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LEUKOTRIENE B_4, C_4, D_4 and E_4 INACTIVATION BY HYDROXYL RADICALS

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Leukotriene B_4 chemotactic activity and leukotriene C_4, D_4 and E_4 slow reacting substance activity were rapidly decreased by hydroxyl radicals generated by two different iron-supplemented acetaldehyde-xanthine oxidase systems. At low Fe^{2+}, leukotriene inactivation was inhibited by catalase, superoxide dismutase, mannitol and ethanol, suggesting involvement of hydroxyl radicals generated by the iron-catalyzed interaction of superoxide and H_2O_2 (Haber-Weiss reaction). Leukotriene inactivation increased at high Fe^{2+} concentrations, but was no longer inhibitable by superoxide dismutase, suggesting that inactivation resulted from a direct interaction between H_2O_2 and Fe^{2+} to form hydroxyl radicals (Fenton reaction). The inactivation of leukotrienes by hydroxyl radicals suggests that oxygen metabolites generated by phagocytes may play a role in modulating leukotriene activity.

Oxidation of arachidonic acid by the lipoxygenase pathway leads to the formation of important mediators of allergic and other inflammatory reactions (1). LTB_4, a dihydroxy derivative, has potent chemokinetic and chemotactic activities (2,3). LTC_4, which contains glutathione in a thioether linkage at the C6 position, and its derivatives LTD_4 and LTE_4 constitute the slow reacting substances (SRS) and cause increased vascular permeability and constriction of peripheral lung airways (1,4,5). A major source of these mediators are leukocytes, namely macrophages (6,7), eosinophils (8) and neutrophils (2). Their inactivation by host control mechanisms would be of great importance in the modulation of their potent biologic activities.

Phagocytes respond to perturbation of the plasma membrane by an appropriately opsonized particle or by certain soluble agents with a burst of respiratory activity (9). Oxygen consumption is increased and the initial

Abbreviations: LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; $OH^{\cdot}$, hydroxyl radical; $O_2^{\cdot-}$, superoxide anion; SOD, superoxide dismutase; SRS, slow reacting substance

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product formed is $O_2^{\cdot-}$. This radical rapidly undergoes a dismutation reaction with its protonated form to yield H_2O_2 as follows:

$$O_2^{\cdot-} + HO_2^{\cdot} + H^+ \longrightarrow O_2 + H_2O_2 \qquad (a)$$

Hydroxyl radicals also are generated by stimulated phagocytes and it is the prevailing view that their formation results from the iron-catalyzed interaction of $O_2^{\cdot-}$ and H_2O_2 as follows:

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH\cdot \qquad (b)$$

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad (c)$$

$$H_2O_2 + O_2^{\cdot-} \longrightarrow O_2 + OH^- + OH\cdot \qquad (d)$$

At high Fe^{2+} concentrations, OH· can be formed in significant amounts by reaction with H_2O_2 (reaction b), without a requirement for the reduction of the Fe^{3+} formed. The strong oxidizing activity of Fe^{2+} and H_2O_2 was first described by Fenton (10) and is known by his name. Since stimulated phagocytes generate OH·, the inactivation of leukotrienes by this potent oxidizing agent was sought, using the aerobic oxidation of acetaldehyde by xanthine oxidase as an oxygen radical generating system (11,12). Our findings have been reported in part in abstract form (13).

MATERIALS AND METHODS

Special reagents. Synthetic LTB_4, LTC_4, LTD_4 and LTE_4 were the generous gifts of Dr. J. Rokach, Merck Frosst Laboratories, Pointe-Claire/Dorval, Quebec. Acetaldehyde was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., xanthine oxidase (bovine milk, 10 mg/ml ≃ 0.4 U/mg in 0.01M ETDA - 2.0M ammonium sulfate) from Boehringer Mannheim Biochemicals, Indianapolis, In., SOD (bovine erythrocytes, lyophilized powder, 12,300 U/mg) from Miles Laboratories, Inc., Miles Research Products, Elkhart, In., and catalase (bovine liver, 60,000 U/mg) from Worthington Biochemical Corp., Freehold, N.J. The xanthine oxidase and catalase were dialyzed against water prior to use. The water was deionized to a resistance of greater than 1.8×10^7 ohms/cm and all salt solutions were passed twice over a Chelex 100 ion exchange resin (12) to remove trace metals. The catalase was heated at 100°C for 15 min and SOD was autoclaved at 120°C for 30 min where indicated.

