

Leukotriene-associated Toxic Oxygen Metabolites Induce Airway Hyperreactivity*

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The effect of toxic oxygen metabolite scavengers was examined in a guinea pig trachealis model of leukotriene (LTD₄)-induced synergism upon histamine contractures. Under both physiologic (2.5 mM) and low (OmM) extracellular calcium conditions, LTD₄ (10⁻⁷ to 10⁻⁹ M) potentiated histamine isometric tension responses. This LTD₄-induced histamine hyperresponse was inhibited by pretreatment with superoxide dismutase. Inhibition of LTD₄ receptor binding by FPL 55712 (10⁻⁵ M) also aborted

this interaction. Actual trachealis superoxide anion (O₂⁻) generation by LTD₄ was observed with a maximal release of 15 nM O₂⁻/CPK unit × 10⁻² over 60 min. Phorbol myristate acetate (PMA) also generated O₂⁻ in this preparation. Trachealis muscle hyperreactivity to histamine induced by 10⁻⁸ M LTD₄ assayed in OmM (Ca⁺⁺)_E was not induced by PMA. It is concluded that exogenous LTD₄ activates toxic oxygen metabolites which interact to induce an acquired hyperreactivity to agonist histamine in trachealis smooth muscle.

Leukotrienes (LT) are highly potent spasmogens of central and small airways in man and guinea pig and may be involved directly in the clinical and inflammatory expression of asthma or may contribute to the fundamental abnormality of hyperreactivity.^{1,2} Since previous work from this laboratory suggested an interaction between leukotrienes and toxic oxygen radicals under conditions of calcium dependent myorelaxation,³ we sought to determine whether toxic oxygen products are associated with leukotrienes and airways hyperreactivity.

MATERIALS AND METHODS

Isometric tension of guinea pig trachealis to agonists was obtained after equilibration at a resting tension of 2.0 g for 60 min at 37°C in Krebs-Henseleit (KH) buffer gassed with 95 percent O₂-5 percent CO₂. KH contained either physiologic extracellular calcium (2.5 mM)_E or was calcium-free/low calcium (OmM). Isometric tension methods and KH composition have been recently detailed.⁴ Cumulative histamine concentration-response curves by successive microliter increments were acquired in a paired manner using the same trachealis without and with synthetic LTD₄; LTD₄ exposure lasted for 20 min prior to, and then throughout, histamine dosing. For acetylcholine (ACH) studies, histamine dosing was similarly performed at the peak (10 min) of ACH tension. Tension responses are expressed as a percentage of the maximal tension produced by a reference standard of histamine (2.72 × 10⁻⁵ M) applied to each trachealis in 2.5 mM (Ca⁺⁺)_E KH before each experiment. Muscles not responding to this standard within ± 10 percent of a mean tension of 1,700 mg were discarded. Molar concentration of agonist causing 50 percent of maximum contraction (EC₅₀) was determined graphically; for (Ca⁺⁺)_E-free studies, EC₅₀ is 50 percent of the same reference histamine standard corrected for the OmM (Ca⁺⁺)_E baseline tension fall to the initial 2.5 mM calcium baseline tension.⁵ Mean differences (± SEM) were analyzed by the Student's *t*-test for paired samples; *p* < 0.05 was determined as the level of significance.

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Superoxide anion (O₂⁻) trachealis release was assayed at 37°C by a modification of the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome-C at 550 nm.^{6,7} The reaction mixture consisted of 120 μm ferricytochrome-C and synthetic LTD₄ (10⁻¹⁰ to 10⁻³ M) to a total volume of 1.5 ml with KH. All samples were assayed in duplicate in the presence or absence of SOD (300 U/ml), in room air, and for 60 min. For inhibition studies, FPL 55712 was incubated 20 min prior to LTD₄. Each reaction tube of 1.5 ml KH contained one trachealis ring (muscle and cartilage) with a mean wet weight of 13 mg. After 60 min, the reaction tubes were immersed in an ice bath, the trachea removed and rapidly frozen at -4°C for creatine phosphokinase (CPK) assay. The remaining ferricytochrome-KH buffer was centrifuged at 1,200 × *g* for 5 min at 4°C, and the supernatant assayed for cytochrome-C reduction in a Beckman DV spectrophotometer at 550 nm. OD values were converted to nmol O₂⁻ using a molar extinction coefficient of 2.1 × 10⁴ cm⁻¹.⁷ The values obtained in the presence of 300U SOD were subtracted from all other values. Appropriate blanks and controls were also assayed.

The remaining trachealis was assayed colorimetrically for muscle CPK activity by creatine formation in a CPK-catalyzed ADP/phosphocreatine reaction using Sigma kit No. 520 (St. Louis, Missouri). Briefly, the trachea is homogenized in 0.35 ml cold KH, and centrifuged at 3,000 × *g* for 20 min at 4°C. Supernatant (0.1 ml) is added to 0.5 ml phosphocreatine, incubated for 20 min and then reacted with 0.2 ml ADP-glutathione solution for 30 min. The reaction is terminated with 0.2 ml of 0.05 mol/L *p*-Hydroxymercuribenzoate. Then to each tube is added 1.0 ml α-naphthol and 1.0 ml, 0.05 percent diacetyl solution and 7.0 ml distilled, deionized water. Maximum color development is permitted for 20 min at 37°C, following which the tubes are centrifuged at 1,200 × *g* for 5 min at 4°C. The supernatants are transferred to cuvettes and absorbance read at 520 nm. The test run is compared to blanks not containing the tracheal ring homogenate. A standard curve is prepared with creatine, whose production is proportional to CPK activity. One CPK unit results in the phosphorylation of 1 nM of creatine/min. Mean CPK units of 30 tracheal rings was 118 ± 6.5 with a coefficient of variation of 6 percent. Final trachealis superoxide formation is expressed as nM O₂⁻/CPK unit × 10⁻² for 60 min reaction time.

