5-Lipoxygenase, but not 12/15-Lipoxygenase, Contributes to Degeneration of Retinal Capillaries in a Mouse Model of Diabetic Retinopathy

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Running Title: Lipoxygenases and Diabetic Retinopathy

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ABSTRACT

**Objective:** Lipoxygenases are regulators of chronic inflammation and oxidative stress generation. We evaluated the role of 5- and 12-lipoxygenases in the development of diabetic retinopathy.

**Research design and methods:** Wild-type (WT) mice, 5-lipoxygenase-deficient (5-LO) mice, and 12/15-lipoxygenase-deficient (12-LO) mice were assessed 1) after 9 months of diabetes for retinal histopathology and leukotriene receptor expression, and 2) after 3 months of diabetes for leukostasis and retinal superoxide generation.

**Results:** Diabetic WT mice developed the expected degeneration of retinal capillaries and pericytes, and increases in both leukostasis and superoxide production (P<0.006). We found no evidence of diabetes-induced degeneration of retinal ganglion cells in these animals. The vascular histopathology was significantly inhibited in 5-LO-deficient mice, but not in 12-LO-deficient mice. Retinas from diabetic 5-LO deficient mice also had significantly less leukostasis, superoxide production and NF-kappaB expression (all p<0.006), whereas retinas from diabetic 12-LO-deficient mice had significantly less leukostasis (P<0.005) but not superoxide production or NF-kappaB expression. Retinas from diabetic wt mice were enriched with receptors for the 5-lo metabolite leukotriene b4.

**Conclusions:** Diabetes-induced histological and biochemical alterations were significantly reduced in 5-LO-deficient mice, but not 12-LO-deficient mice. 5-LO represents a novel pathway for therapeutic intervention of diabetic retinopathy.
Many recent studies support the hypothesis that inflammatory insults to the retina play an important role in development of the early stages of diabetic retinopathy. (1-4) A hallmark lesion of this early retinopathy is degeneration of retinal capillaries. (5-7) This capillary degeneration is believed to be important because when the capillary degeneration is extensive enough, the retina is believed to become ischemic, ultimately leading to retinal neovascularization. The inflammatory response in early diabetic retinopathy includes diabetes-induced increases in 1) cytokine activation, 2) leukostasis, 3) increased vascular permeability and 4) NF-kappaB regulated expression of pro-inflammatory molecules including iNOS, COX2 and ICAM-1. (4; 8-13) The inflammation might damage retinal capillaries through the generation of reactive oxygen species (ROS) occlusion of vessels by leukostasis, as well as promotion of retinal vascular leakage and induction of endothelial cell death. (1; 14-16)

Prior studies have elucidated that metabolites of arachidonic acid, collectively known as the eicosanoids, are critical in the pathogenesis of chronic inflammatory states such as in asthma, arthritis and colitis. (17-19) 5-lipoxygenase metabolites of arachidonic acid, the leukotrienes, play a role in these inflammatory processes. Generation of leukotrienes starts with the release of arachidonic acid from phospholipids by cytosolic phospholipase A2 (cPLA2). (Figure 1) Arachidonic acid is then metabolized to 5-hydroperoxycicosatetraenoic acid (5-HpETE) and, subsequently, to leukotriene A4 (LTA4) by 5-LO, predominantly present in the white blood cell. Notably, LTA4 has a very short half-life. LTA4 is metabolized to leukotriene B4 (LTB4) by LTA4 hydrolase, and LTB4 can then bind to its specific receptors (BLT1 or BLT2). LTB4 is a known leukocyte attractant, and generation of LTB4 has been linked to ROS generation, cytokine activation, and apoptosis. (20-22) The cysteinyl leukotrienes (LTC4, LTD4 and LTE4) are also formed from LTA4 following conjugation with glutathione by LTC4 synthase to generate LTC4. The cysteinyl leukotrienes, signaling through specific cell surface receptors (CysLT1 and CysLT2), can lead to alterations in vascular contractility and permeability. (23-25) The rapid turnover of the leukotrienes makes detection of these molecules difficult in tissues such as the retina, which require time for isolation. Metabolites of 5-LO have not been implemented previously in complications of diabetes.

