

Rat tracheal epithelial responses to water avoidance stress

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Background: Psychologic stress has major effects on many organs and cellular systems. The hypothalamic-pituitary-adrenal axis, corticotropin-releasing factor (CRF), mast cells, and nerves have all been shown to be involved in intestinal epithelial responses to stress. There has been little information in the literature on stress and the lung.

Objective: To investigate Wistar rat tracheal epithelial responses to acute water avoidance stress (1 hour).

Methods: Tracheal tissue was examined in Ussing chambers.

Results: Increases in short-circuit current, but not in conductance, occurred after stress and were inhibited by previous injection of the CRF 1 and 2 receptor antagonist, α -helical CRF-(9-41). Electron microscopic morphologic evidence for tracheal mast cell activation and degranulation was found after stress. Stress and CRF injection both enhanced responses to substance P, but these effects were not inhibited by α -helical CRF.

Conclusion: The data suggest that acute stress affects tracheal epithelium and sensitizes it to enhanced responses to substance P, partly through mast cell activation. Many but not all of these effects are mediated by CRF. These results offer the possibility that stress may be involved in inflammatory diseases of the lung such as asthma. (J Allergy Clin Immunol 2005;116:318-24.)

Key words: Mast cell, hypothalamic-pituitary-adrenal axis, short-circuit current, corticotropin-releasing factor, substance P

The role of stress in asthma is a controversial subject, which is reflected by the literature on this subject.¹ A recent investigation of college students clearly showed that stress associated with examinations enhanced several outcome measures such as sputum eosinophils, eosinophil-derived neurotoxin levels, and IL-5. The authors concluded that stress could act as a cofactor with inhaled antigen to enhance inflammation and asthma severity.² Asthma exacerbations in children increase significantly within 1 to 2 days of a major stressful event. This effect

Abbreviations used

α -Helical CRF: α -Helical CRF-(9-41)

CRF: Corticotropin-releasing factor

Isc: Short-circuit current

PD: Potential difference

SP: Substance P

WAS: Water avoidance stress

was statistically evident for as long as 5 to 7 weeks.³ Indeed, it has become increasingly evident in recent years that the effects of acute and chronic stress on physiologic function can be important in the initiation, development, and/or perpetuation of many human diseases.⁴ The intestine has long been thought of as a target for stress. The role of stress as a determinant in intestinal disease is well established both experimentally and clinically⁵⁻⁷ and may well be relevant to the study of asthma.⁸ Irritable bowel syndrome, as in asthma, is characterized by smooth muscle hyperreactivity.⁹ The major prospective determinant for irritable bowel syndrome after acute infectious diarrhea is accompanying poor psychosocial circumstances.¹⁰ Activation of mast cells colocalized with enteric nerves^{11,12} is a significant hallmark of disease. Finally, experimental acute and chronic stress causes intestinal hypersecretion, permeability, mucin release, and smooth muscle hyperreactivity.^{5-7,13-16} These effects seem to be mediated peripherally by neuropeptides such as substance P and neurotensin, as well as by the autonomic nervous system, and centrally by corticotrophin-releasing factor (CRF). Despite this evidence for stress effects on the intestine, little attention has been paid experimentally to the possible effects of stress on lung function,¹⁷ although the effect of stress on the development and expression of atopy has begun to receive attention.¹⁸ This report represents one of the first such attempts. In it we show that acute stress, mediated in part through the action of CRF, has a stimulatory effect on ion secretion by rat tracheal epithelium, and is accompanied by evidence for mast cell activation and degranulation. At the same time, both stress and CRF prepare (sensitize) tissues for enhanced subsequent responses to substance P (SP). These studies may have important implications for the role of stress in lung diseases such as asthma.

METHODS

Details of the methods can be found in the Journal's Online Repository (www.mosby.com/jaci).

