Glomerular Arachidonate Lipoxygenation in Rat Nephrotoxic Serum Nephritis

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Abstract

Arachidonate lipoxygenation to monohydroxylated eicosatetraenoic acids (HETE) was studied in rat nephrotoxic serum nephritis (NSN). A single infusion of nephrotoxic serum enhanced conversion of [^{3}H]arachidonic acid ([^{3}H]C20:4) to [^{3}H]12-HETE in glomeruli isolated from nephritic rats compared with controls. The percent conversion of [^{3}H]arachidonic acid was 1.95±0.2% in control glomeruli and 14.2±2% in nephritic glomeruli 2 d after induction of disease. No significant changes in the conversion of [^{3}H]C20:4 to [^{3}H]5-, 8-, and 9-HETE were noted. Extraction of glomerular HETE by alkaline hydrolysis, to evaluate possible reacylation of HETE after their production, confirmed the presence of 12-HETE and did not provide evidence of 5-HETE synthesis. Increased glomerular 12-HETE synthesis in nephritic rats was also demonstrated by high pressure liquid chromatography-UV detection and by 12-HETE radioimmunoassay. The enhanced glomerular 12-HETE synthesis commenced as early as 3–5 h after administration of nephrotoxic serum and peaked at day 2 with 10-fold enhancement of 12-HETE production. Increments of glomerular 12-HETE persisted on day 7 and returned toward control levels by day 14. Platelet depletion, induced by antiplatelet antisera, did not decrease glomerular 12-HETE synthesis in NSN, thereby eliminating platelets as the cellular origin of 12-HETE. Glomerular epithelial and mesangial cells are the most likely sources of enhanced 12-lipoxygenase activity. The enhanced arachidonate 12-lipoxygenation in glomerular immune injury could have important proinflammatory effects in the evolution of glomerulonephritis since 12-HETE has important effects on leukocyte function.

Introduction

Arachidonate lipoxygenation to monohydroxylated eicosatetraenoic acids (HETE)[1] occurs in a variety of mammalian cells, with platelets and leukocytes being among the most extensively studied (1, 2). Lipoxygenase activity has also been identified in rat kidney glomeruli, glomerular epithelial and mesangial cells, and to a lesser extent in cortical tubules (3–5). The role of HETE in the pathophysiology of inflammation has been under extensive study. Among the well-characterized proinflammatory effects of HETE are histamine secretion by mast cells (6), neutrophil degranulation, chemotaxis, and chemokinesis (7, 8). In antibody-mediated glomerular injury (nephrotoxic serum nephritis [NSN]), there is enhanced cyclooxygenation of arachidonic acid to prostaglandins and thromboxanes by isolated glomeruli (9). Using inhibitors of thromboxane synthetase or arachidonate cyclooxygenase, we have shown that prostaglandins and thromboxane alter renal hemodynamics in NSN (9). The present study was undertaken to evaluate the arachidonate lipoxygenation in glomeruli isolated from rats with NSN and to characterize which forms of HETE are synthesized over a 2-wk period of the disease.

Methods

Induction of NSN. NSN was induced in male Sprague-Dawley rats, 250–300 g, by a single intravenous administration of 1 ml of rabbit serum raised against rat particulate glomerular basement membrane (GBM) (10). Immunoglobulin concentrations in nephrotoxic serum (NTS) were determined by single radial immunodiffusion using goat anti-rat IgG (Cappel Laboratories, Cochranville, PA) and rat IgG (Sigma Chemical Co., St. Louis, MO) (11). 1 ml of NTS contained 12.4–16.4 mg of IgG. Control rats received 1 ml of serum obtained from rabbits immunized with complete Freund's adjuvant. Nephrotoxicity was assessed by the development of proteinuria and by morphologic evaluation of glomerular lesions using routine methods of light microscopy, immunofluorescence, and electron microscopy.