RESULTS

Cumulative histamine concentration-tension responses in 2.5 mM (Ca⁺⁺)_E following LTD₄ (10⁻⁹ to 10⁻⁷ M) incubation were hyperresponsive, exhibiting

Table 1— EC_{50} and Maximal Tension (Mean \pm SEM) in 2.5 mM $(Ca^{++})_E$

Condition	N	EC_{50} (10^{-7} M)	p^*	Maximal isometric tension (%)†	p^*
Histamine	25	6.48 ± 0.42	—	96 ± 1.9	—
Histamine + LTD ₄					
10^{-9} M	8	3.55 ± 0.70	<0.05	96 ± 1.6	>0.8
10^{-8} M	6	1.20 ± 0.26	<0.01	111 ± 3.0	<0.001
10^{-7} M	19	0.96 ± 0.19	<0.001	135 ± 3.5	<0.001
Histamine + LTD ₄ (10^{-7} M) + SOD (300 U/ml)	11	5.20 ± 0.75	>0.4 ($<0.001\ddagger$)	99 ± 2.3	>0.7 ($<0.001\ddagger$)

*Paired *t*-test, compared to histamine alone.

†Percent of exogenous histamine standard.

‡Paired *t*-test, compared to histamine + LTD₄ (10^{-7} M)

an increase in both maximal tension and a leftward shift of the EC_{50} (Fig 1; Table 1). The maximal mg isometric tension values for histamine alone and for histamine with LTD₄ (10^{-7} M) were $1,710 \pm 195$ and $2,308 \pm 305$, respectively ($p < 0.01$). Although acetylcholine alone (10^{-5} M) increased the isometric tension baseline by 53

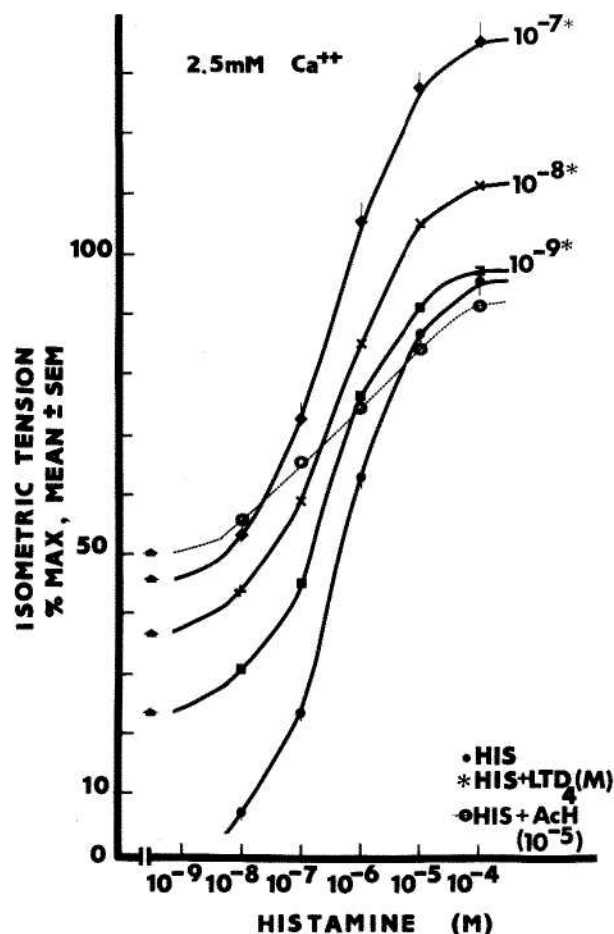


FIGURE 1. Histamine cumulative concentration-isometric tension responses with and without LTD₄ in 2.5 mM $(Ca^{++})_E$: interaction reveals a LTD₄ (10^{-7} to 10^{-9} M) dose-dependent hyperresponse in maximal isometric tension (data presented as percentage of maximal response to exogenous standard). A leftward shift in EC_{50} paralleled these observations (Table 1). The effect of each LTD₄ dose upon the resting isometric baseline is shown by arrows. Data are mean \pm SEM.

percent (Fig 1), the maximal response to histamine before preincubated with acetylcholine was not greater than with histamine alone at 10^{-4} M ($p > 0.5$, $n = 6$).

These data were reanalyzed as the histamine tension component only of these responses by subtracting the tension caused by LTD₄ from the total tension, *eg*, LTD₄ + histamine. A significant histamine hyperresponse was observed following LTD₄ 10^{-7} M incubation, both in percentage of maximal tension and in a leftward shift of the EC_{50} (Fig 2; Table 2). In contrast, 10^{-5} M acetylcholine did not enhance the histamine contractures (Table 2).

