TOXIC OXYGEN PRODUCTS ALTER CALCIUM HOMEOSTASIS IN AN ASTHMA MODEL

EARLE B. WEISS, M.D.,
and the technical assistance of
PRAKASH C. MULLICK, Ph.D.
Worcester, Mass.

From the Division of Respiratory Diseases, St. Vincent Hospital, and University of Massachusetts Medical School, Worcester, Mass.

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Toxic oxygen products alter calcium homeostasis in an asthma model

Earle B. Weiss, M.D., and the technical assistance of Prakash C. Mullick, Ph.D. *Worcester, Mass.*

After anaphylactic or synthetic leukotriene C_4 contractions in guinea pig trachealis muscle, an accelerated initial rate and greater total myorelaxation are induced in these muscle preparations when they are immersed in calcium-free medium, $O(Ca^{++})_E$. Inhibition of the late phase of anaphylaxis (ANA) by FPL 55712 (10^{-5} mol/L) eliminated the post-ANA $O(Ca^{++})_E$-augmented myorelaxation, suggesting a causal role for SRS-A products. Hypoxia or superoxide dismutase/catalase pretreatment also abolished the post-ANA or leukotriene C_4 $O(Ca^{++})_E$-augmented myorelaxation. The data support the hypothesis that toxic oxygen products generated with SRS-A and/or LTC_4 induce an alteration in Ca^{++} homeostasis in airway smooth muscle. In this model of allergic asthma, airway smooth muscle alteration after ANA may contribute to the pathogenesis of asthma and/or airway hypersensitivity associated with allergic asthma. (J ALLERGY CLIN IMMUNOL 75:692-97, 1985.)

One possible cause of asthma is a primary defect of ASM. Previous work from this and other laboratories indicated an acquired abnormality of calcium homeostasis in ASM hyperreactivity.[1, 2] We found an increased sensitivity of RIT to extracellular calcium $(Ca^{++})_E$ after in vitro ANA in guinea pig trachealis.[1] In this study subphysiologic extracellular calcium conditions were required to distinguish an effect on RIT in contrast to previous investigations of experimental asthma in which ASM hyperreactivity was analyzed by agonist-contractile responses.[3] Additionally, the potential contribution of resting basal muscle tone to airway hyperreactivity has been questioned.[4] During our experiments we observed that the initial rate and total magnitude of relaxation of RIT in a calcium-free medium ($O[Ca^{++}]_E$) was enhanced after anaphylaxis (Fig. 1, *A*). This article validates this finding and also

Abbreviations used

ANA:	Anaphylaxis
LT:	Leukotriene
$0(Ca^{++})_E$:	Zero calcium
SOD:	Superoxide dismutase
CAT:	Catalase
ASM:	Airway smooth muscle
RIT:	Resting isometric tension
KH:	Krebs-Henseleit
Ri:	Initial velocity of relaxation
Rm:	Maximal relaxation

From the Division of Respiratory Diseases, St. Vincent Hospital, and University of Massachusetts Medical School, Worcester, Mass.

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Reprint requests: Earle B. Weiss, M.D., Director, Division of Respiratory Diseases, St. Vincent Hospital, 25 Winthrop St., Worcester, MA 01604.

presents the effects of hypoxia and select, free radical oxygen inhibitors on postanaphylactic myorelaxation.

