

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

Rose A. Gubitosi-Klug,<sup>1</sup> Ramaprasad Talahalli,<sup>1</sup> Yunpeng Du,<sup>2</sup> Jerry L. Nadler,<sup>3</sup> and Timothy S. Kern<sup>2,4</sup>

**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 mice, 5-lipoxygenase-deficient mice, and 12/15-lipoxygenase-deficient 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 wild-type 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-lipoxygenase-deficient mice, but not in 12/15-lipoxygenase-deficient mice. Retinas from diabetic 5-lipoxygenase-deficient mice also had significantly less leukostasis, superoxide production, and nuclear factor- $\kappa$ B (NF- $\kappa$ B) expression (all  $P < 0.006$ ), whereas retinas from diabetic 12/15-lipoxygenase-deficient mice had significantly less leukostasis ( $P < 0.005$ ) but not superoxide production or NF- $\kappa$ B expression. Retinas from diabetic wild-type mice were enriched with receptors for the 5-lipoxygenase metabolite leukotriene B<sub>4</sub>. Diabetes-induced histological and biochemical alterations were significantly reduced in 5-lipoxygenase-deficient mice, but not 12/15-lipoxygenase-deficient mice.

**CONCLUSIONS**—5-Lipoxygenase represents a novel pathway for therapeutic intervention of diabetic retinopathy. *Diabetes* 57:1387–1393, 2008

From the <sup>1</sup>Department of Pediatrics, Case Western Reserve University/Rainbow Babies and Children's Hospital, Cleveland, Ohio; the <sup>2</sup>Department of Medicine, Case Western Reserve University, Cleveland, Ohio; the <sup>3</sup>Department of Internal Medicine, University of Virginia, Charlottesville, Virginia; and the <sup>4</sup>Veterans Administration Medical Center Research Service 151, Cleveland, Ohio.

Corresponding author: Rose Gubitosi-Klug, MD, PhD, Division of Pediatric Endocrinology and Metabolism, Case Medical Center, 11100 Euclid Ave., Rainbow Babies and Children's Hospital, Room 737, Cleveland, OH 44106. E-mail: rose.gubitosi-klug@case.edu.

Received for publication 29 August 2007 and accepted in revised form 20 February 2008.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 17 March 2008. DOI: 10.2337/db07-1217.

ICAM, intracellular adhesion molecule; LT, leukotriene; NF- $\kappa$ B, nuclear factor- $\kappa$ B; 12S-HETE, 12S-hydroxy-5,8,10,14-eicosatetraenoic acid.

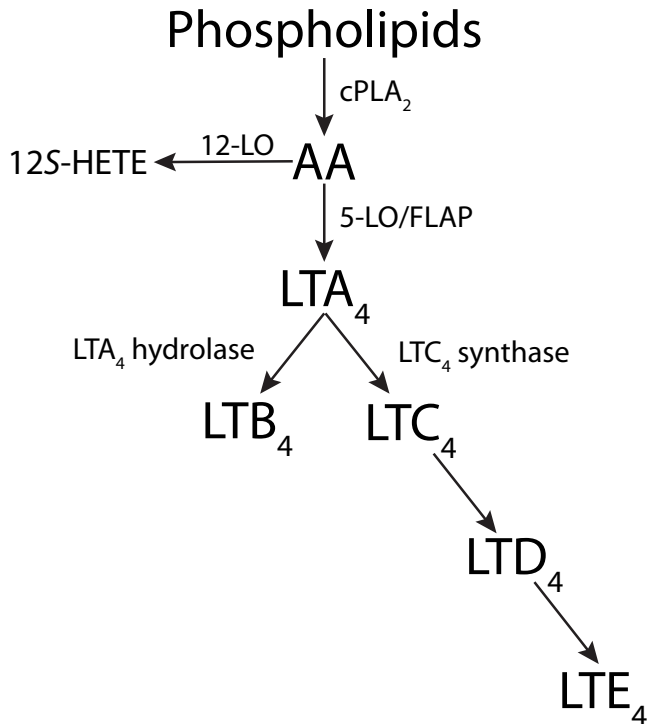
© 2008 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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) vascular permeability, and 4) nuclear factor- $\kappa$ B (NF- $\kappa$ B)-regulated expression of proinflammatory molecules, including inducible NO (nitric oxide) synthase, cyclooxygenase-2, and intracellular adhesion molecule-1 (ICAM-1) (4,8–13). The inflammation might damage retinal capillaries through the generation of reactive oxygen species, occlusion of vessels by leukostasis, 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 cPLA<sub>2</sub> (cytosolic phospholipase A<sub>2</sub>) (Fig. 1). Arachidonic acid is then metabolized to 5-HpETE (5-hydroperoxyeicosatetraenoic acid) and, subsequently, to leukotriene A<sub>4</sub> by 5-lipoxygenase, predominantly present in white blood cells. Notably, leukotriene A<sub>4</sub> has a very short half-life. Leukotriene A<sub>4</sub> is metabolized to leukotriene B<sub>4</sub> by leukotriene A<sub>4</sub> hydrolase, and leukotriene B<sub>4</sub> can then bind to its specific receptors (BLT1 or BLT2). Leukotriene B<sub>4</sub> is a known leukocyte attractant, and generation of leukotriene B<sub>4</sub> has been linked to reactive oxygen species generation, cytokine activation, and apoptosis (20–22). The cysteinyl leukotrienes (leukotrienes C<sub>4</sub>, D<sub>4</sub>, and E<sub>4</sub>) are also formed from leukotriene A<sub>4</sub> after conjugation with glutathione by leukotriene C<sub>4</sub> synthase to generate leukotriene C<sub>4</sub>. 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-lipoxygenase have not been implemented previously in complications of diabetes.