Platelet depletion. To significantly reduce platelet involvement in NSN, we administered 2 ml of antiplatelet antisera (kindly provided by Drs. A. Rehan and K. Johnson, University of Michigan, Ann Arbor, MI) 12 h before the injection of 1 ml of NTS in selected animals. Platelet counts were obtained 36 h after treatment with antiplatelet antisera. The number of platelets was determined on EDTA whole blood using an automated hematology analyzer (Ortho Diagnostic Systems Inc., Johnson & Johnson, Raritan, NJ).

Glomerular biochemical studies. At 3–5 h and on days 2, 7, and 14 after a single infusion of NTS, nephritic and control rats were nephrectomized, and the kidneys were placed in phosphate-buffered saline containing 150 mM NaCl, 10.5 mM Na_2HPO_4, and 3.8 mM NaH_2PO_4, pH 7.4, at 4°C. After the cortices were minced to a pastelike consistency, glomeruli were isolated using a technique previously developed for glomerular biochemical studies (12). Isolated glomeruli were suspended in preweighed siliconized glass culture tubes in 0.5 ml modified Earle's balanced salt solution, pH 7.4, containing 25 mM HEPES, 5,8,11,14-eicosatetraenoic acid (C20:4 arachidonic acid; Sigma Chemical Co.), at a concentration of 5 μg/ml, and the divalent cation ionophore A23187 (3 μM; Sigma Chemical Co.). In experiments assessing the conversion of radiolabeled arachidonic acid to lipoxygenase metabolites, octatritiated C20:4 (500–600 × 10^3 cpm, 60 Ci/mmol sp act; New England Nuclear, Boston, MA) was also added in the incubation media. To assess the extent of nonenzymatic conversion of [^{3}H]C20:4 to [^{3}H]HETE, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA; Sigma Chemical Co.) was also included in a number of glomerular incubations at a con-

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1. *Abbreviations used in this paper:* GBM, glomerular basement membrane; HETE, monohydroxylated eicosatetraenoic acids; HPLC, high pressure liquid chromatography; NDGA, nordihydroguaiaretic acid; NSN, nephrotoxic serum nephritis; NTS, nephrotoxic serum.

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centration of 33 μM. Incubations were performed at 37°C for 5 and 45 min. Glomerular suspensions were then acidified with 0.1 N HCl to pH 3–3.5 and extracted three times with 3 vol of ethyl acetate. Extracts were combined, dried under N_2, reconstituted in 300 μl of hexane, and injected in a gradient high pressure liquid chromatograph (Varian Associates, Inc., Instrument Group, Palo Alto, CA). In several experiments, we hydrolyzed membrane fatty acids using the technique of Bonser et al. (13) to evaluate possible reacylation of HETE into membrane lipids over the 45-min incubation. Glomeruli were extracted by the addition of 2.4 ml chloroform/methanol (1:1 vol/vol) containing 0.001% butylated hydroxyanisole and hydroxytoluene. After acidification with 0.1 ml of 2% formic acid, the suspension was vortexed and centrifuged, and the organic layer was removed and dried under N_2. The lipid residue was resolubilized in 80% methanol with 0.2 N NaOH, pH ≅ 13, and heated overnight at 45°C under N_2. The hydrolyzed lipids were reextracted with chloroform/methanol (3:1, vol/vol), acidified to pH 4 with formic acid, vortexed, cooled, and centrifuged. The organic layer was removed, evaporated under N_2, solubilized in hexane, and injected into the chromatograph.

Radiometric high pressure liquid chromatography (HPLC). Samples obtained from glomerular incubations in the presence of octatritiated C20:4 were chromatographed with coinjected 5-, 8-, 9-, and 12-HETE standards (Upjohn Co., Kalamazoo, MI) on a normal-phase silica analytical column (30 × 4 mm) packed with 5-μm particles (micropak Si-5; Varian Associates, Inc.). Two solvent reservoirs were employed: hexane/isopropanol/acetic acid (100:8:0.5) and hexane/acetic acid (125:1). An initial 10-min isocratic elution of 100% hexane/acetic acid (125:1) removed unconverted arachidonic acid and nonpolar lipids and was followed by a gradient elution from 100 to 70% hexane/acetic acid (125:1) in 110 min to elute HETE. Eluates were monitored via a variable wavelength detector (V-UV 10; Varian Associates, Inc.) at a wavelength of 240 μm and collected in a fraction collector at a rate of 1 ml/min. The radioactivity of collected fractions was subsequently determined by liquid scintillation counting and expressed as percent of total radioactivity added in the original glomerular suspension per milligram glomerular weight.

