normal (Figure 5B). To ensure that these effects were not caused by nonspecific cytotoxicity of apocynin or NAC, we assessed cell viability in HRECs after 24 hours
exposure to apocynin or NAC, using the MTT assay. Apocynin or NAC at the used concentration did not affect HREC viability (94±4% vital cells), indicating that the decrease in the number of adherent leukocytes to 15-HETE-activated HRECs was indeed consecutive to inhibition of NADPH oxidase activity but not to cell death.

In addition to stimulation of leukostasis by 15-HETE, it also promoted retinal endothelial angiogenesis. This is clearly depicted in two representative in vitro angiogenic assays, capillary-like structure formation and cell migration (Figures 6 and 7). As shown in Figure 6A and quantified in Figure 6B, the length of capillary like structure formed by HREC in the presence of 15-HETE was significantly increased by 4-fold compared with control. To investigate the contribution of NADPH oxidase in the formation of capillary-like structure induced by 15-HETE treatment, NADPH oxidase inhibitors were added to HRECs before 15-HETE-treatment. Thereafter, the mean length of tubes between cells was measured. We found that apocynin as well as NAC significantly abrogated 15-HETE-induced tube formation. Similarly, 15-HETE significantly increased HREC migration rate, an early step in angiogenesis. This is graphically shown in Figure 7A, where the capacitance of all treated cells was increased from ~1 nF to 3 nF, wounding phase, then recovered at differential rates during the migration, healing phase. The mean migration rate of HRECs treated with 15-HETE was significantly higher (P < 0.01) than the rate obtained with vehicle treated control. Whether or not NADPH oxidase is involved in 15-HETE-induced endothelial cell migration was then investigated using two complementary approaches. First, a NADPH oxidase inhibitor, apocynin, significantly inhibited 15-HETE-induced HREC migration, without affecting cell viability (Figure 7A). Second, HRECs were transiently transfected with NOX2 siRNA or scrambled siRNA (Figures 7B and C) and then
treated with 15-HETE as before. NOX2 siRNA, but not scrambled siRNA, significantly inhibited 15-HETE-induced HREC migration (Figure 7D).

To further understand the contribution of NADPH oxidase in mediating endothelial barrier dysfunction induced by 12/15-LOX lipid metabolites, we injected 12-HETE intravitreally into NOX2(-/-) mice. At one week after injection, retinal fluorescein leakage of NOX2(-/-) mice was statistically less than 12-HETE-injected WT littermates, (Figure 8). Taken together, these results are confirming that NADPH oxidase is involved in the 12-HETE induced disruption of the inner BRB integrity.
DISCUSSION

The current therapeutic interventions to treat DR are still heavily relying on controlling systemic hyperglycemia. However, many diabetic patients develop retinopathy despite having a good control (37). Furthermore, the recent targeted treatments strategies, corticosteroids and anti-VEGF therapies, as well as laser photocoagulation are limited by their off-target effects. Therefore, it is worthwhile to explore new therapeutic avenues to improve DR. To the best of our knowledge, the current study provides the first preclinical evidence pertaining to the involvement of 12/15-LOX derived bioactive lipid metabolites in the pathogenesis of vascular barrier breakdown in the early stages of DR. Effectively, our study is the first to screen bioactive lipid profile in HRECs under hyperglycemic condition. The key findings of this screen are: 1) the products of 12/15-LOX pathway were significantly up-regulated under hyperglycemic condition with 15-HETE exhibiting the most significant increase. 2) 15-HETE activates retinal endothelial cells through NADPH oxidase system leading to increases in leukocyte adhesion, hyperpermeability and finally neovascularization, the cardinal signs of DR.

