Increased Leukotriene B_4 Synthesis in Immune Injured Rat Glomeruli

Mohamed A. Rahman, M. Nakazawa, Steven N. Emancipator, and Michael J. Dunn
Department of Medicine, Hines Veterans Administration Hospital and Loyola Medical Center, Hines, Illinois and Maywood, Illinois 60141; and Department of Medicine and Institute of Pathology Case Western Reserve University, and Division of Nephrology, University Hospitals of Cleveland, Cleveland, Ohio 44106

Abstract

We examined glomerular synthesis of the 5-lipoxygenase metabolite, LTB_4, in normal and immune-injured rat glomeruli. Glomeruli isolated from normal rats and from rats with nephrotoxic serum nephritis (NSN), passive Heymann nephritis (PHN) and cationic bovine gamma globulin (CBGG)-induced glomerulonephritis were incubated with the calcium ionophore A23187 (3 μM). Lipids in the glomeruli and media were extracted with ethyl acetate, and were purified and fractionated by HPLC. Immunoreactive-LTB_4 (i-LTB_4) was determined by radioimmunoassay on HPLC fractions with a detection limit of 50 pg of i-LTB_4. A large peak of i-LTB_4 that comigrated with authentic LTB_4 was found exclusively in glomeruli isolated from the CBGG-injected rats. Addition of the lipoxygenase inhibitor BW755C (50 μg/ml) to glomerular incubation resulted in > 90% inhibition of i-LTB_4. Synthesis of i-LTB_4 by glomeruli from normal, NSN and PHN rats was undetectable. Glomerular LTB_4 synthesis by CBGG-injected rats was confirmed by radiometric HPLC and by gas chromatography mass-spectroscopy (GC-MS) analysis.

In order to rule out synthesis of LTB_4 by neutrophils entrapped in the glomeruli, a group of rats received 1,000 rad total body x irradiation, with shielding of the kidneys before induction of CBGG glomerulonephritis. Despite > 95% reduction in total leukocyte count, glomerular synthesis of LTB_4 remained enhanced. Augmented glomerular synthesis of the proinflammatory lipid, LTB_4, in the CBGG model of glomerular disease could have an important role in the development of glomerular injury and proteinuria.

Introduction

Several lipoxygenase products of arachidonic acid are synthesized in the kidney, especially in glomeruli. Murine and human glomeruli have lipoxygenase activity and metabolize arachidonic acid to 12-hydroxyeicosa tetraenoic acid (12-HETE) and 12- and 15-HETE, respectively (1, 2). Enhanced glomerular synthesis of 12-HETE has been demonstrated in rats with antibody-mediated glomerular injury, nephrotoxic serum nephritis (NSN)[1] (3). This enhancement of enzymatic activity could play a role in the development of glomerular disease, since 12-HETE has a proinflammatory effect on neutrophils.

In recent years much interest has focused on the 5-lipoxygenase pathway of oxidative arachidonic acid metabolism. This pathway produces 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is converted to several leukotrienes. Leukotriene C_4 (LTC_4) and leukotriene D_4 (LTD_4) were previously known as slow reacting substance of anaphylaxis (4, 5). Leukotriene B_4 (LTB_4) is produced by stimulated inflammatory cells, mainly neutrophils and macrophages (6), and is present in perfusate obtained from antigen challenged lung (7). LTB_4 is a powerful chemotactic and chemokinetic agent for granulocytes, stimulates the generation of reactive oxygen radicals in neutrophils and induces leukocyte adhesion to the endothelial surface (8–10). When injected intradermally with PGE_2, LTB_4 increased permeability of dermal vessels to a degree dependent on its dose, although LTB_4 alone had no effect on capillary permeability (11–14). Therefore LTB_4 is considered to be a major mediator of inflammation, including enhanced vascular permeability, and could play a part in the pathogenesis of glomerulonephritis.

The aims of the present study were: first, to examine the ability of normal glomerular cells to metabolize arachidonic acid through the 5-lipoxygenase pathway and synthesize LTB_4; second, to examine glomerular synthesis of LTB_4 in infiltrative and noninfiltrative models of immune glomerular disease. The infiltrative model we examined was the heterologous phase of NSN, which is characterized by heavy glomerular infiltration with neutrophils. Two noninfiltrative, nonproliferative models were examined; passive Heymann nephritis (PHN) and cationic bovine gamma globulin (CBGG)-induced glomerulonephritis, both pathologically similar to human membranous nephropathy.

Methods

Materials. Bovine gamma globulin (BGG), and fluoresceinated goat antisera to BGG, rat IgG, and rat complement C3 were obtained from U. S. Biochemicals, Cleveland, OH; 1-ethyl-3-[(3-dimethylaminopropyl-carbodiimide hydrochloride] (EDC) and complete Freund's adjuvant were from Sigma Chemical Co., St. Louis, MO, and ethylene diamine was from Fisher Scientific, Pittsburgh, PA. Frozen rat kidneys were obtained from Pel-Freeze Biological, Inc., Rogers, AR. Indomethacin was from Merck Sharp and Dohme, West Point, PA. LTB_4 standards were kindly provided by Dr. J. Gleason, SmithKline & French, Philadelphia, PA, and anti-LTB_4 antiserum was graciously provided by Dr. A. Rosenthal of Merck Sharp and Dohme. [^{3}H]-Leukotrienes B_4, C_4, and D_4, and [^{3}H]arachidonic acid were from New England Nuclear (NEN), Boston, MA. BW755C was generously provided by Dr. S. Moncada, The Wellcome Research Laboratory, Kent, England.