While synthesis of leukotrienes can occur entirely in the white blood cell, recent studies support transcellular synthesis of leukotrienes. (26; 27) Specifically, LTA4 synthesized in the white blood cell can pass transcellularly and be further metabolized by different target cells. Depending on the enzymatic machinery of the target cell, local, tissue-specific generation of leukotrienes can occur.

Leukocyte type12-lipoxygenase has been implicated in the pathogenesis of diabetic macrovascular complications. (28) The major product of 12-lipoxygenase metabolism of arachidonic acid is 12S-hydroxy-5,8,10,14-eicosatetraenoic acid (12S-HETE). (Figure 1) 12S-HETE has a role in various biological processes including LDL oxidation in atherogenesis, cancer cell growth, and neuronal apoptosis following oxidative stress. (28-31) 12S-HETE has pro-inflammatory effects such as chemotaxis and regulation of leukocyte adherence. (32; 33)

In this study, we investigated whether lipoxygenases had a role in the development of diabetic retinopathy. Diabetes was induced in WT mice and mice deficient in either 5-LO or 12-LO, and the retinas were analyzed 1) histologically for capillary degeneration and leukocyte adherence, 2) biochemically for reactive oxygen species generation, and 3) immunologically for expression of NF-kappaB and leukotriene receptor.

METHODS

**Animals.** Both 5-LO and 12-LO deficient mice were previously generated on a mixed background and backcrossed to C57BL/6. A breeding pair of 5-LO deficient mice (B6.129S2-Alox5tm1Fun/J) and WT C57BL/6 mice (control mice per Jackson Laboratories) were purchased from Jackson Laboratories.
Breeding pairs for 12-LO deficient mice and the appropriate WT control mice were provided by Dr. Jerry Nadler. (34) When the mice were 20-25 g body weight (approximately 2 months of age), they were randomly assigned to become diabetic or remain as non-diabetic. All animal experiments were in accordance with the guidelines for Treatment of Animals in Research outlined by the Association for Research in Vision and Ophthalmology. Diabetes was induced by 5 sequential daily intraperitoneal injections of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 45 mg/kg of body weight. Insulin was given as needed to achieve slow weight gain without preventing hyperglycemia and glucosuria (typically 0-0.2 units of NPH insulin subcutaneously, 0-3 times per week). The animals remained insulin-deficient but not grossly catabolic. The animals had free access to both food and water and were maintained under a 14h on/ 10h off light cycle. Food consumption and body weight were measured weekly. Glycated hemoglobin was measured every 2-3 months to estimate the average level of hyperglycemia. (Variant kit, Bio-Rad, Hercules, CA). Specifically, gene deletions had no effect on the hyperglycemia or the health of the animals. Retinas were harvested at 3 months of diabetes duration for leukostasis and superoxide measurement, and at 9 months of diabetes duration for retinal histopathology.

**Isolation of retinal blood vessels.** Retinal vasculatures were isolated as described by us previously. (13; 35; 36) Briefly, freshly isolated eyes were fixed with 10% neutral buffered formalin. Following dissection, retinas were rinsed in water overnight and then incubated with 3% Difco crude trypsin (BD Biosciences, Sparks, MD) containing 0.2 M NaF at 37°C for 1 h. After trypsin digestion, nonvascular cells were removed by gentle brushing, and the isolated vasculature was dried to a microscope slide, stained with hematoxylin and periodic acid-schiff, and analyzed for pathology.

**Quantitation of acellular capillaries.** Acellular capillaries were quantitated in eight fields in the mid-retina (200x magnification) in a masked manner. Acellular capillaries were identified as capillary-sized vessel tubes having no nuclei anywhere along their length and were reported per square millimeter of retinal area. Tubes with a diameter <20% of the diameter of adjacent capillaries or length less than 40 micrometers were identified as strands and not counted as acellular capillaries. The number of acellular capillaries can vary by batch of animals requiring strict use of genetically-matched control animals.