Inactivation of leukotrienes. The components of the reaction mixture (see legends to figures and tables) in a total volume of 0.5 ml were added to 12 x 75 mm polystyrene test tubes with either acetaldehyde or H_2O_2 added last. The tubes were incubated at 37°C in a shaking water bath for the periods indicated, and the reaction stopped by the addition of 385 µg catalase in 0.05 ml. Tubes containing LTB_4 were assayed for chemotactic activity and those containing LTC_4, LTD_4 or LTE_4 for SRS activity as previously described (14). Activity in each experimental tube was compared to controls containing

the leukotriene in buffer alone, and the percent inactivation determined. The data are reported as the mean ± SE of the combined experiments and differences were analyzed for significance using Student's two tailed t-test for independent means (not significant, $p > 0.05$).

RESULTS

Table I demonstrates the partial inactivation of LTB_4 and LTD_4 by the iron-supplemented xanthine oxidase system at pH 5.5, at two iron concentrations. At the lower concentration (2×10^{-6}M), inactivation was greatly decreased or abolished by deletion of acetaldehyde, xanthine oxidase or Fe^{2+} or by the addition of catalase, SOD or the OH· scavengers mannitol or ethanol. When the iron concentration was raised to 5×10^{-5}M, the properties of inactivation were comparable except for the lack of inhibition by SOD. The results were similar with LTC_4 and LTE_4 (data not shown). Inactivation by the xanthine oxidase system at the high iron concentration was rapid with about 70% inactivation of LTB_4 at 30 min and 80% inactivation of LTC_4, LTD_4 and LTE_4 at 15 min (Fig. 1). Acetaldehyde and xanthine oxidase could be replaced by H_2O_2 in the high iron system, and, as with the xanthine oxidase system, inactivation was inhibited by mannitol and ethanol but not by SOD (data not shown).

When the 0.02M sodium acetate buffer pH 5.5 generally employed was replaced by 0.01M sodium phosphate buffer pH 7.0, LTB_4 inactivation by the xanthine oxidase system was 47.0 ± 4.9% (n=9) and 57.4 ± 5.7% (n=11) in 30 min and LTD_4 inactivation was 47.3 ± 6.4% (n = 4) and 69.9 ± 7.7% (n = 4) in 15 min at the low and high iron concentrations respectively.

DISCUSSION

This paper describes the inactivation of leukotrienes by OH·. The aerobic oxidation of acetaldehyde by xanthine oxidase in the presence of iron was employed as a source of OH·, chemotaxis as a measure of the activitity of LTB_4 and guinea pig ileal contraction as a measure of the activity of LTC_4, LTD_4 and LTE_4. Two mechanisms for the generation of OH· by the xanthine oxidase system were employed, which differed in the concentration of iron required. At high Fe^{2+} concentration (5×10^{-5}M), leukotriene in-

Table I

LTB_4 and LTD_4 Inactivation by the Xanthine Oxidase System*

Supplements	Chemotactic activity (% inactivation) LTB_4		SRS activity (% inactivation) LTD_4	
	Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}
Acet + XO + Fe^{2+}	42.5 ± 4.3 (9)	69.2 ± 3.9 (11)	53.8 ± 3.1 (7)	77.8 ± 9.1 (3)
- Fe^{2+}	9.3 ± 3.5 (8)†	9.3 ± 3.5 (8)†	16.3 ± 3.2 (3)†	16.3 ± 3.2 (3)†
- XO	3.1 ± 1.6 (3)†	4.0 ± 1.3 (3)†	5.4 ± 2.3 (3)†	5.2 ± 2.4 (3)†
- Acet	6.0 ± 3.1 (3)†	2.6 ± 2.0 (3)†	2.9 ± 1.8 (3)†	4.7 ± 1.8 (3)†
+ Catalase	5.4 ± 3.1 (3)†	7.9 ± 4.0 (3)†	8.3 ± 2.5 (3)†	13.0 ± 7.0 (3)†
+ Heated catalase	37.1 ± 6.2 (2)	60.3 ± 8.9 (2)	48.5 ± 5.6 (3)	65.3 ± 5.6 (2)
+ SOD	11.0 ± 3.3 (6)†	65.9 ± 3.9 (11)	13.1 ± 5.4 (3)†	82.1 ± 9.5 (3)
+ Heated SOD	35.8 ± 9.1 (2)	73.2 ± 5.9 (2)	47.9 ± 8.1 (3)	76.5 ± 9.1 (3)
+ Mannitol (10^{-1}M)	14.0 ± 7.4 (3)†	14.2 ± 7.9 (2)†	6.9 ± 1.8 (3)†	23.6 ± 3.4 (2)†
+ Mannitol (10^{-2}M)	32.7 ± 5.4 (3)	47.3 ± 4.7 (2)	26.1 ± 6.5 (3)†	53.9 ± 11.4 (2)
+ Mannitol (10^{-3}M)	43.6 ± 3.5 (3)	60.2 ± 8.0 (2)	43.2 ± 7.6 (3)	75.7 ± 12.2 (2)
+ Ethanol (10^{-1}M)	15.7 ± 6.3 (2)†	12.5 ± 6.8 (3)†	8.1 ± 2.3 (3)†	20.5 ± 5.7 (3)†
+ Ethanol (10^{-2}M)	36.3 ± 4.9 (2)	55.9 ± 8.2 (3)	25.0 ± 2.5 (3)†	59.4 ± 9.4 (2)
+ Ethanol (10^{-3}M)	46.3 ± 5.3 (2)	64.7 ± 4.8 (3)	39.7 ± 3.7 (3)†	81.5 ± 8.1 (2)