Preparation of anti-rat glomerular basement membrane (anti-GBM) serum. Particulate GBM was prepared from frozen rat kidneys

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Address reprint requests to Dr. Rahman, Division of Nephrology, Hines VA Hospital, Room G335, Hines-Loyola Medical Center, Hines IL 60141.

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1. *Abbreviations used in this paper:* CBGG, cationic bovine gamma globulin; GBM, glomerular basement membrane; LTB_4, leukotriene B_4; 5-HPETE, 5-hydroperoxy-eicosatetraenoic acid; NSN, nephrotoxic serum nephritis; PHN, passive Heymann nephritis.

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as previously described (15, 16). Female New Zealand white rabbits received intradermal injections of rat GBM, 5 mg of GBM per rabbit, in complete Freund's adjuvant as we have previously described (3). The injections were repeated after 2 wk. 1 mo after the initial injection the nephrotoxicity of the rabbit serum was evaluated by the development of proteinuria after injections of 1 ml serum i.v. in 300 g male Sprague-Dawley rats. Immunofluorescent examination of glomeruli from injected rats confirmed linear staining of GBM for rabbit IgG and rat C3. 1 ml of rabbit serum contained 14.4 mg of IgG by radial immunodiffusion (3).

Preparation of anti-Fx1A antibody. Fx1A tubular antigen was prepared from Sprague-Dawley renal cortex (17). New Zealand white rabbits were immunized with 10 mg Fx1A in complete Freund's adjuvant in the rear foot pads. 2 wk later, a booster immunization with the same preparation was given in multiple subcutaneous sites and repeated biweekly. Rabbits were periodically bled and antibody against tubular antigen assessed by indirect immunofluorescence. Antiserum against Fx1A was heat-inactivated (56°C, 30 min) and absorbed with 0.1 ml normal rat serum and subsequently with 1 ml packed rat blood cells per ml antiserum. The IgG fraction of the rabbit antiserum was prepared by precipitation with half-saturated $(NH_4)_2SO_4$ followed by DEAE cellulose column chromatography in 0.0175 M potassium phosphate buffer, pH 6.30. This preparation is referred to as Heymann antibody; its specificity was confirmed by indirect immunofluorescence (18).

Preparation of the CBGG. BGG was cationized as previously described (19, 20). 2 g BGG was dissolved in 20 ml 0.01 M NaCl, mixed with 40 ml ethylenediamine, and the pH adjusted to 7.0 with 2 N HCl. 4 g EDC was added and the pH was maintained at 7.0. The mixture was left overnight at room temperature, then dialyzed against 0.15 M NaCl for 24 h. Cationic BGG prepared by this procedure has a pI ranging from 9.5 to 11.5.

Experimental models. Sprague-Dawley rats, 200–300 g were divided into four groups: a control group, a NSN group, a PHN group, and a group with CBGG-induced membranous nephropathy. In each of the four groups kidney tissues were examined by light, immunofluorescence and electron microscopy, and glomeruli were isolated for LTB_4 measurement.

NSN was induced in rats by injecting 1 ml of anti-GBM serum i.v.; the rats were sacrificed 48 h later (3). To induce PHN, 60 mg of IgG fraction of anti-Fx1A was injected into rats in three divided doses given 1 h apart, via tail vein (18). Rats were sacrificed 18 d later, during the peak of the autologous phase. For induction of the CBGG model, rats were immunized in the foot pads with 1 mg CBGG in complete Freund's adjuvant. 7 d later the rats received daily intravenous antigen challenge with increasing doses of CBGG for 5 d (day 1: 2 mg, day 2: 4 mg, day 3: 6 mg, day 4: 8 mg, day 5: 10 mg) and were killed 3–4 d after the last intravenous injection of antigen. The pathologic features of the CBGG model have been described recently (20).

X-Irradiation to the CBGG model. To exclude the possibility that circulating or infiltrating neutrophils were the source of measured LTB_4, leukocytes were depleted by total body x-irradiation (21). 5 d after foot pad immunization with CBGG each rat received a total dose of 1,000 rad (Theratron model 780; Atomic Energy of Canada, Ltd., Ottawa, Canada); an 8 cm thick cerrobend shield was placed around the abdomen and the lumbar spine region shielding the kidneys. 4 days later, blood leukocytes were ennumerated and rats received four intravenous injections of CBGG; 2, 4, 6, and 8 mg, 12 h apart. This protocol was adapted to develop the disease in a shorter period of time due to the decreased life span of the irradiated rats. One day after the last CBGG injection proteinuria was examined, a blood sample was drawn for measurement of total white blood cell and neutrophil count, and the rats were killed.

In vivo inhibition of LTB_4 synthesis with BW755C. To determine the effects of 5-lipoxygenase inhibition in the CBGG model of glomerulonephritis we administered the cyclo- and lipoxygenase inhibitor BW755C to rats for 3 d after the last antigenic challenge. Six rats were preimmunized and challenged for four successive days starting a week later, as described above. On days 11–13, treated rats were given 100 mg/kg per d of BW755C as a solution in normal saline per os in two divided doses; the dose, route, and timing were adapted from a previous report of the in vivo use of BW755C in rats (22). Six control rats were given normal saline per os. Rats were maintained on drug or vehicle during a 24-h urine collection, and a blood sample was taken at the midpoint of the urine collection. Protein and creatinine content of urine and serum creatinine were assessed, and glomeruli isolated from kidneys after sacrifice were incubated to determine LTB_4 production.

Measurement of proteinuria. Urine was collected from individual rats over the 24 h immediately before death. Protein excretion was determined by multiplying the urine volume by the concentration of protein in the urine measured as the turbidity induced by 3% sulfosalicylic acid. Bovine serum albumin solutions at various concentrations were used as protein standards.