**Quantitation of pericyte ghosts.** Pericyte ghosts were quantitated in eight fields in the mid-retina (400x magnification) in a masked manner. Pericyte ghosts were identified as spaces in the capillary basement membranes from which pericytes had disappeared. Approximately 1,000 capillary cells in eight field areas in the mid-retina were evaluated in a masked manner, and the number of pericyte ghosts was reported per 1,000 capillary cells. Ghosts on any acellular capillary were excluded.

**Quantitation of cells in the ganglion cell layer.** Cells in the ganglion cell layer were quantitated by light microscopy in formalin-fixed paraffin retinal sections stained with hematoxylin and eosin. Sections through all eyes were cut tangentially through the pupil and optic nerve area. Pictures were taken of the ganglion cell layer (GCL; both sides of the optic nerve) at 400x magnification. The nuclei in the GCL (not including nuclei of blood vessels in that layer) were counted and expressed per 100 micrometers linear length of retina.

**Quantitative measurement of leukostasis.** The number of leukocytes adherent to the microvasculature was determined at 3 months of diabetes. Following cardiac catheterization, anesthetized mice (100 mg/ml Ketaset:100 mg/ml Xylazine = 5:1) were exanguinated by perfusion with PBS. Fluorescein-coupled concanavalin A lectin (20 µg/ml in PBS) (Vector Laboratories, Burlingame, CA) was then infused as previously described. (1; 13) Flat-mounted retinas were viewed via fluorescence microscopy, and brightly fluorescent leukocytes were counted in the entire retina.

**Superoxide measurement.** Fresh retinas from animals were analyzed for superoxide production as previously described. (10) Briefly, retinas were placed in 0.2 ml Krebs/HEPES buffer and allowed to
equilibrate in the dark at 37°C under 95% O₂/5% CO₂ conditions for 30 min. To each tube, 0.5 mM lucigenin (Sigma Chemical Company, St. Louis, MO) was added and the photon emission was detected for 10s by a luminometer (Analytical Luminescence Laboratory, San Diego, CA). Retinal protein was quantified per sample (Bio-Rad, Herculean, CA) and the luminescence was expressed per milligram protein.

**Western blot analysis.** Mouse retinas were sonicated in RIPA buffer (25 mmol/l Tris, pH 7.4, 1 mmol/l EDTA, 150 mmol/l NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 1 mmol/l phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml aprotinin). Whole retinal lysate protein content was quantified. (Bio-Rad, Herculean, CA) Equivalent amounts of sample proteins were loaded, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The blots were probed with primary antibodies for leukotriene B4 receptors (BLT1 (Cayman Chemicals, Ann Arbor, MI) and BLT2 (Santa Cruz Biotechnology, Santa Cruz, CA)) and the species-specific secondary antibody. After extensive washing, protein bands were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA) and evaluated by densitometry. Membranes then were stripped and reprobed with antibody against tubulin (Sigma, St. Louis, MO) to confirm equal protein loading.

**Immunohistochemistry of retinal slices.** The paraffin embedded sections were deparaffinized using 3 changes of xylene. The tissue sections were then subjected to an antigen retrieving protocol in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) by microwaving for 15 minutes (3 times, 5 minutes each). The tissue endogenous peroxidases were quenched using 3.0% hydrogen peroxide for 10 minutes, and non-specific binding sites were blocked using 1.5% normal goat serum for 20 minutes (Vector Laboratories, Burlingame, CA). The tissue sections were then incubated overnight with rabbit polyclonal antibody (1:100 in PBS) for NF-kappaB p65 (Santa Cruz Biotechnology Inc Santa Cruz, CA). Unbound primary antibody was washed off using PBS containing 0.05% Tween 20. Biotinylated secondary anti-rabbit antibody (1:200 dilutions for 30 min, Vector Laboratories, Burlingame, CA) and ABC reagent (30 min at RT, Vector Laboratories, Burlingame, CA) were applied to the sections. DAB substrates with nickel enhancer were used to stain the sections. Processing time was identical among experimental groups. Sections were washed, counterstained with nuclear fast red, dehydrated and permanently mounted using Permount solution. Sections were scored based on the intensity of the nuclear staining on a scale of 1 to 4 with 1 (lightest gray), 2 (light gray), 3 (medium gray) and 4 (black).