Although synthesis of leukotrienes can occur entirely in



**FIG. 1.** Synthesis of lipoxygenase metabolites. Synthesis of lipoxygenase metabolites begins with hydrolysis of arachidonic acid (AA) from phospholipids by cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>). Then, 12/15-lipoxygenase (12-LO) converts the released arachidonic acid to 12S-HETE. Alternatively, 5-lipoxygenase (5-LO) interacts with 5-lipoxygenase activating protein (FLAP), leading to the conversion of arachidonic acid to leukotriene (LT) A<sub>4</sub>. Leukotriene A<sub>4</sub> can be further metabolized either by leukotriene A<sub>4</sub> hydrolase to generate leukotriene B<sub>4</sub> or by leukotriene C<sub>4</sub> synthase to produce leukotriene C<sub>4</sub>. Leukotriene C<sub>4</sub> can subsequently be metabolized to the other cysteinyl leukotrienes, leukotriene D<sub>4</sub> and E<sub>4</sub>.

the white blood cell, recent studies support transcellular synthesis of leukotrienes (26,27). Specifically, leukotriene A<sub>4</sub> 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 type 12-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) (Fig. 1). 12S-HETE has a role in various biological processes, including LDL oxidation in atherosclerosis, cancer cell growth, and neuronal apoptosis after oxidative stress (28–31). 12S-HETE has proinflammatory 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 wild-type mice and mice deficient in either 5-lipoxygenase or 12/15-lipoxygenase, 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- $\kappa$ B and leukotriene receptor.

## RESEARCH DESIGN AND METHODS

Both 5-lipoxygenase- and 12/15-lipoxygenase-deficient mice were previously generated on a mixed background and backcrossed to C57BL/6. A breeding pair of 5-lipoxygenase-deficient mice (B6.129S2-Alox<sup>5<sup>tm</sup>1Fnn</sup>/J) and wild-type

C57BL/6 mice (control mice per Jackson Laboratories) were purchased from Jackson Laboratories. Breeding pairs for 12/15-lipoxygenase-deficient mice and the appropriate wild-type control mice were provided by J.N. (34). When the mice were 20–25 g body weight (~2 months of age), they were randomly assigned to become diabetic or remain nondiabetic. 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 five sequential daily intraperitoneal injections of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 45 mg/kg body wt. 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 14 h on/10 h 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' diabetes duration for leukostasis and superoxide measurement, and at 9 months' 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. After dissection, retinas were rinsed in water overnight and then incubated with 3% Difco crude trypsin (BD Biosciences, Sparks, MD) containing 0.2 mol/l 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 (200 $\times$  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 <40  $\mu$ m 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.

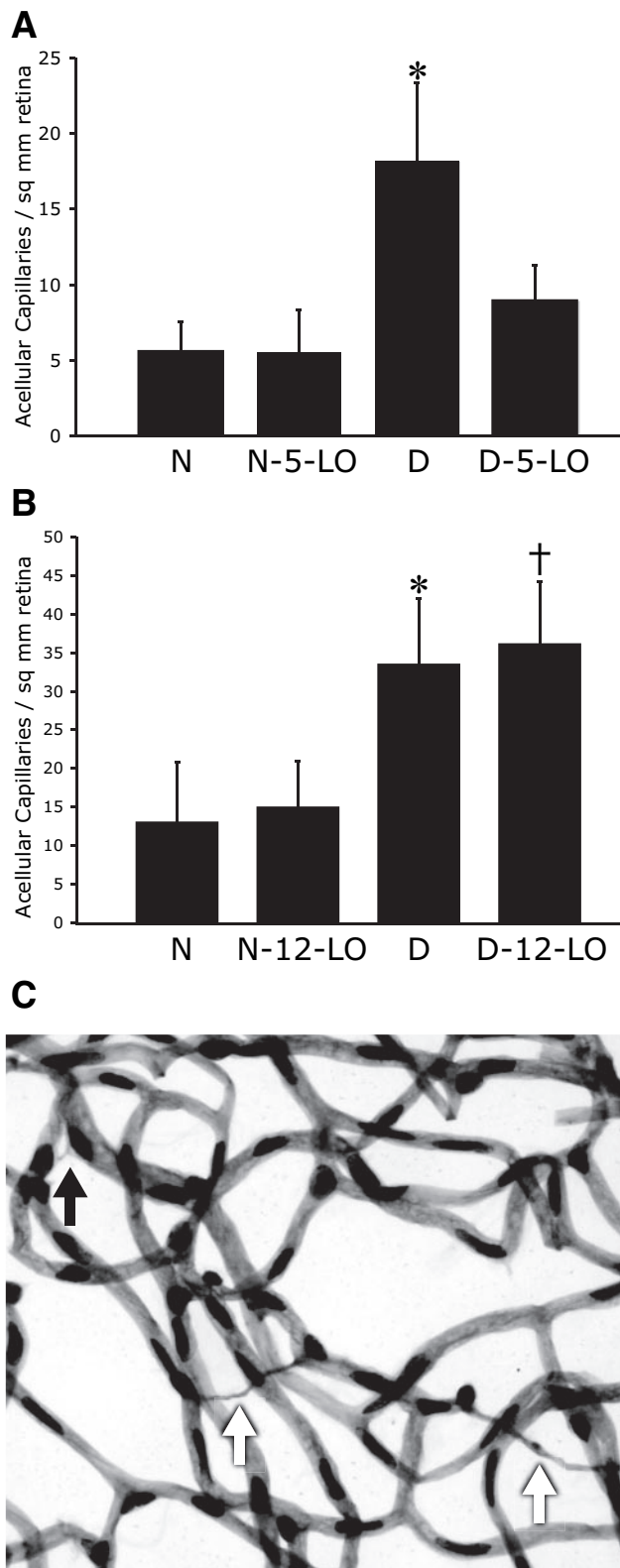
**Quantitation of pericyte ghosts.** Pericyte ghosts were quantitated in eight fields in the mid-retina (400 $\times$  magnification) in a masked manner. Pericyte ghosts were identified as spaces in the capillary basement membranes from which pericytes had disappeared. We evaluated ~1,000 capillary cells in eight field areas in the mid-retina in a masked manner, and we reported the number of pericyte ghosts 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 (both sides of the optic nerve) at 400 $\times$  magnification. The nuclei in the ganglion cell layer (not including nuclei of blood vessels in that layer) were counted and expressed per 100  $\mu$ m linear length of retina.