MATERIAL AND METHODS

The experimental in vitro model consisted of a dual (paired) immersion of guinea pig trachealis muscle into a calcium-free buffer, observing the effect on RIT. Fig. 1, *B* details this paired technique and the method of the passive Schultz-Dale anaphylactic reaction. Isometric tension methods have been reported.[5] Adult male Hartley guinea pigs (Elm Hill Breeding Laboratory, Chelmsford, Mass.) weighing 450 to 600 gm were sacrificed by lethal injection. The trachea was removed and cut into rings. For tension measurements the cartilage was cut, and one end was fastened with no. 50 cotton thread to a fixed clamp; the other end was fastened with a thin piece of platinum wire to a Grass (Grass Instrument Co., Quincy, Mass.) FT03C force displacement transducer amplified by a Hewlett-Packard 8805B amplifier (Hewlett-Packard Co., Palo Alto, Calif.) to record isometric tension changes, in milligrams, on a precalibrated Hewlett-Packard 7754A thermal tip polygraph. Full scale was 5 gm. The muscle was oriented parallel to the direction of the force displacement. The strips were suspended at 37° C under 2 gm of initial tension in a 20 ml chamber (Harvard Apparatus Co., Inc., Millis, Mass.) containing 10 ml of a KH buffer aerated with 95% O_2 and 5% CO_2. Optimal length-tension relationships for these experiments were determined by exposing the muscle to increasing tensions of 0.25, 0.5, 1.0, 2.0, and 3.0 gm against histamine, 1.0 μg/ml; verification of the 2 gm RIT was conducted periodically. Daily variation in mean maximal tension to histamine in our preparation is ≤10% of maximal tension. All tracheas were analyzed for force development, and those exhibiting baseline instability (±5%), inconsistent rates, or erratic responses were discarded. P_{O_2}, P_{CO_2}, and pH were monitored in an 813 blood gas analyzer (Instrumentation Laboratories, Inc., Lexington, Mass.); P_{CO_2} was 40 ± 3 torr, and P_{O_2} was 480 ± 60 torr. The pH of the equilibrated KH buffer was 7.40 ± 0.02.

KH buffer contained (millimolars) NaCl, 118.1; KCl, 4.7; $NaHCO_3$, 24.8; $CaCl_2$, 2.52; $MgSO_4$, 2.4; KH_2PO_4, 1.10; and glucose, 10.0 in distilled, deionized water. Ca^{++}-free KH buffer omitted the $CaCl_2$ and contained 0.1 mmol/L of EGTA. Drugs included histamine dihydrochloride, acetylcholine, diphenhydramine HCl, EGTA, SOD, and CAT (Sigma Chemical Co., St. Louis, Mo.). FPL 55712 (sodium salt) was a gift of Hoffmann-LaRoche, Inc., Nutley, N. J.; synthetic LTC_4 (free acid) was a gift of Merck Frosst Laboratories, Montreal, Canada. Data are expressed as the mean (±SEM) Ri over the first 30 sec of relaxation in $O(Ca^{++})_E$ in milligrams per sec, and the mean (±SEM) Rm in milligrams at 15 min of relaxation in $O(Ca^{++})_E$. The actual time observed to a stable maximal relaxation was 10.5 ± 1.5 min for both control and ANA. Differences of means were analyzed by the Student's paired t test. In all studies after an ANA or LTC_4 contraction, and before $O(Ca^{++})_E$ immersion, elution with 2.5 mmol/L Ca^{++} KH buffer was conducted until the postcontractile RIT baseline was within ±3.0% of the precontractile RIT baseline.