12-HETE HPLC-UV detection and radioimmunoassay (RIA). Samples obtained from glomerular incubations with C20:4 were analyzed by HPLC as described above. Changes in UV absorbance at 240 μm were continuously recorded during each chromatographic run on a Servoriter II (Texas Instruments Inc., Data Systems Group, Dallas, TX) chart recorder connected to the variable wavelength detector. Peak heights (centimeters) corresponding to 12-HETE retention times, obtained during chromatography of unknown samples, were measured and compared with the peak heights of known amounts of 12-HETE standards chromatographed under identical conditions. The mass of 12-HETE (nanograms) corresponding to 12-HETE peaks of unknown samples was thus estimated on the basis of a standard curve obtained from peak heights of the chromatographed 12-HETE standards.

Eluted 12-HETE fractions were subsequently evaporated under N_2 and reconstituted in 0.5 ml of phosphate-buffered saline containing 1% gelatin. These samples were assayed in triplicate dilutions using a 12-L-HETE antibody, generously provided by Dr. L. Levine, Brandeis University, Waltham, MA, at a dilution of 1:1,600, with [^{3}H]12-L-HETE (New England Nuclear) as the radioactive ligand. The 12-HETE RIA methodology was similar to prostaglandin RIA methods reported by this laboratory (12). Since the 12-L-HETE antibody cross-reacts with other HETE (14), HPLC of each sample before RIA was found to be a necessary preparative step. Recoveries of [^{3}H]12-HETE (2–3 × 10^3 cpm) added to unknown samples before extraction and chromatographic analysis were generally between 68 and 72%.

For statistical comparisons of 12-HETE values obtained in glomeruli isolated from NTS-treated and control rats, studied in parallel, the *t* test for unpaired observations was employed. Data are presented as the mean±SEM.

Results

Morphologic evaluation of glomeruli in kidney biopsies of NTS-treated rats revealed strong linear deposition of the infused rabbit anti-rat immunoglobulin on the GBM. In addition, there was detachment of the endothelial lining, mesangial cell proliferation, and the presence of polymorphonuclear leukocytes (day 2), which were gradually replaced by monocytes (day 14). Platelets were present in the glomerular vasculature, especially on days 7 and 14. Proteinuria, ranging from 80 to 120 mg/d, was present on day 2. Two typical HPLC radiochromatograms obtained from glomeruli incubated with [^{3}H]C20:4 are shown in Fig. 1 *A* and *B*. Eluted radioactivity, expressed in counts per minute per milligram glomerular weight, is plotted against retention times. In the radiochromatogram obtained from control glomeruli (Fig. 1 *A*), unconverted [^{3}H]C20:4 was eluted during the isocratic phase of the run, followed by [^{3}H]12-HETE eluting at the retention time of coinjected 12-HETE standard. There were insignificant activities at retention times corresponding to coinjected 5-, 8-, and 9-HETE standards. Using identical chromatographic conditions, we have previously shown (3) that the compound synthesized by glomeruli incubated with C20:4 and coeluting with 12-HETE standard has a mass spectrum identical to that of standard 12-HETE. A typical HPLC radiochromatogram obtained from glomeruli isolated from NTS-treated rats at day 2 of the disease is shown in Fig. 1 *B*. There was a marked increment in the synthesis of [^{3}H]12-HETE, with no significant changes in that of 5-, 8-, and 9-HETE. The peaks preceding those of [^{3}H]12-HETE were impurities present in the [^{3}H]-C20:4 standard, since they persisted when incubation media containing [^{3}H]C20:4 but not glomeruli were extracted and chromatographed under identical conditions. An unidentified

