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Fifteen-S-hydroxyeicosatetraenoic acid (15-S-HETE) specifically antagonizes the chemotactic action and glomerular synthesis of leukotriene B4 in the rat

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Fifteen-S-hydroxyeicosatetraenoic acid (15-S-HETE) specifically antagonizes the chemotactic action and glomerular synthesis of leukotriene B4 in the rat. In models of experimental glomerulonephritis, there is temporal concordance between the shift in the glomerular cellular infiltrate from neutrophils (PMN) to macrophages/monocytes and the suppression of glomerular leukotriene B4 (LTB4) generation. Since macrophages are a rich source of 15-lipoxygenase (15-LO) products, we investigated whether the principal product of arachidonate 15-lipoxygenation, 15-S-hydroxyeicosatetraenoic acid (15-S-HETE), was capable of antagonizing the proinflammatory actions of LTB4 in the rat. PMN exhibited chemotaxis to LTB4 in a dose dependent manner with an LC_{50} of 10^{-8} M. When rat neutrophils were pre-treated with 15-S-HETE, chemotaxis to LTB4 was inhibited in a dose dependent manner (maximal at 30 μM 15-S-HETE) but, the same concentration did not inhibit chemotaxis to n-formyl-1-methionyl-1-phenylalanine (FMP). 12-S-HETE (30 μM) did not inhibit chemotaxis to LTB4. Glomeruli from rats injected with nephrotoxic serum three hours earlier generated increased levels of LTB4; prior exposure of such glomeruli to 15-S-HETE totally normalized LTB4 production. The glomerular production of 15-S-HETE and LTB4 was also determined 3 hours, 72 hours and 2 weeks after administration of nephrotoxic serum. Whereas there was an early, short-lived, burst of LTB4 followed by a return to baseline levels, the production of 15-S-HETE increased steadily over the two week period and was present in amounts fivefold greater than LTB4. Thus, these studies assign a role for locally generated 15-LO derivatives in arresting LTB4-promoted PMN infiltration and suppressing LTB4 synthesis. Coupled with our previous demonstration of counterregulatory interactions between lipoxins and cysteinyl leukotrienes, the current studies provide further support for a generalized anti-inflammatory role for 15-LO products through specific antagonism and/or inhibition of leukotriene synthesis and biologic activities.

Leukotriene B4 [(5S,12R)-dihydroxy-6,14,-cis-8,10,-trans-eicosatetraenoic acid (LTB4)], a product of the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism, is a potent chemoattractant whose primary target appears to be the polymorphonuclear leukocyte (PMN). LTB4 is the major 5-LO product released during experimental glomerulonephritis [1]. Recent studies have suggested that in nephrotoxic serum nephritis, LTB4-promoted PMN infiltration correlates strongly with the reduction in glomerular filtration rate [2]. Additionally, early inhibition of 5-LO abrogates the late development of proteinuria in the same model [3]. Interestingly, increased glomerular synthesis of LTB4 is seen early in nephrotoxic serum nephritis and subsequently falls below that of control animals [1], suggesting that LTB4 production during inflammation is under tight regulatory control. The mechanism whereby such regulation might occur has not been elucidated. Fifteen-S-hydroxyeicosatetraenoic acid (15-S-HETE) is a product of 15-lipoxygenase metabolism of arachidonic acid and is produced in many tissues, including the kidney [4]. Fifteen-S-HETE is known to have anti-inflammatory properties: Administration of 15-S-HETE has been shown to cause regression of psoriatic lesions in humans [5] and to significantly reduce the clinical severity of a canine arthritis model [6]. Recently, it has been shown that preincubation with 15-S-HETE can inhibit the generation of LTB4 from stimulated human PMNs [7]. Fifteen-S-HETE appears to be rapidly incorporated and stored in the phosphatidyl inositol fraction of membrane lipids, and subsequently is released upon cellular activation to generate alternate profiles of intracellular messengers and eicosanoids, in particular lipoxins A4 and B4 [7].

