Superoxide Anion Generation during Airway Anaphylaxis

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Abstract. Extracellular release of superoxide anion (O_2^-) during in vitro anaphylaxis in guinea pig trachealis was found to exhibit both a time- and a concentration-dependent generation of O_2^-. Trachealis O_2^- release was unaffected by treatment with diphenhydramine HCl (10^{-5} M, 10^{-6} M). While indomethacin (10^{-5} M) augmented anaphylaxis tension it did not enhance O_2^- release. However, the semiselective SRS-A antagonist FPL 55712 (10^{-5} M) resulted in essentially complete inhibition of O_2^- generation. These data indicate that O_2^- generation occurring during acute airway anaphylaxis is associated with the activation of SRS-A products.

Introduction

Leukotrienes (LT) are highly potent bronchoconstrictors in man and guinea pig and appear to be important putative mediators of immunologic anaphylaxis [1, 2]. Recent work from this laboratory indicated that LTD_4-induced histamine hyperreactivity in guinea pig trachealis in vitro was inhibited by superoxide dismutase (SOD), implying a role for toxic oxygen radicals in this interaction [3, 4]. Since exogenous LTD_4 was found to concurrently cause the extracellular release of superoxide anion (O_2^-) we sought to determine whether anaphylaxis (ANA) was also associated with O_2^- generation in guinea pig trachealis.

Materials and Methods

Male Hartley guinea pigs weighing 400–500 g were sacrificed with pentobarbital sodium (500 mg/kg). The trachealis was rapidly excised, cut into rings, and isometric tension responses 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% O_2–5% CO_2; full details of isometric tension methods and KH composition have been recently detailed [5]. Passive in vitro sensitization (Schultz-Dale reaction) was accomplished as follows: each trachealis ring was placed in a 6.0-ml plastic tube containing a final total volume of 1.5 ml KH buffer. 0.02 ml of normal saline reconstituted rabbit anti-chicken egg albumin antiserum (ICN, Cleveland, Ohio) was added for 20 min at 37 °C, then excess antibody was eluted with multiple fresh KH washes. ANA was initiated with 0.1 ml antigen, 5× recrystallized chicken egg albumin (14.7% N_2), 0.1–1,000 μg/ml final bath concentration.

