# CYCLOOXYGENASE BLOCKADE ELEVATES LEUKOTRIENE $E_4$ PRODUCTION DURING ACUTE ANAPHYLAXIS IN SHEEP

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The metabolism of arachidonic acid by both cyclooxygenase and lipoxygenase pathways produces several potent mediators of immune-induced and other inflammatory reactions (1–5). Inhibition of the cyclooxygenase pathway appears to account for the major effects of the nonsteroidal antiinflammatory drugs (NSAID).[1] However, inhibition of one pathway of arachidonic acid metabolism may alter the rates of formation and quantities of products of other pathways, and therefore, some of the effects of the NSAID might be secondary to changes in the formation of lipoxygenase products.

Acute systemic anaphylaxis and other immediate hypersensitivity reactions are accompanied by the release of several mediators, including histamine, platelet activating factor, prostaglandins, thromboxane, and leukotrienes (3–6). Slow reacting substance of anaphylaxis (SRS-A) is now known to consist of three sulfidopeptide leukotrienes (SPLT), $LTC_4$ (leukotriene $C_4$), $LTD_4$, and $LTE_4$, which are produced following the action of the enzyme 5-lipoxygenase on arachidonic acid. These SPLTs have several properties which suggest that they may participate in allergic reactions, including bronchoconstriction and alteration in vascular permeability. The other major product of the 5-lipoxygenase pathway, $LTB_4$, has potent effects on chemotaxis, adherence, and other actions of leukocytes (3–5).

We examined the production of several eicosanoids formed during systemic anaphylaxis in anesthetized sheep, in vivo. Our results show that cyclooxygenase blockade by indomethacin is associated with a marked increase in the concentration of $LTE_4$ in lung lymph. These studies suggest that some of the pharmacologic effects of NSAID may be related to increased elaboration of 5-lipoxygenase products, in addition to cyclooxygenase blockade.

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[1] *Abbreviations used in this paper:* ASE, *Ascaris suum* extract; HETE, hydroxyeicosatetrenoic acid; LT, leukotriene; NSAID, nonsteroidal antiinflammatory drug, RP-HPLC, reverse-phase HPLC; SPLT, sulfidopeptide leukotriene; TX, thromboxane.

## Materials and Methods

*Sheep Anaphylaxis.* Suffolk sheep weighing ~30 kg were immunized with a mixture of 5 mg *Ascaris suum* extract (ASE) and alum injected subcutaneously in areas drained by axillary and inguinal lymph nodes, 2 wk before each experiment. One day before each study, the sheep were injected intradermally with progressive dilutions of ASE, followed by intravenous administration of 23 mg of Evans' blue dye. Sensitized sheep were defined as those showing 5 mm bluing at the site injected with $\leq 0.1$ μg ASE. Sheep not immunized with ASE were considered to be nonsensitized controls if there was <5 mm bluing at the site injected with 100 μg ASE.

During general anesthesia (with 1% halothane in oxygen and mechanical ventilation, which maintained a constant tidal volume) a flow-directed pulmonary artery catheter was introduced via the left external jugular vein. A right thoracotomy was performed and the efferent duct of the caudal mediastinal lymph node was cannulated to obtain pulmonary lymph. A left thoracotomy was performed to place a flow meter transducer around the systemic and pulmonary arteries. Hemodynamics, and the flow rate and protein concentrations of lung lymph were monitored for 1–2 h until stable. Anaphylaxis was induced by intravenous administration of 3.0 mg ASE in 5 ml PBS.

