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Dexamethasone Reduces Tachykinin but not ACh Airway Hyperreactivity after O_3

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Abstract. We investigated whether dexamethasone pretreatment affected the acute increase in airway reactivity produced by high-level ozone exposure. Reactivity to intravenous IV substance P (SP), IV acetylcholine (ACh), or aerosolized capsaicin (CAP) before and 1 hr after ozone exposure (3 ppm for 2 hr) was determined by measuring specific airway resistance in anesthetized, spontaneously breathing guinea pigs, half of whom had been pretreated for 2 days pre-ozone with dexamethasone (2 mg/kg intramuscularly [IM] daily). The amount of IV SP, IV ACh, or inhaled capsaicin necessary to increase baseline specific airway resistance by 100% (ED_{200}ACh or ED_{200}SP) or 35% (ED_{135}CAP) was determined by interpolation from dose–response curves. Compared to their pre-ozone status on the day of exposure, we found that dexamethasone-pretreated animals manifested significantly less of an increase in airway reactivity postozone to IV SP or inhaled CAP than did untreated animals. Changes in logEDs of the pretreated group were 0.18 ± 0.03 (mean ± SE) for SP and 2.20 ± 0.11 for CAP compared to 0.27 ± 0.04 and 3.38 ± 0.34, respectively, for the untreated groups post-ozone ($p < 0.05$ and $n = 4$ for each). In contrast, dexamethasone pretreatment had no effect on IV ACh reactivity postozone: changes in logED_{200}ACh were 0.27 ± 0.08 and 0.28 ± 0.04 for the pretreated and untreated groups, respectively ($n = 4$). In animals pretreated with captopril to block possible dexamethasone stimulation of angiotensin-converting enzyme synthesis that could influence tachykinin reactivity, we found that the corticosteroid effect on post-ozone SP reactivity was as marked as that seen in animals without captopril ($n = 4$). Because these reactivity studies were consistent with the possibility that dexamethasone may ameliorate ozone-induced, tachykinin hyperreactivity by stimulating airway neutral endopeptidase (NEP), we measured NEP activity by high-performance liquid chromatography (HPLC) of each tracheal

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homogenate made from other groups of animals. Homogenates from ozone-exposed, dexamethasone-pretreated animals demonstrated significantly greater NEP activity (81 ± 24%) than that from ozone-exposed, untreated animals ($p < 0.05$, $n = 5$). We conclude that corticosteroid pretreatment reduces the acute increase in airway reactivity to exogenous and endogenous tachykinins caused by ozone. This reduction may be at least partly due to stimulation of airway NEP activity, perhaps most of which is nonmucosal in that ozone acutely inactivates mucosal NEP.

Key words: Airway resistance—Airway smooth muscle—Asthma—Bronchoconstriction—Enkephalinase—Guinea pig—Metalloendopeptidase—Substance P receptors.

Introduction

It has been shown by many investigators that airway mucosal cells produce factors that affect airway muscle tone in a variety of species [21]. In particular, the respiratory mucosa [6] is rich in neutral endopeptidase (NEP; also called enkephalinase [EC 3.4.24.11]), an enzyme that affects guinea pig airway response to substance P in vivo [20] and in vitro [19].

As are the cilia on respiratory mucosal epithelial cell membranes, neutral endopeptidase (NEP) may be particularly vulnerable to airway luminal oxidant injury. Acute hyperreactivity due to ozone exposure is associated with several signs of airway mucosal injury. Loss or matting of respiratory epithelial cilia is common after high-level ozone exposure [15]. It has also been reported that ozone-induced mucosal injury in guinea pigs is associated with inactivation of airway NEP and an increase in reactivity to substance P [16, 17]. This observation, coupled with more recent studies of this disorder, have led us to speculate that oxidant-induced increases in airway reactivity to tachykinin may be caused by inactivation of respiratory mucosal NEP.

Although corticosteroid treatment is of proven benefit in asthma, its effect on ozone-induced airway disease is uncertain. Therefore, we investigated whether dexamethasone pretreatment affected the acute hyperreactivity in guinea pigs produced by high-level ozone exposure. Reactivity to various bronchoconstrictors was determined before and 1 hr after ozone (3 ppm for 2 hr) by measuring specific airway resistance in anesthetized, spontaneously breathing animals to avoid the potential effects that endotracheal intubation and anesthesia could have on our findings. To assess reactivity to nontachykinin and to both exogenous and endogenous tachykinin stimulation, the bronchoconstrictors used were intravenous acetylcholine (ACh), intravenous substance P, and aerosolized capsaicin, an agent known to release endogenous tachykinins that are catabolized by airway NEP [12]. To block potential dexamethasone stimulation of angiotensin-converting enzyme synthesis and its possible influence on tachykinin reactivity [3, 18] post-ozone, some of the animals were pretreated with captopril before intravenous substance P reactivity testing. Because these reac-

tivity studies were consistent with the possibility that dexamethasone pretreatment may ameliorate acute, ozone-induced, airway hyperreactivity by stimulating airway NEP activity, we also measured NEP activity in airway homogenates using high-pressure liquid chromatography in the presence of amastatin, an antagonist of airway aminopeptidase activity.