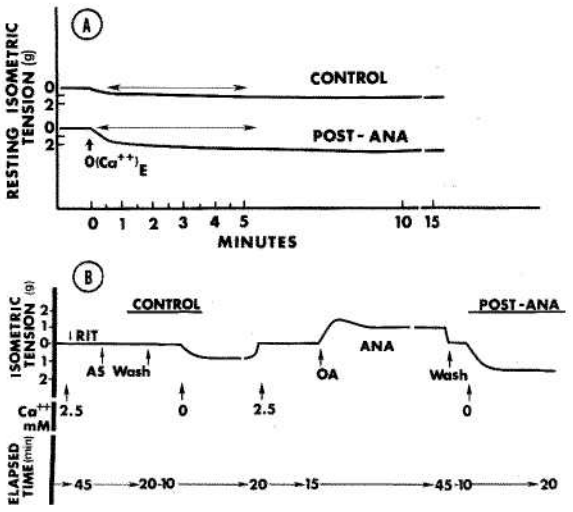

FIG. 1. A, Representative tracing *(redrawn)* of trachealis relaxation in $O(Ca^{++})_E$ under control versus post-ANA conditions. *Vertical arrow* is $O(Ca^{++})_E$ exposure; *horizontal arrows* indicate initial 2.0 gm resting baseline. **B,** Scheme of paired experimental design. After a 45-minute initial equilibration at 37° C in 10 ml of KH buffer with 2.5 mmol/L Ca^{++}, the muscle is incubated for 20 min with antiserum (AS), a 1:10 normal saline dilution of reconstituted rabbit anti-chicken egg-albumin (ICN Pharmaceuticals, Irvine, Calif.). It is then eluted with 3 to 5 washes of 10.0 ml of fresh KH buffer (2.5 mmol/L Ca^{++}) to the same stable RIT baseline. Thereafter, the muscle is immersed in Ca^{++}-free KH buffer containing 0.1 mmol/L EGTA and RIT recorded to (and beyond) Rm, usually 20 min. The *same* muscle is restored to 2.5 mmol/L Ca^{++} KH buffer and reequilibrated for 15 min at the initial RIT baseline. Antigen ovalbumin *(OA, arrow)* challenge follows with 5 X recrystallized egg-albumin (14.7% N_2), final concentration of 100 μg/ml (ICN). These concentrations yield mean maximal anaphylactic contractions of 1200 ± 130 mg. In vitro ANA is recorded for 45 min, and then eluted (KH buffer, 2.5 mmol/L Ca^{++}) to within ±5% of RIT baseline. (Noneluted anaphylactic contraction persists for 60 to 90 min.) The final $O(Ca^{++})_E$ immersion is repeated on the identical post-ANA muscle. Total elapsed time 175 to 190 min.

RESULTS

Table I presents the various effects of this study on trachealis relaxation. First, as observed in item A, Table I after ANA, a higher initial rate and total Rm in calcium-free buffer occurred. Second, to test the effect of antiserum on trachealis relaxation, a group of normal trachealis muscles was exposed to rabbit anti-chicken egg-albumin antiserum for 20 min, and then the excess antiserum was eluted. These muscles were then immersed twice in calcium-free buffer. The second, paired immersion was performed 135 min after the first calcium-free exposure to correspond with the elapsed duration of the paired anaphylactic experiment. Since the initial rate and maximal amounts of muscle relaxation (item B, Table I) were equivalent

TABLE I. Trachealis relaxation in $O(Ca^{++})_E$, mean ± SEM

Paired condition	No.	Initial mean rate (Ri) (mg isometric tension/sec)	p	Maximal relaxation (Rm) (mg at 15 min)	p
A Normal control (+antiserum) vs. post-ANA	16	8.7 ± 2.1 14.6 ± 2.9	<0.001	775 ± 83 1179 ± 84	<0.001
B 1st. normal control immersion vs. 2nd. normal control immersion	10	8.5 ± 1.1 7.8 ± 1.5	>0.5	663 ± 119 683 ± 84	=0.8
C Histamine (2.7×10^{-5} mol/L)	5	6.3 ± 0.8	>0.5	488 ± 63	>0.2
Acetylcholine (2.6×10^{-5} mol/L)	5	5.2 ± 0.5	>0.8	462 ± 199	=0.3
D Normal control (+antiserum) vs. premature termination of ANA	5	6.7 ± 1.5 7.9 ± 1.4	>0.3	425 ± 86 500 ± 212	>0.4
E Normal control + FPL 55712 (10^{-5} mol/L) vs. ANA + FPL 55712 (10^{-5} mol/L)	8	9.2 ± 1.1 8.1 ± 1.6	>0.5	844 ± 256 675 ± 260	>0.3
F Normal control vs. LTC_4 (2.3×10^{-8} mol/L)	8	11.8 ± 2.1 18.9 ± 3.5	<0.001	1012 ± 170 1244 ± 245	<0.05
G Normal control (+antiserum) in O_2 vs. normal control (+antiserum) in N_2	10	10.5 ± 1.2 9.3 ± 1.1	>0.4	685 ± 96 562 ± 80	>0.2
Normal control (+antiserum) in O_2 vs. ANA in N_2	7	11.1 ± 1.7 4.4 ± 1.3	<0.02	684 ± 122 428 ± 86	<0.02
H Normal control (+antiserum) + SOD/CAT vs. ANA + SOD/CAT	13	10.5 ± 0.5 9.9 ± 0.5	=0.5	1042 ± 43 1130 ± 42	>0.2