LOX is an enzyme that catalyzes the addition of oxygen to PUFA containing 1,4-pentadiene structure. The most common analogs are 5-LOX, 12-LOX, and 15-LOX, with the number indicating at which carbon the oxygen is inserted. Of note, species-specific difference between orthologous LOX isoforms has been described for the murine 12-LOX, which is an arachidonate 15-LOX in human. This difference indicates that care should be taken if experimental data on LOX metabolism are being transferred from one species to another (38, 39). Accordingly, we have used 12-HETE in all murine experiments versus 15-HETE in all human cell experiments. Although a large body of data indicates that LOX plays a role in the pathogenesis of various diseases, including cancer and atherosclerosis, novel physiological roles
continue to emerge. In relation to retinal vasculopathies, lipoxygenases present themselves as attractive candidates for therapeutic targeting. This input has originated partly from pathological studies showing the elevated level of 15-HETE in epiretinal membranes of proliferative vitreoretinal and proliferative DR patients (40). These initial observations have been supported by LC/MS analysis of HETEs in biopsied vitreous samples from diabetic subjects (41), and reinforced in postmortem human retinas by additional pathological studies showing a marked increase in the protein levels of both leukocyte and platelet 12/15-LOX in diabetics compared with non-diabetics (21). Furthermore, it has been demonstrated that 5- or 12-LOX deletion reduced diabetes-induced leukostasis. However, deletion of 5-LOX but not 12-LOX reduced capillary degeneration (42), suggesting that 5-, 12-, and 15-HETEs are each required for different stages of DR. Additionally, we have previously demonstrated in a model of OIR that 12/15-LOX and its products are important regulators of retinal NV through modulation of VEGF and PEDF expression (21). In the present study, we provide evidence for the role of 12/15-LOX pathway in compromising retinal barrier function early during diabetes. Hence, inhibition of 12/15 LOX could potentially benefit the two main hallmark features of DR, i.e., barrier function disruption and pathological NV, which independently can lead to loss of vision among diabetics.

Next, the direct effects of specific 12/15-LOX-derived metabolites, 12- or 15-HETE, on retinal vasculature were demonstrated by two complementary approaches. First, the intravitreal injection of 12-HETE engendered many of the features characteristic of the early DR, including pro-inflammatory response and edema. Second, consistent with previous findings (43), 12- or 15-HETE enhanced several in vitro endothelial cell activities that are relevant to barrier function and angiogenesis, including reduced resistance, adhesion response to PMNs, migration, and tube formation.
Accordingly, the direct relationship between HRECs and eicosanoids has been explored through *in vitro* study. Previously, Cheranov et al. (44) reported that 15-HETE induced migration and tube formation of HRECs through Src-mediated Rac1 activation. Rac1 in non-phagocytic cells is a component of the multi-subunit enzyme, NADPH oxidase, which is composed of the catalytic subunits, NOX2 (formerly known as gp91phox) as well as p22phox; and the cytoplasmic subunits, p47phox and p67phox. Our previous studies using animal and tissue culture models have shown that NOX2 is expressed at low levels in normal retinas and in retinal endothelial cells maintained in control conditions, but is substantially increased in retinal vessels of animals with diabetic or ischemic retinopathy and in retinal endothelial cells exposed to high glucose or hypoxia (23-26). Furthermore, ROS derived from this enzyme stimulate diverse redox signaling pathways leading to angiogenesis-related gene induction as well as endothelial cells migration and proliferation (45). Therefore, in gaining more insight into the mechanisms of eicosanoid-induced angiogenesis, we expanded upon these studies to portray the causal relationship between NADPH oxidase and 12- or 15-HETE. Our *in vitro* studies demonstrated a couple of notable findings: first, the potential angiogenic activity of 12 or 15-HETE in mediating both retinal endothelial cell migration as well as tube formation is regulated in a NADPH oxidase-dependent manner. Second, we broadened the scope to include other prominent features associated with endothelial dysfunction such as the secretion of inflammatory cytokines and PMNs adhesion. 12- or 15-HETE activate retinal endothelial cells toward a pro-inflammatory phenotype characterized by increased release of IL-6, IL-8, and MCP-1, key regulators of leukocyte recruitment, and the subsequent augmentation of leukocyte adherence through a NADPH oxidase-dependent mechanism. Together, our results point out that retinal microvascular changes occurred after 12 or 15-HETE challenge are mediated by NADPH
oxidase activity. Figure 9 illustrates the interplay between these pathways and cross talk with other cellular processes during DR.

While the introduction of anti-VEGF therapies, ranibizumab and aflibercept, has shifted the treatment paradigm for DR from laser photocoagulation in favor of this targeted approach, there are still major unmet needs and gaps in the understanding of underlying biological processes. The potential long-term consequences of intraocular VEGF suppression on the retina have to be taken into account because VEGF is a survival factor for retinal neurons (46). Thereby, targeting a pathway that has crucial roles in compromising the vascular integrity in early DR would provide long-term retinal vascular benefits without affecting neighboring neurons. Our current and previous studies (22) suggest 12/15-LOX as a potential therapeutic target that mediates microvascular dysfunction in DR probably via NADPH oxidase dependent mechanism. Therefore, Inhibition of activated retinal 12/15-LOX system would allow the occurrence of baseline level of VEGF to exert its direct neuroprotective effects and thus may provide a therapeutic strategy in modulating pathological pathways of DR when VEGF level is not correlated with disease severity.
REFERENCES