Immunofluorescence microscopy (IF). A small sample of cortical tissue was snap frozen in cold 2-methyl butane immersed in liquid nitrogen. Frozen tissue was sectioned at 4 μm in a Spencer Cryostat (American Optical, Buffalo, NY) and stored at −70°C. Staining was done as previously described (3, 18, 20). Sections were layered with goat antiserum to rat IgG (rhodamine conjugated), bovine IgG, rabbit IgG or rat complement C3 (fluorescein conjugated), all diluted at 1:20. There was no cross-reactivity between any of the antisera among themselves or with rat IgG, bovine IgG, or rat serum (for C3) in double immunodiffusion. Coded sections (to prevent observer bias) were examined in a microscope (Ernst Leitz Inc., Wetzlar, West Germany).

Electron microscopy (EM). Small (1 mm in each dimension) portions of renal cortex were immersion fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate adjusted to pH 7.3. The tissue was postfixed in ($Os\ O_4$) and embedded in Spurr's epoxy. Ultrathin (50 nm) sections were cut, picked up on uncoated nickel grids, and stained with uranyl acetate and lead citrate. Specimens (coded to prevent observer bias) were evaluated in an electron microscope (201 C; Philips Medical Electronics Div., Piscataway, NJ) for the number and size of electron dense deposits in subendothelial, intramembranous, subepithelial, and mesangial glomerular sites, in a manner similar to that previously described (18, 20).

Light microscopy (LM). Tissue was immersion fixed in 10% formalin buffered to pH 7.5 with 0.01 M sodium phosphate for 24 h. The tissue was then processed through graded alcohols and xylenes and infiltrated in molten Paraplast (Fisher Scientific). The tissue was embedded, sectioned at 2 μm and stained with periodic acid silver-methenamine, and with hematoxylin and eosin. Coded sections were examined in a light microscope (American Optical, Buffalo, NY).

Glomerular isolation and incubation. After small portions of the cortex were taken for electron, light and immunofluorescence microscopy, the remainder of the kidney tissue was placed in ice-cold PBS (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.6) and used for glomerular isolation. Glomeruli were isolated by a differential sieving technique (20). The cortex was separated from the medulla and minced to a pastelike consistency. The minced cortical paste was passed through a 106-μm metal sieve to remove large tubular fragments and suspended in PBS. This suspension was triturated and poured over a 75-μm sieve. The glomeruli trapped on the 75-μm sieve were collected and suspended in PBS. The purity of the glomerular preparation was assessed by light microscopy and varied between 92 and 98%. The isolated glomeruli were transferred to a siliconized glass tube and were suspended in Earle's balanced salt solution with 20 mM Hepes, 3.0 μM calcium ionophore (A23187) and 2 mM reduced glutathione, at a total volume of 1 ml. In some experiments we added the cyclo- and lipoxygenase inhibitor BW755C (50 μg/ml) or [^{3}H]-arachidonic acid, specific activity 80 Ci/mmol (5×10^5 cpm/ml of media yielding 1.8 ng arachidonate/ml). The glomerular suspension was incubated for 30 min at 37°C with mild agitation.

Extraction and purification of lipids. Incubation was terminated by acidification to pH 3.5 with 0.1 N HCl and lipids were extracted three times with 3–4 vol of ethyl acetate. The extracted lipids were dried under nitrogen and reconstituted in 100% methanol and then in 5%

methanol in water. The reconstituted lipids were further purified on ODS silica cartridges, pretreated with 1 ml methanol followed by 3–5 ml of water immediately before use. After application of the sample, the cartridge was washed with 2–3 ml of water then the sample was eluted with 1 ml of methanol. Samples were stored in methanol under N_2 at −60°C until separation by reverse phase HPLC and LTB_4 measurement by RIA.

HPLC and RIA for LTB_4. Samples obtained from glomerular incubation in the presence of calcium ionophore (A23187) were analyzed by HPLC-RIA. Reverse phase HPLC was carried out on an instrument model 5000; Varian Instruments, Palo Alto, CA fitted with a Nucleosil C-18 column. The column was eluted at 0.9 ml/min, with a linear gradient from 93.4% 0.01 M phosphate buffer (pH 7.4), 6% methanol, and 0.6% t-amyl alcohol, to 99.4% methanol with 0.6% t-amyl alcohol (% by volume) over 100 min (23). Fractions were collected at 1-min intervals, dried under nitrogen and reconstituted in 0.5 ml of PBS containing 1% gelatin. Immunoreactive LTB_4 was determined in each fraction using LTB_4 antibodies. The sensitivity and the cross-reactivity of the antibodies were previously described (24).

Radiometric-HPLC. Samples obtained from glomerular incubation with A23187 and [^{3}H]arachidonic acid were analyzed by HPLC as described above. The radioactivity in each fraction was determined by liquid scintillation counting and was expressed as counts per minute per milligram glomerular protein.

GC-MS. HPLC fractions that comigrated with authentic LTB_4 and were shown to contain i-LTB_4 peaks, and fractions eluted 2 min before and 2 min after the LTB_4 retention time, were further studied by GC-MS by Dr. Ian Blair, Vanderbilt University, Nashville, TN (25). Briefly, after esterification of the fatty acid esters in the HPLC fractions with pentafluorophenol, the bis-trimethylsilyl ether was generated by heating with bis(trimethylsilyl)trifluoroacetamide in pyridine (1:1) at 60°C for 1 h. Derivatized samples were chromatographed on a 6 m DB-1 capillary column (0.25 mm i.d.) with an injection temperature of 100°C and a 5°C/min thermal column program; elution was with helium as carrier gas at an inlet pressure of 90 kPa. Ions were detected in a Nermag R1010C mass spectrometer operating in negative ion mode with an ion-source temperature of 210°C at 89 eV, at a source pressure of 30 Pa. Synthetic [^{2}H]LTB_4 served as an internal standard. The capillary column chromatography separates the various isomers of 5-,12-dihydroxyeicosatetraenoic acids (26).