**Quantitative measurement of leukostasis.** The number of leukocytes adherent to the microvasculature was determined at 3 months of diabetes. After cardiac catheterization, anesthetized mice (100 mg/ml ketaset-to-100 mg/ml xylazine ratio of 5:1) were exsanguinated by perfusion with PBS. Fluorescein-coupled concanavalin A lectin (20  $\mu$ g/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% O<sub>2</sub>/5% CO<sub>2</sub> conditions for 30 min. To each tube, 0.5 mmol/l 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) and the luminescence was expressed per milligram protein.

**Western blot analysis.** Mouse retinas were sonicated in radioimmunoprecipitation assay 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  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml aprotinin). Whole retinal lysate protein content was quantified (Bio-Rad). 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 B<sub>4</sub> receptors BLT1 (Cayman Chemicals, Ann Arbor, MI) and BLT2 (Santa Cruz Biotechnology) and the species-specific secondary antibody. After extensive washing, protein bands were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology) and evaluated by densitometry.



**FIG. 2.** Inhibition of diabetes-induced acellular capillary formation by 5-lipoxygenase (5-LO) deficiency. **A:** Wild-type diabetic (D) mice demonstrated an increase in the number of acellular capillaries compared with wild-type nondiabetic (N) mice ( $*P < 0.005$ ), whereas diabetic 5-lipoxygenase-deficient (D-5-LO) mice 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 wild-type and 12/15-lipoxygenase-deficient mice demonstrated a statistically significant increase in acellular capillary formation in both the diabetic wild-type (D) mice and in the

**TABLE 1**  
Pericyte ghost counts

	Nondiabetic	Diabetic
Wild type (pericyte ghosts/1,000 cells)	11.0 ± 2.0	22.0 ± 2.5*
5-Lipoxygenase-deficient (pericyte ghosts/1,000 cells)	12.3 ± 3.1	14.8 ± 3.0
12-Lipoxygenase-deficient (pericyte ghosts/1,000 cells)	13.2 ± 2.4	18.2 ± 2.4†

Data represents six to eight animals per experimental group.  $*P < 0.005$  compared with nondiabetic wild type mice;  $†P < 0.01$  compared to non-diabetic wild-type or 12-lipoxygenase-deficient mice.

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 three changes of xylene. The tissue sections were then subjected to an antigen-retrieving protocol in sodium citrate buffer (10 mmol/l sodium citrate, 0.05% Tween 20, pH 6.0) by microwaving for 15 min (three times, 5 min each). The tissue endogenous peroxides were quenched using 3.0% hydrogen peroxide for 10 min, and nonspecific binding sites were blocked using 1.5% normal goat serum for 20 min (Vector Laboratories). The tissue sections were then incubated overnight with rabbit polyclonal antibody (1:100 in PBS) for NF- $\kappa$ B p65 (Santa Cruz Biotechnology). 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) and ABC reagent (30 min at room temperature; Vector Laboratories) were applied to the sections. DAB (3,3'-diaminobenzidine) 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).

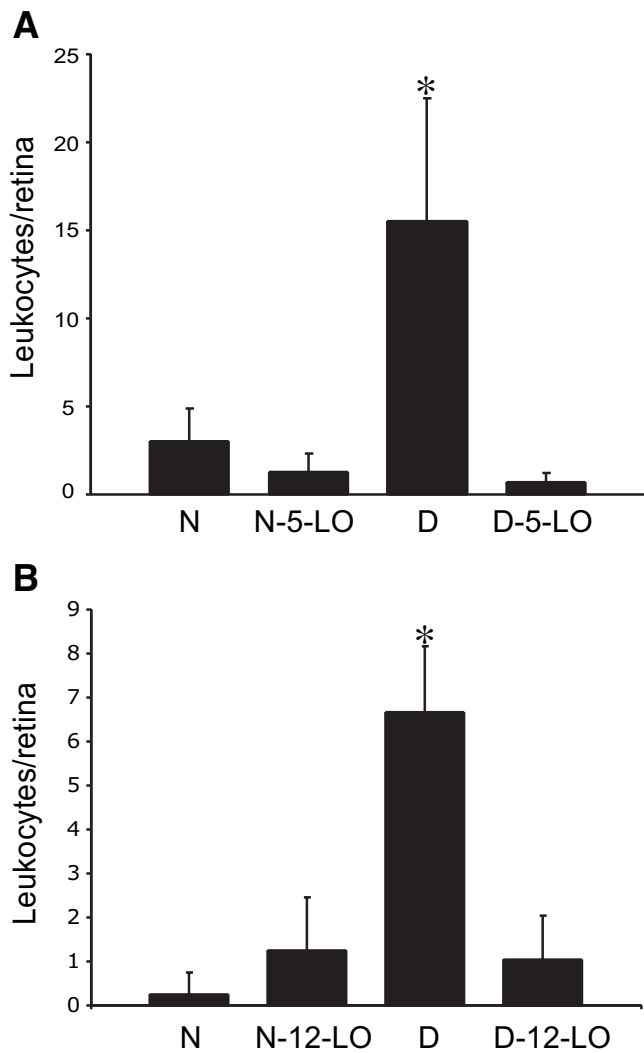
**Statistical analysis.** All results are expressed as the means ± SD. Because of modest group sizes, the data were analyzed by the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U* test. Differences were considered statistically significant when the *P* values were  $< 0.05$ .

## RESULTS

**Animals.** The degree of hyperglycemia, as denoted by glycated hemoglobin, did not vary among diabetic groups (glycohemoglobins:  $11.8 \pm 1.9$  vs.  $12.8 \pm 1.4\%$  for 5-lipoxygenase-deficient diabetic vs. wild-type diabetic mice;  $11.2 \pm 1.4$  vs.  $12.9 \pm 0.8\%$  for 12/15-lipoxygenase-deficient diabetic vs. wild-type diabetic mice).