Methods and Materials

Animals

Male Hartley strain guinea pigs (600–750 g body weight) were used in the study. Baseline specific airway resistance (sRaw, in ml/cmH_2O/ml/sec) and airway reactivity to intravenous acetylcholine (ACh, n = 4), to aerosolized capsaicin (n = 4), or to intravenous substance P (in the presence or absence of captopril pretreatment, n = 4 each) were determined before experimentation. Animals were then exposed to ozone (3 ppm) for 2 hr while awake, and spontaneously breathing at rest. Half of these guinea pigs had been pretreated with dexamethasone (2 mg/kg IM per day) for 2 days (experimental group), while the other half received a similar quantity of 0.9% saline IM (control group). One hr postozone, reactivity testing to ACh, capsaicin, or substance P was repeated. In other animals exposed to either ozone or room air, each trachea was removed and homogenized to measure airway NEP activity in these cases.

Procedures

Airway Studies in Vivo. sRaw and reactivity to intravenous substance P, acetylcholine, or aerosolized capsaicin were measured as previously described in intact, unanesthetized, spontaneously breathing guinea pigs. Each animal was positioned in a 2-compartment Lucite chamber designed to keep its head fixed (with mouth closed) and isolated from it body and the plethysmograph. Flow at the snout was measured using a pneumotachograph (no.0, Fleish Instruments [Pres Lausanne, Switzerland]) connected to a differential pressure transducer (model MP45-1, Validyne [Northridge, CA]). Animals were placed in the plethysmograph, which was equipped with another transducer for measuring changes in box pressure. The pneumotachograph was calibrated using a rotometer (model 1355-01C1AAA, Brooks Instrument, Hartfield, PA) to pass known airflows through it. Changes in box pressure were calibrated (with box tightly sealed) by rapidly delivering boluses of air from a syringe. Airflow and box pressure signals were displayed simultaneously on an X-Y oscilloscope (model 502A, Tektronix [Portland, OR]). The angle described during the rapid inspiratory phase of the animals breathing was measured and sRaw was calculated from it. Prior to ozone, sRaw and airway reactivity were determined on at least 2 occasions to establish reproducibility of each animal's responsiveness to bronchoconstrictor. Thirty minutes before reactivity was tested, each animal was pretreated with propranolol (1 mg/kg in 0.9% normal saline injected IV) to decrease variation in airway bronchoconstrictor responsiveness within and between animals due to endogenous beta-adrenergic stimulation [5]. All animals thus tested showed reproducibility and were included in the experimental protocol.

Substance P or acetylcholine was delivered via a 0.61 mm OD, 0.28 mm ID polyethylene canula that had been previously placed in an external jugular vein. SRaw was measured continuously before and after rapid bolus administration of substance P dissolved in 1% acetic acid in 0.9% normal saline (NS). Substance P was given at 2 min intervals in increasing doses until sRaw was at least 200% of the baseline value. Animals pretreated with captopril were given 5 mg IV in 0.5 ml NS [4, 18]. Acetylcholine was delivered as previously described [15]. After each injection, the jugular catheter (dead space ~0.08 ml) was immediately flushed with 0.2 ml of isotonic heparinized saline (1000 U heparin/10 ml). Reactivity to inhaled capsaicin was determined using a nebulizer

(model 1700, Hudson [Temecula CA]) driven by compressed-air source at 28 psi (nebulizer output: 276 ± 12 μl/min; aerosol particle size: 4.28 ± 1.62 μm aerodynamic mass median diameter). After baseline sRaw and responses to diluent alone were determined, 10 breaths of 10^{-8} M capsaicin (dissolved in 10% ethyl alcohol and then diluted to final concentration in NS [2]) were given, and the peak sRaw occurring within the next 30 sec thereafter was recorded. The concentration of capsaicin was then increased 100-fold, and the delivery was repeated eveyr 5 min until baseline sRaw had increased by at least 35%. Inhalation of the diluent alone in each case (10% ethyl alcohol diluted in NS) did not affect sRaw in the guinea pigs.

Animals were exposed to ozone awake and spontaneously breathing at rest in a 1 m^3 Rochester chamber. Ozone was generated by passing 100% O_2 through an ozonizer (type III, Sander [Frankfurt, Germany]) regulated by a variable voltage supply. The ozone level was monitored using an ultraviolet ozone analyzer (model 1003 AH, Dasibi [Glendale, CA]) calibrated by comparison to a reference source from the Illinois Environmental Protection Agency.