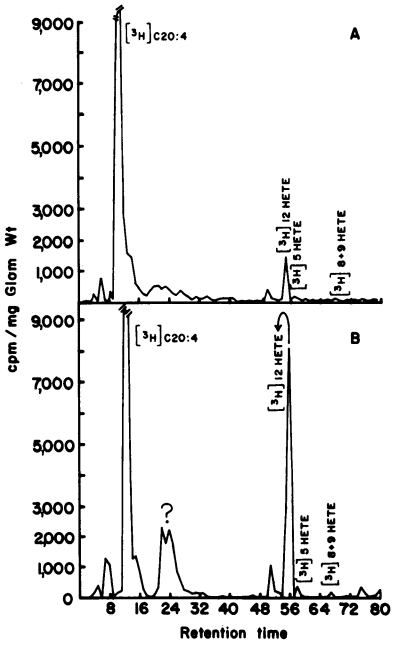

Figure 1. Radiometric high pressure liquid chromatograms obtained after incubation of glomeruli obtained from control (*A*) and NTS-treated rats (*B*) and incubated with [^{3}H]C20:4. Total activity in eluates, expressed as counts per minute per milligram glomerular (glom) weight (ordinate), is plotted against retention times in minutes of co-chromatographed HETE standards. Note that control glomeruli (*A*) synthesize primarily [^{3}H]12-HETE and insignificant amounts of [^{3}H]5-, 8-, and 9-HETE. The synthesis of [^{3}H]12-HETE in glomeruli from NTS-treated rats is enhanced (*B*). An unidentified peak (?) was noted in the radiochromatogram obtained from the latter.

peak was also present in the radiochromatogram obtained from glomeruli isolated from NSN rats. We considered the possibility that the absence of a [^{3}H]5-HETE peak may have been due to rapid reacylation of 5-HETE into membrane lipids. After 5-min incubations, [^{3}H]5-HETE was not detected, although [^{3}H]12-HETE was present. We also extracted HETE by alkaline hydrolysis of glomeruli after 45-min incubations with [^{3}H]C20:4. Whereas [^{3}H]12-HETE was released from the hydrolyzed membranes, no [^{3}H]5-HETE could be found. The conversion of [^{3}H]C20:4 to [^{3}H]12-HETE by isolated glomeruli at the four time points of the 14-d study period is shown in Fig. 2. Results are expressed as percent conversion of [^{3}H]C20:4 added in glomerular suspensions. Enhanced conversion was observed as early as 3–5 h after NTS (5.1±0.9% compared with 2.35±0.6%, $P < 0.01$, $n = 3$) and increased further on day 2 (14.2±2% compared with 1.95±0.2%, $P < 0.005$, $n = 4$). The augmentation of [^{3}H]12-HETE synthesis persisted on day 7 (6.1±1.1% compared with 2.2±0.18%, $P < 0.01$, $n = 3$) and to a lesser extent on day 14 (3.9±0.9% compared with 1.7±0.4%, $P < 0.01$, $n = 3$). Incubation of glomeruli in the presence of the lipoxygenase inhibitor NDGA partially inhibited [^{3}H]12-HETE synthesis (Fig. 2).

The glomerular 12-HETE synthetic rates determined by HPLC-UV detection and HPLC-RIA at 3–5 h and on days 2, 7, and 14 after induction of NSN are shown in Table I. Control glomeruli synthesized amounts of 12-HETE that ranged from 1.83±0.25 to 2.11±0.17 and 1.34±0.37 to 1.96±0.20 ng/mg glomerular wt per 45 min by UV detection and RIA, respectively. After administration of NTS, enhanced glomerular 12-HETE synthesis, determined both by UV detection and RIA, commenced at 3–5 h and peaked on day 2 with 7- to 10-fold increments of 12-HETE synthesis as determined by HPLC-RIA and HPLC-UV, respectively. The increments persisted, but to a lesser extent, on day 7, and returned toward control values by day 14 (Table I).