**Retinas from 5-lipoxygenase-deficient mice, but not 12/15-lipoxygenase-deficient mice, are protected from diabetes-induced capillary degeneration.** Wild-type mice diabetic for 9 months demonstrated a significant increase in the number of degenerate acellular capillaries compared with nondiabetic wild-type mice ( $P < 0.005$ ) (Fig. 2A–C). Capillary degeneration was significantly inhibited in diabetic 5-lipoxygenase-deficient mice, whereas diabetic 12/15-lipoxygenase-deficient mice were not protected from the vascular degeneration ( $P < 0.006$ ) (Fig. 2A and B). Similarly, an increase in the degeneration of retinal pericytes was seen in diabetic wild-type compared with nondiabetic wild-type mice ( $P < 0.005$ ) (Table 1 and Fig. 2C). Diabetic 5-lipoxygenase-deficient mice were protected from significant pericyte loss, whereas the number

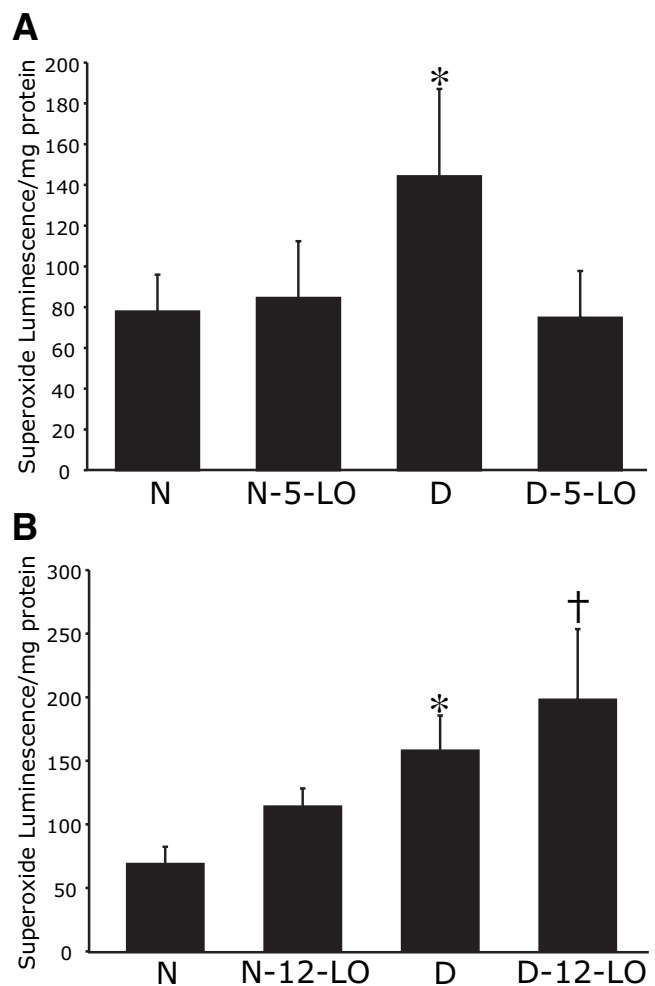
diabetic 12/15-lipoxygenase-deficient (D-12-LO) mice ( $*P < 0.008$  and  $†P < 0.006$  vs. nondiabetic mice). Results represent 6–8 retinas per group. **C:** Early diabetic lesions in the mouse retinal microvasculature are visualized after trypsin digestion as described in RESEARCH DESIGN AND METHODS. Representative acellular capillaries (open arrows) and pericyte ghosts (solid arrow) are depicted.



**FIG. 3.** Leukostasis is diminished in 5-lipoxygenase- and 12/15-lipoxygenase-deficient mice. **A:** Wild-type diabetic (D) mice had a significantly increased number of adherent leukocytes compared with nondiabetic wild-type (N) mice ( $*P < 0.005$ ). The number adherent leukocytes in diabetic 5-lipoxygenase-deficient (D-5-LO) mice was not increased and was indistinguishable from nondiabetic 5-lipoxygenase-deficient (N-5-LO) mice. **B:** As with 5-lipoxygenase-deficient mice, diabetic 12/15-lipoxygenase-deficient (D-12-LO) mice likewise did not demonstrate a diabetes-induced rise in the number of adherent leukocytes ( $*P < 0.005$ ). Data represent six to eight animals per group.

of pericyte ghosts in diabetic 12/15-lipoxygenase-deficient mice was greater than in nondiabetic 12/15-lipoxygenase-deficient mice, and it was similar to diabetic wild-type mice ( $P < 0.01$ ) (Table 1). Ganglion cell counts were not different among nondiabetic and diabetic animals in any group (wild-type, 5-lipoxygenase-deficient, or 12/15-lipoxygenase-deficient) (Table 2).

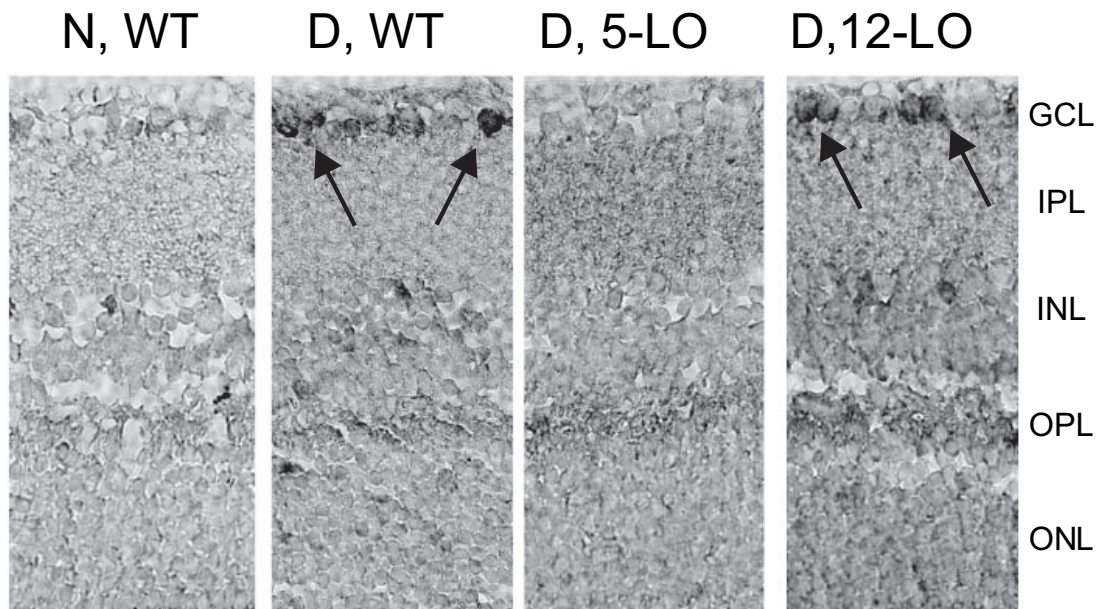
**Inhibition of leukocyte adherence in lipoxygenase-deficient mice.** Leukocyte adherence to the vascular wall was quantified after perfusion with fluorescently labeled concanavalin A. The number of leukocytes in the microvasculature of the diabetic wild-type mouse retina was significantly increased compared with the nondiabetic wild-type mouse retina ( $P < 0.005$ ) (Fig. 3A and B). In contrast, retinas from mice with 5-lipoxygenase or 12/15-lipoxygenase deficiency were protected from diabetes-induced increase in leukocyte adherence (Fig. 3A and B).