Dose–response curves to either intravenous substance P, acetylcholine or capsaicin aerosol were constructed by plotting baseline sRaw and the peak values of sRaw for each dose of the bronchoconstrictors used. The dose of substance P or ACh (in μg) that doubled baseline sRaw (i.e., ED_{200}SP or ED_{200}ACh) was determined by interpolation from these graphs. For capsaicin, the dose (in M) that increased baseline sRaw by 35% (i.e., ED_{135}CAP) was determined. If this change did not occur with capsaicin, the value was represented by the maximum dose of capsaicin used (10^{-4} M). Changes in $\log ED_{200}$SP, $\log ED_{200}$ACh, and $\log ED_{135}$CAP (log value preozone − log value 1 hr postozone) for the corresponding experimental and control subgroups were compared using the Student's t-test. Differences were considered significant for p values less than 0.05.

Histopathologic Evaluation. Histologic sections of airways were made for each case. Eight μm thick frozen sections were cut and stained with hematoxylin and eosin. They were each examined by light microscopy to ensure that none of the cases studied had respiratory tract infections evident histopathologically.

Biochemical Assays of Neutral Endopeptidase Activity in Tracheal Tissue. Each guinea pig tracheal segment (200–250 mg in weight) was homogenized (Brinkman Instruments [Boston, MA] in 10 volumes of ice-cold 50 mM Tris-HCl buffer, pH 7.4. These whole tissue homogenates were sonicated twice at 75 W for 10 sec, and then centrifuged at 135,000 g for 1 hr.The pellet was resuspended in 3 ml Tris-HCl buffer containing 0.1% Triton X-100. This was homogenized and placed in a shaker bath at 4°C for 2 hr. The suspension was again centrifuged at 135,000 g. Employing a modification of methods described by Kuwada and Kotayama [13], the initial supernatant was assayed along with the secondary pellet and supernatant (the latter contained all of the activity in the tissue). Aliquots were incubated for 10 min with the chromogenic substrate succinyl $(Ala)_3$-p-nitroaniline (SA_3NA) in the presence of 1 μM phosphoramidon and/or 100 μM amastatin, an antagonist of aminopeptidase activity. Chromophores were detected in the effluent fractions by absorbance measurement at 314 nm. The enzyme preparation was incubated with SA_3NA (0.5 mM), 50 mM Tris-HCl, and 0.1% Triton X-100, pH 7.4 in a final volume of 80 μl. When specific inhibitors were used, they were preincubated with enzyme for 15 min at 4°C. Reactions were run at 37°C for 10 min while being gently shaken and then stopped with 20 μl of 50% trichloroacetic acid. Samples were spun in a microcentrifuge (Eppendorf: model 5451c) at 14,000 g for 10 min at 4°C.

Supernatants were directly analyzed using a high-performance liquid chromatography (HPLC) system (Waters Associates, Milford, MA) to determine hydrolytic products of substrate cleaved by the enzyme. They were applied to a Novapak C-18 reverse phase column (3.9 × 150 mm, Phenomenex, Rancho Palos Verdes, CA) and separated under isocratic conditions (40% methanol and 60% NaH_2PO_4 [10 mM], pH 3.0). Products detected at 314 nm were identified by coelution with standards. Products were quantitated by comparing integrated peak areas to peak areas of known amounts of authentic standards. Authenticity was based upon the sole production of ANA from substrate and its inhibition by 1 μM phosphoramidon. Protein concentrations were measured using a microprotein assay (Bio-Rad Laboratories [Richmond, CA]. Specific activity was expressed in pmoles of alanine p-nitroaniline (ANA) produced/min/mg protein.