for both immersions, it is concluded that neither the initial calcium-free exposure nor the antiserum caused the enhanced postanaphylactic myorelaxation. We also observed that this myorelaxation phenomenon required 20 to 25 min of agonist contraction for the enhanced relaxation process to develop and that it persisted for 3 hr after termination of ANA. Third, to determine whether a nonimmune, isometric contraction of equivalent maximal tension and duration could be culpable, 2.6×10^{-5} mol/L of acetylcholine or 2.7×10^{-5} mol/L of histamine (maximal concentrations) was incubated for 45 min with normal trachealis and then fully eluted and exposed to calcium-free buffer. The data of item C, Table I indicate no effect by these agonists. Hence, these isolated agonist contractures do not induce an accelerated myorelaxation (Ri or Rm) in $O(Ca^{++})_E$. Comparative control values for trachealis relaxation (not shown) were of the same order of magnitude as item B, Table I.

We then examined the effect of time of anaphylactic contraction and antigen concentration. When ANA was prematurely terminated after 2 min of a maximal contraction (mean = 1200 ± 130 mg) by rapid KH buffer elution, these muscles failed to exhibit the typical postanaphylactic pattern (item D, Table I). Therefore, the postanaphylactic enhanced myorelaxation in $O(Ca^{++})_E$ is related to the duration of ANA. A dose-response relationship was demonstrated to exist between ovalbumin (0.1 to 1000 μg/ml) and anaphylactic tension. Maximum anaphylactic tension was observed at ovalbumin concentrations above 100 μg/ml. The concentration resulting in 50% of the maximal response for ovalbumin was 0.6 μg/ml for Rm and 5 μg/ml for Ri (Fig. 2).

Since a typical anaphylactic contraction consists of an early, rapid phase caused by the release of preformed histamine and a slow, prolonged contracture caused by generation of SRS-A and related products,[6] each phase was studied for its role. Diphenhydramine (10^{-6} mol/L) antagonism of the initial, rapid tonic phase of ANA (60% at 2 min) did not attenuate the subsequent postanaphylactic enhanced myorelaxation: Ri control 10.7 ± 1.7 versus Ri post-ANA 17.4 ± 2.9 ($p < 0.01$); Rm control 686 ± 87 versus Rm post-ANA 986 ± 123 ($p < 0.01$) (n = 11, all containing 10^{-6} mol/L of diphenhydramine).

Thereafter, the role of SRS-A on postanaphylactic myorelaxation was assessed via the semiselective SRS-A antagonist FPL 55712 that was preincubated at 10^{-5} mol/L for 15 min before and during ovalbumin challenge. FPL 55712 caused complete inhibition of the increased postanaphylactic relaxation (Ri and Rm) in calcium-free buffer with muscles that had been challenged with ovalbumin (item E, Table I). This inhibitor did not affect the Ri or Rm in control trachealis muscles not challenged with ovalbumin. FPL

55712 (10^{-5} mol/L) inhibited the prolonged SRS-A phase by 70% after 12 min compared to a typical spontaneous anaphylaxis decay of only 15% to 20% in this same time. The observation of FPL 55712 inhibition in our anaphylactic model must be viewed with some caution, since at $>10^{-6}$ mol/L, other activities may be operative including antagonism to prostaglandins $F_{2\alpha}$ and inhibition of allergic mediator release.[7]

Because LTs are the major SRS-A component, synthetic LTC_4 (2.3×10^{-8} mol/L) was examined. After a 45-minute LTC_4 contracture in normal trachealis, an enhanced myorelaxation in calcium-free buffer was observed similar to that observed after ANA (item F, Table I).