These relationships were also examined under calcium-free KH conditions with 10^{-7} M LTD₄. A leftward shift of the histamine-response curve ensued with both

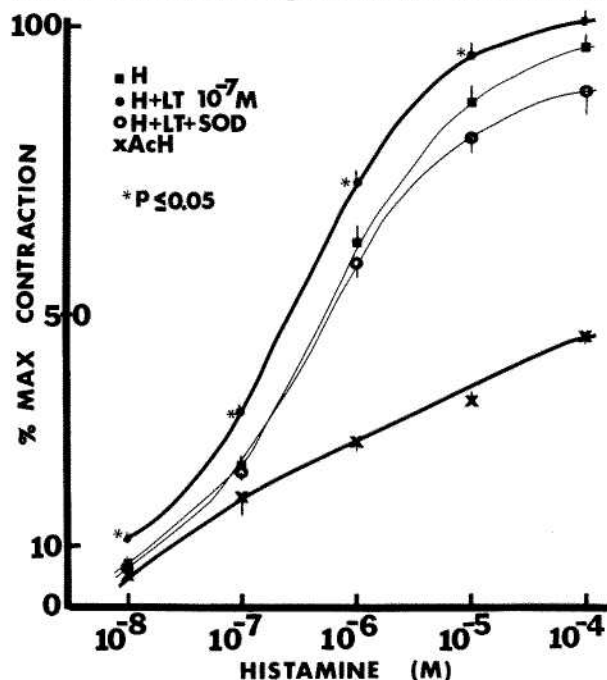


FIGURE 2. Histamine component only of the histamine/LTD₄ interaction: an increase in maximal tension and leftward shift of EC_{50} was observed after subtracting the LTD₄ component of isometric tension from the total LTD₄ + histamine isometric tension. Acetylcholine (ACH) response is blunted. Inhibition of the hyperresponse in the histamine component was observed with SOD (300 U/ml) (Table 2).

Table 2—Dose-% Responses, Histamine Component only (2.5 mM $[Ca^{++}]_E$); Mean \pm SEM

	N	Histamine concentration (m)					EC_{50} ($\times 10^{-7}$ m)
		10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
Histamine alone	24	7.1 ± 0.62	23.8 ± 1.3	63.0 ± 2.6	87.0 ± 2.6	96.1 ± 1.9	6.48 ± 0.42
Histamine alone vs							
Histamine + LTD ₄ (10^{-7} M)*	—	<0.05	<0.01	<0.05	<0.05	>0.3	<0.01
Histamine + LTD ₄ (10^{-7} M)	17	10.2 ± 0.83	32.2 ± 2.0	72.2 ± 2.5	94.0 ± 3.0	100.2 ± 2.5	4.20 ± 0.56
Histamine + LTD ₄ (10^{-7} M) vs							
Histamine + LTD ₄ (10^{-7} M) + SOD (300 U/ml)*	—	<0.05	<0.01	<0.01	<0.01	<0.05	<0.01
Histamine + LTD ₄ (10^{-7} M) + SOD (300 U/ml)	14	6.9 ± 1.2	23.0 ± 1.6	59.2 ± 2.9	80.0 ± 2.1	88.0 ± 3.9	6.60 ± 0.41
Histamine + acetylcholine (10^{-5} M)	6	7.2 ± 0.2	18.0 ± 3.0	28.1 ± 1.6	35.0 ± 1.0	46.0 ± 0.3	6.37 ± 1.27

*Paired *t*-test.

a greater maximal percentage response and lower EC_{50} (Fig 3; Table 3). This synergism occurred despite no active tension development by 10^{-7} M LTD₄ in OmM (Ca^{++})_E. These data in OmM (Ca^{++})_E for LTD₄ are identical to those previously reported.⁸ The specificity of the LTD₄ effect in OmM (Ca^{++})_E was demonstrated by pretreatment of the trachealis with acetylcholine (5.5×10^{-7} M, an equivalent concentration) instead of LTD₄. No enhancement of the histamine response