*Eicosanoid Assays.* Levels of thromboxane $B_2$ ($TXB_2$) and of the prostacyclin metabolite, 6-keto-$PGF_{1\alpha}$ were determined by RIA as described previously (7, 8), using antisera provided by Dr. L. Levine. Blood was collected in tubes containing 38 mg sodium EDTA and 100 μg indomethacin, and lymph was collected in tubes containing 140 IU heparin; 0.1–0.2 ml portions of plasma and lymph were assayed for eicosanoids directly, or after dilution, as needed. LTs were assayed in lymph and plasma using a slightly modified RIA described previously (9). The antiserum to SPLT reacts with $LTC_4$, $LTD_4$, aind $LTE_4$. Because significant SPLT activity was found only in sheep lymph and was shown by HPLC to consist almost entirely of $LTE_4$, the RIA for SPLT was done primarily to detect $LTE_4$. The concentrations of $LTE_4$ were calculated by comparison to standard curves based on the displacement of [$^3$H]$LTC_4$ by $LTE_4$. In this assay, 50% inhibition of binding of [$^3$H]$LTC_4$ occurred at $LTE_4$ concentrations of 1.5 ng in a final volume of 1.0 ml per assay tube. In a similar assay for $LTB_4$, the binding of [$^3$H]$LTB_4$ was inhibited 50% by 0.7 ng $LTB_4$ in an assay volume of 1.0 ml. All samples and standards were stored at −80°.

*Reverse-phase HPLC (RP-HPLC).* A Waters Associates (Milford, MA) system for RP-HPLC, consisting of an M6000 pump, a model 440 absorbance detector and a C18μ-Bondapak column was used. The LTs were extracted from lymph onto Sep-pak C18 cartridges (Waters Associates) previously rinsed with methanol and water. After applying the lymph, the columns were rinsed with water and hexane, and LTs were eluted with methanol. The methanol was removed in a stream of $N_2$, and the residue was reconstituted in the HPLC mobile phase. The LT samples were applied and eluted isocratically with a solvent consisting of 67% methanol, 33% water, and 0.08% glacial acetic acid, neutralized to apparent pH 5.5 with 58% aqueous ammonia. Fractions of 1 ml were collected at a flow rate of 1 ml/min. The fractions were prepared for RIA by removing the solvent in vacuo using a Speed Vac concentrator (Savant, Hicksville, NY) and dissolving the residue in Isogel-Tris buffer, pH 7.4.

*Statistical Methods.* Data were analyzed by two-way analysis of variance with groups and time as factors.

*Materials.* [$^3$H]$TXB_2$, [$^3$H]6-keto-$PGF_{1\alpha}$, and [$^3$H]$LTC_4$, were obtained from New England Nuclear, Boston, MA. Standards of $LTC_4$, $LTD_4$, and $LTE_4$ were generously provided by Dr. J. Rokash, Merck-Frosst, Montreal, Canada. Standard $TXB_2$ and 6-keto-$PGF_{1\alpha}$ were purchased from the Upjohn Co., Kalamazoo, MI. Solvents for HPLC were of spectral grade (Burdich and Jackson, Muskegon, MI).

## Results

*Cyclooxygenase Products.* After monitoring and sampling devices were installed, baseline measurements were made at 30 or 60 min before challenge with ASE. Indomethacin, 10 mg/kg, was given intravenously to one group of sheep