In view of these observations and previous observations suggesting that rat PMNs may not exhibit chemotaxis to LTB4 [8–10], we undertook the present studies to answer the following questions. First, do rat PMNs exhibit a chemotactic response to LTB4 in a concentration dependent manner similar to human PMNs? Secondly, dose pre-treatment of rat PMNs with 15-S-HETE results in inhibition of chemotactic movement toward LTB4? Thirdly, does 15-S-HETE treatment inhibit the generation of LTB4 by isolated glomeruli from rats with glomerular immune injury? Fourthly, is there evidence for glomerular production of 15-S-HETE during glomerular immune injury?

Methods

Determination of chemoattractant activity

Rat peritoneal exudate PMNs were used as the responding cells in the chemotaxis assay. PMN were obtained by peritoneal

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lavage four hours after a peritoneal injection of 10 ml thioglycolate broth culture medium without indicators (Difco Laboratories, Detroit, Michigan, USA). Cells were washed and suspended in Dulbecco's phosphate buffered saline (PBS) (Whittaker Bioproducts, Inc. Walkersville, Massachusetts, USA) at a concentration of 2×10^6 viable PMN per ml. Chemotactic assays were performed utilizing a multiwell chamber as described previously [11]. Responding cells were placed in the upper chamber and the potential chemoattractant was placed in the lower chamber separated by a polycarbonate filter with 3 micron pore diameters. Cell migration took place during a 30-minute incubation period in a humidified incubator at 37°C. After incubation, the filters were fixed and stained with Diff Quick. The cells that migrated through the filter were counted by oil immersion light microscopy (1000×). The chemotactic activity was determined by counting the PMNs which migrated toward the test chemoattractant in 10 oil immersion light microscopic fields. Each individual value represents the average of triplicate measurements. For all the experiments, the positive control was n-formyl-l-methionyl-l-phenylalanine (FMP) (10^{-5} M) and the negative control was PBS.

Control PMN were preincubated in PBS or 15-S-HETE for 20 minutes at 37°C in a humidified incubator. 15-S-HETE was provided by Dr. Alan Brash (Vanderbilt University, Department of Clinical Pharmacology) and 12-S-HETE was purchased from Biomol Research Laboratories, Plymouth Meeting, Pennsylvania, USA. Fifteen-S-HETE and 12-S-HETE each were prepared as stock solutions in absolute ethanol and were added to the appropriate volume of cell suspension to achieve the desired concentration. In each case, the final ethanol concentration to which cells were exposed was not more than 1%. Eicosanoid purity and quantity was checked by ultraviolet spectroscopy. Pretreatment with 15-S-HETE, 12-S-HETE, and 1% ethanol did not alter neutrophil viability which was measured as 95% by trypan blue vital dye exclusion.

Determination of LTB4 production by isolated glomeruli

Glomeruli were isolated from 200 g Munich Wistar rats three hours after intravenous injection of either nephrotoxic serum ($N = 3$) prepared as previously described [12], or with normal rabbit serum ($N = 3$) (control). Glomerular isolation was achieved by a modification of the differential sieving technique, aimed at optimizing glomerular viability. In brief, the removed kidneys were immediately placed in a physiologic buffer (Buffer A) consisting of 105 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2 mM Na_2HPO_4, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, 1 mM L-alanine, 10 mM HEPES, and 0.2% BSA at pH 7.4, which was prebubbled with 95% O_2/5% CO_2 for 30 minutes. The cortical tissue was then minced at 4°C and transferred to another beaker containing a solution of 0.3 mg/ml collagenase Sigma, Type I, (Sigma Chemical, St. Louis, Missouri, USA) in Buffer A, and bubbled in this solution with 95% O_2/5% CO_2 for 45 minutes at 37°C. Differential sieving was then carried out by passing cortical tissue sequentially through 150 and 75 micron sieves, with warming and gentle centrifugation. The glomerular preparation that was obtained was >95% pure. The isolated glomeruli from single animals were then pre-incubated with either buffer A (control) or 15-S-HETE for 20 minutes at 37°C. Thereafter, the calcium ionophore A23187 (2 μM) was added to each preparation for 45 minutes. In a similar manner glomeruli were isolated from single animals in order to determine the time course of LTB4 production. Glomeruli were isolated as described above from rats that had been previously injected with either normal rabbit serum (control; $N = 3$) or nephrotoxic serum [12] three hours ($N = 3$), 72 hours ($N = 3$) and two weeks ($N = 3$) earlier. The isolated glomeruli were then exposed to the calcium ionophore A23187 (2 μM) for 45 minutes. The reaction was stopped by the addition of three volumes of absolute ethanol. Subsequently the extraction and quantitation of LTB4 was performed by high-pressure liquid chromatography and radioimmunoassay (RIA) as described [1], and expressed as picograms per mg of glomerular protein. For the LTB4 RIA the coefficient of correlation was −0.9862 for the standard curve points 25, 50, and 100 picograms; and −0.9457 for the standard curve points 100, 200, and 400 picograms. The sensitivity of the standard curve was 12.5 picograms. The interassay coefficient of variation was 8.6% at 30 picograms per milliliter. The intra-assay coefficient of variation was 3.8% at 28 picograms per milliliter.