**FIG. 4.** Retinas from 5-lipoxygenase-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 RESEARCH DESIGN AND METHODS. **A:** Retinas from diabetic wild-type (D) mice generated significantly more superoxide than nondiabetic wild-type (N) mice ( $*P < 0.006$ ). However, this enhanced generation of superoxide production was not seen in diabetic 5-lipoxygenase-deficient (D-5-LO) mice. **B:** Retinas from both diabetic wild-type (D) mice and diabetic 12/15-lipoxygenase-deficient (D-12-LO) mice generated increased amounts of superoxide when compared with nondiabetic wild-type mice (N) and nondiabetic 12/15-lipoxygenase-deficient (N-12-LO) mice, respectively ( $*P$  and  $†P < 0.01$ ). Data represents six to eight freshly isolated retinas per group.

**Suppression of superoxide generation in 5-lipoxygenase-deficient mice.** Oxidative stress in the diabetic retina was evaluated by measuring superoxide generation. In wild-type mice, diabetes caused a nearly twofold increase in superoxide production ( $P < 0.006$ ) (Fig. 4A and B). Retinas from 5-lipoxygenase-deficient mice did not show the diabetes-induced increase in superoxide generation (Fig. 4A), whereas retinas from 12/15-lipoxygenase-deficient mice did generate increased levels of superoxide in diabetes ( $P < 0.01$ ) (Fig. 4B).

**Suppression of NF- $\kappa$ B expression in diabetic 5-lipoxygenase-deficient mice.** We examined the expression of the p65 subunit of NF- $\kappa$ B by immunohistochemical analysis of paraffin-embedded sections of mouse retina. Retinas from diabetic wild-type mice demonstrated a threefold increase in expression of NF- $\kappa$ B in nuclei of cells in the ganglion cell layer compared with retinas from



**FIG. 5.** Inhibition of diabetes-induced NF- $\kappa$ B expression by 5-lipoxygenase deficiency. Sections of mouse retina were analyzed for expression of NF- $\kappa$ B using immunohistochemistry as described in RESEARCH DESIGN AND METHODS. Increased expression of NF- $\kappa$ B in the ganglion cell layer (GCL) was detected in the diabetic wild-type retina (D, WT) compared with nondiabetic wild-type retina (N, WT), especially in nuclei. Diabetic 5-lipoxygenase-deficient retina (D, 5-LO), but not 12/15-lipoxygenase-deficient retina (D, 12-LO), inhibited the diabetes-induced increase in NF- $\kappa$ B expression. Sections are representative of the results from four to six retinas per group. INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

nondiabetic wild-type mice (staining scores as described in RESEARCH DESIGN AND METHODS:  $3.6 \pm 0.5$  vs.  $1.2 \pm 0.4$  for diabetic wild-type vs. nondiabetic wild-type mice,  $P < 0.005$ ) (Fig. 5). Likewise, retinas from diabetic 12/15-lipoxygenase-deficient mice demonstrated an increase in NF- $\kappa$ B expression in the ganglion cell layer (staining score:  $3.3 \pm 0.5$ ,  $P < 0.005$  compared with nondiabetic mice) (Fig. 5). Retinas from diabetic 5-lipoxygenase-deficient mice did not express NF- $\kappa$ B in the ganglion cell layer (grading score:  $1.4 \pm 0.5$ ) (Fig. 5).

**Increased expression of leukotriene B<sub>4</sub> receptors in the diabetic mouse retina.** Because our experiments suggested a selective role for 5-lipoxygenase in the pathogenesis of diabetic retinopathy, we examined the retina for receptors of leukotriene B<sub>4</sub>, the 5-lipoxygenase metabolite critical for leukocyte recruitment. Whole retinal lysates were probed for BLT1 receptors. Increased expression of BLT1 receptors were noted in retinas from diabetic wild-type mice compared with nondiabetic wild-type mice ( $P < 0.005$ ) (Fig. 6).

## DISCUSSION

Early in diabetic retinopathy, markers of inflammation (e.g., cytokine activation, increased adherence of leukocytes to the vessel wall, and increased expression of cyclooxygenase-2 and other proinflammatory 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 retinopathy was unknown, but their role in other chronic inflammatory conditions prompted our investigations. In this study, we demonstrate a selective role for 5-lipoxygenase in the pathogenesis of the capillary degeneration that is characteristic of diabetic retinopathy.