Results

Proteinuria. Table I shows the 24-h protein excretions in the three models of glomerular injury and in control rats. All rats that received NTS and CBGG developed significant proteinuria in excess of 60 mg/24 h. Rats with PHN developed mild proteinuria, 37±13.8 mg/24 h, and control rats excreted 8±0.7 mg/24 h.

Glomerular morphologic changes. LM examination of glomeruli from rats given CBGG was not different from normal controls; no mesangial or endocapillary proliferation, basement membrane changes, crescents or cellular infiltration were seen. On the other hand, glomeruli from rats given anti-GBM serum showed tuft necrosis, hypercellularity, and neutrophilic infiltration. Rats given anti-Fx1A had normocellular glomeruli without crescents, infiltration or necrosis, but capillary walls were thickened and focal epimembranous spikes were evident. Immunofluorescence (Fig. 1) demonstrated characteristic linear staining of rabbit IgG and rat C3 on the GBM in rats given anti-GBM serum (*A*), intense granular glomerular capillary wall deposits of rabbit IgG, rat IgG and C3 in glomeruli from rats given anti-Fx1A (*B*), and granular capillary wall deposition of BGG, rat IgG and C3 in rats given CBGG (*C*); mesangial deposits were not prominent in any rat. Control rats were negative for all immune reactants. By EM, rats with NSN had areas of polymorphonuclear and mononuclear cell infiltration, and detachment of endothelial lining (Fig. 2 *A*). Electron dense deposits were not evident, but a few foci of tuft necrosis were observed. All rats given anti-Fx1A antibody had numerous electron dense deposits in the subepithelial layer of the capillary basement membrane, and foot processes were diffusely broadened (Fig. 2 *B*). Intramembranous deposits were seen rarely, and subendothelial and mesangial deposits were absent. EM of kidney tissues removed from rats given CBGG revealed broadening of the foot processes and numerous regularly spaced electron dense subepithelial deposits, as well as a few mesangial deposits (Fig. 2 *C*). There was no thickening of capillary basement membrane and no neutrophil or monocyte infiltration.

Table I. Results of Proteinuria in Control Rats and Rats with Experimental Models of Glomerular Disease

Experimental model	No. of rats	Proteinuria
		mg/24 h
Control	9	8±0.7
NSN	10	106±11.6*
PHN	11	37±13.8*
CBGG	11	112±12.7*

* $P < 0.01$ vs. control.

Glomerular LTB_4 production. Immunoreactive LTB_4 (i-LTB_4) was measured by RIA on HPLC fractions with a detection limit of 50 pg of i-LTB_4. The elution profile of authentic leukotrienes measured by flow photometry at 280 nm indicated retention times of 53, 57, and 61 min for LTC_4, LTD_4, and LTB_4, respectively (Fig. 3 *A*). The amount of i-LTB_4 in the HPLC fraction that comigrates with authentic LTB_4 from both control rats (n = 9 rats in three experiments) (Fig. 3 *B*) and those with NSN (n = 10 rats in three experiments) (Fig. 3 *C*), did not exceed the background noise of the assay, which resulted from cross reactivity of LTB_4 antibodies with other lipid fractions. Likewise, glomeruli isolated from rats with PHN did not synthesize measurable amounts of i-LTB_4 (n = 11 rats in four experiments, data not shown). In contrast to control, NSN, and PHN rats, the synthesis of i-LTB_4 was greatly enhanced in glomeruli isolated from rats immunized and challenged with CBGG (n = 11 rats in four experiments) (Fig. 3 *D*) in which we detected 12.4±2.3 ng i-LTB_4/mg glomerular protein, that comigrated with authentic LTB_4.

In different experiments glomeruli isolated from CBGG injected rats were incubated with [^{3}H] C 20:4. Lipids extracted from glomerular incubations were chromatographed, and the counts per minute per milligram glomerular protein in each fraction plotted against retention time. A large radioactive peak, 2803 (cpm/mg glomerular protein)·ml was detected, comigrating with authentic LTB_4, while no radioactive peaks comigrating with authentic LTC_4 or LTD_4 were seen (Fig. 4). Glomeruli from normal rats did not reveal any leukotriene peak (data not shown).

Glomerular incubation with lipoxygenase inhibitor. Glomeruli isolated from rats injected with CBGG were incubated with the cyclo- and lipoxygenase inhibitor BW755C, 50 μg/ml. In two separate experiments, glomerular LTB_4 synthesis decreased from 11.8±4.4 ng/mg glomerular protein without inhibitor to 0.71±0.30 ng/mg in the presence of BW755C.