Determination of 15-S-HETE production by isolated glomeruli

Glomeruli were isolated as described above from rats that had been previously injected with either normal rabbit serum (control; $N = 3$) or nephrotoxic serum [12] three hours ($N = 3$), 72 hours ($N = 3$) and two weeks ($N = 3$) earlier. The isolated glomeruli were then exposed to the calcium ionophore A23187 (2 μM) for 45 minutes. The reaction was stopped by the addition of three volumes of ethanol. Subsequently the extraction and quantitation of 15-S-HETE was performed by high-pressure liquid chromatography and RIA. For the 15-S-HETE RIA the coefficient of correlation was −0.9577 for standard curve points 16, 40 and 100 picograms; and −0.9497 for standard curve points 100, 250 and 625 picograms. The sensitivity of the standard curve was 16 picograms. The interassay coefficient of variation was 15.4% at 73 picograms per milliliter. The intra-assay coefficient of variation was 7.2% at 100 picograms per milliliter.

Statistics

One way analysis of variance was performed between groups; $P < 0.05$ was considered to be significant.

Results

Rat neutrophils exhibited a chemotactic response to LTB4 in a concentration dependent manner (Fig. 1). The peak response was observed at a concentration of 10^{-6} M LTB4 while the half maximal response was observed at a concentration of 10^{-8} M (LC_{50}). These findings are consistent with the responses observed with human neutrophils [13].

Pre-treatment of neutrophils with 15-S-HETE (30 μM) for 20 minutes resulted in complete inhibition of chemotaxis to LTB4 (Fig. 1). These results are representative of three separate experiments. In each of these experiments, migration to PBS, which served as a negative control, was negligible. The effect of pre-treatment with 15-S-HETE on chemotaxis to LTB4 was titratable with no effect observed with pre-treatment with 10 μM, a significant intermediate effect with 15 μM, and a maximal effect with 30 μM of 15-S-HETE (Fig. 2).

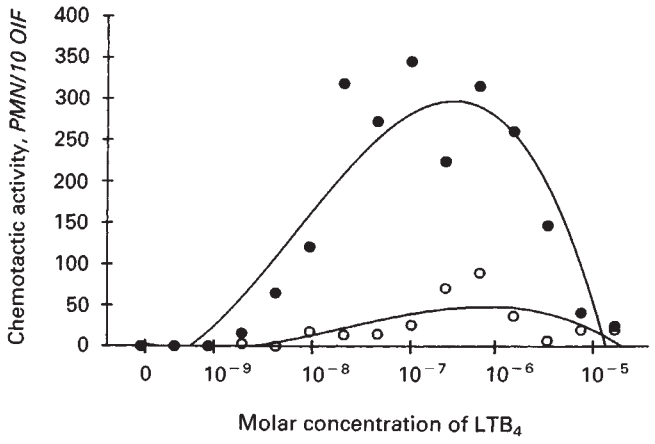

Fig. 1. *The chemotactic activity of LTB4.* The chemotactic activity is expressed as the mean number of neutrophils per 10 oil immersion light microscopic fields (OIF) ($N = 3$). The molar concentration of LTB4 is shown on the horizontal axis. Symbols are: (●) PMNs that were pre-treated with PBS (control); (○) PMNs that were pre-treated with 15-S-HETE (30 μM) for 15 minutes.