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Animals

Specific pathogen-free male Wistar rats (300–420 g; Charles River, St Constant, Quebec, Canada) were maintained on a 12-hour: 12-hour light-dark cycle. Rats were provided with food and water ad libitum and handled daily for 1 week before the study to minimize effects of stress occasioned by human handling. All experiments were approved by the Animal Care Committee at McMaster University.

Stress model

Water avoidance stress (WAS) was chosen as the model of stress. This model appears to have similar effects and modes of action insofar as intestinal responses and the hypothalamic-pituitary-adrenal axis are concerned.^{5,19} Stress sessions were performed between 1000 and 1300 hours to minimize diurnal variations in response.

Drugs

Corticotrophin-releasing factor and α -helical CRF-(9–41) (α -helical CRF), a CRF 1 and 2 antagonist (Peninsula Lab, Belmont, Calif), were dissolved in PBS according to the manufacturer's instructions, aliquoted, and kept frozen at -70°C until used. The neutral endopeptidase inhibitor, phosphoramidon, was obtained from Sigma Chemical Co (St Louis, Mo) and dissolved in PBS. SP (Peninsula Lab) was dissolved in PBS, aliquoted, and stored at 0°C before use. All drugs were used fresh. Immediately before the experiments, the drugs were diluted in PBS to the appropriate concentrations.

Ussing chamber experiments

Trachea. The trachea was removed, immersed in 37°C oxygenated Krebs buffer, opened along the anterior margin, and immediately mounted in Ussing chambers. Ussing chambers measure current across living tissue. In the case of tracheal tissue, the current measured reflects chloride ion secretion by the epithelium. Tissue was mounted in an Ussing chamber and the spontaneous potential difference (PD) clamped at 0 volts (WP Instruments automated voltage clamp; WP Instruments, Nacro Scientific, Mississauga, Ontario, Canada). The injected current, the short-circuit current (Isc in $\mu\text{A}/\text{cm}^2$), required to maintain 0 PD, was continuously measured and indicates net active ion transport. At intervals, the PD across the tissue was measured to allow calculation of tissue ion conduction (indicates net passive ion flux across the preparation) using the Ohm law and the PD and Isc values. Baseline values for Isc, an indicator of ion secretion, and G, an indicator of ion permeability, were measured 15 minutes after mounting the tissues, and then every 5 minutes for 40 minutes. The tissue was considered equilibrated after 15 minutes. Abnormal baseline values of $G > 50$ millisiemens/ cm^2 were considered damaged and were excluded.

Colon. Tissue was treated as described elsewhere.⁵

Tracheal responses to SP *in vitro*

Substance P in amounts of 10 μL to a final concentration of 10^{-6} mol/L was added to the luminal or the serosal buffer of the Ussing chambers 50 minutes after mounting. After adding SP to the luminal buffer, immediate, sharp increases in Isc were observed. The Isc was calculated as the difference between the baseline Isc value and the maximum value within 10 seconds of addition of SP. In all experiments, phosphoramidon (final concentration: 10^{-5} mol/L) was added to the chamber buffer 10 minutes before adding SP.

Electron microscopy

The tissue was fixed in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 2 hours at room temperature, washed, left overnight at 4°C in 0.2 mol/L sodium cacodylate buffer, pH 7.4,

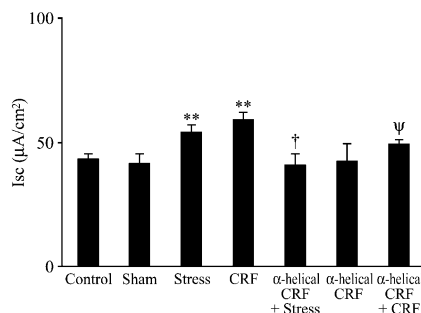


FIG 1. Effect of WAS on tracheal baseline Isc. Rats were subjected to WAS or sham stress for 1 hour or injected intraperitoneally with CRF (50 $\mu\text{g}/\text{kg}$) or α -helical CRF (250 $\mu\text{g}/\text{kg}$) 30 minutes before stress or CRF protocol. Bars represent means $\pm$ SEMs; $n = 4$ to 12 rats/group. ** $P < .01$ vs control; † $P < .05$ vs stress; Ψ $P < .05$ vs CRF.