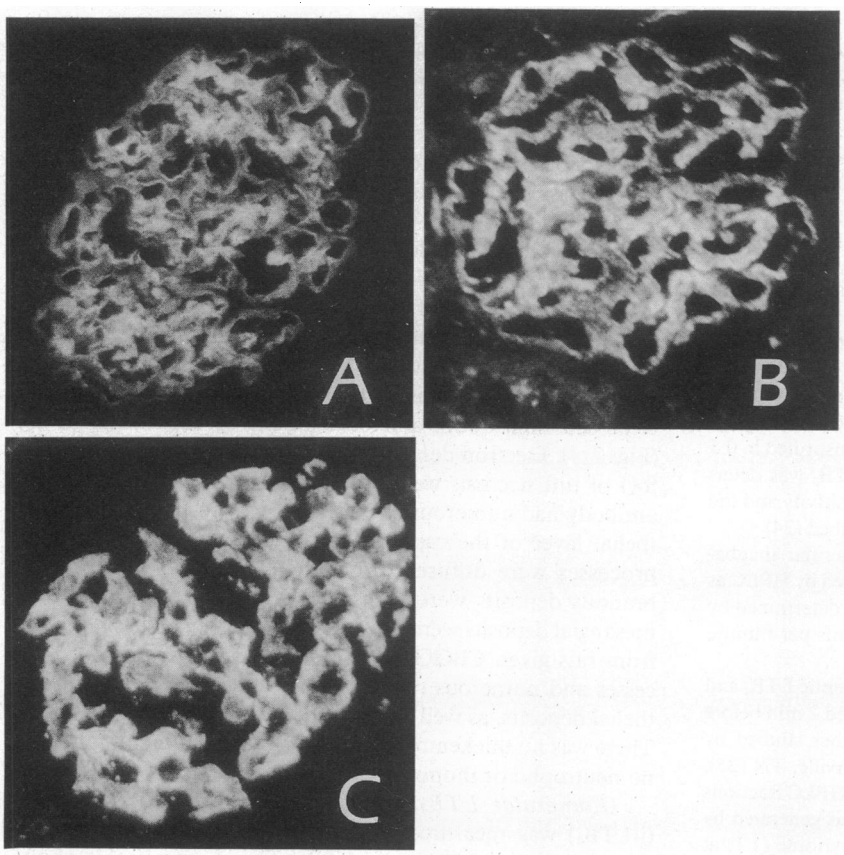

Figure 1. Immunofluorescence micrographs. Representative staining for rat C3 in glomeruli from rats with NSN (*A*), PHN (*B*), and CBGG-induced glomerulonephritis (*C*). Linear staining as seen for C3 in A was also observed for rat IgG and rabbit IgG in rats with NSN. Rats with PHN manifest granular staining for rat and rabbit IgG similar to the pattern present for C3 depicted in B. Similarly, rats with CBGG glomerulonephritis had granular deposits of BGG and rat IgG in capillary walls, in a distribution similar to the pattern for C3 depicted in C. (× 400).

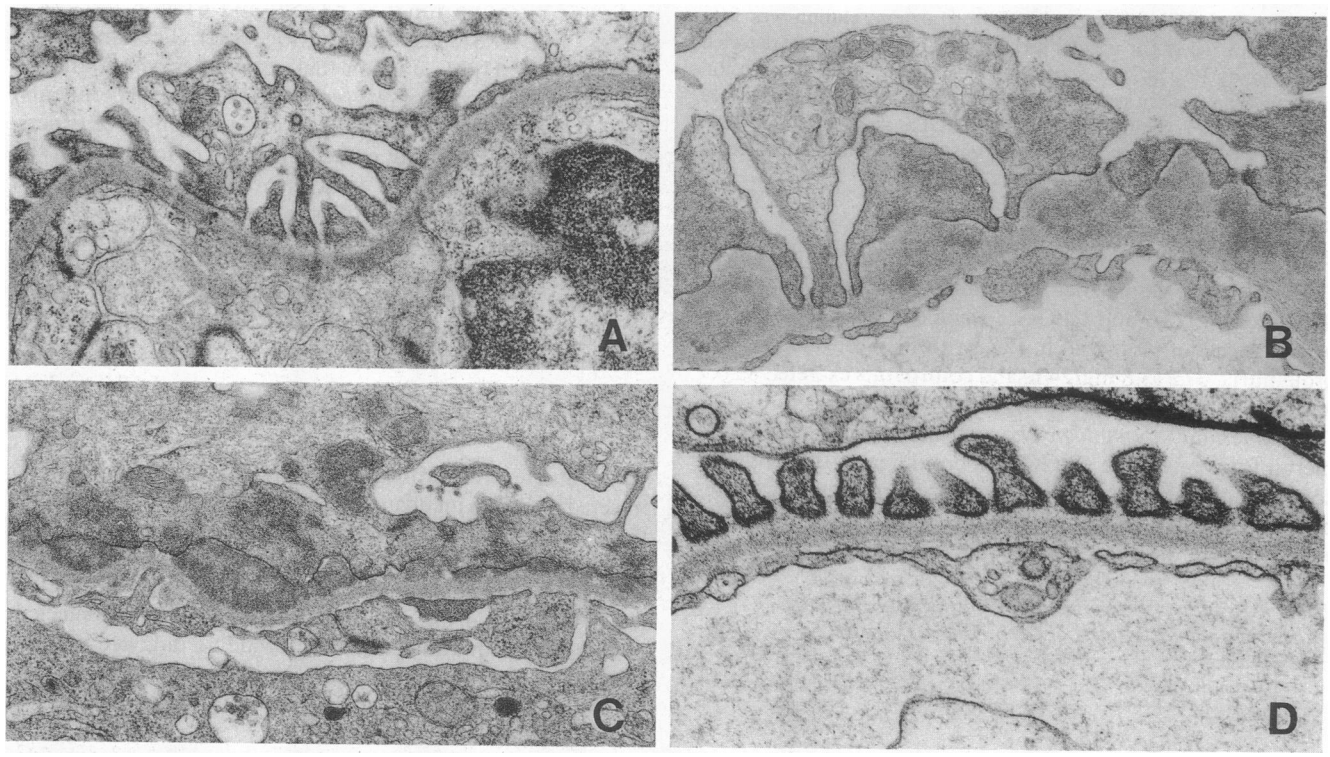

Figure 2. Electron micrographs. Ultrastructurally, rats with NSN reveal leukocytic infiltration of glomeruli with abutment of leukocytes on basement membranes denuded of endothelium, and foci of fibrin (*A*). Rats with PHN have large, coalescent epimembranous electron dense deposits, and a few smaller scattered intramembranous deposits (*B*). The deposits present in glomeruli of rats with CBGG-induced glomerulonephritis are also in epimembranous sites, but are smaller and more discrete than those in PHN, without new basement membrane formation (*C*). An electron micrograph of a section of capillary wall from a normal rat (*D*) is included for comparison. (*a–c* × 20,000; *d* × 30,000.)