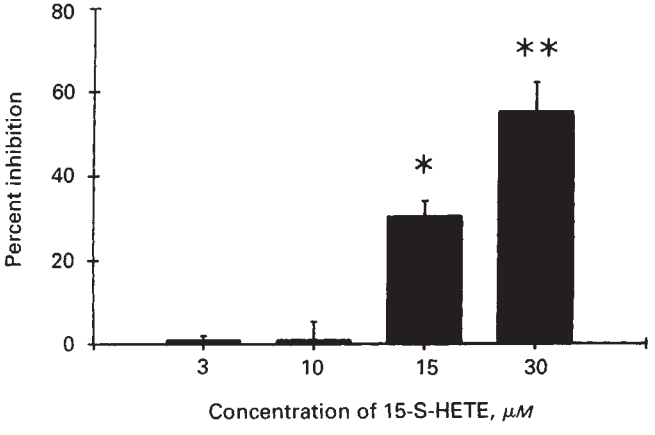

Fig. 2. *The effect of pre-treatment of PMNs with 15-S-HETE on chemotactic response to LTB4.* The data is expressed as percent inhibition of migration as compared to control PMNs which were not pre-treated. The micromolar concentrations of 15-S-HETE are shown on the horizontal axis. $*P < 0.05$, $**P < 0.005$ versus control (one way ANOVA).

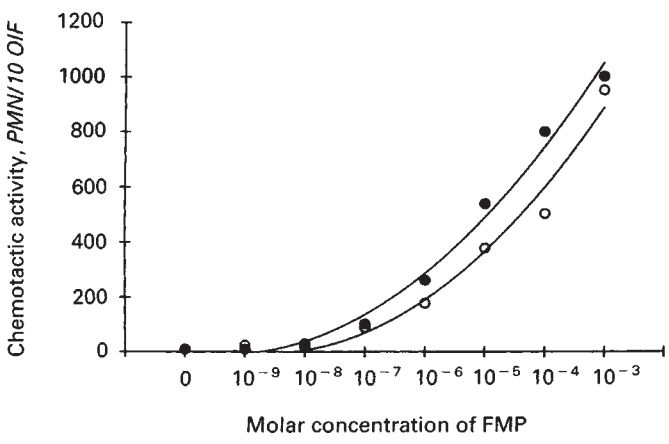

Fig. 3. *Effect of pre-treatment of PMNs with 15-S-HETE on PMN chemotactic response to FMP.* No significant effects are demonstrated. Chemotactic activity is defined in Figure 1.

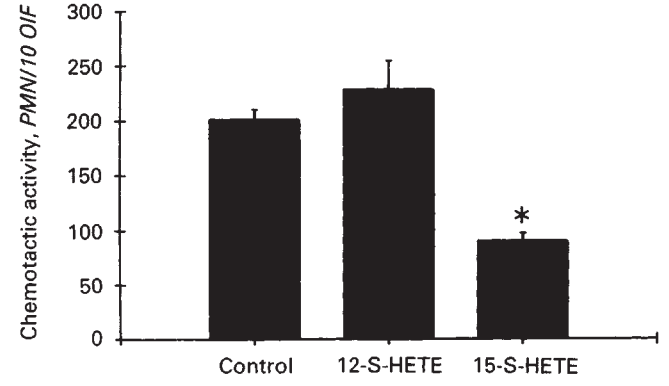

Fig. 4. *The effect of pre-treatment of PMNs with 12-S-HETE or 15-S-HETE.* PMNs were pre-incubated with either PBS (control), 12-S-HETE (30 μM) or 15-S-HETE (30 μM). Chemotactic activity is defined in Figure 1. ($N = 3$). $*P < 0.005$ versus control (one way ANOVA).

To determine the specificity of this effect to LTB4, we examined the effect of pre-treatment with 15-S-HETE on neutrophil migration to FMP. Figure 3 demonstrates the dose response curve of chemotaxis to FMP by control neutrophils and neutrophils that were pre-incubated with 15-S-HETE. There was no significant difference between the two.

To determine if the inhibition of LTB4-evoked chemotaxis was specific for 15-S-HETE, neutrophils were pretreated with 12-S-HETE at the same concentration (30 μM) and the above experiments repeated. Twelve-S-HETE had no effect on LTB4-promoted chemotaxis (Fig. 4). In addition, pretreatment with vehicle (1% ethanol) had no effect on neutrophil migration (data not shown).