O_2^- trachealis release was assayed at 37 °C by a modification of the superoxide dismutase-inhibitable reduction of ferricytochrome-C at 550 nm [6, 7]. The reaction mixture consisted of one tracheal ring (mean wet weight 13 mg), 120 μM ferricytochrome-C, antiserum and ovalbumin with the anaphylactic effluent assayed at time points specified in the text. All samples were assayed in duplicate in the presence or absence of bovine, erythrocytic SOD (300 U/ml), in room air. For inhibition studies, FPL 55712 (10^{-5} M) (gift of Hoffmann-La Roche, Nutley, N.J.) or diphenhydramine HCl (10^{-5} M, 10^{-6} M) was incubated 30 min prior to inducing ANA with ovalbumin; the effect of indomethacin (10^{-5} M) (Sigma Chemical Co., St. Louis, Mo.) was tested similarly. After the appropriate time periods, the reaction was terminated by immersion of the reaction tubes to an ice bath, the trachea removed and rapidly frozen at –4 °C for subsequent creatine phosphokinase (CPK) assay. The remaining ferricytochrome-KH buffer was centrifuged at 1,200 g for 5 min at 4 °C and the supernatant then assayed for cytochrome-C reduction in a Beckman DV spectrophotometer at 550 nm. The OD values were converted to nmol O_2^- using a molar extinction coefficient of 2.1×10^4 cm^{-1} [7]. O_2^- production was calculated as the difference between the amount of ferricytochrome-C reduction obtained between parallel samples in the presence or absence of SOD. Appropriate blanks and controls in the presence of ferricytochrome-C but without the addition of the stimulating agent were examined in parallel with the experimental samples. Neither antigen ovalbumin, antibody, SOD nor trachealis alone affected this O_2^- assay. The remaining trachealis was analyzed colorimetrically for muscle CPK activity by creatine formation in a CPK-catalyzed ADP/phosphocreatine reaction using Sigma kit No. 520 (Sigma). Final trachealis superoxide generation is expressed as nM O_2^-/ CPK unit $\times 10^{-2}$ over specified reaction times. Differences between observations were analyzed by Student's t test for grouped data with $p < 0.05$ taken as significant; results are expressed as mean $\pm$ SEM.
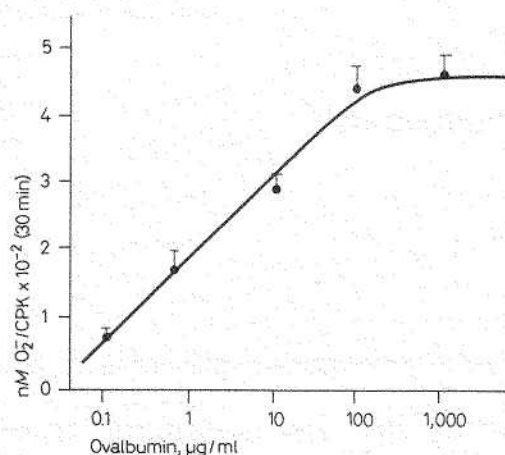
Fig. 1. Concentration response of ovalbumin versus extracellular O_2^- generation in guinea pig trachealis at 30 min of ANA. The EC_{50} is 4.5 μg/ml. Semilogarithmic plot; data are mean $\pm$ SEM.
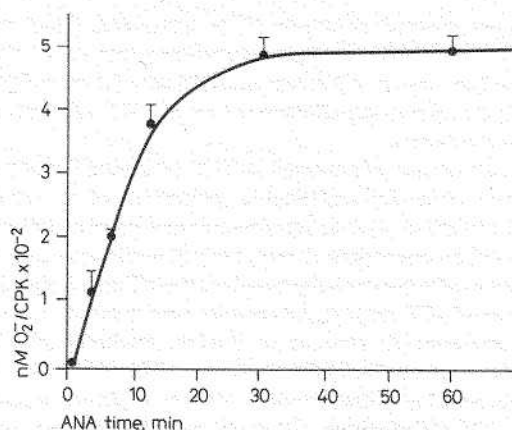

Fig. 2. O_2^- release in guinea pig trachealis during in vitro ANA measured over a 60-min interval using 100 μg/ml ovalbumin. Actual O_2^- generation was measured by removing an aliquot of ANA reactants and assaying at 3, 6, 12, 30, and 60 min. Maximum O_2^- release (4.65 nM/CPK $\times 10^{-2}$) occurred by 30 min.

Results

A typical anaphylactic contracture consists of an initial rapid phase (5 min) and a prolonged tonic phase of 90 min, with maximal tension occurring at approximately 45 min; maximal ANA tension was 1,200 $\pm$ 130 mg. As depicted in figure 1, an ovalbumin concentration-O_2^- response was observed from 0.1 to 1,000 μg/ml and with an EC_{50} of 4.5 μg/ml. The maximal O_2^- extracellular release was 4.65 nM/CPK $\times 10^{-2}$ after 30 min of ANA. This is one-third of the maximum release observed with synthetic LTD_4 (10^{-6} M, maximal concentration) of 15.1 $\pm$ 1.1 nM

Table I. Effect of diphenhydramine and FPL 55712 on O_2^- generation

Condition	n	n$M O_2^-$/CPK $\times 10^{-2}$ (30 min)[1]
Control ANA	5	4.85 $\pm$ 0.29
Diphenhydramine HCl, M		
10^{-5}	4	4.15 $\pm$ 0.59[2]
10^{-6}	4	4.45 $\pm$ 0.10[2]
FPL 55712, M		
10^{-5}	4	0.35 $\pm$ 0.08[3]

[1] Over 30 min reaction time.
[2] Not significant (p > 0.7), compared to control ANA.
[3] Significant (p < 0.001), compared to control ANA.

O_2^-/CPK $\times 10^{-2}$ at 30 min and whose EC_{50} was 2.5×10^{-8} M [4]. SOD (300 U/ml) had no effect on ANA tension development.

The extracellular release of O_2^- over the time course of ANA reveals trace amounts generated in the first few minutes of ANA, becoming maximal at 30 min (fig. 2). In these time studies an ovalbumin concentration of 100 μg/ml was employed.