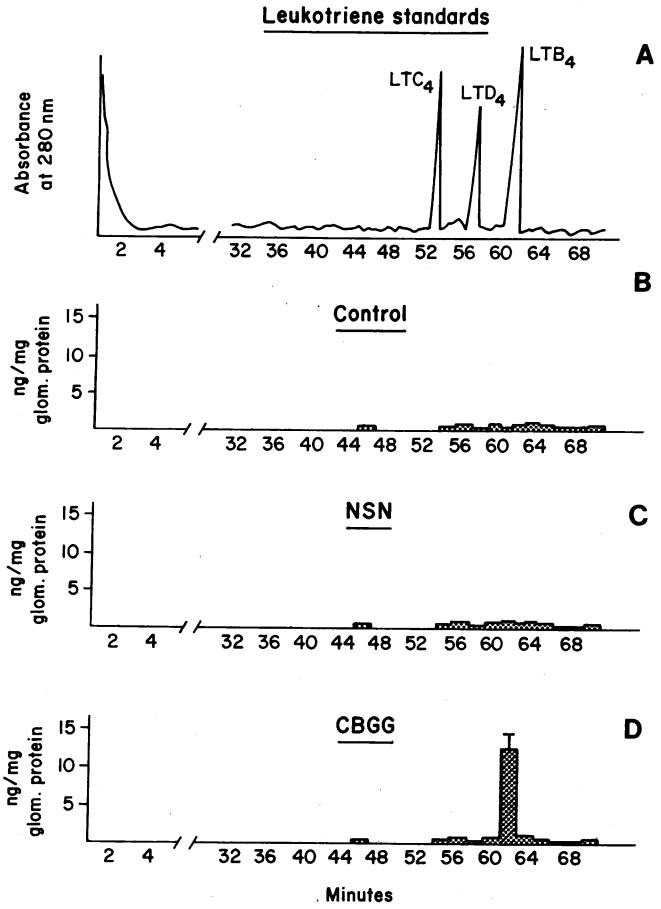

Figure 3. Determination of LTB_4 by HPLC-RIA. The retention times of LTC_4, LTD_4, and LTB_4 standards are 53, 57, and 61 min, respectively (*A*). The results of RIA of LTB_4 on HPLC fractions are shown by hatched bars in control (*B*), NSN (*C*), and CBGG-injected rats (*D*). Data are the means±SE of three or four separate experiments, expressed in ng/mg glomerular protein and plotted against retention time in minutes. Control and NSN synthesized undetectable amounts of i-LTB_4. Glomeruli from CBGG injected rats synthesized 12.4±2.3 of i-LTB_4.

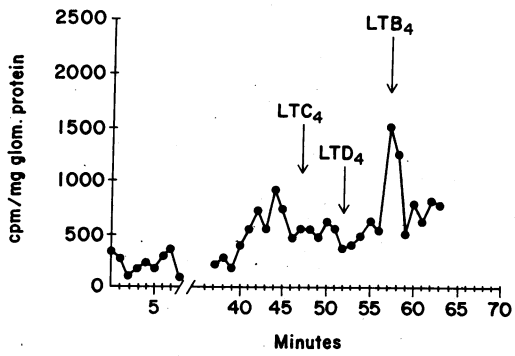

Figure 4. Radiometric HPLC obtained after incubation of glomeruli from CBGG rats. Counts per minute per milligram glomerular protein in each fraction is plotted against retention time in minutes. The retention time of leukotriene standards are indicated by arrows. A large peak of radioactivity (integral under the curve is 2803 (cpm/mg glom. protein) · ml), comigrated with standard LTB_4. No LTC_4 or LTD_4 peaks were seen.

GC-MS. To verify the structural characteristics of the i-LTB_4 in glomeruli isolated from CBGG injected rats, selected samples of extracted glomeruli and incubation medium were subjected to GC-MS analysis after HPLC purification. Fractions that comigrated with standard LTB_4 and contain peaks of i-LTB_4 were identified as authentic LTB_4 based on an intense negative ion at m/z 479, identical to the spectrum of standard LTB_4. There was close agreement in the quantification of LTB_4 by HPLC-RIA and by GC/MS (Table II).

X-irradiation to the CBGG model. Some rats were X-irradiated before intravenous injection of CBGG to minimize the possibility that glomerular LTB_4 was derived from circulating leukocytes. Irradiated rats developed 118±14 mg proteinuria/24 h, comparable to nonirradiated rats. Glomerular i-LTB_4 production was 8.2 ng/mg glomerular protein (pooled from four rats). There was more than a 95% reduction in circulating leukocytes prior to induction of glomerulonephritis on day 4 postirradiation ($0.67\pm0.23 \times 10^3$ WBC/μl blood, $0.15\pm0.02 \times 10^3$ neutrophils/μl and $66.0\pm20.6 \times 10^3$ platelets/μl) and at sacrifice on day 7 postirradiation ($0.05\pm0.28 \times 10^3$ WBC/μl, $0.12\pm0.04 \times 10^3$ neutrophils/μl and $26\pm3.0 \times 10^3$ platelets/μl) compared to nonirradiated controls ($13.6\pm1.0 \times 10^3$ WBC/μl, $6.86\pm1.7 \times 10^3$ neutrophils/μl, and $318\pm28 \times 10^3$ platelets/μl).

