Effects of a Two-Year Inhalation Exposure of Rats to Coal Dust and/or Diesel Exhaust on Tension Responses of Isolated Airway Smooth Muscle[1-3]

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SUMMARY This study was performed to determine whether chronic inhalation exposure of rats to levels of coal dust (CD) and/or diesel exhaust (DE) similar to those experienced by underground miners affects the pharmacologic characteristics of the animal's airway smooth muscle. Animals were exposed for 2 yr to CD alone (2 mg/m^3 of respirable particulates), DE alone (2 mg/m^3 of respirable particulates), or CD and DE (CD+DE) in combination (1 mg/m^3 CD plus 1 mg/m^3 DE). Concentration-response relationships for tension changes induced with acetylcholine, 5-hydroxytryptamine, potassium chloride, and isoproterenol were assessed *in vitro* on isolated preparations of rat airway smooth muscle (trachealis). Compared with control animals, the maximal contractile responses to acetylcholine of tissues from CD−, DE−, and CD+DE-exposed animals were significantly increased; the effects of CD and DE exposure were additive. The CD+DE exposure, but not the individual treatments, resulted in a significant increase in the maximal relaxation response elicited by isoproterenol; this interaction may have resulted from the addition of, or the synergism between, the nonsignificant effects of CD and DE alone. No treatment altered the sensitivity (EC_{50} values) of the muscles to the agonists used. The results indicate that chronic exposure to CD, DE, and CD+DE produces differential modifications in the behavior of rat airway smooth muscle. These findings may have some bearing on humans exposed to these substances. AM REV RESPIR DIS 1985; 131:651-655

Introduction

The health hazards associated with inhalation of diesel engine emissions in the general population and in an occupational setting and the toxicity of inspired coal dust (CD) in miners have been studied extensively in recent years. The introduction of diesel-powered equipment into coal mining operations has stimulated new evaluations of the possibility that exposure of miners to permissible levels of CD and diesel exhaust (DE) together might result in an occupationally related pulmonary toxicity that is not evident as a result of exposure to either agent alone. With respect to clinical studies of workers exposed to these agents, it has been difficult to establish unequivocally whether inhalation of DE in combination with coal or other dusts presents an additional hazard attributable to DE (1-7).

An alternative means of assessing the potential toxicity of combined CD plus DE exposure involves the use of laboratory animals. Karagianes and coworkers (8) reported recently that the inhalation of CD and DE for 20 months yielded lesions in rat lungs that resembled simple coal worker's pneumoconiosis. No published data are available on the possible interactive effects of the 2 agents on respiratory function in animals, although the literature on the effects of DE on such parameters is vast (9). To our knowledge, the effects of chronic inhalation of CD and DE, alone or together and at workplace levels or higher, on the pharmacologic characteristics of airway smooth muscle have not been studied. This information is needed insofar as alterations in airway resistance, which is determined in part by airway smooth muscle, have often been reported to occur in humans and in animals after exposure to these substances. Therefore, the purpose of the present investigation was to ascertain whether chronic inhalation exposure of rats to levels of CD and DE characteristic of deep coal mines affects the ability of large airway smooth muscle (trachealis) to contract or relax in response to pharmacologic agents.

Methods

Animal Exposures

A detailed description of exposure chamber design and treatment protocols is available elsewhere (10). Briefly, rats (Fischer 344 SPF; Charles River Laboratories, Wilmington, MA), after immunization against Sendai virus and a 2-wk quarantine period, were exposed in inhalation chambers to filtered air (controls), CD, DE, or CD plus DE (CD+DE). Exposures were for 7 h per day, 5 days per wk for 2 yr. The CD was produced by micronization of samples from the Pittsburgh seam. The DE was generated from No. 2 diesel fuel by a 4-cylinder, 4-cycle Caterpillar diesel engine, which is identical to those used in some deep mines in the United States. A repetitive 8-mode duty cycle was used to generate emissions characteristic of DE exposure in deep mines.