and then washed 3 times in the same buffer. Samples were postfixed in 2% osmium tetroxide for 1 hour, dehydrated in graded ethanol, and embedded in Spurr resin. The sections were photographed at a magnification of $3000\times$ in a transmission electron microscope (JEOL 1200 EX, Tokyo, Japan). At least 20 mast cells from each rat were analyzed by 2 investigators who were blind to the experiments. Interobserver error did not vary by more than 3%. Activation of mast cells was defined by the presence of granules with altered or absent electron-dense content.

Fecal pellet output

The number of fecal pellets expelled by each rat per hour was used as an indirect measure of colonic motility, as previously described.^{13,20}

Experimental design and pharmacological treatments

A pilot study showed that colonic epithelial abnormalities were maximal at 1 hour after initiation of WAS. The rats were injected intraperitoneally with CRF (50 $\mu\text{g}/\text{kg}$) 1 hour before mounting the tissue. Others had previously determined that this dose was maximal in intestinal Isc responses.⁵ The dose (250 $\mu\text{g}/\text{kg}$) of α -helical CRF has been shown to block intestinal responses to WAS effectively⁵ and was injected intraperitoneally 30 minutes before the WAS protocol.

Measurement of serum corticosterone

Levels were determined by the HPLC method of Wong et al²¹ with some minor modifications.

Statistics

The data were expressed as means $\pm$ SEMs. A P value of less than .05 was considered significant. Differences between the values were tested by using the Student t test or the Scheffe method after ANOVA.²²

RESULTS

Corticosterone levels

For corticosterone levels, see Fig E1 in the Online Repository (www.mosby.com/jaci). The results from the HPLC standards were linear over a dose range of 10 to 1000 ng/mL carbon-treated serum. Control values in normal rats were 102.6 ± 19.5 . It was evident that the sham animals were undergoing mild stress, because their levels were 270 ± 56.1 , which differed significantly from

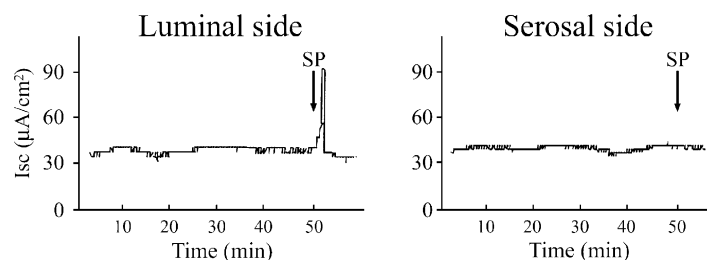


FIG 2. Effect of SP (10^{-6} mol/L) added to the luminal or serosal sides of trachea on I_{sc} generation in Ussing chamber. SP was added 50 minutes after mounting. These tracings are representative of 3 similar experiments.

the controls ($P < .05$). The levels in stressed animals (785.1 ± 121.8) differed significantly both from sham ($P < .01$) and control ($P < .01$) groups. The responses to an intraperitoneal injection of CRF (50 μ g/kg) in PBS 1 hour before mounting the tissues in Ussing chambers were 912.6 ± 201.8 , which again differed significantly from control ($P < .01$). The injection of α -helical CRF before the stress or CRF protocol reduced the levels to approximately the same as those of the sham (234.2 ± 29.4). This level, however, differed significantly from the control values ($P < .05$).

Defecation during stress and response to α -helical CRF

See Fig E2 in the Online Repository (www.mosby.com/jaci). Measurement of the number of fecal pellets per hour corresponded to values expected in controls (0.5 ± 0.3), sham (1.1 ± 1.0), or stress (6.4 ± 0.7) and those animals pretreated with α -helical CRF (3.8 ± 1.0). The number seen in the stress group differed significantly from both control and sham groups ($P < .01$), whereas the animals pretreated with α -helical CRF had a reduced but not significantly reduced number of pellets.