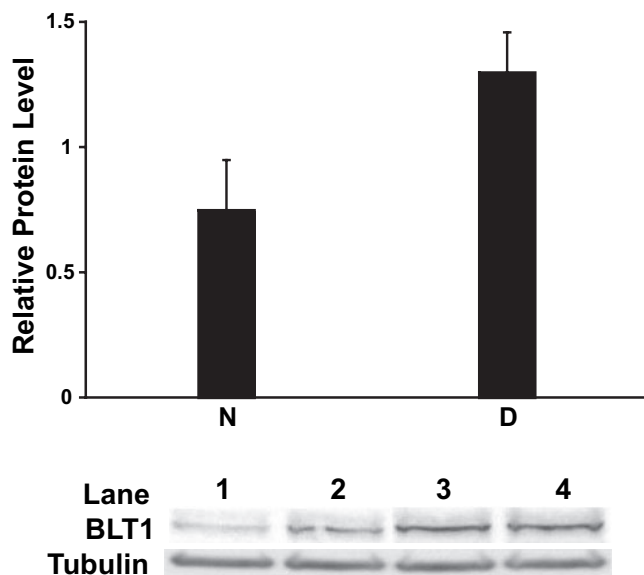
Deficiency of 5-lipoxygenase, but not 12/15-lipoxygenase, inhibited the diabetes-induced degeneration of retinal capillaries. Degeneration of retinal capillaries in diabetes is largely considered to be a cardinal event in the pathogenesis of diabetic retinopathy (5–7).

The generation of superoxide and oxidative stress in the retina under diabetic conditions has been previously correlated to the pathological development of acellular capillaries by us and other investigators (15,38). Our current results are consistent with this association. Superoxide production was inhibited in retinas from diabetic mice in whom capillary degeneration was inhibited (i.e., 5-lipoxygenase-deficient mice), and not inhibited in retinas from diabetic mice in whom capillary degeneration was not inhibited (i.e., 12/15-lipoxygenase-deficient mice). Thus, 5-lipoxygenase 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 endothelial NO synthase (39). Recent reports suggest that mitochondrial superoxide generation is important in the induction of biochemical abnormalities of hyperglycemia (40) and in the pathogenesis of diabetic retinopathy (38). Given the predominant expression of 5-lipoxygenase in the white blood cell, the inhibition of

**TABLE 2**  
Cell counts in the ganglion cell layer

	Nondiabetic	Diabetic
Wild type (cells/100 $\mu$ m retinal length)	$10.8 \pm 1.8$	$11.5 \pm 1.9$
5-Lipoxygenase-deficient (cells/100 $\mu$ m retinal length)	$10.0 \pm 2.3$	$9.9 \pm 1.6$
12-Lipoxygenase-deficient (cells/100 $\mu$ m retinal length)	$8.7 \pm 0.9$	$9.4 \pm 1.5$

Data represents six to eight animals per experimental group.



**FIG. 6.** Expression of BLT1, an leukotriene B<sub>4</sub> receptor, is increased in wild-type diabetic mice. Western blot analysis of isolated retinas demonstrated an increase in expression of the leukotriene B<sub>4</sub> receptor BLT1 in retinas from diabetic wild-type (D) mice (lanes 3 and 4) compared with nondiabetic wild-type (N) mice (lanes 1 and 2) (\**P* < 0.005). Immunoblots are representative of the results from six to eight retinas per group. Tubulin expression was used for relative comparison by densitometry as described in RESEARCH DESIGN AND METHODS.

retinal superoxide generation in 5-lipoxygenase-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-lipoxygenase activation has been associated with leukotriene B<sub>4</sub>-mediated stimulation of leukocyte NADPH oxidase (41). Reduced activation of NADPH oxidase may account for reduced superoxide generation in our 5-lipoxygenase-deficient mice. Accordingly, the persistent generation of superoxide in the diabetic 12/15-lipoxygenase-deficient mice might be due to the fact that they can still produce 5-lipoxygenase metabolites. 5-Lipoxygenase has been noted to be expressed in some nonmyeloid cell types (42,43), and transcellular synthesis of leukotrienes can occur (26,27); therefore, it remains to be studied how retinal cells participate in superoxide generation.

Whereas superoxide generation and acellular capillary formation were selectively inhibited only in the diabetic 5-lipoxygenase-deficient mice, leukocyte adherence was inhibited in both 5- and 12/15-lipoxygenase-deficient diabetic mice. Both 5- and 12/15-lipoxygenase 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- and 12/15-lipoxygenase-deficient animals may explain the observed decrease in leukocyte adherence in the knockouts. We cannot exclude a possibility that leukostasis does increase eventually in 12/15-lipoxygenase-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 leukotriene B<sub>4</sub> receptor, BLT1, in the mouse retina under diabetic conditions is

consistent with a retinal response to leukotriene B<sub>4</sub>. Postreceptor signaling through BLT1 has been previously linked to activation of NF- $\kappa$ B (44,45). Importantly, NF- $\kappa$ B has been implicated in capillary degeneration in diabetic retinopathy. Diabetes induces NF- $\kappa$ B activation in multiple cells throughout the retina, including neuronal and vascular cells (4,13). The detection of the p65 subunit of NF- $\kappa$ B in retinal cells of diabetic wild-type animals and diabetic 12/15-lipoxygenase-deficient animals but not in diabetic 5-lipoxygenase-deficient animals suggests that 1) 5-lipoxygenase metabolites participate in NF- $\kappa$ B 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- $\kappa$ B activation.

Interestingly, recent reports demonstrate that 5-lipoxygenase can also generate anti-inflammatory metabolites of docosahexaenoic acid, the resolvins, and that these metabolites can signal through BLT1 (44). One might propose a "competition" among pro- and anti-inflammatory mediators at this receptor. In addition, the importance of 5-lipoxygenase and its metabolites in human disease has been broadened with the recent associations of polymorphisms in the gene for FLAP (5-lipoxygenase activating protein) with susceptibility to stroke (25,46,47). This calls further attention to the 5-lipoxygenase cascade as regulators of inflammation as well as critical links between pathological chronic inflammation and genetic risk.

Although 12/15-lipoxygenase apparently does not play a key role in the degeneration of retinal capillaries in early diabetic retinopathy, other investigations implicate 12/15-lipoxygenase 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-lipoxygenase deficiency on early diabetic retinopathy in our mouse model suggests a role for the proinflammatory metabolites of 5-lipoxygenase in the pathogenesis of diabetic retinopathy. This novel role for 5-lipoxygenase 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.