Gas, vapor, and aerosol concentrations in the inhalation chambers were within permissible exposure limits for underground coal mines; a portion of these analyses has been reported elsewhere (10). Respirable particulate levels for the 3 treatment groups were kept at 2 mg/m^3, the current federal permissible limit for underground coal mines, by varying the total particulate levels (10). Thus, the composition of respirable particulates in the CD+DE mixture was 1 mg/m^3 of CD plus 1 mg/m^3 of DE.

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Experimental Design

To eliminate bias, the treatment code assigned to the 4 groups of animals was not broken until all the experiments (see below) were completed and the data were analyzed. However, when the animals were received we could not help but notice that the fur of some animals had a brown or grey tint. The animals were received from Cincinnati for testing (in Morgantown) in 2 installments, a week apart, of 20 animals (5 of each exposure group) per delivery. Experiments were begun 1 day after the arrival of the animals. To minimize the possible reversal of treatment effects with time, and since all animals could not be studied simultaneously, animals were killed and studied 3 at a time in continuous morning, afternoon, and evening sessions until all animals were used. The selection of animals from the control and treatment groups for the sessions was sequential, e.g., ABC, DAB, CDA, etc. Because these animals were to be used for many studies by others, only the smooth muscle of the trachea could be allocated for these experiments.

Concentration-Response Relationships of in vitro *Trachealis Preparations*

These experiments were in accord with the "Guiding Principles in the Care and Use of Animals" (11) and other national and international codes. The animals were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally) and killed by aortic phlebotomy. After a midline incision was made in the neck, the trachea from the thyroid cartilage to the point where it enters the thorax was removed, placed in modified Krebs-Henseleit solution (composition below), and cleaned of fat and connective tissue under a dissecting microscope. The trachea was slit longitudinally through its ventral aspect, and opposing ends of the cut cartilage rings were reflected and pinned down. After inspection for gross morphologic alterations, the trachea was cut into segments containing 2 adjacent rings. One end of the cut cartilage was attached by ligature to a holder and the other end was attached to a force-displacement transducer for the measurement of isometric tension responses. Each preparation was mounted in a separate 20-ml organ bath containing modified Krebs-Henseleit solution. A resting tension of 200 mg was applied. The tissues were incubated for 1 h prior to drug additions, during which time the bathing solution was changed every 15 min. The modified Krebs-Henseleit solution contained (mM): NaCl, 113; KCl, 4.8; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 5.7. The solution was gassed with 95% O_2–5% CO_2 to give a pH of 7.4 at 37° C.

Concentration-Response Studies

Concentration-response curves for contraction induced by acetylcholine, KCl, and 5-hydroxytryptamine were obtained by adding cumulative amounts of these agonists to the organ bath. Histamine was not used since it does not contract the rat trachea. To obtain concentration-response curves for isoproterenol-induced relaxation, the tissues were first contracted with 30 mM KCl, a concentration that approximates the EC_{50} (the effective concentration of agonist producing 50% of the maximal response) for KCl. Isoproterenol was added cumulatively after the response to KCl reached a plateau. Each preparation was used to provide only one concentration-response curve for a single agonist. Because regional differences in the pharmacologic characteristics of airway smooth muscle have been noted (12), the 4 paired ring preparations obtained from each trachea were randomized with respect to the agonist used.

Statistical Analysis

Geometric mean EC_{50} values (13) were obtained from regression analysis of probit-transformed data and are presented in the table as -log EC_{50}. All results are presented as mean ± SEM.