In view of the fact that 15-S-HETE had been shown to decrease production of LTB4 by neutrophils [7], we examined the effect of 15-S-HETE on LTB4 production by isolated glomeruli. Figure 5 demonstrates the production of LTB4 measured from isolated glomeruli obtained from rats three hours after injection of nephrotoxic serum, and stimulated with 2μM calcium ionophore A23187. The data is represented as a percent of the total LTB4 measured from control (rabbit serum treated) glomeruli that were stimulated in an identical manner [total LTB4 measured, mean values ($N = 3$): control, 203.5 pg/mg protein, nephrotoxic serum, 454.8 pg/mg protein, nephrotoxic serum followed by pre-incubation with 15-S-HETE, 219.0 pg/mg protein]. Clearly, nephrotoxic serum nephritis resulted in increased production of LTB4 at three hours. Pre-incubation of glomeruli with 15-S-HETE for twenty minutes resulted in marked abrogation of LTB4 production ($P < 0.005$). Finally, to explore the physiologic relevance of 15-S-HETE during experimental glomerulonephritis we determined the production of 15-S-HETE by glomeruli isolated from rats three hours, 72 hours and two weeks after administration of nephrotoxic serum and stimulated with 2 μM calcium ionophore A23187. As shown in Figure 6, the production of LTB4 by isolated glomeruli followed the pattern that has been described previously [1, 14, 15]: there was an early peak in the first three

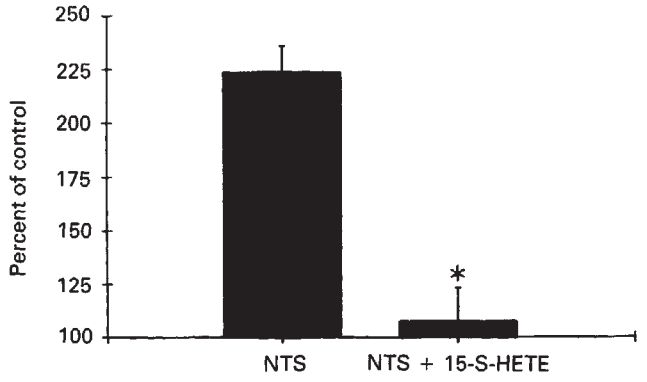

Fig. 5. *Production of LTB4 by isolated glomeruli.* The data represent production of LTB4 by isolated glomeruli obtained from rats three hours after injection of nephrotoxic serum, pre-treated with either PBS or 30 μM 15-S-HETE and stimulated with 2 μM calcium ionophore A23187. The data are expressed as an increase in LTB4 production over that measured from control (rabbit serum treated) glomeruli that were stimulated in an identical manner ($N = 3$). *$P < 0.02$.

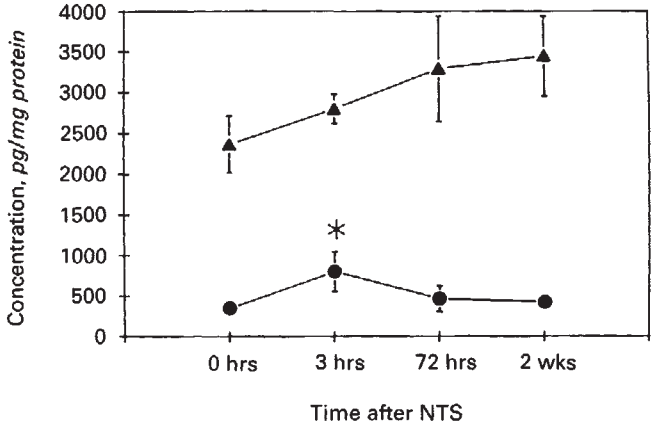

Fig. 6. *Production of 15-S-HETE (▲) and LTB4 (●) by isolated glomeruli.* The data represent production of 15-S-HETE and LTB4 by isolated glomeruli obtained from rats at 0 hour, 3 hours, 72 hours and two weeks after injection of nephrotoxic serum and stimulated with 2 μM calcium ionophore A23187. ($N = 3$). *$P < 0.05$ versus 72 hours (one way ANOVA).

hours following injury and a subsequent return to baseline levels. The production of 15-S-HETE, however, was fivefold greater than LTB4 production and increased steadily over the two week period following initial injury.