#### ACKNOWLEDGMENTS

This work was supported by grants to R.A.G.K. from the National Eye Institute (K08EY16833) and the Endocrine Fellows Foundation, to T.S.K. from the Medical Research Service of the Department of Veteran Affairs and National Institutes of Health (R01EY00300), and to J.L.N. from the National Institutes of Health (HL PO1 55798). Histology service was provided by the Case Western Reserve University Visual Science Research Center Core Facilities (P30EY11373).

These data were presented in part at the Association for Research in Vision and Ophthalmology, Fort Lauderdale, Florida, May 2005; the 66th Scientific Sessions of the American Diabetes Association, Washington, D.C., June 2006; and the 67th Scientific Sessions of the American Diabetes Association, Chicago, Illinois, June 2007.

#### REFERENCES

- Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, Schraermeyer U, Kociok N, Fauser S, Kirchhof B, Kern TS, Adamis AP: A central

- role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB J* 18:1450–1452, 2004
2. Kern TS, Miller CM, Du Y, Zheng L, Mohr S, Ball SL, Kim M, Jamison JA, Bingaman DP: Topical administration of nepafenac inhibits diabetes-induced retinal microvascular disease and underlying abnormalities of retinal metabolism and physiology. *Diabetes* 56:373–379, 2007
  3. Mohr S: Potential new strategies to prevent the development of diabetic retinopathy. *Expert Opin Investig Drugs* 13:189–198, 2004
  4. Zheng L, Howell SJ, Hatala DA, Huang K, Kern TS: Salicylate-based anti-inflammatory drugs inhibit the early lesion of diabetic retinopathy. *Diabetes* 56:337–345, 2007
  5. Aguilar E, Friedlander M, Gariano RF: Endothelial proliferation in diabetic retinal microaneurysms. *Arch Ophthalmol* 121:740–741, 2003
  6. Cogan DG, Kuwabara T: The mural cell in perspective. *Arch Ophthalmol* 78:133–139, 1967
  7. Engerman RL: Pathogenesis of diabetic retinopathy. *Diabetes* 38:1203–1206, 1989
  8. Antonetti DA, Barber AJ, Khin S, Lieth E, Tarbell JM, Gardner TW: Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. Penn State Retina Research Group. *Diabetes* 47:1953–1959, 1998
  9. Antonetti DA, Lieth E, Barber AJ, Gardner TW: Molecular mechanisms of vascular permeability in diabetic retinopathy. *Semin Ophthalmol* 14:240–248, 1999
  10. Du Y, Miller CM, Kern TS: Hyperglycemia increases mitochondrial superoxide in retina and retinal cells. *Free Radic Biol Med* 35:1491–1499, 2003
  11. Gardner TW, Antonetti DA, Barber AJ, LaNoue KF, Nakamura M: New insights into the pathophysiology of diabetic retinopathy: potential cell-specific therapeutic targets. *Diabetes Technol Ther* 2:601–608, 2000
  12. Kowluru RA: Retinal metabolic abnormalities in diabetic mouse: comparison with diabetic rat. *Curr Eye Res* 24:123–128, 2002
  13. Zheng L, Szabo C, Kern TS: Poly(ADP-ribose) polymerase is involved in the development of diabetic retinopathy via regulation of nuclear factor-kappaB. *Diabetes* 53:2960–2967, 2004
  14. Jousen AM, Murata T, Tsujikawa A, Kirchhof B, Bursell SE, Adamis AP: Leukocyte-mediated endothelial cell injury and death in the diabetic retina. *Am J Pathol* 158:147–152, 2001
  15. Kowluru RA, Chan PS: Oxidative stress and diabetic retinopathy [Review]. *Exp Diabetes Res* 2007:43603, 2007
  16. Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T, Clermont AC, Aiello LP, Ogura Y, Adamis AP: Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. *Proc Natl Acad Sci U S A* 96:10836–10841, 1999
  17. Cuzzocrea S, Rossi A, Mazzon E, Di Paola R, Genovese T, Muia C, Caputi AP, Sautebin L: 5-Lipoxygenase modulates colitis through the regulation of adhesion molecule expression and neutrophil migration. *Lab Invest* 85: 808–822, 2005
  18. Rubin P, Mollison KW: Pharmacotherapy of diseases mediated by 5-lipoxygenase pathway eicosanoids. *Prostaglandins Other Lipid Mediat* 83:188–197, 2007
  19. Sharma JN, Mohammed LA: The role of leukotrienes in the pathophysiology of inflammatory disorders: is there a case for revisiting leukotrienes as therapeutic targets? *Inflammopharmacology* 14:10–16, 2006
  20. Islam SA, Thomas SY, Hess C, Medoff BD, Means TK, Brander C, Lilly CM, Tager AM, Luster AD: The leukotriene B4 lipid chemoattractant receptor BLT1 defines antigen-primed T cells in humans. *Blood* 107:444–453, 2006
  21. Lundeen KA, Sun B, Karlsson L, Fourie AM: Leukotriene B4 receptors BLT1 and BLT2: expression and function in human and murine mast cells. *J Immunol* 177:3439–3447, 2006
  22. Pompeia C, Freitas JJ, Kim JS, Zyngier SB, Curi R: Arachidonic acid cytotoxicity in leukocytes: implications of oxidative stress and eicosanoid synthesis. *Biol Cell* 94:251–265, 2002
  23. Austen KF: The mast cell and the cysteinyl leukotrienes. *Novartis Found Symp* 271:166–175 [discussion 176–178, 198–199], 2005
  24. Letts LG: Leukotrienes: role in cardiovascular physiology. *Cardiovasc Clin* 18:101–113, 1987
  25. Osher E, Weisinger G, Limor R, Tordjman K, Stern N: The 5 lipoxygenase system in the vasculature: emerging role in health and disease. *Mol Cell Endocrinol* 252:201–206, 2006