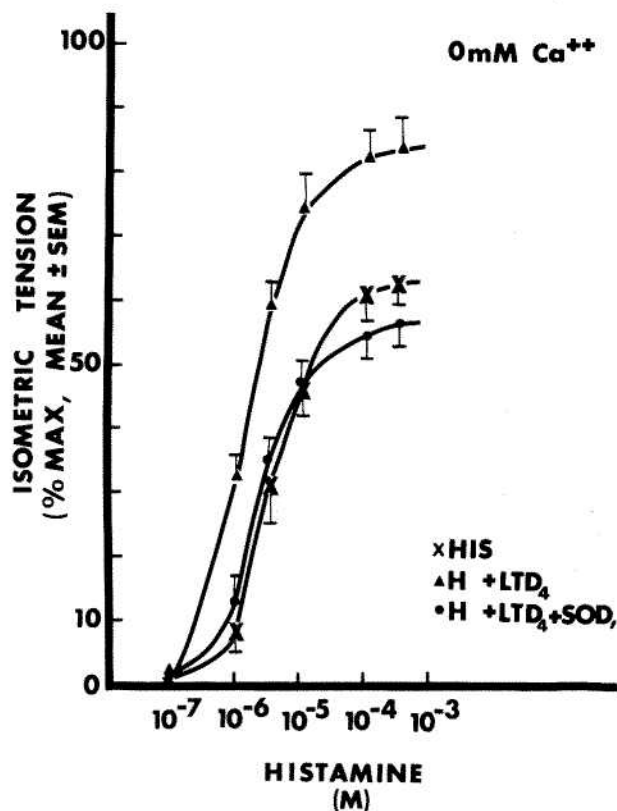


FIGURE 3. Histamine LTD₄ interactions in zero mM (Ca^{++})_E: removal of extracellular calcium results in persistence of LTD₄-induced histamine hyperresponse. In contrast to studies in 2.5 mM (Ca^{++})_E, LTD₄ (10^{-7} M) does not increase baseline tension in OmM (Ca^{++})_E. Therefore, depicted curves are essentially the histamine component only. As also shown in Table 3, there is a statistically different increase in percentage of maximal tension and leftwards shift of EC_{50} for histamine exposed to LTD₄ (10^{-7} M). Superoxide dismutase (300 U/ml) also fully inhibited this interaction.

developed (Table 3).

Recent work from this laboratory³ indicated that enhanced trachealis myorelaxation under OmM (Ca^{++})_E conditions following leukotriene contraction was inhibited by specific enzymes presumed to protect cells against activated oxygen products. Hence, the above-cited interactions between LTD₄ and histamine were retested in the presence of superoxide dismutase (SOD). SOD (300 U/ml) was incubated in OmM (Ca^{++})_E KH 10 min prior to and during the identical histamine and histamine-LTD₄ cumulative-concentration conditions. As shown in Figure 3, SOD fully inhibited the LTD₄ (10^{-7} M) synergism upon histamine (Table 3). SOD had no effect upon the resting baseline tension, the exogenous histamine standard, or the histamine dose-response (Table 3). Denaturation ($100^\circ\text{C} \times 10$ min) of SOD eliminated its inhibitory effect upon the LTD₄-histamine interaction (Table 3); the denatured enzyme did not affect resting trachealis tone or the cumulative histamine-response curve.

In a separate group of experiments, the effect of SOD alone or catalase (350 U/ml) alone was tested to determine which toxic oxygen species was associated with the observed LTD₄-induced histamine hyperreactivity. Only SOD inhibited the LTD₄-induced histamine hyperreactivity; catalase had no effect.

The semiselective SRS-A/LT antagonist FPL 55712 (10^{-5} M) was incubated for 10 min prior to and during these LTD₄-histamine studies in OmM (Ca^{++})_E buffer solution. Complete inhibition of LTD₄ sensitization ensued despite the absence of any LTD₄ tension development in low (Ca^{++})_E KH; (EC_{50} $6.8 \pm 1.7 \times 10^{-6}$ M, $p > 0.3$ compared to histamine, $[n = 8]$, Table 3). Hence, inhibition of LTD₄ binding to its receptor site may interfere with the subsequent production of toxic O₂ products. Additionally, ascorbic acid pretreatment (5.7×10^{-5} M) fully inhibited the LTD₄-induced synergism to histamine (Table 3).

We then examined the inhibiting influence of superoxide dismutase (300 U/ml) upon the histamine-LTD₄ interactions at physiologic extracellular calcium concentrations (2.5 mM Ca^{++})_E. Significant inhibition was

Table 3— EC_{50} and Maximum Tension (Mean \pm SEM) in $OmM (Ca^{++})_E$

Condition	N	EC_{50} ($10^{-6}M$)	p^*	Maximal tension (%)	p^*
Histamine	7	5.58 ± 0.9	—	63.0 ± 4.3	—
Histamine + LTD ₄ ($10^{-7}M$)	10	1.57 ± 0.28	<0.01	84.1 ± 5.4	<0.01
Histamine + Acetylcholine ($5.5 \times 10^{-7}M$)	5	5.26 ± 0.75	>0.8	60.2 ± 1.8	>0.6
Histamine + LTD ₄ ($10^{-7}M$ + SOD†)	9	4.46 ± 0.65	>0.3 ($<0.01\ddagger$)	57.2 ± 4.0	>0.3 ($<0.01\ddagger$)
Histamine + SOD	8	5.66 ± 0.9	>0.9	66.0 ± 6.0	>0.7
Histamine + LTD ₄ ($10^{-7}M$) + SOD (Denatured)	7	1.55 ± 0.39	<0.01	77.4 ± 5.5	<0.05
Histamine + LTD ₄ ($10^{-7}M$) + Ascorbic Acid ($5.7 \times 10^{-5}M$)	5	6.58 ± 0.7	>0.3 ($<0.001\ddagger$)	55.0 ± 3.1	>0.2 ($<0.01\ddagger$)

*Compared to histamine alone.

†SOD = 300 U/ml

‡ = Compared to histamine + LTD₄ ($10^{-7}M$)

observed in 2.5 mM Ca^{++}_E upon the cumulative total LTD₄ ($10^{-7}M$)-histamine response or the histamine component alone response (Fig 2, 4; Tables 1, 2). Thus, with both forms of analysis, SOD inhibited the increased isometric tension responses and the leftward shifted EC_{50} s.