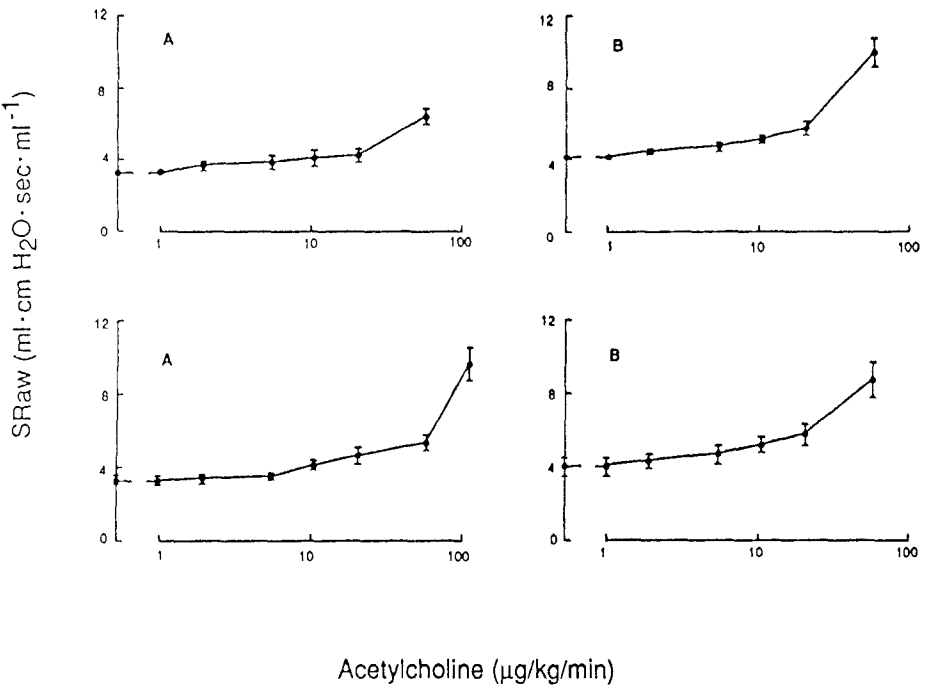

Fig. 1. Mean dose–response curves demonstrate effect of ozone on airway reactivity to intravenous ACh in control (untreated: top row) or dexamethasone pretreated guinea pigs (bottom row). Curves were obtained before ozone (**A**), and at 1 hr (**B**) after exposure (n = 4 for each). Response was measured as specific airway resistance (sRaw) before and after each dose of bronchoconstrictor delivered. Each graph point represents mean ± SEM of measurements made at a given dose.

Drugs. For the experiments in vivo, all solutions were freshly made on the day of use. Acetylcholine, capsaicin, and substance P were all purchased from Sigma Chemical Co (St. Louis, MO); captopril was kindly provided by Squibb Pharmaceutical Corp. (Rahway, NJ).

Statistical Analysis. Mean ± SEM values for each animal subgroup of sRaw and of the change in $\log ED_{200}SP$, $\log ED_{200}ACh$, or $\log ED_{135}CAP$ after exposure were compared using independent tests. Mean ± SEM values of NEP activity from tracheal homogenates of each subgroup were also calculated and compared using the t-test. Differences between subgroups were considered significant for p 0.05.

Results

In neither the experimental (dexamethasone pretreated) nor control (untreated) animal groups was there a significant change in sRaw values 1 hr after ozone exposure. Furthermore, dexamethasone pretreatment itself had no significant effect on sRaw or airway reactivity in unexposed animals.

Mean data concerning changes in SRaw of the groups to intravenous ACh before and after ozone exposure are shown in Fig. 1. One hour after exposure

to ozone, there was no difference between the 2 groups in the increase in their muscarinic reactivity. The delta log ED_{200}ACh was 0.27 ± 0.08 for the experimental group and 0.28 ± 0.04 for the control group at this time (Fig. 3).

In contrast to the lack of effect of dexamethasone pretreatment on muscarinic reactivity post-ozone, animals that had been pretreated with dexamethasone showed significantly less of an increase in reactivity to intravenous substance P after ozone exposure than did the control group (Figs. 2, 3). The delta log ED_{200}SP was 0.18 ± 0.03 for experimental animals and 0.27 ± 0.04 for the controls ($p < 0.05$). There was no difference in sRaw post-ozone between these 2 groups.

Fig. 4 shows data on the changes in reactivity to capsaicin post-ozone in both the experimental and control groups. Mean data for the dexamethasone pretreated group was significantly different from that for the untreated animals. Although the experimental group still demonstrated an increase in capsaicin-induced reactivity post-ozone, the degree of increase in the dexamethasone-pretreated group was less than that seen in controls (Figs. 3, 4).

Data on the ozone-induced changes in substance P reactivity of captopril-pretreated animals are shown in Fig. 5. By itself, captopril pretreatment significantly increased intravenous substance P reactivity in both groups ($p < 0.05$). However, when comparing subgroups pretreated with captopril, it appeared that the corticosteroid effect was as marked postozone on substance P reactivity as that seen in animals without captopril (Fig. 3).

NEP activity of tracheal homogenates from ozone-exposed, dexamethasone-pretreated animals was substantially greater (81 ± 24%) than that from ozone-exposed, untreated animals ($p < 0.05$, $n = 5$). The mean value postozone for guinea pigs who received dexamethasone was 18.3 ± 2.6 nmoles of alanine p-nitroaniline/min/mg protein. This activity was determined in the presence of 10 μM amastatin and was abolished by 1 μM phospharamidon.