Effects of stress, CRF, and α -helical CRF on colonic epithelial physiology

See Fig E3 in the Online Repository (www.mosby.com/jaci). Baseline I_{sc} responses 15 minutes after mounting were approximately the same in control (30.7 ± 6.3) and sham groups (37.7 ± 6.1). WAS significantly increased I_{sc} relative to sham ($P < .05$) and control groups ($P < .01$). Intraperitoneal injection of CRF (25 μ g/kg in saline) confirmed that this produced significant increases in I_{sc} responses. α -Helical CRF had no effect on colonic I_{sc}⁵ and reduced the effects of stress to baseline.⁵

Effects of stress, CRF, and α -helical CRF on tracheal epithelial physiology

Stress caused increases in I_{sc} compared with both sham ($P < .05$) and control groups ($P < .01$; Fig 1). The effect of intraperitoneal injection of CRF (50 μ g/kg) 1 hour before the tissue was mounted in the Ussing chamber was significant compared with sham ($P < .01$) and control ($P < .01$) groups. This concentration has been shown by others⁵ to have maximal effects on colon epithelial

physiology. The time point of 1 hour was chosen to examine the effect of CRF on the trachea because the stress exposure lasted for 1 hour. We saw no difference at 2 or 4 hours from that seen at 1 hour. There was no significant difference between any of the groups in conductance values (see Table E1 in the Online Repository at www.mosby.com/jaci). Pretreatment with α -helical CRF reduced the I_{sc} seen in stress to the levels of control and sham ($P < 0.05$). The effects of α -helical CRF (42.8 ± 6.4) alone did not differ significantly between control (43.5 ± 1.9) and sham (41.8 ± 3.4) groups. No changes in conductance were seen as a result of treatment with α -helical CRF in any of the groups examined.

Effects of stress and CRF on SP-induced tracheal epithelial I_{sc}

We wished to establish whether stress sensitized tissues for responses to other agonists such as SP. SP effects on the generation of tracheal epithelial I_{sc} have been shown by others to be effective only if delivered on the luminal side.²³⁻²⁵ We confirmed these results (Fig 2) and established that the effect of SP at a concentration of 10^{-6} mol/L was optimal on tissue equilibrated for 15 minutes. No differences were seen with or without the addition of phosphoramidon. As seen in Table I, both stress ($P < .01$) and sham stress ($P < .05$) significantly increased the response to standardized amounts of SP compared with controls. Similarly, CRF injection (50 μ g/kg) intraperitoneally 1 hour before the experiment significantly increased responsiveness of the tissue to SP ($P < .01$). No changes in conductance were seen at any time in these experiments.

Effects of α -helical CRF on stress and CRF effects

Results of experiments with α -helical CRF are shown in Fig 3. α -Helical CRF, which in earlier experiments at this concentration (250 μ g/kg) was effective in reducing stress effects, had no effect on the sensitization induced by stress for subsequent SP responses. Surprisingly, the control experiment of injection of α -helical CRF by itself sensitized the trachea equally as well as stress or CRF so that there were no significant differences compared with control between the stress, CRF, α -helical CRF plus

TABLE I. Effect of stress and intraperitoneal CRF injection on tracheal responses to SP†

	ΔIsc ($\mu\text{A}/\text{cm}^2$)
Control	47.9 ± 4.4
Sham	$105.1 \pm 21.7^*$
Stress	$107.1 \pm 6.4^{**}$
CRF	$129.5 \pm 4.1^{**}$

†Values are means $\pm$ SEMs. The ΔIsc was calculated as the difference between the baseline Isc value and the maximum value within 10 seconds of addition of SP.