Three parameters were subjected to a statistical evaluation of treatment effects on concentration-response curves: EC_{50} values, maximal tissue responses (e.g., contraction or relaxation), and slopes of concentration-response curves. A linear model was fitted by means of the SAS General Linear Model® procedure of the S.A.S. Institute Inc. (Cary, NC) to allow for the evaluation of independent and combined effects (as an interaction term,

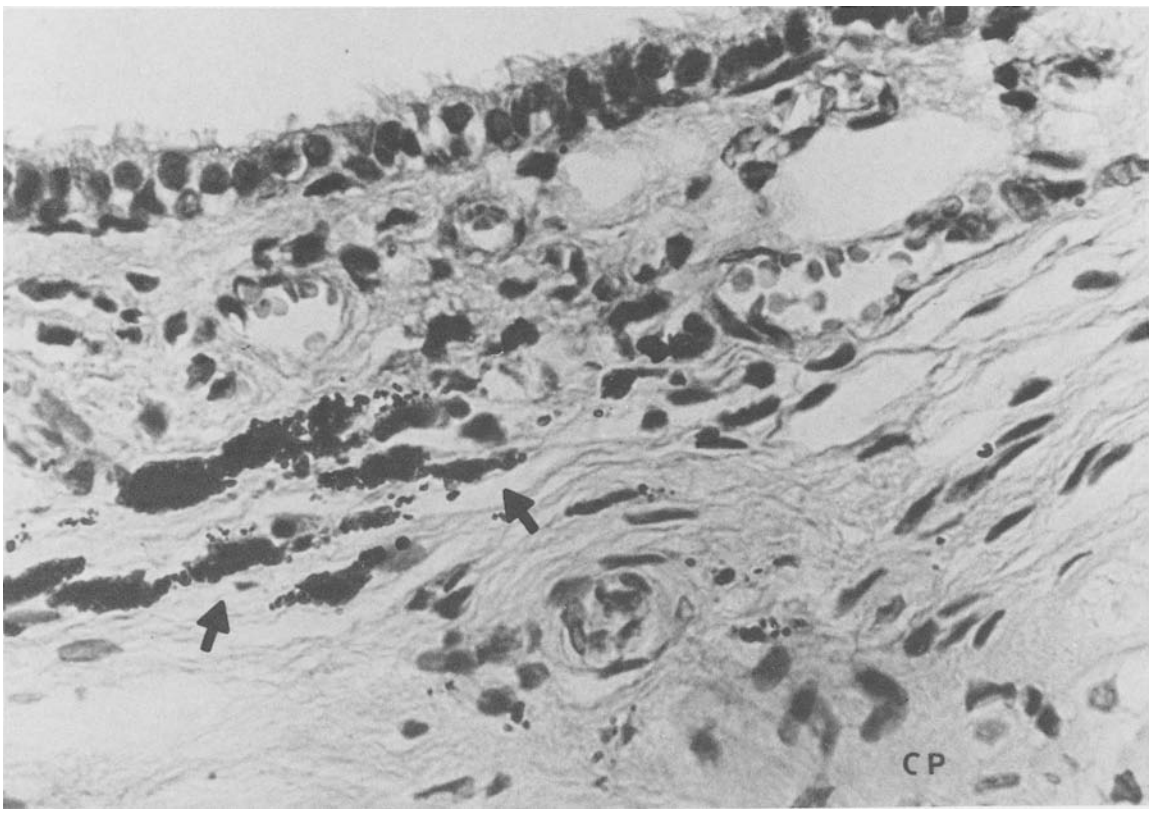

Fig. 1. Section from the trachea of a rat exposed to coal dust for 24 months. Clumps of macrophages containing coal dust particles (*arrows*) are seen in the mucosa adjacent to a cartilagenous plate (CP). The trachea was perfused with formaldehyde solution, embedded in paraffin, sectioned at 7-μm thickness, and stained with hematoxylin-eosin (magnification: ×400).

a_3) of CD and DE on these parameters. The model was:

$$\text{predicted response} = a_0 + a_1[CD] + a_2[DE] + a_3[CD] \times [DE]$$

where [CD] = 2 mg/m³ for CD alone, 1 mg/m³ when combined with DE, and zero for air-breathing control animals, and [DE] = 2 mg/m³ for DE alone, 1 mg/m³ when combined with CD, and zero for air-breathing control animals. Thus, for a_1: [CD] = 0 for air-breathing control animals, [CD] = 2 for CD alone, [CD] = 1 for CD+DE. For a_2: [DE] = 0 for air-breathing control animals, [DE] = 2 for DE alone, [DE] = 1 for CD+DE. For a_3: [CD] × [DE] = 0 for air-breathing control animals and for CD alone and DE alone; [CD] × [DE] = 1 for CD+DE. This model implies a linear effect of [CD] and of [DE] on each response, and permits the exploration of the interaction between CD and DE when applied together.