Discussion

We have found in this study that dexamethasone pretreatment significantly reduces the increase in airway reactivity to intravenous substance P and to inhaled capsaicin caused by ozone. In contrast, dexamethasone pretreatment had no effect on muscarinic airway hyperreactivity in guinea pigs 1 hr after ozone. In animals pretreated with captopril to block possible dexamethasone stimulation of angiotensin-converting enzyme synthesis and its consequent influence on tachykinin reactivity post-ozone, we found that the corticosteroid effect was as marked as that seen in animals without captopril. Furthermore, homogenates of airway tissue from ozone-exposed, dexamethasone-pretreated animals demonstrated significantly greater NEP activity by chromatographic analysis than did those from ozone-exposed, untreated animals.

There are a number of potential mechanisms by which dexamethasone may act to decrease acute, ozone-induced hyperreactivity to tachykinins. These include stimulation of airway beta-adrenergic responsivity, stimulation of angio-

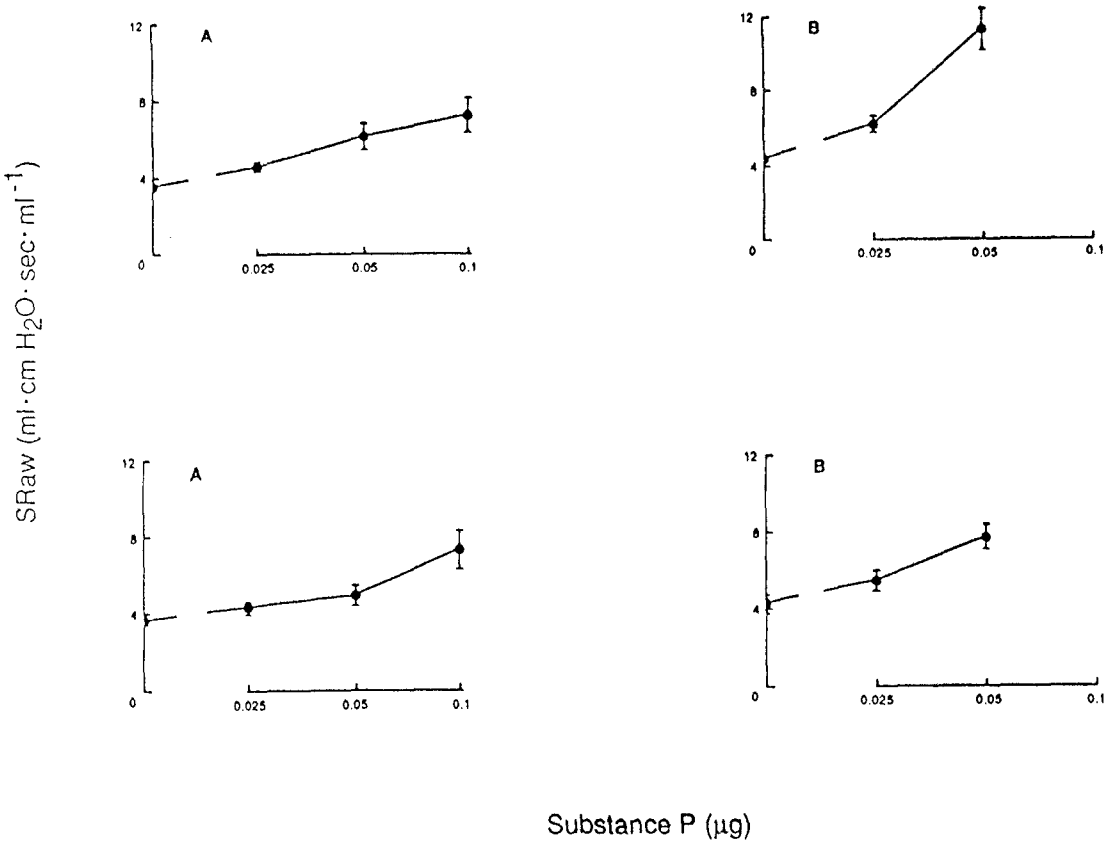

Fig. 2. Mean dose–response curves demonstrate effect of ozone on airway reactivity to intravenous substance P in control (top row) or dexamethasone-pretreated guinea pigs (bottom row). Curves were obtained before ozone (**A**), and at 1 hr (**B**) after exposure (n = 4 for each). Response was measured as specific airway resistance (sRaw) before and after each substance P dose. Each graph point represents mean ± SEM of measurements made at a given dose.