*The reaction mixture contained 0.02M sodium acetate buffer pH 5.5, either 20 ng LTB_4 or LTD_4 and the supplements, where indicated, as follows: acetaldehyde (Acet), 4 X 10^{-3}M; xanthine oxidase (XO), 10 µg; ferrous sulfate (Fe^{2+}) 2 X 10^{-6}M (low Fe^{2+}) or 5 x 10^{-5}M (high Fe^{2+}); catalase 30 µg; superoxide dismutase (SOD), 2.5 µg and mannitol or ethanol at the concentrations indicated. Incubation was for 30 min. Each value represents the mean ± SE of (n) experiments.

†Significantly different from the complete Acet + XO + Fe^{2+} system, $P < 0.05$; all others not significant.

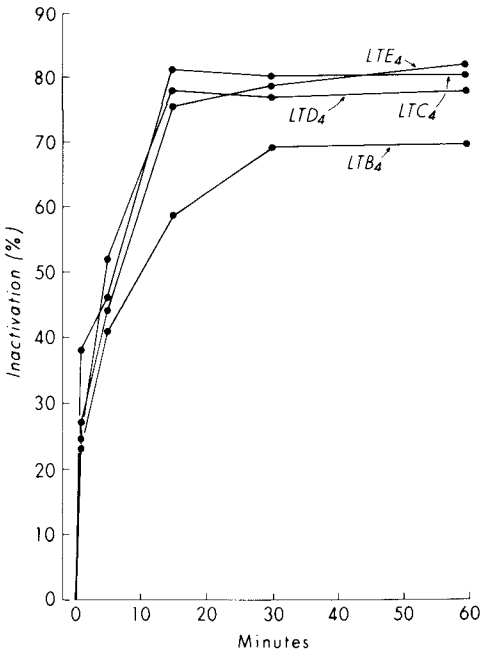

Figure 1: Time course of leukotriene inactivation by the acetaldehyde-xanthine oxidase-Fe^{2+} system. The reaction mixture was as described for the high Fe^{2+} (5×10^{-5}M) system in Table I except that the time of incubation was varied as indicated. The data are the mean of three experiments with either LTB_4 (20 ng), LTC_4 (20 ng), LTD_4 (20 ng) or LTE_4 (250 ng).

activation was inhibited by the OH· scavengers mannitol and ethanol and by catalase but not SOD, and the xanthine oxidase system could be replaced by H_2O_2. This suggests that H_2O_2 generated by the xanthine oxidase system can react stoichiometrically with Fe^{2+} at high concentrations to generate OH· in amounts sufficient to inactivate leukotrienes, as shown in reaction b (Fenton's reagent). The reaction was generally conducted in 0.02M acetate buffer pH 5.5 as earlier studies of OH· - dependent iodination had indicated that these conditions were optimal for the generation of available OH· by Fenton's reagent (15). However inactivation also was observed when 0.01M phosphate buffer pH 7.0 was employed.

When the iron concentration was decreased to 2×10^{-6}M, the xanthine oxidase system retained its capacity to inactivate leukotrienes; however in contrast to the high iron system, inactivation was inhibited by SOD, as well as by catalase and the OH· scavengers mannitol and ethanol. In this

instance, the reduction of ferric ion formed by Fenton's reagent is required for continued OH· formation and $O_2^{-}\cdot$ performs this function as shown in reactions b, c and d (Haber-Weiss reaction). Inactivation of LTB_4 and LTD_4 was greatly decreased when iron was deleted from the xanthine oxidase system; however some inactivation, particularly of LTD_4 (16.3%), was still observed. We have previously shown that LTC_4 and LTD_4 are inactivated by H_2O_2 alone at relatively high concentrations (14) and H_2O_2 would be anticipated as a product of the xanthine oxidase system (16). Work is now in progress regarding the nature of the products formed on the oxidation of leukotrienes by OH·.

This report of the inactivation of leukotrienes by OH·, a product of the respiratory burst of leukocytes, together with the earlier report of the inactivation of leukotrienes by the peroxidase - H_2O_2 - halide system of phagocytes (14,17,18), as well as by H_2O_2 alone at high concentration (14), indicates that leukocytes not only generate leukotrienes but also can inactivate them by a number of mechanisms. This suggests the role of phagocytes in the modulation of the activity of these potent mediators in inflammatory reactions.

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