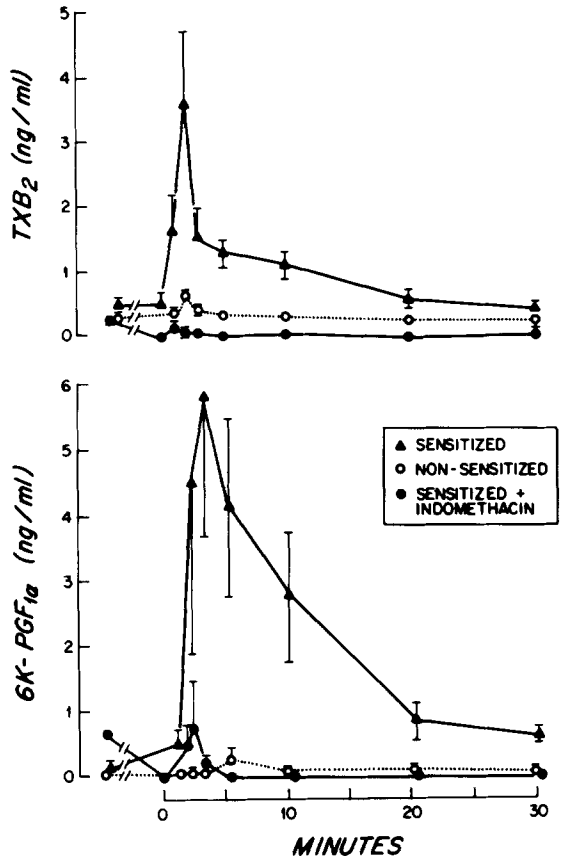


FIGURE 1. Levels of two cyclooxygenase products in sheep arterial plasma from antigen-challenged sensitized sheep, nonsensitized sheep, and sensitized sheep pretreated with indomethacin. Blood samples were obtained from a catheter in the ascending aorta, and $TXB_2$ and 6K-$PGF_1$ concentrations were determined by RIA of plasma. A sample was obtained from each animal either 30 or 60 min before antigenic challenge at $t = 0$, and at several time intervals after challenge. Means ± SEM are shown.

60 min before challenge. After ASE challenge of sensitized sheep, there was a rapid increase in arterial plasma levels of $TXB_2$ and 6-keto-$PGF_{1\alpha}$ (Fig. 1); these values returned to baseline levels within 30 min. In some animals (data not shown), selected measurements were made for an additional 3.5 h after challenge; no significant changes from baseline were observed. Statistical analyses showed significant differences in $TXB_2$ levels of sensitized ($n = 7$) compared with both nonsensitized ($n = 4$) and indomethacin-treated ($n = 4$) groups, $p = 0.0001$. For 6-keto-$PGF_{1\alpha}$, the sensitized group ($n = 7$) also differed from both nonsensitized ($n = 3$; $p = 0.007$) and indomethacin-treated ($n = 3$, $p = 0.004$) groups. Both $TXB_2$ and 6-keto-$PGF_{1\alpha}$ were measured in venous plasma and in pulmonary lymph from two to three sheep in each group (data not shown); in each case, the findings paralleled the results shown for arterial plasma.

*LT in Pulmonary Lymph.* Levels of SPLT became markedly elevated in pulmonary lymph within 20 min after induction of anaphylaxis in sensitized sheep

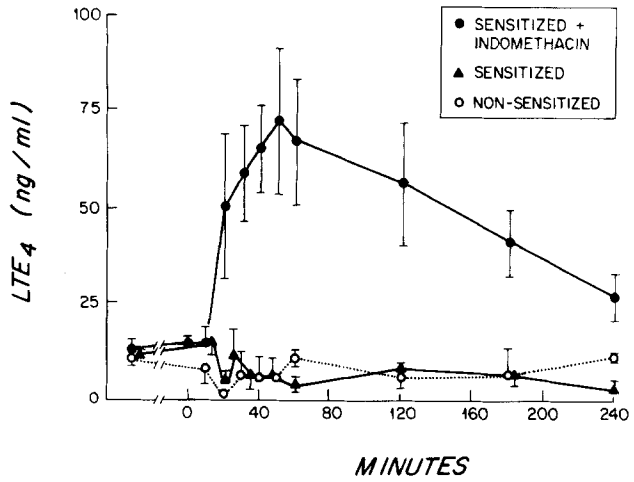


FIGURE 2. $LTE_4$ levels in lung lymph from antigen-challenged sensitized sheep, nonsensitized sheep, and sensitized sheep pretreated with indomethacin. Lymph was collected from a canula placed into the efferent duct of the caudal mediastinal lymph node. Samples were collected 30 or 60 min before and at several times after antigenic challenge at $t = 0$.

that had been pretreated with indomethacin (Fig. 2). The levels reached a maximum between 40 and 60 min, and gradually declined over the 4-h period of observation. Levels of SPLT did not become elevated above prechallenge levels in the other two groups of sheep. The concentrations of SPLT are reported as $LTE_4$ equivalents based on the results of RP-HPLC analyses described below. We also analyzed arterial and venous plasma for SPLT and $LTB_4$ and lymph for $LTB_4$ over the time course of these experiments in two or three sheep from each of the three groups. Low or undetectable levels of these compounds were found, and there was no increase in these levels after antigen challenge.