Because of these observations, actual extracellular superoxide anion (O_2^-) generation was measured. The trachealis released O_2^- in a concentration-dependent mode following LTD₄ exposure (Fig 5) with a maximal O_2^- generation of 15.1 ± 1.1 nM O_2^- /CPK unit $\times 10^{-2}$ over 60 min. This is a mean generation rate of 0.25 nM O_2^- /min (per CPK unit $\times 10^{-2}$) at LTD₄ $10^{-6}M$. The LTD₄ EC_{50} was $2.5 \times 10^{-8}M$, with a threshold concentration of $5 \times 10^{-10}M$. A unit dose of synthetic LTC₄ ($10^{-7}M$) resulted in 6.6 nM O_2^- /CPK 10^{-2} unit per 60 min. Interestingly, neither histamine, acetylcholine or PGF_{2 α} (all $10^{-5}M$) released any O_2^- . Addition of FPL 55712 (10^{-5} to $10^{-7}M$) resulted in inhibition of LTD₄-induced O_2^- generation; EC_{50} with FPL 55712 ($10^{-5}M$) was $7.0 \times 10^{-6}M$ ($p < 0.01$) (Fig 5). This inhibition by FPL 55712 was competitive, as demonstrated by: a) mean maximal O_2^- response to $10^{-3}M$ LTD₄ was not statistically different ($p > 0.5$) compared to $10^{-3}M$ LTD₄ plus FPL 55712 ($10^{-5}M$), b) equivalency (Student's *t*-test) of the slopes of the regression lines derived by the method of least squares employing the linear portion (between 20 and 80 percent) of the respective dose-response curves, and c) calculation of the weighted log (x-1) regression line (Schild plot) with reference to the negative logarithm of the molar concentration of FPL 55712; the derived linear equation slope was -1.0021 ± 0.110 , not significantly different from -1.0 .⁹

We then compared LTD₄-induced extracellular superoxide anion release by the trachealis preparation to that of 4 β -phorbol 12-myristate 13-acetate (PMA), known to stimulate O_2^- generation from polymorphonuclear leukocytes.⁶ A concentration-response was

observed between PMA (1 to 6 $\mu g/ml$) and O_2^- release, with an EC_{50} of 1.6 $\mu g/ml$. PMA exhibited a maximal intrinsic potency approximately one-third that of LTD₄, eg, PMA 5.6 vs LTD₄ 15.0, nM O_2^- /CPK unit $\times 10^{-2}$, at 60 min of incubation. At these concen-

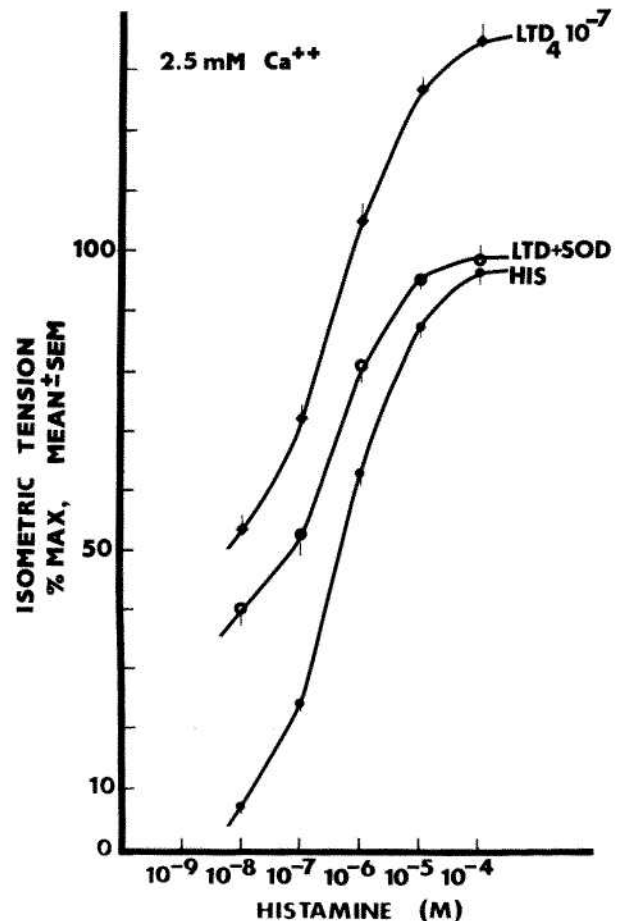


FIGURE 4. Interaction of histamine with LTD₄ ($10^{-7}M$) in 2.5 mM $(Ca^{++})_E$ and the effect of pretreatment with superoxide dismutase (SOD): SOD (300 U/ml) inhibited the LTD₄-induced histamine hyperresponse. Both maximal isometric tension and EC_{50} returned to control levels following SOD (Tables 1, 2).