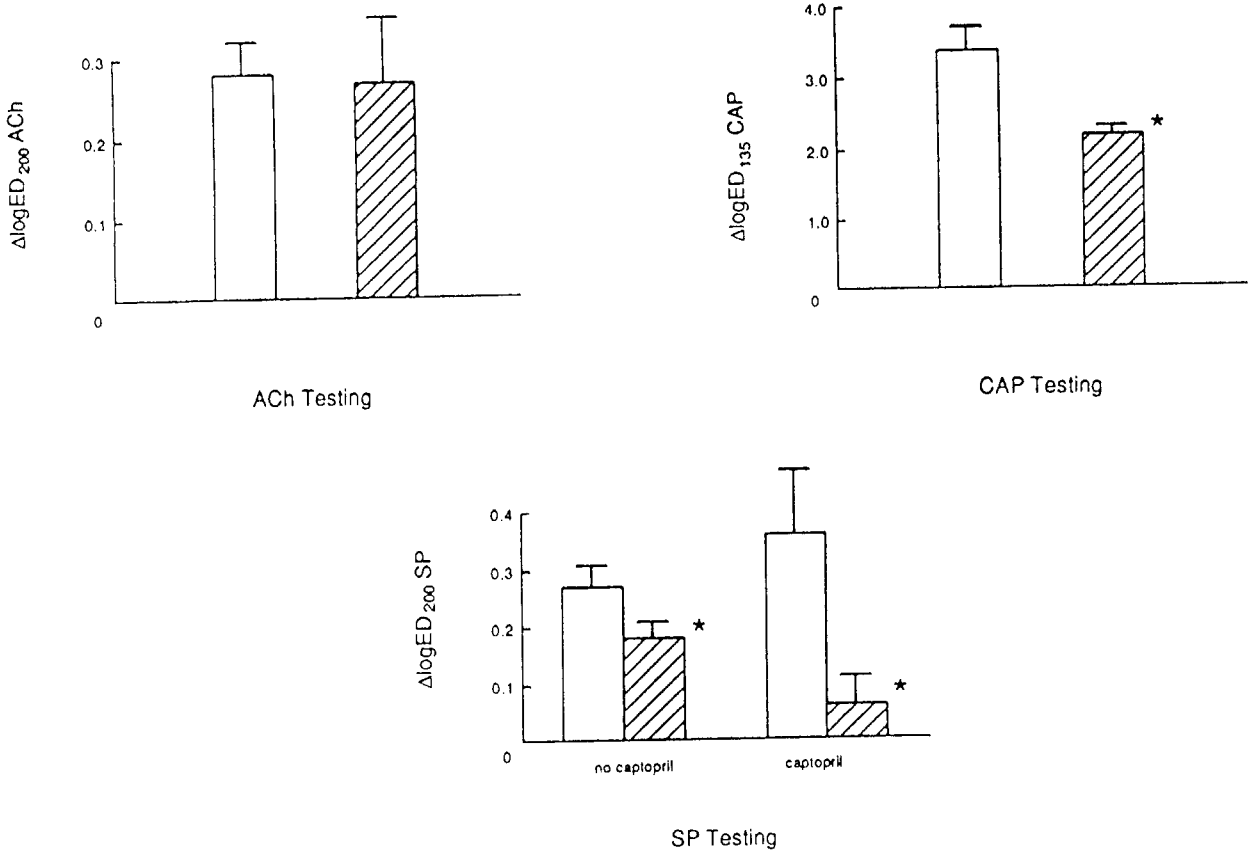

Fig. 3. Comparison of mean values (± SE) for changes post-ozone (Δ) in log EDs for intravenous ACh, aerosolized capsaicin, or intravenous substance P (± captopril) testing of dexamethasone pretreated (cross-hatched bar) and untreated groups (white bar). Asterisks denote values for dexamethasone group significantly less than corresponding untreated group ($p < 0.05$ and $n = 4$ for each).