It is uncertain whether $LTE_4$ arises from $LTC_4$ and $LTD_4$ in situ in pulmonary tissue and lymphatics, in the canula during collection, or whether it arises at both sites. We have found that both $LTC_4$ and $LTD_4$ are largely (>80%) converted to $LTE_4$ in pulmonary lymph incubated in vitro for 60 min at 37 °C. However, in one experiment, lymph was collected from an indomethacin-treated sheep during anaphylaxis, and immediately diluted in five volumes of ethanol. Analysis by HPLC and RIA using the methods for the experiments shown in Fig. 3 showed that >90% of the SPLT was $LTE_4$, and that $LTC_4$ and $LTD_4$ together accounted for <10% of the sum of these three SPLT. These results show that formation of $LTE_4$ from its precursor SPLT must have occurred before the lymph was collected for analyses. It is possible that formation of $LTE_4$ occurs in the lymphatic system or, to some extent, in the canula draining the lymph. Regardless of the site(s) of formation of $LTE_4$, there is a marked elevation of SPLT concentration in lung lymph from indomethacin-treated sheep.

*Characterization of SPLT in Lymph by RP-HPLC.* To further define the material detected in lymph from indomethacin-treated sheep, pooled lymph from each of two animals that had elevated SPLT levels was extracted and fractionated separately by RP-HPLC. As shown in Fig. 3, all of the SPLT activity detected by RIA (>1 ng/ml $LTE_4$ equivalents) was found in two or three fractions

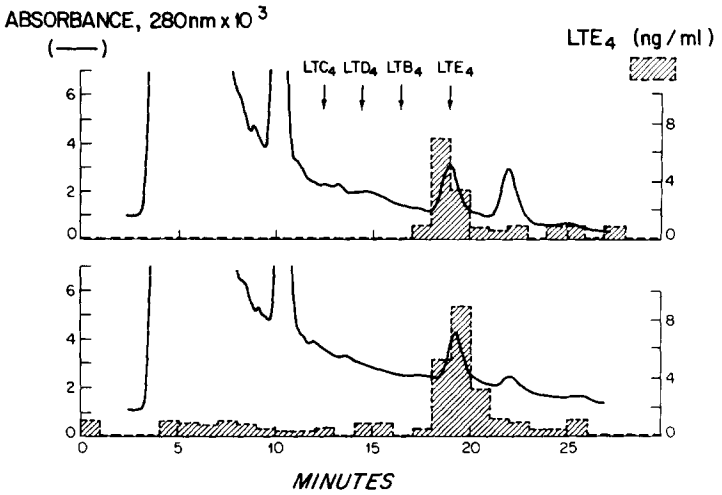


FIGURE 3. HPLC of pulmonary lymph from antigen-challenged sensitized sheep pretreated with indomethacin. Lymph was obtained 20–120 min after challenge, and was pooled from each of two sheep; pooled samples were processed separately. Following chromatographic separation, each fraction was tested for SPLT by RIA. SPLT activity was detected primarily in fractions corresponding to the elution position of the $LTE_4$ standard; these fractions also contained a peak of absorbance at 280 nm.

corresponding to the position of the $LTE_4$ standard. In both cases, a peak of absorbance at 280 nm was found in fractions of lymph corresponding to elution position of the $LTE_4$ standard.