25


ACKNOWLEDGMENTS

This Publication was made possible by 5R01EY023315 grant from the NIH and NPRP grant # 4-1046-3-284] from the Qatar National Research Fund (a member of Qatar Foundation). This study was also supported in part by National Center for Research Resources, National Institutes of Health Grant S10RR027926. The authors would like thank Dr. Krishna Maddipati for his assistance with the lipid analysis and discussion.
**FIGURE LEGENDS**

**Figure 1.** Characterizing the effect of hyperglycemia on altering the bioactive-lipid profile in human retinal endothelial cells (HRECs) using Liquid chromatography coupled to mass spectrometry (LC/MS). **A)** Fold change in lipidomic profile of HRECs under hyperglycemia, D-glucose (30 mM) for 5-days, compared to normo-osmotic control, D-glucose (5 mM) plus L-glucose (25 mM). **B)** Fold change of upregulated metabolites detected in the previous screen in the presence or absence of exogenous arachidonic acid AA (20 µM) under hyperglycemia compared to the corresponding controls, D-glucose (5 mM) plus L-glucose (25 mM), with or without AA (20 µM). Data shown are the mean ± SD of three independent experiments.

**Figure 2.** Hyperglycemia induced 15-lipoxygenase protein expression as well as phospholipase A2 (PLA2) activity. **A)** HRECs were treated with high glucose, D-Glucose (30 mM), or normo-osmotic control for 5 days. Western blot was performed as described in methods using 15-Lipoxygenase (15-LOX), 5-Lipoxygenase (5-LOX), and actin antibodies followed by densitometric analysis. Ratio of the band intensity of 15-LOX or 5-LOX relative to the actin were reported as fold increase in relation to normo-osmotic control (L-Glucose), which was arbitrarily set at 1.0. **B)** HRECs were treated with 15-HETE or vehicle and the change in the resistance was monitored as described in methods using ECIS. Normalized Transcellular Electrical Resistance (TER) for 15-HETE treatment was compared to vehicle treated endothelial monolayer. **C)** Treatment of HRECs with D-glucose (30 mM) for 5 days stimulated the activity of PLA2 compared to normo-osmotic control. Data shown are the mean ± SD of three independent experiments. **D)** *In vivo* detection of phosphorylated PLA2 (active form) in serial sections from human diabetic or normal retinas around blood vessel regions (yellow arrowhead),
perivascular in the area of glial cells, detected by its marker Glial fibrillary acidic protein (GFAP, white arrows), as well as in the outer segment of the photoreceptors (yellow arrow).

Figure 3. Evolving role of 12/15-lipoxygenase -(LOX) in compromising retinal barrier function during diabetes. A) Fluorescein angiography (FA) of normal wild type (WT), normal 12/15-LOX−/−, diabetic WT, and diabetic 12/15-LOX−/− mice together with their binary images. Data are representative pictures taken at constant interval of every mouse studied in each group. The fluorescence intensity of FA per mouse retina was calculated by the ImageJ software after conversion to binary images then normalized to that of normal WT which was arbitrarily set at 100. B) Western blot of total retinal albumin among the studied groups followed by densitometric analysis. Ratios of albumin band intensities relative to the actin for each group were compared with normal WT control, which was arbitrarily set at 1.0. Data shown for the comparison are the mean ± SD of 4-6 mice studied in each group.

Figure 4. Direct effects of 12/15-lipoxygenase -(LOX) derived metabolites on retinal vasculature. A) Fluorescein angiography (FA) of normal WT mice injected intravitreally with vehicle as a control, or 12-HETE. One week later, FA was performed to evaluate changes of retinal vasculature. The relative fluorescence intensity of FA per mouse retina was calculated by the ImageJ software then normalized as a percentage to that of vehicle-injected control which was arbitrarily set at 100%. B) Western blot of total retinal albumin among the studied groups followed by densitometric analysis. Ratio of the albumin band intensity relative to actin for 12-HETE-injected group was compared to vehicle-injected control, which was arbitrarily set at 1.0. C) Western blot analysis of retinal ICAM-1, VCAM-1, CD45, and NOX2 after intraocular injection with either 12-HETE (0.1 µM), or Vehicle followed by densitometric analysis. Ratios
of band intensities of ICAM-1, VCAM-1, CD45, and NOX2, respectively, relative to the actin for 12-HETE-injected group were compared with vehicle-injected control, which was arbitrarily set at 1.0. Data shown for the comparison are the mean ± SD and representative of 4-6 mice studied in each group.