In vivo inhibition of LTB_4 synthesis with BW755C. Treatment with BW755C resulted in microhematuria, and the urine of these rats contained up to 7 mg/24 h hemoglobin, measured from the optical density of the urine at 410 nm versus a turbidity blank at 700 nm. The hemoglobin values were subtracted from the total protein determined by sulfosalicylic acid. CBGG rats had 77±8.3 mg protein in the 24-h urine collection, whereas rats given BW755C excreted only 28.4±11.8 mg/24 h ($P < 0.01$). However, the endogenous creatinine clearance in the treated rats was 0.24±0.07 ml/min, compared with 0.55±0.05 ml/min in the saline-treated group ($P < 0.01$). There was no correlation between proteinuria and endogenous creatinine clearance between the treated and control groups. Moreover, while the creatinine clearance was reduced to 44% of the control group, the proteinuria was reduced to 37% of the control value in treated rats. LTB_4 synthesis in the treated rats was 1.06 ng/mg glomerular protein, versus 2.96 ng/mg in untreated controls.

Table II. Comparison of LTB_4 Determined by HPLC-RIA and GC/MS

Rats with CBGG-glomerulonephritis	Retention on column	LTB_4 extracted from medium	
		HPLC-RIA	GC/MS
	min	*ng/ml*	
Normal	61	117.2	97.7
Normal	59	1.6	1.1
Normal	63	0.2	0.2
Leukocyte depleted*	61	37.8	38.8
Leukocyte depleted*	59	17.2	0.8

* These rats received 1000 R total body x-irradiation before induction of glomerulonephritis.

Discussion

Arachidonic acid is the substrate for cyclo- and lipoxygenase enzymes. Glomerular synthesis of the cyclooxygenase metabolites, prostaglandins, and thromboxane, has been studied extensively. These eicosanoids modulate renal hemodynamics, and the inflammatory response in glomerular disease (20, 27–29). On the other hand, the data on the role of the lipoxygenase metabolites in glomerular inflammation are few and the capacity of the glomeruli to synthesize 5-lipoxygenase products has not been established.

In this study, we demonstrate glomerular synthesis of the 5-lipoxygenase metabolite LTB_4 in the CBGG-induced model of immune glomerular disease. Our studies were always conducted with calcium ionophore added to isolated glomeruli, in order to stimulate 5-lipoxygenase. This model, pathologically similar to human membranous nephropathy, is a nonproliferative and noninfiltrative model, and injury is independent of serum complement and circulating leukocytes (30). The enhanced glomerular synthesis of LTB_4 was demonstrated by HPLC/RIA, by radiometric HPLC after incubation of isolated glomeruli with tritiated arachidonic acid, and by GC/MS, which unequivocally demonstrated the presence of authentic LTB_4 in the HPLC fractions shown to contain i-LTB_4. Furthermore, synthesis of immunoreactive LTB_4, measured by HPLC-RIA, was inhibited by the cyclo- and lipoxygenase inhibitor BW755C. Although we included reduced glutathione in the incubation medium, we did not observe any sulfidopeptide leukotriene products or their major metabolite, LTE_4. We cannot exclude the possibility that some sulfidopeptide leukotriene is produced by glomeruli, but it is clear that the major product of 5-lipoxygenase activity in glomeruli is LTB_4. We could not detect LTB_4 production by normal glomeruli, even with addition of ionophore. This is not incompatible with the recent report by Cattell and her colleagues, since they pooled glomeruli from many more rats, totaling 5 mg glomerular protein to obtain 120 pg/mg LTB_4 produced by normal glomeruli without addition of ionophore (31). We detected as much as 12 ng/mg LTB_4 produced by glomeruli from rats with CBGG nephritis containing only 0.5 mg protein; this is clearly different from the synthetic rate of normal glomeruli, whether or not stimulated with ionophore.

The enhanced glomerular LTB_4 synthesis in the CBGG model could be derived from endogenous glomerular cells, or circulating neutrophils or monocytes trapped in the glomeruli during glomerular isolation. Although there were no infiltrating monocytes or neutrophils in glomeruli demonstrable by light and electron microscopy in this model, it is conceivable that there were some circulating cells present in the glomeruli not demonstrated by routine morphologic examination (21, 32). The contribution of these cells to the glomerular LTB_4 should be minimal, if any. This is supported by our finding of increased glomerular LTB_4 synthesis associated with CBGG-induced glomerulonephritis even when circulating leukocytes are reduced by > 95% through bone marrow irradiation. Furthermore, glomerular synthesis of LTB_4 in rats with NSN was below the detection limit of our assay, despite heavy glomerular infiltration with neutrophils. Therefore, the most likely source of enhanced LTB_4 synthesis in the CBGG model is endogenous glomerular cells. Glomerular epithelial and mesangial cells are capable of synthesizing prostaglandins, thromboxane, and 12-HETE (33). They are a possible source of LTB_4; however, their capacity to synthesize 5-lipoxygenase products has not been established. A bone marrow-derived macrophage, recently described as resident in the mesangium, increases in immune glomerular injury (32), and could be a source of LTB_4. These cells may metabolize arachidonic acid through the 5-lipoxygenase pathway similar to monocytes and macrophages. Endothelial cells in the glomeruli may also be a source of the LTB_4 we observed, but this is very unlikely since endothelial cells are relatively poor producers of LTB_4, generating i-LTB_4 in the pg range/2×10^6 cells/h, even with 10 μM ionophore (34).