Platelet depletion experiments were conducted to minimize platelets as the source of the 12-HETE in the glomeruli of NSN rats. Normal platelet counts in control rats ranged from 0.9 to 1.3×10^6/ml whole blood. 36 h after receiving 2 ml of antiplatelet antisera, platelet counts ranged from 0.01 to 0.26×10^6/ml. Seven platelet-depleted rats with NSN developed proteinuria (98±10 mg/d) of the same magnitude as five NSN controls (92±12 mg/d). The glomerular synthesis of 12-HETE measured by RIA, after HPLC purification of the sample, was similarly increased in 24-h NSN rats regardless of platelet depletion (Fig. 3).

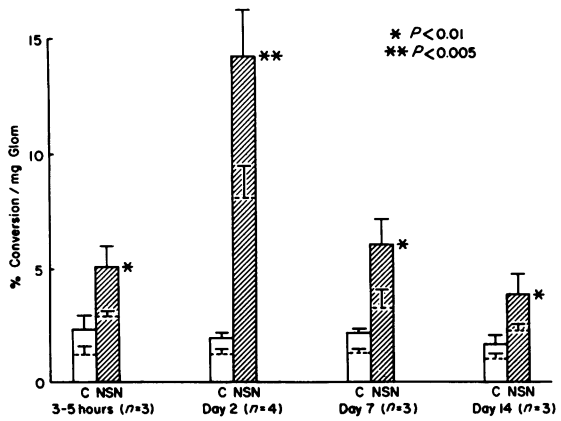

Figure 2. Semiquantitative evaluation of glomerular 12-HETE synthesis at 3–5 h and on days 2, 7, and 14 after administration of NTS. Glomeruli from control (C) and NSN rats were incubated with [^{3}H]C20:4 and the percent conversion to [^{3}H]12-HETE per milligram glomerular weight, mean±SEM, is shown at all time points of the study. Enhanced conversion to [^{3}H]12-HETE in NSN glomeruli was noted with a peak at day 2. NDGA partially inhibited [^{3}H]12-HETE synthesis (dashed lines). Statistical comparisons are between C and NSN at each time of study.

Discussion

We and others have demonstrated that isolated glomeruli, glomerular epithelial cells, and mesangial cells can convert arachidonic acid to prostaglandins, thromboxane, and 12-HETE (3–5). Glomerular synthesis of thromboxane A_2, as well as prostaglandins, is augmented in NSN, and glomerular hemodynamics are partially regulated by these arachidonate metabolites (9, 15). Other biologically active mediators of the immune response in NSN include vasoactive amines (16) and alpha adrenergic catecholamines (17).

The results of the present study indicate that the NSN model of glomerular immune injury also activates the lipoxygenase pathway within immunologically injured glomeruli. Of the four arachidonate lipoxygenation products evaluated, 12-HETE appeared to be the only HETE synthesized in control as well as in NSN glomeruli. Normal glomeruli synthesized measurable quantities of 12-HETE in the nanogram range. In glomeruli from NTS-treated rats, enhanced 12-HETE synthesis commenced as early as 3–5 h after administration of NTS and persisted over a 14-d period. The enhanced synthesis of 12-HETE was demonstrated by three different analytical methods: radiometric HPLC, HPLC-UV detection, and HPLC-RIA. There was generally good agreement between HPLC-UV detection and HPLC-RIA with respect to increments in glomerular 12-HETE synthesis (Table I). However, at all time points, and especially at the peak of 12-HETE synthesis (day 2), higher values were obtained by HPLC-UV detection than by RIA. This discrepancy is partially attributable to 12-HETE losses (~30%) during steps after HPLC analysis and preceding the RIA, i.e., evaporation of eluates and reconstitution in buffer. Several sources of 12-HETE can be implicated in NSN. During the heterologous phase (3–5 h and day 2), potential sources include injured glomerular, epithelial, or proliferating mesangial cells as well as recruited platelets and polymorphonuclear leukocytes. Leukocytes, however, are known to synthesize 5-, 12-, and 15-lipoxygenase metabolites and therefore are not likely sources of the 12-HETE, since we did not note measurable conversion of [^{3}H]C20:4 to [^{3}H]5-HETE by isolated NSN glomeruli. Alkaline extraction of membrane lipids also did not yield 5-HETE, thereby excluding reesterification of 5-HETE as an explanation for the absence of this fatty acid. During the autologous phase (days 7–14), the inflammatory cellular infiltrates consist primarily of monocytes and macrophages (18, 19). These cell types synthesize primarily 5-lipoxygenase products and are therefore unlikely sources of 12-HETE. The newly identified resident Ia-bearing mesangial macrophages are increased in experimental NSN (20). These cells might also be a source of 12-HETE, although the type(s) of eicosanoids they synthesize has not been identified, and 5-HETE probably predominates. We consider it unlikely that platelets are the sole explanation of increased glomerular 12-HETE in NSN, since