**Statistical analysis.** All results are expressed as means ± SD. Due to modest group sizes, the data were analyzed by the nonparametric Kruskal-Wallis followed by the Mann-Whitney test. Differences were considered statistically significant when the P values were <0.05.

**RESULTS**

**Animals.** The degree of hyperglycemia, as denoted by glycated hemoglobin, did not vary among diabetic groups. (Glycohemoglobins: 5-LO-deficient diabetic mice, 11.8 ± 1.9% vs. WT diabetic mice, 12.8 ± 1.4% ; 12-LO-deficient diabetic mice, 11.2 ± 1.4% vs. WT diabetic mice, 12.9 ± 0.8%)

**Retinas from 5-LO deficient mice, but not 12-LO deficient mice, are protected from diabetes-induced capillary degeneration.** WT mice diabetic for 9 months demonstrated a significant increase in the number of degenerate acellular capillaries compared to non-diabetic WT mice. (P<0.005, Figure 2, A, B and C) Capillary degeneration was significantly inhibited in diabetic 5-LO-deficient mice, whereas diabetic 12-LO-deficient mice were not protected from the vascular degeneration. (P<0.006, Figure 2, A and B) Similarly, an increase in the degeneration of retinal pericytes was seen in diabetic WT mice compared to non-diabetic WT mice. (P<0.005, Table 1 and Figure 2, C) Diabetic 5-LO-deficient mice were protected from significant pericyte loss, whereas the number of pericyte ghosts in diabetic 12-LO-
deficient mice was greater than in non-diabetic 12-LO-deficient mice, and was similar to diabetic WT mice. (P<0.01, Table 1) Ganglion cell counts were not different among non-diabetic and diabetic animals in any groups (WT, 5-LO-deficient or 12-LO-deficient). (Table 2)

**Inhibition of leukocyte adherence in lipoxygenase deficient mice.** Leukocyte adherence to the vascular wall was quantified following perfusion with fluorescently labeled concanavalin A. The number of leukocytes in the microvasculature of the diabetic WT mouse retina was significantly increased compared to the non-diabetic WT mouse. (P<0.005, Figure 3, A and B) In contrast, retinas from mice with 5-LO or 12-LO deficiency were protected from diabetes-induced increase in leukocyte adherence. (Figure 3, A and B)

**Suppression of superoxide generation in 5-LO deficient mice.** Oxidative stress in the diabetic retina was evaluated by measuring superoxide generation. In WT mice, diabetes caused a nearly two-fold increase in superoxide production. (P<0.006, Figure 4, A and B) Retinas from 5-LO-deficient mice did not show the diabetes-induced increase in superoxide generation (Figure 4, A), whereas retinas from 12-LO-deficient mice did generate increased levels of superoxide in diabetes. (P<0.01, Figure 4, B)

**Suppression of NF-kappaB expression in diabetic 5-LO deficient mice.** We examined the expression of the p65 subunit of NF-kappaB by immunohistochemical analysis of paraffin-embedded sections of mouse retina. Retinas from diabetic WT mice demonstrated a three-fold increase in expression of NF-kappaB in nuclei of cells in the GCL compared to retinas from non-diabetic WT mice. (Figure 5, Staining scores as described in Methods: 3.6 ± 0.5 for diabetic WT mice vs. 1.2 ± 0.4 for non-diabetic WT mice, P<0.005) Likewise, retinas from diabetic 12-LO deficient mice demonstrated an increase in NF-kappaB expression in the GCL. (Figure 5, Staining score: 3.3 ± 0.5, P<0.005 compared to non-diabetic mice) Retinas from diabetic 5-LO deficient mice did not express NF-kappaB in the GCL. (Figure 5, Grading score: 1.4 ± 0.5)