  26. Folco G, Murphy RC: Eicosanoid transcellular biosynthesis: from cell-cell interactions to in vivo tissue responses. *Pharmacol Rev* 58:375–388, 2006
  27. Gijon MA, Zarini S, Murphy RC: Biosynthesis of eicosanoids and transcellular metabolism of leukotrienes in murine bone marrow cells. *J Lipid Res* 48:716–725, 2007
  28. Natarajan R, Gerrity RG, Gu JL, Lanting L, Thomas L, Nadler JL: Role of 12-lipoxygenase and oxidant stress in hyperglycaemia-induced acceleration of atherosclerosis in a diabetic pig model. *Diabetologia* 45:125–133, 2002
  29. Bolick DT, Srinivasan S, Whetzel A, Fuller LC, Hedrick CC: 12/15 lipoxygenase mediates monocyte adhesion to aortic endothelium in apolipoprotein E-deficient mice through activation of RhoA and NF-kappaB. *Arterioscler Thromb Vasc Biol* 26:1260–1266, 2006
  30. Jankun J, Aleem AM, Malgorzewicz S, Szkudlarek M, Zawadzky MI, Dewitt DL, Feig M, Selman SH, Skrzypczak-Jankun E: Synthetic curcuminoids modulate the arachidonic acid metabolism of human platelet 12-lipoxygenase and reduce sprout formation of human endothelial cells. *Mol Cancer Ther* 5:1371–1382, 2006
  31. Lebeau A, Terro F, Rostene W, Pelaprat D: Blockade of 12-lipoxygenase expression protects cortical neurons from apoptosis induced by beta-amyloid peptide. *Cell Death Differ* 11:875–884, 2004
  32. Bolick DT, Orr AW, Whetzel A, Srinivasan S, Hatley ME, Schwartz MA, Hedrick CC: 12/15-lipoxygenase regulates intercellular adhesion molecule-1 expression and monocyte adhesion to endothelium through activation of RhoA and nuclear factor-kappaB. *Arterioscler Thromb Vasc Biol* 25:2301–2307, 2005
  33. Li SL, Dwarakanath RS, Cai Q, Lanting L, Natarajan R: Effects of silencing leukocyte-type 12/15-lipoxygenase using short interfering RNAs. *J Lipid Res* 46:220–229, 2005
  34. Bleich D, Chen S, Zipser B, Sun D, Funk CD, Nadler JL: Resistance to type 1 diabetes induction in 12-lipoxygenase knockout mice. *J Clin Invest* 103:1431–1436, 1999
  35. Kern TS, Engerman RL: Pharmacological inhibition of diabetic retinopathy: aminoguanidine and aspirin. *Diabetes* 50:1636–1642, 2001
  36. Tang J, Mohr S, Du YD, Kern TS: Non-uniform distribution of lesions and biochemical abnormalities within the retina of diabetic humans. *Curr Eye Res* 27:7–13, 2003
  37. Mustafa GT, Rosca M, Biemel KM, Reihl O, Smith MA, Viswanathan A, Strauch C, Du Y, Tang J, Kern TS, Lederer MO, Brownlee M, Weiss MF, Monnier VM: Paradoxical effects of green tea (*Camellia sinensis*) and antioxidant vitamins in diabetic rats: improved retinopathy and renal mitochondrial defects but deterioration of collagen matrix glycoxidation and cross-linking. *Diabetes* 54:517–526, 2005
  38. Kanwar M, Chan PS, Kern TS, Kowluru RA: Oxidative damage in the retinal mitochondria of diabetic mice: possible protection by superoxide dismutase. *Invest Ophthalmol Vis Sci* 48:3805–3811, 2007
  39. Li JM, Shah AM: Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol* 287:R1014–R1030, 2004
  40. Brownlee M: The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54:1615–1625, 2005
  41. Serezani CH, Aronoff DM, Jancar S, Peters-Golden M: Leukotriene B4 mediates p47phox phosphorylation and membrane translocation in polyunsaturated fatty acid-stimulated neutrophils. *J Leukoc Biol* 78:976–984, 2005
  42. James AJ, Penrose JF, Cazaly AM, Holgate ST, Sampson AP: Human bronchial fibroblasts express the 5-lipoxygenase pathway. *Respir Res* 7:102, 2006
  43. Zhang YY, Walker JL, Huang A, Keane JF, Clish CB, Serhan CN, Loscalzo J: Expression of 5-lipoxygenase in pulmonary artery endothelial cells. *Biochem J* 361:267–276, 2002
  44. Arita M, Ohira T, Sun YP, Elangovan S, Chiang N, Serhan CN: Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation. *J Immunol* 178:3912–3917, 2007
  45. Back M, Bu DX, Branstrom R, Sheikine Y, Yan ZQ, Hansson GK: Leukotriene B4 signaling through NF-kappaB-dependent BLT1 receptors on vascular smooth muscle cells in atherosclerosis and intimal hyperplasia. *Proc Natl Acad Sci U S A* 102:17501–17506, 2005
  46. Kaushal R, Pal P, Alwell K, Haverbusch M, Flaherty M, Moomaw C, Sekar P, Kissela B, Kleindorfer D, Chakraborty R, Broderick J, Deka R, Woo D: Association of ALOX5AP with ischemic stroke: a population-based case-control study. *Hum Genet* 121:601–607, 2007
  47. Lohmussaar E, Gschwendtner A, Mueller JC, Org T, Wichmann E, Hamann G, Meitinger T, Dichgans M: ALOX5AP gene and the PDE4D gene in a central European population of stroke patients. *Stroke* 36:731–736, 2005
  48. Kang SW, Adler SG, Nast CC, LaPage J, Gu JL, Nadler JL, Natarajan R: 12-lipoxygenase is increased in glucose-stimulated mesangial cells and in experimental diabetic nephropathy. *Kidney Int* 59:1354–1362, 2001
  49. Obrosova IG, Inytska O, Lyzogubov VV, Pavlov IA, Mashtalir N, Nadler JL, Drel VR: High-fat diet induced neuropathy of pre-diabetes and obesity: effects of "healthy" diet and aldose reductase inhibition. *Diabetes* 56:2598–2608, 2007