The influence of different oxygen tensions on anaphylactic myorelaxation is described in the following studies. Anaphylactic-enhanced myorelaxation in calcium-free medium was unaffected by reducing (isocarbic) the P_{O_2} from 480 ± 60 torr to P_{O_2} 80 ± 2.6 torr: Ri control 9.8 ± 1.4 versus Ri post-ANA 15.4 ± 2.2 ($p < 0.01$); Rm control 579 ± 56 versus Rm post-ANA 933 ± 57 ($p < 0.001$) (n = 12). Then, acute isocarbic hypoxia was induced by saturating the muscle chamber at 37° C with a gas mixture of 95% N_2 and 5% CO_2. Within 5 min P_{O_2} decreased to a constant value of 10 ± 4 torr (P_{CO_2} 40 ± 4 torr, pH 7.40 ± 0.05). The typical anaphylactic contraction during 45 min of hypoxia was unaffected in magnitude and duration. However, both RI and Rm phases of myorelaxation after ANA were significantly suppressed by hypoxia ($p < 0.02$) (item G, Table I). Although ASM–agonist myocontraction depends on oxygen, no inhibition of RIT or myorelaxation under hypoxic, $(Ca^{++})_E$-free conditions was observed in control trachealis, i.e., not challenged with ovalbumin[8] (item G, Table I). These observations imply, in part, a requirement for oxygen in the overall process(es) leading to enhanced trachealis relaxation after ANA, since released or activated mediators still induced an anaphylactic contraction despite hypoxic conditions.

Based on this oxygen dependence of postanaphylactic myorelaxation, the influence of specific enzymes known to protect cells against activated oxygen species was evaluated. SOD (500 U/ml) with CAT (500 U/ml) were incubated 30 min before and during ANA, and then relaxation in calcium-free buffer was induced. This combination of SOD and CAT completely inhibited the increased postanaphylactic myorelaxation in calcium-free buffer (item H, Table I). These enzyme concentrations had no differential effect on control RIT or myorelaxation: control Ri 10.2 ± 1.0 versus control Ri + SOD/CAT 10.5 ± 0.5 ($p > 0.8$); control Rm 1157 ± 103 versus control Rm + SOD/CAT 1038 ± 58 ($p > 0.7$) (n = 7). These enzymes also did not influence the magnitude or duration of ANA.

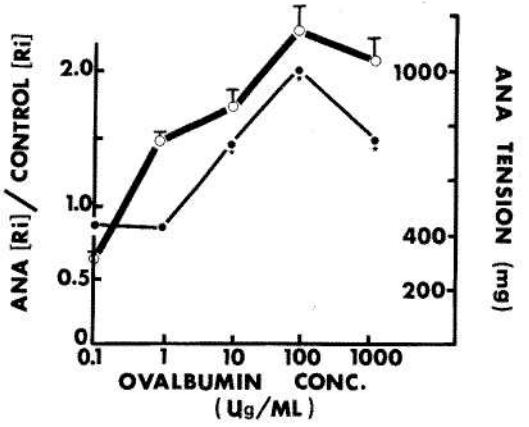

FIG. 2. Ovalbumin concentration versus mean anaphylactic tension (±SEM) *(open circles)* and Ri *(closed circles)* expressed as ANA Ri/control Ri; *asterisk* indicates Ri in ANA statistically different ($p < 0.05$) from control Ri. N = 3 for each point.

Finally, we tested the action of the cited enzymes against an LTC_4 (2.3×10^{-8} mol/L) contraction in normal trachealis. These enzymes (SOD 300 U/ml, CAT 300 U/ml) inhibited the post-LTC_4–associated increased myorelaxation as follows: Ri = control + SOD/CAT 11.6 ± 1.4 versus LTC_4 + SOD/CAT 12.8 ± 2.1 ($p = 0.7$); Rm = control + SOD/CAT 1121 ± 199 versus LTC_4 + SOD/CAT 1228 ± 189 ($p = 0.6$) (n = 8). This data also imply some relationship between synthetic LTC_4 and toxic oxygen radicals.