**Figure 5.** 12- or 15-HETE activates human retinal endothelial cells (HRECs) for leukocyte adhesion in a NADPH oxidase dependent manner. A) Multiplex analysis of cytokine and chemokine production in conditioned media of HRECs treated with vehicle or 12- or 15-HETE (0.1 µM), n=4. B) Reduction of 15-HETE-induced leukocyte adhesion by inhibiting reactive oxygen species (ROS) derived from NADPH oxidase. HRECs were seeded in 24-well plates and treated with 15-HETE (0.1µM, 24 hours) or vehicle, in the presence or absence of apocynin (30 µM) or N-acetyl cysteine (NAC, (50 µM)). Representative photomicrographs for adherent leukocytes among studied groups; vehicle-treated control, LPS as a positive control, 15-HETE, 15-HETE+apocynin, and 15-HETE+ NAC, were taken and quantified for adherent leukocytes. Quantitative data for adherent leukocytes (labelled with red fluorescence dye) were expressed as the mean number of adherent cells per 100 µm ± SD. Numbers represent average of 3 independent experiments.

**Figure 6.** 15-HETE promotes angiogenesis of human retinal endothelial cells (HRECs) through NADPH oxidase. A) Representative photomicrographs for capillary tube formation among studied groups; vehicle-treated control, VEGF as a positive control, 15-HETE, 15-HETE+apocynin, and 15-HETE+N acetyl cysteine (NAC). B) Quantitative data for endothelial cell tube formation expressed as the mean length of formed tube µm ± SD. HRECs were seeded in 96-well plates containing matrigel, and treated with 15-HETE (0.1µM, 24 hours) or vehicle, in
the presence or absence of apocynin (30 µM) or NAC (50 µM). Numbers represent average data from 3 separate experiments.

**Figure 7.** 15-HETE induces human retinal endothelial cell (HREC) migration microvascular via NADPH oxidase dependent pathway. A) Real-time measurement of HRECs migration under 15-HETE treatment in presence of apocynin (Apo., 30 µM) or DMSO using the Electrical Cell Impedance Sensor (ECIS). The migration velocity calculated by dividing the total distance that HRECs moved on the radius of the electrode which is 125-µm divided by the time required for recovering 1 nF capacitance, the confluence point, then normalized as a percentage relative to the migration rate obtained from vehicle-treated cells which was arbitrarily set at 100%. B-D) Inhibition of 15-HETE-induced HREC migration by NOX2 siRNA. Cells were transfected with siRNA or scrambled siRNA for 24 h and then treated with 15-HETE for 15 minutes before wound induction. B) Fluorescent detection of transfection control duplex siRNA (TYE 563 Fluorescently labelled) in living HRECs cells 24 hours post-transfection. C) Measurement of NOX2 expression relative to actin by Western blot after transfection with NOX2 siRNA. D) Real-time measurement of HRECs migration under 15-HETE treatment in presence of NOX2 siRNA or scrambled siRNA using ECIS. Relative migration velocity of 15-HETE treated HRECs in presence or absence of NOX2 siRNA normalized as a percentage to that of vehicle with scrambled siRNA-treated cells, which was arbitrarily set at 100%. Data shown are the mean ± SD of independent three experiments.

**Figure 8.** NADPH Oxidase Mediates the *in vivo* Deleterious Effect of 12-HETE on retinal vasculature. Fluorescein angiography (FA) of normal WT or NOX2(-/-) mice injected intravitreally with 12-HETE. The relative fluorescence intensity of FA per mouse retina
calculated by the ImageJ software and normalized as a percentage to 12-HETE-injected WT mice, arbitrarily set at 100%. Data shown are the mean ± SD and representative of 4-6 mice studied in each group.

**Figure 9. Cascade events involved in the pathogenesis of diabetic retinopathy:**

Hyperglycemia activates the phospholipase A2 to release arachidonic acid from the retinal cells membrane. Arachidonic acid is then converted to 12- or 15-hydroxyeicosatetraenoic acids (HETE) that generates reactive oxygen species through NADPH oxidase, creating a status of oxidative stress. This oxidative stress leads to the activation of retinal endothelial cells through various inflammatory signaling pathways, leading to leukocyte adhesion, hyperpermeability, and ultimately neovascularization (the cardinal signs of diabetic retinopathy).
Table 1. Non-detectable lipid metabolites in the lipidomic profile of HRECs under hyperglycemia.