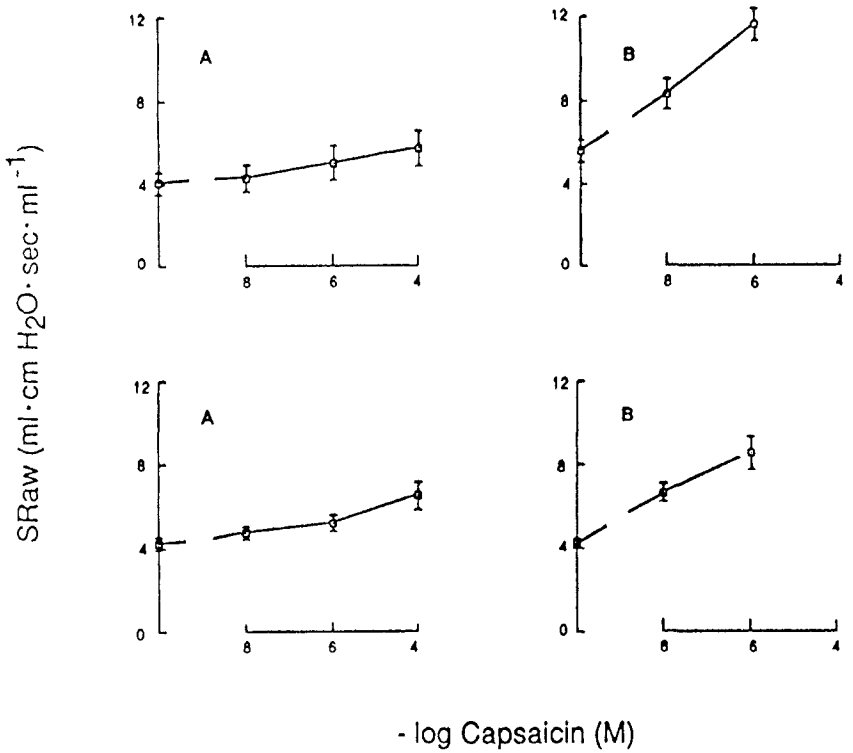

Fig. 4. Mean dose–response curves demonstrate effect of ozone on airway reactivity to aerosolized capsaicin in control (top row) or dexamethasone-pretreated guinea pigs (bottom row). Curves were obtained before ozone (**A**), and at 1 hr (**B**) after exposure (n = 4 for each). Response was measured as specific airway resistance (sRaw) before and after each dose generated from the concentration of capsaicin solution indicated. Each graph point represents mean ± SEM of measurements made at a given dose.

tensin-converting enzyme, and/or reduction in lung vascular permeability. Although corticosteroids have been shown to increase beta-adrenergic receptors in lung cells [9], the former is an unlikely explanation for our observations since all animals were propranolol treated prior to study in vivo. The latter also seems to be an unlikely explanation for our observations. We have previously found that steroid pretreatment does not affect the onset, degree, or time course of ozone-induced nontachykinin hyperreactivity [15]. Other investigators [8] as well as ourselves have concluded that this disorder is not dependent on airway tissue neutrophil infiltration and/or vascular permeability, at least in rodents. In the present study, our finding a differential effect of corticosteroids on exogenous and endogenous tachykinin regarding acetylcholine-mediated hyperreactivity further argues against the possibility that dexamethasone acts on airway reactivity post-ozone by reducing vascular permeability. Such a reduction would permit less intravenous bronchoconstrictor to reach the air-

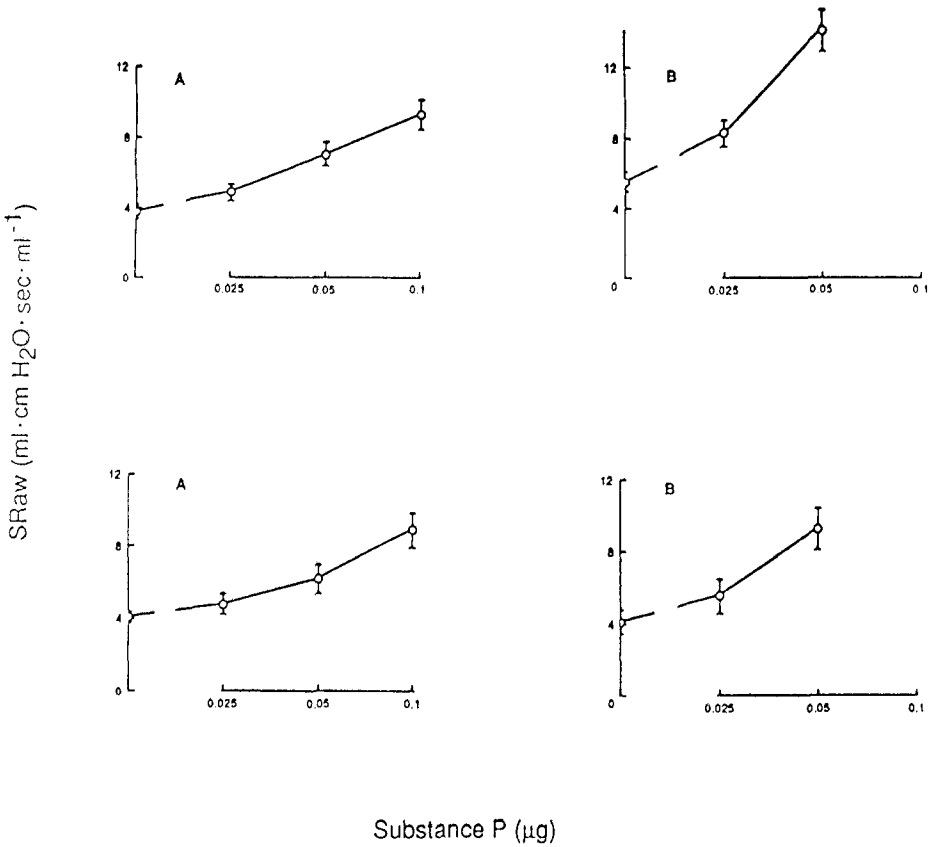

Fig. 5. Mean dose–response curves demonstrate effect of ozone on airway reactivity to intravenous substance P in control (top row) or dexamethasone-pretreated guinea pigs (bottom row) that also received captopril pretreatment. Curves were obtained before ozone (**A**) and at 1 hr (**B**) after exposure (n = 4 for each). Response was measured as specific airway resistance (sRaw) before and after each substance P dose. Each graph point represents mean ± SEM of measurements made at a given dose.