** $P < .01$; * $P < .05$ vs control.

stress, and α -helical CRF groups. Pretreatment with α -helical CRF before injection with CRF did not reduce the enhanced response to SP. In fact, the response of this group showed a significant enhancement over CRF itself ($P < .05$), but not over the α -helical CRF alone.

Effects of stress on mast cell morphology

Acute stress caused mast cell activation (Table II) and in some cases degranulation (Fig 4). There were significant differences between control and stressed animals ($P < .05$) in the trachea as well as in the colon ($P < .01$). Nevertheless, some loss of density of granule contents was also found in both colon and trachea of control animals. If this criterion is used to establish activation, an average of 22% of total mast cell granules in various normal tissues, such as skin, ileum, heart, and cranial dura mater,²⁶⁻³⁰ show signs of activation under normal physiologic conditions.

DISCUSSION

To the best of our knowledge, this is the first report of the effects of psychological stress on lung epithelial physiology. We have shown that in Wistar rats, acute water avoidance stress caused increased tracheal epithelial Isc activity, and that this was accompanied by morphologic evidence of mast cell activation and degranulation, was mimicked by systemic CRF injection, and could be largely reversed by a CRF receptor competitive inhibitor, α -helical CRF.

There are many reports now that have focused on the effects of different forms of acute or chronic psychological stress on various intestinal physiologic parameters.^{6,7,14,15,20,31,32} These include experimental results, obtained mostly¹⁶ (but not exclusively) with animal tissues, and include effects on fluid or ion secretion and absorption, permeability,^{5,15,23,33,34} motility,^{6,13,20} and pain perception.³² Tissue responses have been shown to vary according to the experimental animal, strain, and tissue source, ie, stomach,³⁵ jejunum,^{15,31,33} ileum,³⁶ and colon.^{5,6,13,32}

Thus, a significant body of literature now exists that suggests acute stress effects in the intestine are largely mediated by CRF,^{5,6,37,38} because many of the physiological consequences can be reproduced equally either by intracerebral injection of CRF^{6,7,32,38} or through its

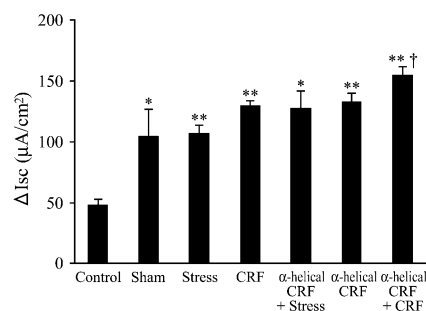


FIG 3. Effect of WAS, CRF, or α -helical CRF injection on SP responses of the trachea in Wistar rats. Rats were subjected to WAS or sham stress for 1 hour or injected intraperitoneally with CRF (50 $\mu\text{g}/\text{kg}$). One hour after the initiation of stress, 90 minutes after α -helical CRF, or 1 hour after CRF injection alone, tracheas were mounted in Ussing chambers. SP (10^{-6} mol/L) was added to the luminal side of the chamber at 50 minutes after mounting. Bars represent means $\pm$ SEMs; ** $P < .01$, * $P < .05$ vs control; † $P < .05$ vs CRF.

peripheral systemic administration.^{5-7,37,38} These effects are mostly mediated through mast cell activation, because pharmacological inhibition of mast cell degranulation prevents many of the consequences of acute stress,^{5,6,32} and mast cell deficient rats fail to exhibit these findings.^{14,15} Mast cell–nerve interactions and communication^{11,39,40} have been shown to be involved in the generation of a variety of intestinal and lung physiologic responses to antigen.^{16,25,39-41} These interactions³⁵ appear to be crucial for the generation of many of the functional intestinal effects of stress, because SP antagonists⁶ block these effects. They are also inhibited by cholinergic, adrenergic, and autonomic ganglion blockade through the use of a variety of well defined pharmacologic agents.⁵