For isoproterenol concentration-response curves, tests for significant treatment effects also used Student's *t* test for independent means. A probability of less than 0.05 was considered significant.

Miscellaneous. Acetylcholine, 5-hydroxytryptamine, and l-isoproterenol were obtained from Sigma Chemical Co. (St. Louis, MO), and sodium pentobarbital was from The Butler Co. (Columbus, OH). All other chemicals were of the highest grades available commercially.

Results

Morphologic Observations

Upon inspection of the internal surface of the tracheal wall during preparation of the tissues for organ bath experiments, dark patches (less than 0.5 mm) were noted localized in the interstices between the cartilage rings of many of the animals treated with CD and/or DE. They were not particularly prevalent in each trachea (no more than 3 were ever seen in the fields examined). They were noted with the following frequency: CD-exposed, 8/10 trachea; DE-exposed, 6/10 (one questionable); CD+DE-exposed, 6/10 (one questionable). No patches were observed in the tracheas of the air-breathing control animals. The patches appeared to be embedded in the wall of the trachea, since gentle scraping with the tip of microforceps failed to dislodge them. Histologic preparations of tracheas prepared from other, identically treated animals revealed that the patches were composed of dust-laden macrophages in the submucosa adjacent to cartilagenous plates (figure 1).

The trachealis of the rat trachea is very small, and it extends beyond the opening of the cartilage ring. It was not possible, therefore, to obtain gravimetric data on the possible treatment-induced alterations in muscle mass. A subjective nonmorphometric appraisal of histologic preparations of tracheas from other, identically treated animals gave no evidence of treatment-induced gross structural alterations in the trachealis.

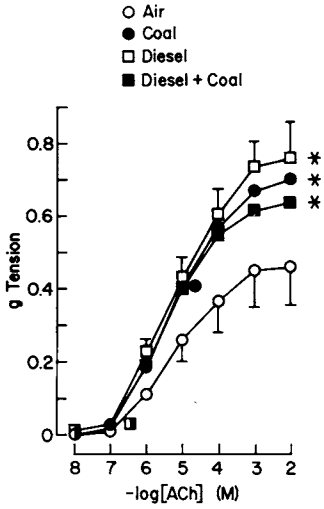

Fig. 2. Acetylcholine (ACh) concentration-response curves for rat trachealis smooth muscle after chronic inhalation exposure of animals to coal dust alone (*closed circles*; n = 10), diesel exhaust alone (*open squares*; n = 10), and coal dust plus diesel exhaust (*closed squares*; n = 9). *Significantly larger than air-breathing control animals (*open circles*; n = 10); g Tension is grams developed contractile tension.

Concentration-Response Relationships

The maximal contractile responses of tracheale from CD-, DE-, and CD+DE-exposed rats to acetylcholine were all increased significantly compared with those from the air-breathing control animals (figure 2). The statistical analysis indicated that the effect of CD+DE treatment resulted from the additive effects of CD and DE and that no interaction between CD and DE had occurred. The lack of a significant effect of any exposure on the EC_{50} value for acetylcholine (table 1) indicates that the sensitivity of the preparations to this agonist was not changed by these treatments. The slopes of the concentration-response curves for acetylcholine also were not affected.

The maximal responses, EC_{50} values, and slopes of 5-hydroxytryptamine concentration-response curves were not affected by any exposure (figure 3 and table 1). Similarly, concentration-response relationships for KCl were not altered significantly (figure 4 and table 1).