ways, and therefore one might expect to find that muscarinic reactivity post-ozone is also decreased by steroids. This was not our experience. In addition, we think it is also unlikely that possible stimulation of angiotensin-converting enzyme synthesis by dexamethasone is responsible for the reduction in tachykinin reactivity postozone we observed, in that dexamethasone had at least as great an effect on animals that had been pretreated with captopril.

Another explanation for our findings is that dexamethasone stimulates airway NEP activity. We pursued this possibility in our study by measuring airway tissue NEP activity chromatographically. Our having found that airway tissue NEP from dexamethasone-treated animals post-ozone is significantly greater than that found in untreated, ozone-exposed animals is supportive of this possibility. Such stimulation of airway NEP by dexamethasone may partly compen-

sate for the loss in NEP activity due to oxidant injury. Because dexamethasone inhibits tissue neutrophil infiltration [15], our data also argue against the possibility that corticosteroids affect acute, ozone-induced airway hyperreactivity by decreasing airway tissue neutrophil infiltration. If such were the case, one might have expected to find that dexamethasone pretreatment decreases airway NEP activity 1 hr post-ozone in that neutrophils themselves have considerable NEP activity [7]. On the contrary, dexamethasone was found to increase airway tissue NEP activity in this study. A final possibility worth considering is that dexamethasone treatment may downregulate substance P receptors in vivo. This effect has been shown to occur in vitro [10, 11]. Because we have no data to address this issue directly, future studies are required to evaluate it.

The precise biochemical events occurring upon ozone injury of the airways are not yet well understood. On the surface of respiratory mucosal cell membranes [6], it would seem that neutral endopeptidase may be quite vulnerable to inhaled environmental pollutants. Our prior work has led us to the conclusion that airway hyperreactivity to intravenous substance P is caused by ozone's effect on airway mucosal NEP. Although we have previously reported that airway NEP hypoactivity appears to be linked to ozone-induced hyperreactivity and that aerosolized NEP can reverse it [16], these studies do not prove that oxidation of respiratory mucosal NEP is a cause of this disorder, however. Because the major site of degradation of intravenous substance P is the vascular endothelium, an alternative explanation is that ozone-induced hyperreactivity to intravenous bronchoconstrictor derives from endothelial damage rather than at the level of the airway luminal surface. Because ozone is a potent and highly reactive airway oxidant, either itself (if toxic ozone levels are achieved within lung vessels) or secondary mediators it generates upon airway mucosal injury may increase vascular permeability and/or alter perfusion of the vascular bed, thereby allowing more intravenous substance P to reach the airways. Although these possibilities merit future investigation, the fact that guinea pig airway tissue behaves in vitro as if its mucosal endopeptidase activity has been reduced by ozone [17] seems to us to favor a different explanation. This impression derives partly from our observations that mucosal denudation of air-exposed airway tissues causes them to become as hyperresponsive to substance P as ozone-exposed intact tissues, and that mucosal denudation of ozone-exposed airway tissues does not further increase their substance P responsiveness [17]. In other words, removal of ozone-damaged mucosa has no effect on the underlying smooth muscle response to substance P, whereas removal of mucosa from air-exposed tissue, with presumably normal neutral endopeptidase activity, increases substance P responsiveness.

Taken with our past work, the present findings suggest to us that the protection provided by corticosteroid pretreatment against acute, ozone-induced hyperreactivity to tachykinins may be due, at least in part, to stimulation of airway NEP activity. Airway mucosal and nonmucosal NEP may be affected by dexamethasone. It has recently been reported that dexamethasone increases NEP activity in airway epithelial-derived cell lines [1, 14]. In our experience, this increase appears to be both dose- and time-dependent, and is

linked to an increase in NEP mRNA synthesis identified by Northern blot analysis [14]. If ozone inhalation inactivates airway mucosal NEP, as our studies indicate, then it may be that corticosteroid protection such as we observed may be afforded by its stimulating nonmucosal airway cell NEP expression. This steroid effect may benefit post-oxidant reactivity to both exogenous and endogenous tachykinins, as our study suggests. These issues and the therapeutic potential of post-oxidant injury corticosteroids merit future investigation.

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