*Table I. Glomerular 12-HETE Synthetic Rates**

	HPLC-UV detection		HPLC-RIA	
	Control	NSN	Control	NSN
3–5 h (n = 4)	1.92±0.12	3.32±0.29‡	1.34±0.37	3.1±0.14‡
Day 2 (n = 5)	2.11±0.17	22.5±4.1‡	1.86±0.14	12.7±1.40‡
Day 7 (n = 4)	1.83±0.25	6.3±0.75‡	1.96±0.20	3.55±0.56‡
Day 14 (n = 4)	1.96±0.24	3.54±0.56§	1.79±0.17	2.38±0.43

* Expressed as nanograms per milligram glomeruli per 45 min. ‡ $P < 0.005$, NSN compared with control. § $P < 0.05$, NSN compared with control.

glomerular 12-HETE synthesis peaked by day 2 when platelets were scarce on electron microscopy and waned significantly by day 14 when platelet involvement in the immune-injured glomeruli was substantial. This impression is reinforced by the platelet depletion experiments, which showed equivalent increments of glomerular 12-HETE synthesis despite >80% reductions of circulating platelet counts. Furthermore, we have dissociated the increased glomerular thromboxane synthesis in NSN from platelet deposition and platelet thromboxane release (9). Intrinsic glomerular cells proliferate within 24 h of the onset of NSN, before infiltration of blood-borne cells, and these glomerular, mesangial, and epithelial cells synthesize exclusively 12-HETE in cell culture. We theorize that glomerular immune injury stimulates mesangial and/or epithelial 12-HETE production.

The significance of the enhanced 12-HETE synthesis in glomeruli in anti-GBM disease remains to be explored. The well-described effects of 12-HETE, namely, chemotaxis and chemokinesis (8), increase in the expression of C_3b receptors (21, 22), and release of neutrophil lysosomal enzymes (7) could be important in the pathophysiology of glomerulonephritis. These actions of 12-HETE, if expressed in vivo, could alter glomerular capillary and basement membrane permeability to macromolecules. Furthermore, the hydroperoxy precursor of 12-HETE stimulates leukotriene synthesis in leukocytes (23). Finally, the recently described actions of 12-HETE on migration of vascular smooth muscle cells (24) raise the possibility of direct effects on mesangial cell contractility and proliferation.

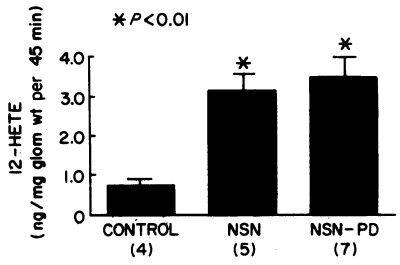

Figure 3. 12-HETE synthesis by glomeruli after platelet depletion. 12-HETE was measured by RIA after HPLC sample preparation 24 h after induction of NSN. NSN are platelet depleted. Both NSN and platelet-depleted NSN are significantly different from control but are not different from each other.

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