At no time did we see any increase in conductance, a reflection of the integrity of the tracheal epithelium, whereas this is invariably seen as a stress response in the intestine.^{5,34} This probably reflects a general difference between trachea and intestine, because antigen exposure of tracheal tissue from sensitized rats, despite causing large increases in Isc ²⁵ and mast cell degranulation,²¹ never showed increased conductance.^{25,30}

Stress effects are complex end results of a range of interacting signals between a variety of systems and cells of different types. Indeed, the results obtained with α -helical CRF inhibition of stress on corticosterone levels are instructive, because the levels of corticosterone were reduced only to those found in sham animals, and still differed significantly from those of controls. This suggests that factors in addition to CRF and its pathway are operative in the production of corticosterone and not inhibited by this antagonist of CRF receptors 1 and 2.^{42,43} Indeed, behavioral changes apparently mediated by CRF 1 receptors can still be seen in transgenic CRF knockout mice.⁴⁴ Furthermore, stress-enhanced inflammation in a hapten sensitization model of colitis was shown not to be caused by CRF.⁴⁵

Corticotropin-releasing factor is known to have both proinflammatory^{29,46} and anti-inflammatory⁴⁷⁻⁵⁰ effects.

TABLE II. Mast cell activation during stress†

Tissue	Condition	Total granules	Mast cell activation	
			granular change	% Total activations§
			Total	low density‡
Trachea	Control	2631	600	22.5 ± 2.0
	Stress	3234	1163	35.3 ± 2.9*
Colon	Control	2728	619	22.0 ± 2.0
	Stress	2352	940	41.0 ± 1.7**

†At least 20 mast cells from each rat were evaluated (n = 4 in each group).

‡Loss of granule electron density content.

§Indicates the percentage of changed granules (means ± SEMs).

**P < .01; *P < .05 vs control.

It has been shown to cause mast cell degranulation when injected into the skin.²⁹ It also appears to be involved in stress-induced cardiac mast cell degranulation,⁵¹ degranulation of cranial dura mater mast cells,⁵² and those of the urinary bladder.⁵³ However, CRF has not previously been shown to be involved in tracheal or lung mast cell activation. Because we have shown that the tracheal Isc responses to stress are inhibited by the α -helical CRF, and because stress increased tracheal mast cell degranulation (Table II; Fig 4), it appears likely that directly or indirectly, CRF causes tracheal mast cell degranulation. Mast cell degranulation is one of the first effects of allergen inhalation. Stress mediated lung mast cell degranulation is another mechanism for enhancement of the consequence of allergen inhalation in the sensitized host and would further promote airways inflammation. Although rat tracheal and lung mast cells are a mixture of mucosal and connective tissue types,⁵⁴ both seem to respond to stress and CRF, as evidenced by the fact that injection of CRF into rat skin caused degranulation of primarily connective tissue-type mast cells,²⁹ and stress caused elevations of rat mast cell protease II,⁶ a specific marker of mucosal mast cells.

Our results also showed that both acute stress and the peripheral systemic injection of CRF enhanced the action of SP on epithelial cell secretion. Joachim et al⁵⁵ recently showed that stressed mice exhibited increased bronchial hyperreactivity and that this and the increase in airways inflammation was caused by SP. Our experimental results were anticipated in view of the data of McAlexander and Udem,⁵⁶ who showed that CRF caused enhancement of tachykinin-induced contractions of guinea pig tracheo-bronchial smooth muscle. However, our results could not be predicted, because there are now several reports showing that CRF can have a protective effect.⁴⁷⁻⁵⁰ These results range from experiments involving antigen-induced plasma extravasation and that induced by SP or vagal stimulation, all the way to stress-induced worsening of a hapten model of colitis⁴⁸ both in hypo Lewis (LEW/N) and hyper (Fischer, 344/N) CRF responders to stress.^{57,58} Interpretation of the role of CRF in the modulation of stress effects is complicated by our own results, which clearly indicated not only that effects on stress-induced Isc increases were inhibited totally by the