Table 4—Dose-response Histamine with LTD₄ or PMA (in OmM [Ca⁺⁺]_E) as a Percent of Maximal Standard Response ± SEM

Histamine concentration (M)	Histamine alone (n=7)	Histamine + LTD ₄ (10 ⁻⁸ M) (n=7)	Histamine + PMA (6 μg/ml) (n=6)	p*
1 × 10 ⁻⁶	15.1 ± 3.4	31.0 ± 4.4	10.2 ± 1.7	<0.001
5 × 10 ⁻⁵	33.0 ± 2.0	43.1 ± 4.7	25.0 ± 3.8	<0.01
1 × 10 ⁻⁵	40.3 ± 3.1	55.3 ± 3.0	38.3 ± 2.7	<0.001
1 × 10 ⁻⁴	47.0 ± 2.2	66.0 ± 3.0	47.0 ± 1.5	<0.001
1 × 10 ⁻³	48.0 ± 2.3	70.1 ± 2.1	53.0 ± 2.1	<0.001

*Paired *t*-test, histamine + LTD₄ (10⁻⁸ M) vs histamine + PMA (6 μg/ml)

Table 5—EC₅₀:histamine Responses with LTD₄ or PMA in OmM (Ca⁺⁺)_E

Condition	N	Mean EC ₅₀ (10 ⁻⁶ M) ± SEM	p*
Histamine alone	7	5.58 ± 0.9	—
Histamine + LTD ₄ (10 ⁻⁸ M)	7	2.76 ± 0.6	<0.01 histamine alone vs histamine + LTD ₄ (10 ⁻⁸ M)
Histamine + PMA (6 μg/ml)	6	5.30 ± 1.2	>0.6 histamine vs histamine + PMA (6 μg/ml)
			<0.05 histamine + LTD ₄ (10 ⁻⁸ M) vs histamine + PMA (6 μg/ml)

*Paired *t*-test.

trations, PMA did not induce any tension, and hence no histamine release. Similarly, LTD₄ (10⁻⁷M) was free from histamine-releasing ability as evidenced by failure of diphenhydramine [10⁻⁶M] to inhibit an LTD₄ contracture: (LTD₄ [10⁻⁷M] maximal tension 1433 ± 238 vs LTD₄ [10⁻⁷M] + diphenhydramine [10⁻⁶M] =

1533 ± 281, *p* > 0.6).

Concentrations of PMA (6 μg/ml) and LTD₄ (1 × 10⁻⁸M) equipotent in extracellular O₂⁻ release (eg, 6nM O₂⁻/CPK × 10⁻² at 15 min incubation) were retested for their ability to induce trachealis tension hypersensitivity as follows: histamine with PMA, or histamine with LTD₄. No synergism was observed between PMA and histamine incubated for 20 min and then assayed for tension responses in OmM calcium buffer solution (Tables 4, 5; Fig 6). However, with 10⁻⁸M LTD₄, a leftward shift of the histamine response occurred. Hence, while PMA releases O₂⁻ in the trachealis preparation, it does not induce a hyperresponse to histamine. In contrast, LTD₄ released O₂⁻ and concurrently induced histamine hyperreactivity, a process specifically inhibited by superoxide dismutase.

Lastly, another O₂⁻ generation system, hypoxanthine (1mM)-xanthine oxidase (4 U/ml) produced 31 ± 3.0 nM O₂⁻ (n=6) over 10 min, but was ineffective in contracting the trachealis muscle.

DISCUSSION

This study confirms an enhancement of histamine contractures upon guinea pig airways smooth muscle induced by leukotriene-D₄. The sulfidopeptide leukotrienes are spasmogenic upon airways smooth muscle with effects upon both small and central airways. In human airways they are 200 to 3,000 times more potent than histamine, with several studies indicating their extremely potent bronchoconstrictor activity.^{2,10-13}

It is currently not clear whether leukotrienes *per se* cause hyperreactivity to other mediators (eg, histamine) or stimuli. Lee and colleagues¹⁴ have reported that LTE₄, but not C₄ or D₄, augmented guinea pig trachealis histamine contractures after elution of LTE₄ and recovery of the tissues to resting isometric tension. Creese and Bach⁸ observed an LTD₄ enhancement of histamine and acetylcholine reactivity in guinea pig

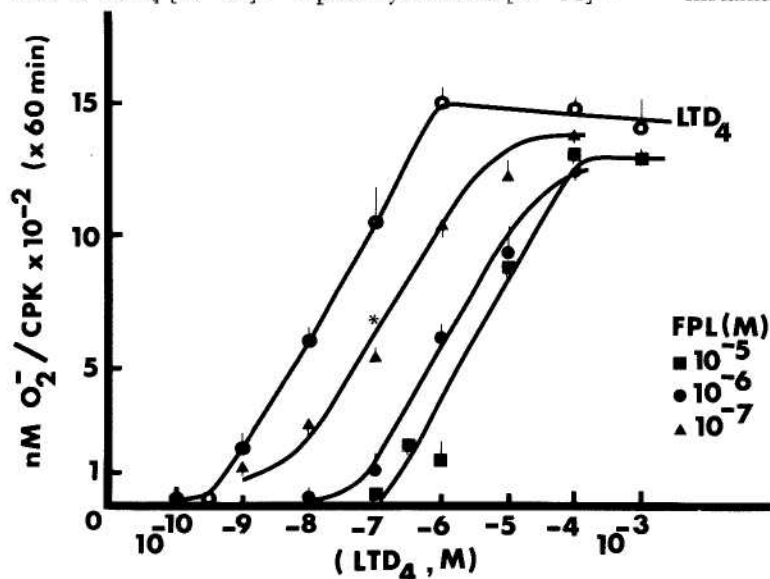


FIGURE 5. Generation of superoxide anion (O₂⁻) by synthetic LTD₄ in trachealis: a dose-response between LTD₄ and nmol O₂⁻ (expressed per unit of muscle CPK × 10⁻⁵) over 60 min of incubation is demonstrated. The EC₅₀ was 2.5 × 10⁻⁸M. Competitive inhibition of O₂⁻ generation by FPL 55712 (10⁻⁵ to 10⁻⁷M) occurred. * = effect of a single dose of LTC₄ (10⁻⁷M).