**Increased expression of LTB4 receptors in the diabetic mouse retina.** Since our experiments suggested a selective role for 5-LO in the pathogenesis of diabetic retinopathy, we examined the retina for receptors of LTB4, the 5-LO metabolite critical for leukocyte recruitment. Whole retinal lysates were probed for BLT1 receptors. Increased expression of BLT1 receptors were noted in retinas from diabetic WT mice compared to non-diabetic WT mice. (P<0.005, Figure 6)

**DISCUSSION**

Early in diabetic retinopathy, markers of inflammation such as cytokine activation, increased adherence of leukocytes to the vessel wall and increased expression of COX2 and other pro-inflammatory molecules are detected. (1-3) Moreover, interventions to block these abnormalities have inhibited the development of vascular lesions of diabetic retinopathy in animals. (2; 35; 37) These findings support the hypothesis that inflammation plays a causative role in development of diabetic retinopathy. The possible participation of lipoxygenase metabolites in the development of the retinopathy was unknown, but their role in other chronic inflammatory conditions prompted our investigations. In this present study, we demonstrate a selective role for 5-LO in the pathogenesis of the capillary degeneration that is characteristic of diabetic retinopathy. Deficiency of 5-LO, but not 12-LO, inhibited the diabetes-induced degeneration of retinal capillaries. Degeneration of retinal capillaries in diabetes is largely considered to be a cardinal event in the pathogenesis of diabetic retinopathy. (5-7)

The generation of superoxide and oxidative stress in the retina under diabetic conditions previously has been correlated to the pathologic development of acellular capillaries by us and other investigators. (15; 38) Our present results are consistent with this association. Superoxide production was inhibited in retinas from diabetic mice in whom capillary degeneration was
inhibited (i.e., 5-LO-deficient mice), and not inhibited in retinas from diabetic mice in whom capillary degeneration was not inhibited (i.e., 12-LO-deficient mice). Thus, 5-LO apparently contributes to the diabetes-induced increase in retinal superoxide. Multiple cellular sources can generate superoxide including mitochondria, NADPH oxidase, xanthine oxidase, cytochrome P450 and uncoupled eNOS. (39) Recent reports suggest that mitochondrial superoxide generation is important in the induction of important biochemical abnormalities of hyperglycemia (40) and in the pathogenesis of diabetic retinopathy. (38) Given the predominant expression of 5-LO in the white blood cell, the inhibition of retinal superoxide generation in 5-LO-deficient animals suggests that the white blood cells within retinal vessels contribute to the retinal superoxide generation in diabetes either directly or indirectly in a paracrine manner. In macrovascular disease, it is hypothesized leukocyte-generated superoxide via NADPH oxidase can have effects on endothelial cell–generated superoxide. (39) Indeed, 5-LO activation has been associated with LTB4-mediated stimulation of leukocyte NADPH oxidase. (41) Reduced activation of NADPH oxidase may account for reduced superoxide generation in our 5-LO-deficient mice. Accordingly, the persistent generation of superoxide in the diabetic 12-LO-deficient mice might be due to the fact that they can still produce 5-LO metabolites. 5-LO has been noted to be expressed in some non-myeloid cell types (42; 43), and transcellular synthesis of leukotrienes can occur (26; 27), so, it remains to be studied how retinal cells participate in superoxide generation.

While superoxide generation and acellular capillary formation were selectively inhibited only in the diabetic 5-LO-deficient mice, leukocyte adherence was inhibited in both 5-LO-deficient and 12-LO-deficient diabetic mice. Both 5-LO and 12-LO metabolites have been shown to increase ICAM-1 expression (17; 32), and ICAM-1 expression is increased in diabetic retinopathy. Decreased ICAM-1 expression by 5-LO–deficient and 12-LO-deficient animals may explain the observed decrease in leukocyte adherence in the knockouts. We cannot exclude a possibility that leukostasis does increase eventually in 12-LO-deficient mice, but our data suggests that alterations in leukostasis early in diabetes are not sufficient to predict the development of long-term retinopathy.