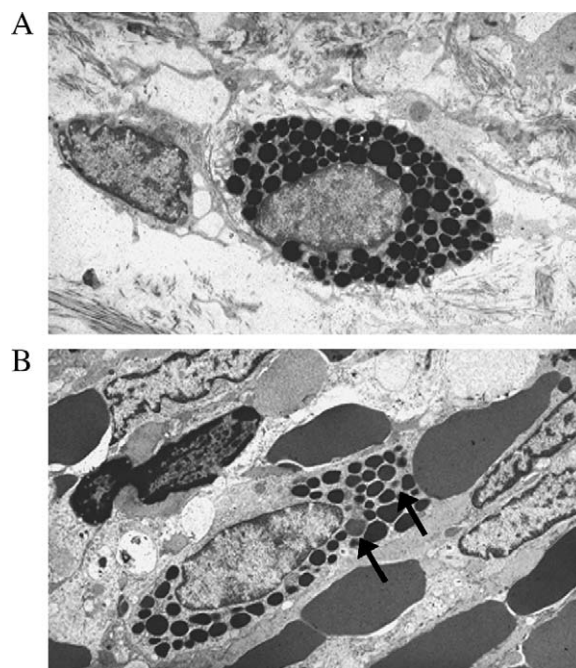


FIG 4. Representative electron micrographs of mast cells in rat tracheal epithelium from control (A) and stress (B) rats. Tracheas were fixed 15 minutes after mounting in Ussing chambers. Mast cells from control groups showed some signs of activation. Mast cells from stress groups were more activated, as indicated by loss of granule density (arrows point to such granules in B). Magnification: 3000 $\times$.

α -helical CRF inhibitor but also that the stress-induced enhancement of the effect of SP was not inhibited at all by the α -helical CRF inhibitor. Further complexity to interpretation was added by the observation that the α -helical CRF inhibitor alone enhanced the SP effect but notably had no effect itself on Isc generation either in the colon or trachea. Last, α -helical CRF did not reduce the activity of CRF in this model but actually enhanced it, confirming its activity independent of CRF. We can conclude that although stress effects on Isc may be mediated largely by CRF, its effects on enhancement of SP may be through additional and different mechanisms.

Several possible explanations may be entertained to explain these unexpected findings. First, α -helical CRF has weak intrinsic activity^{37,42} in several different systems.^{43,59} It might have influenced SP receptor number or affinity or have agonist activity on new, as yet undiscovered CRF receptors expressed in target tissue. That the stress effect itself is, however, not wholly dependent on CRF is further evidenced by our observations that corticosterone levels after stress in α -helical CRF pretreated animals were not reduced to levels seen in controls, but rather only to those seen in sham animals.

The biological significance and relevance of these experiments to asthma lie in the primary observation that acute stress can cause moderate effects on tracheal function. It seems reasonable to argue teleologically that it would be detrimental to the host if hypersecretion by the

tracheal epithelium occurred in response to acute stress, whereas intestinal hypersecretion would not be deleterious to the host's survival. Therefore, it may be expected that these tracheal responses would indeed be moderate and likely to be subject to a significant array of physiologic mechanisms of inhibition and regulation. However, stress activation of epithelium¹⁵ may cause upregulation of cytokines such as IL-8 and possibly eotaxin to account for the findings of Liu et al² that stress enhances sputum eosinophil numbers and products.

Our observation of profound enhancement of the effect of SP as a result of stress may have great significance in terms of understanding the mechanisms of lung diseases such as asthma. SP and other neuropeptides may have major effect in the generation of bronchial smooth muscle contraction experimentally and in asthma.⁶⁰ Extensive reduction of thresholds for this activity would likely have profound deleterious effects in asthma. It is becoming increasingly recognized that several signals may have to occur simultaneously, or in prescribed succession, and only when these occur in genetically vulnerable individuals may this constellation of stimuli lead to disease.^{61,62} In this scenario, acute stress occurring in a particular context of infection and genetic susceptibility could have significant detrimental consequences to the host.⁶³

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