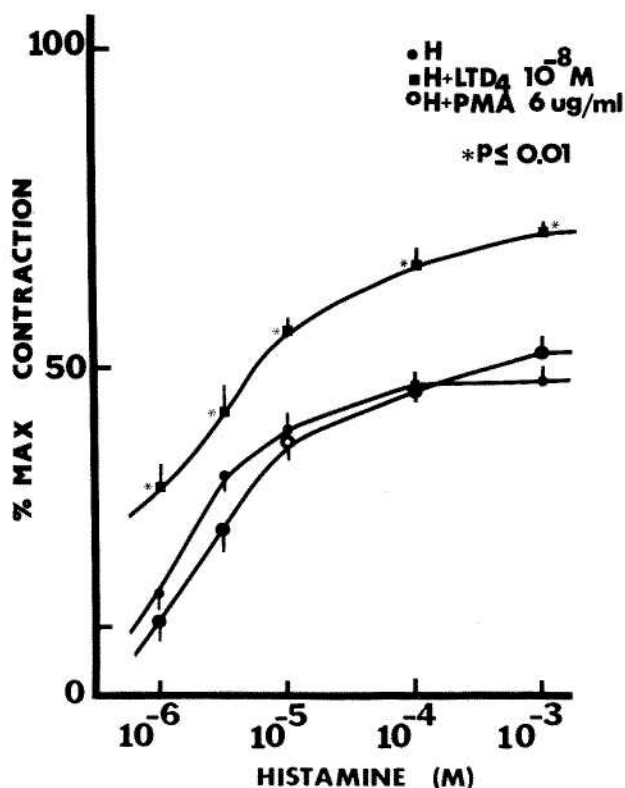


FIGURE 6. Interaction of histamine and PMA: concentrations of PMA (6 $\mu\text{g/ml}$) and LTD₄ (10^{-8}M) which released equivalent amounts of O_2^- over 60 min in the trachealis preparation were tested for their ability to induce trachealis hyperresponsiveness. As shown, LTD₄ (10^{-8}M) caused a significant upward and leftward shift of histamine dose-response curve; PMA did not (Tables 4, 5).

trachealis, but only under low extracellular calcium conditions. Our study demonstrates histamine hyperresponsiveness to LTD₄ under physiologic (2.5 mM) and low (OmM) extracellular calcium conditions by: a) an absolute increase in the maximal isometric tension developed by the guinea pig trachealis to histamine, and b) a leftward shift in EC_{50} following exposure to submaximal and maximal concentrations of LTD₄. Since this histamine hyperresponse occurred under physiologic extracellular calcium conditions, inflammatory or immunogenic release of leukotrienes *in vivo* may contribute to bronchial hyperreactivity. The dependency of LT-induced contractions to extracellular calcium has been described in guinea pig ileum and trachealis muscle.^{4,15} LTD₄ may also utilize an intracellular source of calcium to initiate smooth muscle contraction.¹⁶ Since the contractile state of smooth muscle is a function of the cytoplasmic concentration of free Ca^{++} , the persistence of this hyperresponse under low extracellular calcium concentrations suggests alterations (independent of $[\text{Ca}^{++}]_E$) in plasma membrane permeability or intracellular calcium sequestration as part of the mechanism of leukotriene-induced airways reactivity. Other models utilizing low $(\text{Ca}^{++})_E$ conditions have reported a similar increase in airways smooth muscle sensitivity.^{17,18}