The CD or DE exposures alone appeared to increase the maximal response to isoproterenol, but these effects were not significant. The inhalation of both agents together resulted, however, in a significantly larger maximal relaxation response than was observed in the air-breathing control animals (figure 5, *left panel*, and table 1). If the effects of CD alone and DE alone were real but insignificant because of a type II error, then the significant effect produced by CD+DE exposure is explained as an additive effect. However, if the individual effects are truly zero, the observed effect is explained by the statistical model to have occurred through a synergistic interaction between CD and DE. When these data were normalized to examine the fractional extent to which the KCl-induced tone was reduced by isoproterenol (figure 5, *right panel*), a similar relationship held, i.e., that only CD+DE inhalation affected significantly the response to isoproterenol. These effects are not attributable to the level of KCl-induced tone, as responses to KCl (figure 4 and table 1) were themselves not affected by any of the inhalation exposures. No significant effects of the inhalants on the EC_{50} of isoproterenol were observed. However, the slope of the isoproterenol concentration-response curve (table 1) was reduced significantly in preparations removed from CD+DE-exposed rats. This alteration cannot be explained by a synergism or addition of the effects of CD and DE alone.

Discussion

The results indicate that a 2-yr inhalation exposure of rats to CD and/or DE modified some aspects of the pharmacologic responsiveness of the tracheal smooth muscle. The type of alterations that resulted were agonist-specific and, to some extent, treatment-dependent. Thus, (*1*) CD and DE exposures alone and in combination increased the maximal contractile responses of the preparations to acetylcholine; combined treatment produced an additive effect. (*2*) On the other hand, a significant increase in the maximal relaxation response to isoproterenol was seen when CD and DE were inhaled together but not after inhalation of the individual agents. The results suggest that an interaction of some kind occurred when CD and DE were inhaled together, insofar as 1 mg/m³ of CD plus 1 mg/m³ of DE produced a significant effect, whereas 2 mg/m³ of either CD or DE did not. However, it is difficult to be certain of this since the mean responses to CD alone and to DE alone, although not significantly different from the air-breathing control group, were elevated. Thus it may be that type II er-

TABLE 1

ANALYSIS OF TREATMENT EFFECTS ON CONCENTRATION-RESPONSE CURVES

Agonist/Treatment	$-\log EC_{50}$ (M)	Maximal Response	Slope*
Acetylcholine		g Contraction	
Air	4.98 ± 0.18	0.46 ± 0.11	0.94 ± 0.05
Coal	5.13 ± 0.75	0.70 ± 0.06†	0.88 ± 0.04
Diesel	5.10 ± 0.10	0.76 ± 0.11†	0.87 ± 0.04
Diesel + coal	5.16 ± 0.10	0.70 ± 0.05†	0.93 ± 0.05
5-Hydroxytryptamine		g Contraction	
Air	6.11 ± 0.07	0.12 ± 0.01	2.05 ± 0.09
Coal	6.20 ± 0.07	0.07 ± 0.02	2.00 ± 0.01
Diesel	6.07 ± 0.10	0.17 ± 0.06	2.04 ± 0.13
Diesel + coal	6.29 ± 0.08	0.11 ± 0.03	2.08 ± 0.24
KCl		g Contraction	
Air	1.46 ± 0.01	0.67 ± 0.13	7.68 ± 0.31
Coal	1.47 ± 0.01	0.61 ± 0.09	7.58 ± 0.43
Diesel	1.49 ± 0.01	0.65 ± 0.10	7.57 ± 0.32
Diesel + coal	1.46 ± 0.01	0.78 ± 0.11	7.48 ± 0.23
Isoproterenol		g Relaxation	
Air	7.66 ± 0.10	0.15 ± 0.03	1.21 ± 0.04
Coal	7.66 ± 0.11	0.21 ± 0.03	1.19 ± 0.05
Diesel	7.68 ± 0.08	0.22 ± 0.04	1.23 ± 0.07
Diesel + coal	7.98 ± 0.28	0.25 ± 0.03†	0.96 ± 0.05†
		% Inhibition‡	
Air		91.1 ± 5.3	
Coal		100.0 ± 4.7	
Diesel		84.9 ± 10.3	
Diesel + coal		105.2 ± 3.9†	

* ΔProbit/Δ(− log[Agonist]). There were no significant effects of any of the treatments on the correlation coefficients (0.98 to 0.99) of the regression analyses that yielded these slopes.