Regardless of the cellular source of LTB_4, we consider deposited immune complexes to be the likely stimulus. Lianos recently reported transient increases in i-LTB_4 synthesis within hours after induction of the heterologous phase of NSN, but noted that LTB_4 synthesis did not persist until the onset of proteinuria (35). Lianos and Noble also reported similar transient i-LTB_4 synthesis in the heterologous phase of PHN and active Heymann nephritis, again not associated with proteinuria either temporally or in degree (36). Although PHN is an immune complex disease pathologically similar to the CBGG model, we did not observe an increment in glomerular LTB_4 synthesis in proteinuric rats with PHN or with NSN, in agreement with Lianos et al. (35, 36). In contrast, in CBGG-induced glomerulonephritis there is more 5-lipoxygenation of arachidonic acid than in the other models and heightened LTB_4 synthesis is sustained for at least a few days after onset of proteinuria. Hence, ongoing formation or deposition of immune complexes which persists in active models of glomerulonephritis but occurs only transiently in the passive models might be a key factor in stimulating glomerular 5-lipoxygenase.

Data on the effects of LTB_4 on glomerular function are scarce. Physiologic effects of LTB_4 are most extensively studied in leukocytes. LTB_4 is a potent chemotactic lipid, equipotent on molar basis to formyl-methionyl-leucyl-phenylalanine and complement component C5a, the two most potent chemotactic agents known (37, 38). In vivo, LTB_4 shows chemotactic activity towards polymorphonuclear leukocytes, monocytes, and eosinophils. Also, LTB_4 can cause secretion of lysosomal enzymes from neutrophils (10, 39), enhance complement receptor expression (40) and induce expansion of human suppressor cells (41).

The lack of glomerular cellular infiltration in the CBGG model, despite the generation of the powerful chemotactic lipid LTB_4, is consistent with the findings of Kreisle et al. (42), who found that rat PMN do not exhibit chemotaxis to LTB_4 at concentrations from 10^{-10} to 10^{-6} M. At these concentrations LTB_4 can elicit a powerful chemotactic response in human PMNs. Thus, any proinflammatory effects of LTB_4 in rat glomerular disease are likely mediated by mechanisms other than recruitment of inflammatory cells into the glomeruli.

We do not expect any effect of LTB_4 on glomerular hemodynamics, since LTB_4 has no demonstrable spasmogenic activity on rat mesangial cells in culture (43), guinea pig ileum (44), and rat and rabbit aorta (45). This is in contrast to the well known contractile effects of the sulfidopeptide leukotrienes, LTC_4 and LTD_4 on glomeruli (46) and mesangial cells in culture (43, 46), and on the renal vasculature (47, 48).

Although LTB_4 has no effect on vascular permeability itself, simultaneous injection of LTB_4 with vasodilators such as PGE_2 or bradykinin increases vascular permeability (11–14).

The vasodilators alone did not increase vascular permeability even at relatively high doses, and the increase in permeability was dependent on the concentration of LTB_4 injected. Although one group found no increase in vascular permeability in the kidney after infusion of both eicosanoids into the renal artery at doses that did induce increased permeability in skin and other sites, these investigators studied whole kidney rather than alterations in glomerular capillary permeability specifically (49). These experiments were, moreover, quite acute, being terminated only 8 min after infusion of eicosanoids. Since increased glomerular synthesis of PGE_2 is a feature of the CBGG-induced model of glomerulonephritis (20), synergism between increased PGE_2 and LTB_4 in promoting increased vascular permeability may well apply to glomerular permeability to macromolecules in this model. We administered BW755C, a combined lipoxygenase and cyclooxygenase inhibitor to a group of CBGG rats and decreased glomerular LTB_4 synthesis by 65%, proteinuria by 63%, and GFR by 56%. Hence, the reduction of proteinuria after inhibition of glomerular LTB_4 could also be attributed to a reduced filtered protein load. Furthermore, BW755C is a cyclooxygenase inhibitor and undoubtedly reduced glomerular PGE_2 production and removed the synergistic actions of PGE_2 and LTB_4 to enhance capillary permeability. The precise definition of the role of LTB_4 in the CBGG model must await the availability of specific 5-lipoxygenase inhibitors and/or LTB_4 receptor antagonists.

Reports of synergy between LTB_4 and vasodilators in inducing vascular permeability ascribe the actions of the eicosanoids to an effect on neutrophils (11, 12, 50). Yet, in the CBGG-induced model of glomerulonephritis, we do not observe a cellular infiltrate (20) and in separate studies reported elsewhere, we have found that enhanced glomerular prostaglandin production and proteinuria develop independently of circulating leukocytes (30). This does not exclude synergistic interaction between PGE_2 and LTB_4 in CBGG-induced glomerulonephritis; rather, it indicates that the functions subserved by neutrophils in the enhancement of cutaneous vascular permeability may be provided by cells other than circulating or infiltrating leukocytes in glomerulonephritis, perhaps endogenous glomerular cells. Glomerular mesangial cells in culture manifest several functions traditionally associated with granulocytes, including expression of receptors for immunoglobulin Fc region and for activated complement components, generation of reactive oxygen intermediates and inflammatory eicosanoids, elaboration of neutral proteases, and even phagocytosis and secretion of interleukin 1 (51, 52). Thus, some mesangial effect of LTB_4 in conjunction with PGE_2 may ultimately cause proteinuria.

In summary, we examined glomerular synthetic capacity of LTB_4 by normal rat glomeruli and by glomeruli isolated from the heterologous phase of NSN, PHN, and CBGG-induced membranous nephropathy. Glomerular synthesis of LTB_4 was exclusively enhanced in the CBGG model independent of infiltrating leukocytes or platelets. This increment of LTB_4 may play an important role in the pathophysiology of glomerular disease.

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