Discussion

Glomerular synthesis of LTB4 is enhanced markedly early in the course of several forms of glomerular immune injury, including those characterized by PMN infiltration, such as early nephrotoxic serum (NTS) nephritis [1, 14–16] and anti-thymocyte (a-Thy1.1 Ag)-antibody nephritis [17], or in the absence of PMN involvement, such as in cationic bovine gamma globulin (CBGG)-induced glomerular injury and passive Heymann nephritis (PHN) [15, 17, 18]. In these studies, LTB4 was measured in the supernates of isolated glomeruli either by high-pressure liquid chromatography and radioimmunoassay [1, 15–18] or by gas chromatography and mass spectroscopy [14]. The cellular origin of augmented LTB4 synthesis in injured glomeruli remains undefined. Lianos [1] and Schreiner, Rovin and Lefkowith [16] suggest non-leukocyte source in NTS nephritis, but implicate PMNs in the enhanced LTB4 generation of PHN and a-Thy1.1 Ag-induced nephritides [17]. While there is discrepancy as to its cellular origin during glomerular injury, all studies concur as to the *transient* nature of LTB4 release (LTB4 levels are undetectable beyond the first 24 to 48 hours of injury [1, 14–18]). In NTS-induced injury, suppression of LTB4 synthesis accompanies the shift in the glomerular infiltrate from one comprised mainly of PMNs to a macrophage/monocyte infiltrate, localized in the mesangium [1, 14–16]. The temporal concordance between the disappearance of PMNs and the suppression of LTB4 generation brings into focus the potential role of locally generated anti-chemotactic mediators in arresting LTB4-promoted PMN infiltration and possibly suppressing LTB4 synthesis. Since macrophages are a rich source of 15-LO products [4, 19–21], we investigated whether the principal product of arachidonate 15-lipoxygenation, 15-S-HETE, was capable of exerting counterregulatory effects to antagonize the proinflammatory actions of LTB4 in the rat. This became particularly relevant in view of the recent demonstration of the modulation of PMN activation by their prior incubation with 15-S-HETE and of the anti-chemotactic properties of the 15-LO product, lipoxin A4 [22].

At the outset, it was essential to demonstrate that rat PMNs do indeed respond to the chemotactic activity of LTB4, in view of past studies suggesting lack of activity for LTB4 in this species [8–10]. As demonstrated in Figure 1, rat neutrophils exhibit chemotaxis to LTB4 in a dose dependent manner similar to the response seen with human neutrophils [13]. The tapering of the response curve at supraphysiologic concentrations of agonist is also described for the response of human PMNs, and the peak response concentration and the LC50 are highly consistent with those described previously for human leukocytes [13].

When rat neutrophils were pretreated with 15-S-HETE, chemotaxis to LTB4 was inhibited with the peak response observed at 30 μM 15-S-HETE (Fig. 1). The inhibitory effect of 15-S-HETE displayed several characteristics which suggest strongly that it is biologically relevant: (i.) The degree of inhibition of LTB4-provoked chemotaxis was dependent on the concentration of 15-S-HETE (Fig. 2). (ii.) The inhibition of chemotaxis by 15-S-HETE was not due to a generalized toxic effect on PMN function, since the response of these cells to the chemotactic action of FMP, even at the highest concentration of 15-S-HETE tested, was still robust and displayed the expected dose-dependence (Fig. 3). Further, we did not observe a decrement in trypan blue dye exclusion with pre-treatment with 15-S-HETE. (iii.) The inhibitory action of 15-S-HETE appears to be specific to this eicosanoid, since a structurally similar compound, 12-S-HETE, was without effect on LTB4-induced chemotaxis (Fig. 4). Thus, both LTB4 and 15-S-HETE display reciprocal specificity in their biological interactions (Figs. 3 and 4).