† Significantly different from air-breathing animals.

‡ Values expressed as percent inhibition of the tension induced with 30 mM KCl before isoproterenol was added.

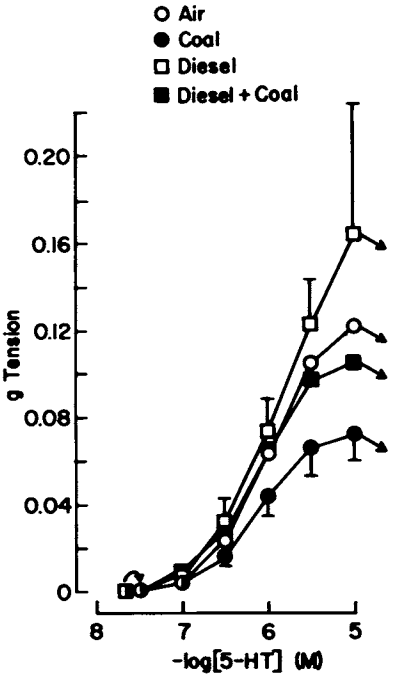

Fig. 3. 5-Hydroxytryptamine (5-HT) concentration-response curves for rat trachealis smooth muscle after chronic inhalation exposure of animals to coal dust alone (*closed circles*; n = 10), diesel exhaust alone (*open squares*; n = 10), and coal dust plus diesel exhaust (*closed squares*; n = 10). Open circles are air-breathing controls (n = 9). The arrows indicate that higher concentrations than those shown elicited gradual declines in tension; g Tension is grams developed contractile tension.

ror prevented the individual effects of CD and DE from being clearly discerned, whereas the combined effect of CD and DE was sufficiently large to attain significance. It follows that it is not possible to determine absolutely how CD and DE act, individually and in concert, on the tissues examined here. (*3*) The maximal contractile responses of the preparations to KCl and to 5-hydroxytryptamine were not affected by any treatment.

Treatment-induced hypertrophy of the trachealis, and/or alterations in the tissue length-tension relationship (since all of the preparations were placed under the same resting tension) could explain the greater contractions of the preparations to acetylcholine and could have contributed to the greater relaxations evoked in response to isoproterenol. There is little evidence to support the view that these considerations obtain, however, for the reasons that no apparent structural alterations in the trachealis were observed and that hypertrophy of the muscle would have resulted in increased maximal responses to KCl and 5-hydroxytryptamine in a manner similar to that observed for acetylcholine, but this was not observed.

No inhalation treatment affected the EC_{50} values for the 3 agonists, suggesting, indirectly, that the affinities of the muscarinic, 5-hydroxytryptamine and beta-adrenergic receptors for these agonists were not modified. The reduced slope of the isoproterenol concentration-response curve observed after combined CD and DE exposure is not readily explained. The lack of treatment effects on the EC_{50} for KCl suggests very indirectly that no substantial membrane electrophysiologic changes occurred in the smooth muscle cells.

The cellular alterations and the chemical factors in CD and DE responsible for the observed changes are unknown and cannot be determined from present results. So many biochemical events occur to initiate contraction and relaxation in response to agonists that it is inappropriate to speculate on the underlying mechanisms.

A major purpose of the present study was to ascertain whether any changes occur after inhalation of CD and DE together that are not observed after treatment with either agent alone. Only 1 such interaction between the 2 agents was observed: the maximal response and slope of isoproterenol concentration-response curves were altered significantly. Because neither treatment alone caused significant changes in these parameters, it is concluded that CD and DE interact to modify responsiveness to isoproterenol.