The total amount of $LTE_4$ measured by RIA in fractions 19–21 in the experiments shown in the upper and lower panels of Fig. 3 was ~12 and 18 ng, respectively. These values were not corrected for losses during extraction and chromatography. In other experiments, authentic $LTE_4$ was extracted from sheep lymph with yields of 40–60%. We also estimated the quantities of $LTE_4$ detected in each experiment based on the integrated optical density of the absorbance peaks at the position of the $LTE_4$ marker in Fig. 3. We assumed a molar extinction coefficient for $LTE_4$ of $4.0 \times 10^4/M \cdot cm$ a value previously reported (10) for $LTC_4$. The amount of $LTE_4$ in the two experiments was estimated to be $22 \pm 5$ ng, uncorrected for yields. This value is in reasonable agreement with the quantities measured by RIA in the reconstituted fractions. Therefore, the SPLT measured in lymph from indomethacin-pretreated sheep appears to be $LTE_4$ on the basis of its RP-HPLC mobility and its optical absorbance properties.

## Discussion

These studies showed that acute anaphylaxis, in sheep pretreated with an amount of indomethacin sufficient to block the expected elevation of plasma cyclooxygenase metabolites by >90%, was associated with marked elevations in SPLT levels in pulmonary lymph. Nearly all of the SPLT measured in lymph by RIA was shown to have the mobility of $LTE_4$ on RP-HPLC. The maximum levels of $LTE_4$ of 75 ng/ml in sensitized sheep treated with indomethacin (Fig. 2) represents at least a fivefold increase above levels in the other two groups of

sheep ($p = 0.0001$, for the comparison between indomethacin-treated sensitized sheep and the other two groups). It is unclear why increased levels of SPLT are found in lymph while no corresponding increases are found in plasma. It is possible that the larger volume of blood draining the lung may contain lower levels of SPLT because of dilution.

We did not detect $LTB_4$ in lymph in any of our experiments using an RIA of nearly 12-fold greater sensitivity for $LTB_4$ than that of the SPLT RIA for $LTE_4$. Therefore, levels of $LTE_4$ must have exceeded levels of $LTB_4$ in lymph from indomethacin-treated sheep by at least 12-fold. It seems unlikely that our failure to detect $LTB_4$ in lymph is related to rapid degradation of this compound in the lung. First, we have shown that both $LTB_4$ and $LTE_4$ resist degradation in sheep lymph and heparinized blood in vitro at 37°C for 1 h (D. Robinson, and C. Melvin, unpublished experiments). Second, others (11) have reported that $LTB_4$ was not metabolized by isolated perfused rat lung administered either intravascularly or by inhalation. We therefore tentatively conclude that more SPLTs than $LTB_4$ are produced in sheep lung during anaphylaxis.

The production of greater quantities of SPLT than $LTB_4$ provides some evidence for the type of cells responsible for LT production in our experiments, because the patterns of 5-lipoxygenase products elaborated by certain cells is characteristic. Ionophore-stimulated neutrophils, monocytes, and alveolar macrophages produce more $LTB_4$ than SPLT, whereas eosinophils and mast cells produce substantially larger (6–35-fold) quantities of SPLT than $LTB_4$ (3). The conclusion that greater quantities of SPLT than $LTB_4$ were produced in indomethacin-treated sheep during anaphylaxis is consistent with the mast cell being the source of the LTs produced under these conditions.

The functional changes observed during anaphylaxis in the sheep are complex and will be reported elsewhere. In brief, pretreatment of sensitized sheep with indomethacin altered their physiologic responses to antigenic challenge in comparison to those of sensitized sheep. Indomethacin-treated sheep had reduced pulmonary vasoconstriction, increased cardiac output in the first 5 min after challenge, and an impressive degree of late tachycardia (5–30 min after challenge), associated with a reduced cardiac output (data not shown).

The SPLTs are potentially important mediators of anaphylactic hypersensitivity reactions. Each of these compounds, $LTC_4$, $LTD_4$, and $LTE_4$ is biologically active (3, 4, 12–15). The SPLTs are potent bronchoconstrictors for both peripheral and central airways (4, 15). Both $LTC_4$ and $LTD_4$ stimulate mucous secretion by the bronchial mucosa (16, 17). The SPLTs increase permeability in several vascular beds (3–5). They are potent vasoconstrictors and may markedly reduce cardiac output, probably related to both peripheral and coronary artery constriction (5, 18, 19). Cyclooxygenase inhibition may modify the response of pulmonary and vascular tissues to LTs under certain conditions. For example, the in vitro $LTD_4$-induced contractile response of sheep trachea, but not of bronchi or lung parenchymal strips, was enhanced by meclofenamate (20). On the other hand, the increased pulmonary vascular resistance resulting from injection of $LTD_4$ into sheep pulmonary artery was inhibited by cyclooxygenase blockade (21).