The mechanism(s) underlying the capacity of 15-S-HETE to specifically antagonize the leukoattractant actions of LTB4

remained incompletely defined. The recent evidence by Brezinski and Serhan [7], however, suggests that the specific incorporation of 15-S-HETE into the phosphotidylinositol fraction of cell membrane lipids results in the generation of a 15-S-HETE substituted diacylglycerol (DAG) upon cellular stimulation by agonists which activate phospholipase C (PLC). The generation of this "altered second messenger" would be expected to markedly attenuate cellular responses. Interestingly, the interactions of LTB4 with PMN receptors has been convincingly linked to the activation of PLC and secondary generation of DAG and inositol trisphosphate [23]. Other potential mechanisms underlying the modulation of LTB4 actions by 15-S-HETE include possible effects on PMN LTB4 receptor binding affinity or number, or other interruptions of LTB4 receptor-coupled intracellular signalling pathways. These mechanisms are under investigation.

Additionally, we examined whether the anti-inflammatory action of 15-S-HETE was restricted to the antagonism of LTB4-evoked PMN attraction, or extended to the regulation of LTB4 biosynthesis. To begin an investigation of this question, we tested the capacity of 15-S-HETE to suppress the synthesis of LTB4 in glomeruli isolated from acutely inflamed rat kidneys under the influence of nephrotoxic serum. Since these glomeruli, isolated from the rat three hours after the injection of NTS, are already characterized by a marked PMN infiltrate [16], any effect of 15-S-HETE on LTB4 synthesis would not be a result of its anti-chemotactic action described above. Glomeruli isolated from NTS-treated animals displayed a 225% increase in LTB4 generation, as compared to rabbit serum-injected controls (Fig. 5, left bar). This finding is in concordance with those reported previously by numerous other investigators in this model of injury [1, 14, 15]. In dramatic contrast, however, pre-incubation of similarly isolated glomeruli with 15-S-HETE totally abolished the augmented synthesis of LTB4 by these inflamed glomeruli. Since LTB4 generation is itself a consequence of leukocyte activation [24], the mechanism underlying the inhibition of LTB4 synthesis by 15-S-HETE may well be similar to those described above for its abrogation of LTB4-mediated chemotactic responses. In fact, generation of LTB4 from human neutrophils following activation by the calcium ionophore A23187 is markedly attenuated by prior incubation with 15-S-HETE [7]. Finally, we determined the time course of 15-S-HETE production by isolated glomeruli in rats with nephrotoxic serum nephritis. Interestingly, the 15-S-HETE is produced in relative abundance as compared to LTB4, and in addition, the production of 15-S-HETE increases progressively over the two week period while the levels of LTB4 fall dramatically. The relatively high glomerular generation of 15-S-HETE correlates well with the higher levels required in vitro to achieve the observed antagonistic effects of 15-S-HETE on the actions of LTB4 (Fig. 2). The cellular source of 15-S-HETE produced by isolated glomeruli could not be determined from these experiments and may very well be the macrophage infiltrate as opposed to the endogenous glomerular cells. Indeed, the enhanced production of 15-S-HETE is greatest beyond 72 hours after the lesion is induced, and as such correlates well with the predominance of macrophages in the lesion at that time [16].

In nephrotoxic serum nephritis, suppression of LTB4 synthesis accompanies the shift in the glomerular infiltrate from one composed mainly of neutrophils to a macrophage/monocyte infiltrate, localized in the mesangium [16]. Since the macrophage lesion which follows is a potentially major source of 15-S-HETE [4, 19–21], it is tempting to speculate that 15-S-HETE released locally from macrophages is, in part, responsible for the suppression of LTB4 generation, and further contributes to the suppression of neutrophil infiltration by actively inhibiting LTB4-mediated chemotaxis.

In summary, we have demonstrated that rat PMNs are a target for LTB4-promoted chemotaxis, validating the use of rat models of inflammation to study the roles of LTB4 in glomerular injury. Further, these studies provide evidence that the two lipoxygenase products, LTB4 and 15-S-HETE, display reciprocally specific interactions in vitro, whereby 15-S-HETE antagonizes the chemotactic activity of LTB4 and appears to suppress its biosynthesis in inflamed glomeruli. Coupled with our previous demonstration of counterregulatory interactions between lipoxins and cysteinyl leukotrienes by competition for common binding sites [25], the current studies provide further support for a generalized anti-inflammatory role for 15-lipoxygenase products through specific antagonism and/or inhibition of leukotriene synthesis and biologic activities.

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