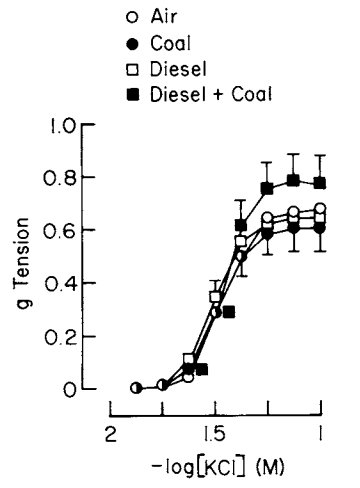

Fig. 4. Potassium chloride (KCl) concentration-response curves for rat trachealis smooth muscle after chronic inhalation exposure of animals to coal dust alone (*closed circles*; n = 10), diesel exhaust alone (*open squares*; n = 10), and coal dust plus diesel exhaust (*closed squares*; n = 10). Open circles are air-breathing control animals (n = 10); g Tension is grams developed contractile tension.

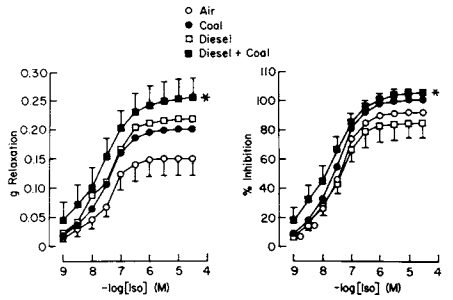

Fig. 5. Isoproterenol (Iso) concentration-response curves for rat trachealis muscle after chronic inhalation exposure of animals to coal dust alone (*closed circles*; n = 9), diesel exhaust alone (*open squares*; n = 10), and coal dust plus diesel exhaust (*closed squares*; n = 9). *Significantly different from air-breathing control animals (*open circles;* n = 10) but not coal dust alone or diesel exhaust alone. Left panel: g Relaxation is grams relaxation of response to 30 mM KCl. Right panel: % Inhibition is percent inhibition of the tension induced with 30 mM KCl before isoproterenol was added.

The relationship of these findings to the potential health hazard in humans is difficult to assess. Studies to date of the effects of CD plus DE exposure on respiratory function in humans have been unable to yield convincing evidence that these agents in concert evoke substantial alterations in ventilatory function (see Introduction) that are not produced by the individual agents or that cannot be otherwise explained. However, chronic inhalation exposure to CD alone has been reported to result in an obstructive impairment in monkeys (14). This finding is, for the most part, entirely consistent with the ability of CD and/or DE treatments to increase the maximal contractile response of isolated rat trachealis (i.e., large airway) muscle to acetylcholine. For example, an increase in responsiveness of the muscles *in vivo* to the excitatory actions of acetylcholine released endogenously by intrinsic cholinergic motor nerves might well result in a relative bronchoconstriction, to which the aforementioned increases in airway obstruction could be ascribed.

The ability of CD plus DE to increase the maximal relaxation response to isoproterenol is puzzling. Aside from the fact that this was the only nonadditive interaction noted, it is difficult to explain how an increase in muscle responsiveness to an inhibitory agent could contribute to pathologic alterations in pulmonary function. Such an increase, however, could enhance the inhibitory effects on airway smooth muscle tone of circulating catecholamines and ameliorate to some degree the increase in airway resistance that would occur as a result of an increased responsiveness of airway smooth muscle to endogenous acetylcholine.

Finally, the presence of dark patches in the wall of the trachea of some treated animals is of interest. In addition to their localization in macrophages (15), black granules have been observed in tracheal and bronchial epithelium of cats (16), and within the mucosa of airways in guinea pigs (17), after acute exposure to diesel exhaust. The localization of patches in the wall of rat trachea, in fairly close proximity to the trachealis, would offer ready access of leachable agents to the smooth muscle where a chronic effect could be exerted. Alternatively, lysosomal enzymes or other pharmacologically active agents released from actively phagocytosing macrophages could contribute to the observed changes.

Acknowledgment

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