The increased expression of the LTB4 receptor, BLT1, in the mouse retina under diabetic conditions is consistent with a retinal response to LTB4. Post-receptor signaling through BLT1 has been previously linked to activation of NF-kappaB. (44; 45) Importantly, NF-kappaB has been implicated in capillary degeneration in diabetic retinopathy. Diabetes induces NF-KappaB activation in multiple cells throughout the retina, including neuronal and vascular cells. (4; 13) The detection of the p65 subunit of NF-kappaB in retinal cells of diabetic WT animals and diabetic 12-LO deficient animals but not in diabetic 5-LO deficient animals suggests that 1) 5-lipoxygenase metabolites participate in NF-kappaB activation in the diabetic retina and 2) that although the absence of 12-lipoxygenase prevents leukostasis, it does not alter other parameters of the inflammatory response, such as NF-kappaB activation.

Interestingly, recent reports demonstrate that 5-LO can generate also anti-inflammatory metabolites of docosahexaneoic acid, the resolvins, and that these metabolites can signal through the BLT1 receptor. (44) One might propose a “competition” among pro-inflammatory and anti-inflammatory mediators at this receptor. In addition, importance of 5-LO and its metabolites in human disease has been broadened with the recent associations of polymorphisms in the gene for FLAP with susceptibility to stroke. (25; 46; 47) This calls further attention to the 5-LO cascade as regulators of inflammation as well as critical links between pathologic chronic inflammation and genetic risk.
Although 12-LO apparently does not play a key role in degeneration of retinal capillaries in early diabetic retinopathy, other investigations implicate 12-LO in the pathogenesis of diabetes-induced macrovascular endothelial cell dysfunction and atherosclerosis, as well as microvascular complications such as nephropathy and peripheral neuropathy. (28; 48; 49) These observations underscore that the pathogenesis of diabetes-related complications is likely tissue-specific.

At this time, the protective effect of 5-LO deficiency on early diabetic retinopathy in our mouse model suggests a role for the pro-inflammatory metabolites of 5-LO in the pathogenesis of diabetic retinopathy. This novel role for 5-LO in the pathogenesis of early diabetic retinopathy affords the potential for development and application of new drugs for the prevention and treatment of diabetic retinopathy.

ACKNOWLEDGEMENTS

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**TABLE 1.**

<table>
<thead>
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<th>Pericyte Ghost Counts</th>
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<td></td>
<td>(pericyte ghosts/1000 cells)</td>
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<tr>
<td>WT</td>
<td>Non-diabetic</td>
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<td>11.0 ± 2.0</td>
<td>22.0 ± 2.5*</td>
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<tr>
<td>5-LO-deficient</td>
<td>12.3 ± 3.1</td>
<td>14.8 ± 3.0</td>
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<td>12-LO-deficient</td>
<td>13.2 ± 2.4</td>
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Data represents 6-8 animals per experimental group.

*P < 0.005 compared to non-diabetic WT
†P < 0.01 compared to non-diabetic WT or 12-LO-deficient
<table>
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<tr>
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<th>Cell Counts in the Ganglion Cell Layer</th>
<th>(cells/100 um retinal length)</th>
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<tr>
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<tr>
<td>WT</td>
<td>10.8 ± 1.8</td>
<td>11.5 ± 1.9</td>
</tr>
<tr>
<td>5-LO deficient</td>
<td>10.0 ± 2.3</td>
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</tr>
<tr>
<td>12-LO deficient</td>
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Data represents 6-8 animals per experimental group.
FIGURE LEGENDS

FIGURE 1. Synthesis of lipoygenase metabolites. Synthesis of lipoygenase metabolites begins with hydrolysis of arachidonic acid (AA) from phospholipids by cPLA₂. 12-LO then converts the released AA to 12S-HETE. Alternatively, 5-LO interacts with 5-lipoxygenase activating protein (FLAP) leading to the conversion of AA to LTA₄. LTA₄ can be further metabolized either by LTA₄ hydrolase to generate LTB₄ or by LTC₄ synthase to produce LTC₄. LTC₄ can subsequently be metabolized to the other cysteinyl leukotrienes, LTD₄ and LTE₄.