Several previous studies showed that cyclooxygenase inhibition by NSAIDs

may enhance formation of lipoxygenase products in vitro. Indomethacin inhibition of $TXB_2$ and HHT synthesis by platelets was associated with increased levels of the 12-lipoxygenase product 12-hydroxyeicosatetraenoic acid (12-HETE). The increased synthesis of 12-HETE was equal to the sum of the reduced synthesis of $TXB_2$ and HHT, suggesting that available free arachidonic acid was shunted to lipoxygenase products (22). Other in vitro studies showed that cyclooxygenase inhibition augmented SPLT activity in guinea pig trachea (23) and human bronchus (24), and also augmented 15-lipoxygenase products in human neutrophils (25). More recently, Ham et al. (26) have shown that $PGE_1$ and $PGE_2$ in the range of $10^{-7}$ M inhibited the formation of $LTB_4$ and 5-HETE in cytochalasin B–treated rat neutrophils stimulated by a chemoattractant peptide. These authors suggested that cyclooxygenase inhibitors may augment the synthesis of 5-lipoxygenase products by removing the inhibition of this pathway by prostaglandins (26). In one in vivo study, cyclooxygenase blockade also was associated with slight (~40%) elevation in $LTB_4$ synthesis by peritoneal exudates in rats (27).

The demonstration in our experiments that cyclooxygenase blockade is associated with increased levels of SPLT in vivo suggests that increased production of lipoxygenase products may account for some of the pharmacologic effects attributed to NSAIDs. The results support the hypothesis that the adverse pulmonary and circulatory reactions to NSAIDs in some patients with aspirin sensitivity may be related to inhibition of cyclooxygenase and enhanced production of SPLTs (28). The vasoconstrictor effects of SPLT in renal, cardiac, and other vascular beds, (29, 30) raise the possibility that increased SPLT production in these organs might contribute to the decreased renal blood flow, impaired renal function, and aggravation of congestive heart failure observed in some patients treated with NSAIDs. Finally, because the LTs are potent mediators of inflammation, the increased production of these compounds during treatment with NSAIDs may reduce the therapeutic efficacy of these drugs.

## Summary

We examined changes in the levels of eicosanoids in blood and pulmonary lymph of anesthetized sheep undergoing acute anaphylaxis. Within 1–3 min of intravenous antigenic challenge of previously sensitized sheep, there were ~7–30-fold elevations in mean arterial plasma levels of thromboxane $B_2$ and 6-keto-prostaglandin $F_{1\alpha}$, respectively, as measured by RIA. Negligible changes in levels of these cyclooxygenase products were found in both nonsensitized sheep and in sensitized sheep treated with indomethacin before antigenic challenge. In contrast, no changes in levels of sulfidopeptide leukotrienes (SPLT) in pulmonary lymph were detectable by RIA during anaphylaxis in sensitized or nonsensitized sheep, but levels of SPLT in indomethacin-treated sensitized sheep increased more than fivefold above levels in lymph from both other groups of animals. The immunoreactive SPLT in lymph from indomethacin-treated sheep was accounted for as $LTE_4$, as demonstrated by mobility on HPLC and absorbance at 280 nm. These results support the possibility that certain undesirable effects of nonsteroidal antiinflammatory drugs, such as cardiopulmonary reactions in aspirin-sensitive individuals, and impaired renal and cardiac function during therapy with these drugs, may be related in part to augmented synthesis of the

5-lipoxygenase pathway products, especially those of the sulfidopeptide class. Increased LT production could also limit the antiinflammatory effectiveness of these drugs in many disease states.

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