Based on our observations, the incitant(s) for this reactivity appear to involve leukotrienes, specifically LTD₄, and superoxide anion, with H_2O_2 apparently not immediately involved. Oxygen-derived free radicals are highly reactive molecular species produced endogenously from the univalent reduction of molecular oxygen. These highly-charged molecules (superoxide O_2^- , hydrogen peroxide H_2O_2 , and hydroxyl radical OH^\bullet) are normally produced in small quantities as oxidative metabolism byproducts and are detoxified by endogenous free radical scavengers, including intracellular superoxide dismutase and catalase. It is proposed that, in a variety of pathologic conditions, production of O_2 -derived free radicals may exceed scavenging capacity, leading to tissue injury, possibly via structural membrane lipid peroxidation. Leukocytes can both form and degrade leukotrienes with lipoxygenase products rapidly degraded metabolically in the presence of oxygen radicals and hydrogen peroxide.¹⁹⁻²² Our observations suggest the possibility that: a) some active oxidative metabolite(s) of LTD₄ induce airways hyperreactivity to histamine, since the LTD₄-histamine interaction is inhibited by superoxide dismutase; or, alternatively b) that the superoxide dismutase inhibition may be via direct removal of toxic O_2^- avoiding reaction with biologic targets which may cause oxidative damage in cell membranes.²³ That this latter mechanism alone is not culpable was shown by our experiments with PMA. Since O_2^- release by PMA did not induce histamine hypersensitivity, we conclude that some interaction of LTD₄ (or a metabolic derivative) with O_2^- is related to the trachealis LTD₄-histamine hyperreactivity. Furthermore, O_2^- itself does not have a direct contractive effect under the conditions of our study. That ω -oxidation products of synthetic LTB₄ may still retain significant biologic activity has been documented.^{24,25} Alternatively, mast cell release of histamine could account for a component of the enhanced smooth muscle tension responses. Histamine release from rat peritoneal mast cells mediated by PMA-activated neutrophils is dependent on oxidative metabolites.²⁶ In normal and *Ascaris* reactor primates, a component of LTD₄ contracture may be due to concomitant histamine activity.²⁷ In contrast, human pulmonary tissue does not exhibit LTD₄ inhibition with mepyramine.^{10,28} While histamine release may have contributed to our observations, we do not believe it played a major role. First, the LTD₄-histamine hyperresponse persisted in OmM $(\text{Ca}^{++})_E$ where LTD₄ (10^{-7}M) did not significantly increase the resting isometric tension baseline (Fig 3). Second, pretreatment of our trachealis with 10^{-6}M diphenhydramine did not inhibit a 10^{-7}M LTD₄ contracture in 2.5 mM $(\text{Ca}^{++})_E$. However, our *in vitro* final LTD₄ bath concentration of 0.05 $\mu\text{g/ml}$ was one-twentieth that delivered in the above-cited primate

study. Higher LTD₄ concentrations may release mast cell histamine; however, this mechanism could involve toxic O₂ radicals as intermediates in the process.

Cellular origins of superoxide anion released by LTD₄ or PMA in our trachealis preparation are not defined. While LTB₄ may amplify the neutrophil release of oxygen metabolites, there currently are no reports of O₂ release in trachealis as we have described. Gay et al⁶ have reported enhancement of chemotactic-factor stimulated neutrophil (PMN) oxidative metabolism by LTB₄. PMNs incubated with 10⁻⁹M LTB₄ and FMLP (N-formyl-methionyl-leucyl-phenylalanine) released 17.7 nm O₂/1.5 × 10⁶ PMN. Bielefeldt Ohmann et al²⁹ have reported formation of approximately 12.3 nM O₂/10⁶ leukocytes for 15 min incubation time following opsonized zymozan stimulation of bovine leukocytes. Our heterogeneous cellular trachealis preparation released a maximum of 15 nM O₂/CPK unit × 10⁻² following LTD₄ equivalent to 0.15 nM O₂/13.0 mg wet weight total trachealis ring. Mean trachealis generation rate was 0.25 nM O₂/min/CPK unit × 10⁻² at LTD₄ 10⁻⁶M, over 60 min. Curnutte et al²³ found cis-unsaturated fatty acids (82 μM arachidonate) stimulate O₂ release at a rate of 105 nM O₂/min/10⁷ human neutrophils. Differences in cell population, methods and potency differences of leukotrienes as O₂ releasers preclude a quantitative comparison of O₂ release among these preparations.

Inhibition of both pharmacologic LTD₄-induced histamine reactivity and LTD₄-O₂ generation (competitively) by FPL 55712 indicates that some specific LTD₄ receptor event(s) is associated with this process. However, the existence of heterogeneous or disparate leukotriene receptors complicates precise interpretation of the action of FPL 55712.³⁰ In addition, the hyperreactivity was also inhibited "non-specifically" by ascorbic acid (5.7 × 10⁻⁵M), presumably acting as an antioxidant. In the absence of an efficient O₂ scavenger, the ascorbate system can act as a sink for superoxide; some human and animal studies of experimental asthma have cited a beneficial role for ascorbic acid.^{31,32}

While leukotrienes can incite neutrophilic influx and stimulated PMNs can generate oxygen radicals and hydrogen peroxide that modify or inactivate certain leukotrienes, there is currently no evidence in airways smooth muscle that arachidonate-derived substrates relate toxic oxygen products generation to subsequent smooth muscle reactivity.³³ It has been proposed that acute pulmonary injuries as increased permeability pulmonary edema or vascular endothelial cell damage might result from local, toxic, neutrophil products, including proteases, arachidonate products and/or oxygen-derived toxic products.³⁴ A protective action by SOD upon free radical-mediated pulmonary vascular permeability has been described in dogs.³⁵ That a similar phenomenon involving

airways smooth muscle exists is prompted by our and other studies: canine airways hyperreactivity induced by ozone correlates with airways inflammation,³⁶ lipoxygenase products augment histamine responses in human bronchi.³⁷

In conclusion, the present *in vitro* observations are strong evidence for a specific link or association between exogenous LTD₄ and reactive oxygen species generation resulting in an acquired airways smooth muscle hyperreactivity to (non-immune) agonist histamine. The significance of these findings to human asthma is yet to be determined.

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