<table>
<thead>
<tr>
<th>NO.</th>
<th>NO.</th>
<th>Lipid metabolite</th>
<th>NO.</th>
<th>NO.</th>
<th>Lipid metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
<td>9-OxoOTrE</td>
<td>51</td>
<td>51</td>
<td>8,9-EpETrE</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>15d-D12,14-PGJ2</td>
<td>52</td>
<td>52</td>
<td>16(17)-EpDPE</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>5-HEPE</td>
<td>53</td>
<td>53</td>
<td>19(20)-EpDPE</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>12-OxoETE</td>
<td>54</td>
<td>54</td>
<td>D17-PGE1</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>5-oxoETE</td>
<td>55</td>
<td>55</td>
<td>8-isoPGF2a</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>15-OxoETE</td>
<td>56</td>
<td>56</td>
<td>11bPGF2a</td>
</tr>
<tr>
<td>7</td>
<td>32</td>
<td>5,6-EpETrE</td>
<td>57</td>
<td>57</td>
<td>7(8)-EpDPE</td>
</tr>
<tr>
<td>8</td>
<td>33</td>
<td>5(S)-HETrE</td>
<td>58</td>
<td>58</td>
<td>10(11)-EpDPE</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td>15-OxoEDE</td>
<td>59</td>
<td>59</td>
<td>13(14)-EpDPE</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td>15(S)-HEDE</td>
<td>60</td>
<td>60</td>
<td>RvD2</td>
</tr>
<tr>
<td>11</td>
<td>36</td>
<td>tetranor PGEM</td>
<td>61</td>
<td>61</td>
<td>RvD5</td>
</tr>
<tr>
<td>12</td>
<td>37</td>
<td>5,6-DiHETE(EPA)</td>
<td>62</td>
<td>62</td>
<td>RvE3</td>
</tr>
<tr>
<td>13</td>
<td>38</td>
<td>5(S),6(S)-DiHETE</td>
<td>63</td>
<td>63</td>
<td>PGA1</td>
</tr>
<tr>
<td>14</td>
<td>39</td>
<td>5(S),15(S)-DiHETE</td>
<td>64</td>
<td>64</td>
<td>LTC4</td>
</tr>
<tr>
<td>15</td>
<td>40</td>
<td>Bicyclo PGE1</td>
<td>65</td>
<td>65</td>
<td>LTD4</td>
</tr>
<tr>
<td>16</td>
<td>41</td>
<td>5,6-DiHETrE</td>
<td>66</td>
<td>66</td>
<td>LTE4</td>
</tr>
<tr>
<td>17</td>
<td>42</td>
<td>8,9-DiHETrE</td>
<td>67</td>
<td>67</td>
<td>N-acetyl LTE4</td>
</tr>
<tr>
<td>18</td>
<td>43</td>
<td>11dh-2,3-dinor TXB2</td>
<td>68</td>
<td>68</td>
<td>9-HEPE</td>
</tr>
<tr>
<td>19</td>
<td>44</td>
<td>2,3-dinor TXB2</td>
<td>69</td>
<td>69</td>
<td>13,14dh-15k-PGF2a</td>
</tr>
<tr>
<td>20</td>
<td>45</td>
<td>RvE1</td>
<td>70</td>
<td>70</td>
<td>11(R)-HEDE</td>
</tr>
<tr>
<td>21</td>
<td>46</td>
<td>LXA5</td>
<td>71</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>47</td>
<td>15-keto PGE2</td>
<td>72</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>48</td>
<td>PGE3a</td>
<td>73</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>49</td>
<td>20-hydroxy LTB4</td>
<td>74</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>50</td>
<td>LXA4</td>
<td>75</td>
<td>75</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1

- L-Glucose
- D-Glucose

Significance levels:
- p < 0.05
- p < 0.001
- NS (not significant)

Substances analyzed:
- 2-HETE
- 13-HODE
- 16-HDoHE
- 17-HDoHE
- 8, 15-DiHETE
- 13-HDoHE
- 11-HETE
- 15-HETE
- 8-HDoHE

Comparisons:
- L-Glucose vs. D-Glucose
- L-Glucose + AA vs. D-Glucose + AA

Significant differences indicated with p-values.
Fig. 8

WT

NOX2<sup>−/−</sup>

FA 12-HETE

FA 12-HETE

Fluorescence Intensity

WT

NOX2<sup>−/−</sup>

12-HETE

p = 0.0301
Diabetes

PLA2

Arachidonic Acid

12/15-LOX

12- or 15-HETE

NADPH oxidase

Retinal Endothelial Cell Activation

Hyper-permeability

Inflammation and Leukocyte Adhesion

Neovascularization

Diabetic Retinopathy