FIGURE 2. Inhibition of diabetes-induced acellular capillary formation by 5-LO deficiency. A: WT diabetic mice (D) demonstrated an increase in the number of acellular capillaries compared to WT, non-diabetic mice (N) (* P<0.005), whereas, diabetic 5-LO-deficient mice (D-5-LO) were protected from the diabetes-induced increase in acellular capillary formation, despite similar degrees of hyperglycemia over the 9-month diabetes duration. B: Long-term experiments in WT and 12-LO-deficient mice, demonstrated a statistically significant increase in acellular capillary formation in both the diabetic WT mice (D) and in the diabetic 12-LO-deficient mice (D-12-LO). (* P < 0.008 and †P<0.006 vs. non-diabetic mice) Results represent 6-8 retinas per group.

C: Early diabetic lesions in the mouse retinal microvasculature are visualized following trypsin digestion as described in Methods. Representative acellular capillaries (open arrow) and pericyte ghosts (solid arrow) are depicted.

FIGURE 3. Leukostasis is diminished in 5-LO-deficient mice and 12-LO-deficient mice. A: WT diabetic mice (D) had a significantly increased number of adherent leukocytes compared to non-diabetic WT mice (N). (*P<0.005). The number adherent leukocytes in diabetic 5-LO-deficient mice (D-5-LO) was not increased and was indistinguishable from non-diabetic 5-LO-deficient mice (N-5-LO). B: As with 5-LO-deficient mice, diabetic 12-LO-deficient mice (D-12-LO) likewise did not demonstrate a diabetes-induced rise in the number of adherent leukocytes. (*P<0.005) Data represents 6-8 animals per group.

FIGURE 4. Retinas from 5-LO-deficient mice were protected from diabetes-induced increases in superoxide generation. At 3 months of diabetes duration, measurement of superoxide generation from freshly-isolated retinas was performed as described in Methods. A: Retinas from diabetic WT mice (D) generated significantly more superoxide than non-diabetic WT mice (N). (*P<0.006) However, this enhanced generation of superoxide production was not seen in diabetic 5-LO-deficient mice (D-5-LO). B: Retinas from both diabetic wild-type mice (D) and diabetic 12-LO-deficient mice (D-12-LO) generate increased amounts of superoxide when compared to non-diabetic WT mice (N) and non-diabetic 12-LO-deficient mice (N-12-LO), respectively. (*P and †P <0.01) Data represents 6-8 freshly isolated retinas per group.

FIGURE 5. Inhibition of diabetes-induced NF-kappaB expression by 5-LO deficiency. Sections of mouse retina were analyzed for expression of NF-kappaB using immunohistochemistry as described in Methods. Increased expression of NF-kappaB in the ganglion cell layer (GCL) was detected in the diabetic WT retina (D, WT) compared to non-diabetic WT retina (N, WT) especially in nuclei. Diabetic 5-LO deficient retina (D, 5-LO), but not 12-LO deficient retina (D, 12-LO), inhibited the diabetes-induced increase in NF-kappaB expression. Sections are
representative of the results from 4 - 6 retinas per group. (IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer)

**FIGURE 6.** Expression of BLT1, an LTB4 receptor, is increased in wild-type diabetic mice. Western blot analysis of isolated retinas demonstrated an increase in expression of the LTB4 receptor, BLT1, in retinas from diabetic WT mice (D; lanes 3 and 4) compared to non-diabetic WT mice (N; lanes 1 and 2). (*P< 0.005) Immunoblots are representative of the results from 6-8 retinas per group. Tubulin expression was used for relative comparison by densitometry as described in Methods.

**NOTE:** The figures for this article can be found using the link entitled “Figures”. (Available at http://dx.doi.org/10.2337/db07-1217.)