These observations imply an effect by SRS-A components, rather than histamine, upon O_2^- release. To evaluate this further, the specific inhibitor diphenhydramine (10^{-5} M, 10^{-6} M) and the semiselective inhibitor FPL 55712 (10^{-5} M) were tested for their action on O_2^- release. The experimental conditions were antigen ovalbumin of 100 μg/ml and measured at 30 min of ANA. At these cited concentrations, ANA tension was inhibited by approximately 80% including the initial rapid phase by diphenhydramine and late tonic phase by FPL 55712 respectively. As shown in table I, diphenhydramine (10^{-5} M) did not inhibit O_2^- generation (p > 0.7). In contrast, FPL 55712 reduced the maximal O_2^- release by 93%, from 4.85 to 0.35 nM O_2^-/CPK $\times 10^{-2}$ at 30 min of ANA (p < 0.001).

Indomethacin (10^{-5} M) increased ANA isometric tension by 26%, measured at 45 min of ANA; ANA = 1,000 $\pm$ 55 mg vs. ANA + indomethacin = 1,260 $\pm$ 56 mg, n = 9, p < 0.01. However, indomethacin (10^{-5} M) had no effect upon O_2^- release in the trachealis preparation: ANA = 4.65 $\pm$ 0.33 vs. ANA with indomethacin = 4.48 $\pm$ 0.32 nM O_2^-/CPK unit $\times 10^{-2}$ (n = 12, p > 0.7), assayed at 30 min.

Discussion

In vitro ANA in guinea pig trachealis was found to be associated with extracellular generation of O_2^-. Since the reaction assay mixture contains SOD we can infer the majority of released free radical oxidants was O_2^- with a minimal component of H_2O_2. As we have observed with synthetic LTD_4, O_2^- release occurred only in the late tonic phase of ANA indicating the association of SRS-A derivatives with toxic O_2^- product release. There appears to be little role for histamine in this process of free radical generation [4]. This finding was substantiated by inhibition of O_2^- formation with FPL 55712, a semiselective SRS-A antagonist, and not by diphenhydramine. The cellular origins of O_2^- released during ANA in this trachealis preparation are not defined in this present study; the preparation does contain neutrophils, mast cells and smooth muscle elements, among others. While LTB_4 has been reported to amplify the neutrophilic (PMN) release of oxygen metabolites, there are currently no reports of O_2^- release in trachealis preparations as we have observed. For example, Gay et al. [6] have reported enhancement of chemotactic-factor stimulated PMN-oxidative metabolism by LTB_4. PMNs incubated with 10^{-9} M LTB_4 and FMLP (N-formyl-methionyl-leucyl-phenylalanine) released 17.7 nM O_2^-/1.5×10^6 PMN. Curnutte et al. [8] observed cis-unsaturated fatty acids (82 μM arachidonate) stimulation of O_2^- release at a rate of 105 nM O_2^-/min/10^7 human PMN. Rat peritoneal mast cells histamine release by PMN-activated neutrophils is reported to be dependent upon oxidative metabolites [9]. Finally, immunologic stimulation of rat peritoneal mast cells and human lung mast cells were shown to generate O_2^-, the release of which paralleled the release of histamine [10]. Hence, PMN and/or mast cells appear the most likely source of O_2^- in our studies of trachealis anaphylaxis. However, differences in cell populations, methods and potency factors preclude quantitative comparisons of O_2^- release among these preparations.

As previously reported, indomethacin enhancement of antigen-induced trachealis isometric tension by cyclooxygenase inhibition was demonstrated in our preparation [11]. However, since no increased release of O_2^- occurred, it appears there is no role for O_2^- in the enhancement of anaphylactic contractures by indomethacin.

We have previously reported that exogenous LTD_4 activates oxygen-derived free radicals which interact to induce an acquired hyperreactivity to agonist histamine in trachealis smooth muscle [4]. The present studies of ANA indicate that immunogenic activation of SRS-A products, e.g. LT, also generate O_2^- in this guinea pig trachealis preparation which may also influence airways smooth muscle reactivity.

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