DISCUSSION

In summary the SRS-A phase of in vitro ANA is associated with an accelerated initial rate and greater Rm when trachealis smooth muscle is subsequently exposed to a calcium-free medium. This process is inhibited by the semiselective SRS-A antagonist FPL 55712 (10^{-5} mol/L), indicating a causal role for SRS-A products. This myorelaxation phenomenon is also induced in normal trachealis after contracture with exogenous LTC_4. We hypothesize that ANA or LTC_4 alters cellular binding or plasma membrane permeability to calcium as observed in RIT muscle tone as previously cited.[1] A supportive observation by Volpi et al.[9] is that exogenous arachidonate metabolites cause a specific, rapid, and significant increase in the permeability of the plasma membrane to $^{45}Ca^{++}$ in rabbit peritoneal neutrophils.

This postanaphylactic enhanced myorelaxation was aborted when ANA was conducted with either hypoxia or, more specifically, with pretreatment by SOD/CAT that presumably impede deleterious actions of free radical O_2^- and H_2O_2. Although release of arachidonic acid-cyclooxygenase toxic and inflammatory products occurs during in vitro ANA,[10] existing information does not clarify the relationship(s) between these metabolites and the concomitant release or action of oxygen-derived free radical or related products. It is known that stimulated polymorphonuclear leukocytes can generate oxygen radicals and hydrogen peroxide that modify or inactivate certain LTs.[11] However, at present there is no direct evidence in ASM that arachidonate-derived products can be related to toxic oxygen product generation.[12]

Nevertheless, we observed that although SOD/CAT pretreatment did not suppress an anaphylactic contraction, these enzymes completely inhibited the typically increased trachealis muscle relaxation after ANA and subsequent immersion in zero-calcium buffer. Similarly, the enhanced myorelaxation in normal trachealis after an LTC_4 contracture (i.e., nonimmune) was also inhibited by pretreatment with these enzymes. This indicates that SRS-A and specifically LTC_4 activate, or are concurrently associated with, toxic O_2 production formation, resulting in the subsequently accelerated trachealis muscle relaxation when exposed to a calcium-free medium.

It has been proposed that acute pulmonary injuries such as increased permeability pulmonary edema or pulmonary vascular endothelial cell damage might result from local, toxic neutrophil products, including proteases, arachidonate products and/or oxygen-derived toxic products.[13] A protective action by SOD on free radical-mediated pulmonary vascular permeability has been described in dogs.[14] That a similar phenomenon involving ASM might exist is prompted by our current observations and other studies. Holtzman et al.[15] have demonstrated that canine airway hyperreactivity induced by ozone inhalation correlated with airway inflammation, implying an association between increased numbers of inflammatory epithelial neutrophils and the ozone-induced hyperreactivity. Other information incriminates inflammatory mediators to ASM reactivity; lipoxygenase products augment the response to histamine in human bronchi[16]; methacholine responses in man can be increased by cyclooxygenase products.[17]

A functional alteration, or damage, in the ASM plasma membrane induced by immune-activated SRS-A resulting in an increased calcium permeability could explain why *specific* allergen-induced asthma in humans subsequently increases the *nonspecific* airway reactivity to agonists such as histamine or acetylcholine.[18] For example, the following model is proposed:

Normal ASM cell + anaphylactic release SRS-A (leukotrienes) → activation of toxic oxygen products → alters smooth muscle Ca^{++} homeostasis (permeability) → hyperesponse to nonspecific stimuli, agonists.

Our data suggest a link or association between anaphylactic-released SRS-A or exposure to exogenous LTC_4 and toxic oxygen metabolite generation resulting in the acquired permeability of trachealis muscle to calcium. This offers a new approach to ASM sensitivity. These observations also extend antigen-antibody hypersensitivity reactions in ASM to other biologic systems documented to exhibit toxic oxygen product injury phenomena, e.g., erythrocyte membrane.[19] However, further studies are necessary to define the precise mechanisms as they might affect ASM reactivity in a disease such as asthma.

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