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The role of endothelin in mediating virus-induced changes in endothelin$_B$ receptor density in mouse airways

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ABSTRACT: Emerging evidence supports a mediator role for endothelin (ET)-1 in airway diseases including asthma. Respiratory tract viral infections, are associated with increased levels of ET and altered ET receptor density and function in murine airways. To determine whether these virus-induced effects are causally linked, perhaps involving ET-1-induced ET_B receptor downregulation, the current study investigated the influence of *in vivo* administration of CGS 26303, an ET-converting enzyme inhibitor, on virus-induced changes in ET-content and ET_B receptor density.

CGS 26303 (5 mg·kg^{-1}·day^{-1}) or placebo was administered to mice *via* osmotic minipumps implanted subcutaneously. Two days after implantation, mice were inoculated with influenza A/PR-8/34 virus or sham-infected, and all measurements were performed on tissue obtained on the fourth day post-inoculation.

Viral infection was associated with elevated levels of immunoreactive ET and decreased densities of ET_B receptors in murine airways. Both of these effects were attenuated in virus-infected mice that had received CGS 26303. Virus-induced increases in wet lung weight were also inhibited by CGS 26303. Importantly, administration of CGS 26303 had no effect on the titres of infectious virus in the lungs and similarly, viral infection had no effect on the plasma levels of free CGS 26303.

In summary, CGS 26303 inhibited the virus-induced changes in both immunoreactive endothelin content and endothelin$_B$ receptor density. These findings are consistent with the postulate that the elevated epithelial expression of endothelin-1 during respiratory tract viral infection is a contributing factor in the downregulation of endothelin$_B$ receptors in airway smooth muscle. Whether inhibitors of endothelin synthesis attenuate virus-induced exacerbations of asthma or airways hyperresponsiveness remains to be established.

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The levels of endothelin (ET)-1 in the airways are significantly elevated in respiratory diseases such as asthma [1] as well as in several animal models of airways disease, including allergic inflammation [2, 3] and respiratory tract viral infection [4]. ET-1, *via* its potent actions on a raft of different cell types within the airways and lungs (for review see [5]), may contribute significantly to airway wall remodelling, oedema, bronchial obstruction, long-lasting bronchoconstriction and the development of airway hyperresponsiveness in asthma and during respiratory tract viral infections.

Respiratory syncitial virus induces the expression of ET-1 in bronchial epithelial cells [6] and the levels of immunoreactive (ir)-ET-1 are elevated in murine airways and lung during influenza A viral infection [4]. Increases in the production of ET-1 by the airway epithelium would be expected to lead to enhanced stimulation of ET_A and ET_B receptors in adjacent tissues such as the airway smooth muscle. However, respiratory tract viral infection in mice was associated with reductions in ET_B receptor density, which was reflected in attenuated ET_B receptor-mediated contractile function [7, 8]. This raises the intriguing possibility that the enhanced levels of ir-ET contribute to the reduction in ET_B receptor density and function, perhaps associated with ET-induced ET_B receptor downregulation. In support of this postulate, ET_B receptors present in murine and rat tracheal smooth muscle were readily desensitized *in vitro* [9]. To further test the link between virus-induced increases in ir-ET and reductions in ET_B receptor density, mice in the current study were treated with an ET-converting enzyme (ECE) inhibitor CGS 26303 to attenuate virus-induced increases in ET. If the postulate holds, then administration of CGS 26303 should be associated with inhibition of virus-induced changes in ET_B receptor density as well as ET content.

Methods

Drug administration

Eight-week-old male CBA/CaH mice, specified pathogen free, were obtained from the Animal Resources Centre (Perth, Australia), housed in a controlled environment (Microbiology Animal House, University of Western

Australia, Nedlands, Australia) and received food and water *ad libitum*. Mice were anaesthetized (60 $mg \cdot kg^{-1}$ pentobarbitone sodium, (*i.p.*)), and an osmotic minipump (Alzet 1007D; Alza Corporation, Palo Alto, CA, USA; 7 day duration) containing CGS 26303 or placebo (0.25 M $NaHCO_3$) was implanted subcutaneously on the back immediately posterior to the scapulae. CGS 26303 was dissolved in 0.25 M $NaHCO_3$ at 8.75 $mg \cdot mL^{-1}$ and was delivered at a rate of 0.5 $\mu L \cdot h^{-1}$ (~5 $mg \cdot kg^{-1} \cdot day^{-1}$).

Respiratory tract virus stock and animal inoculation

Influenza A/PR-8/34 virus was grown in the allantoic fluid of 10-day-old embryonated chicken eggs at 37°C for 3 days as described previously [10]. The allantoic fluid was harvested and contained 2.7×10^6 mL^{-1} egg infectious doses (EID_{50}) of virus as determined by the method of allantois-on-shell titration for infectivity [10]. The virus stock was stored in 0.5-mL aliquots at -85°C. Two days after implantation of osmotic minipumps, mice were anaesthetized with Penthrane (methoxyflurane, 1 mL added to a 500 mL-sealed container; Medical Developments, Melbourne, Australia) and groups of CGS 26303-treated animals and placebo-treated controls were intranasally inoculated with 15 μL of fluid containing 1,000 EID_{50} doses of influenza A virus. The remaining mice were sham-infected using a 15 μL solution of a 1:40 dilution of allantoic fluid from virus-free chicken eggs. On day six of the study (*i.e.* day four post-inoculation), mice were sacrificed by an overdose of pentobarbitone sodium (200 $mg \cdot kg^{-1}$, *i.p*; Rhone Merieux Australia Pty Ltd., Pinkemba, Australia). A 0.4-mL blood sample was taken to determine the levels of free CGS 26303 in the circulation. Plasma samples were centrifuged in Centrifree tubes (Amicon, Beverly, MA, USA) and the concentrations of CGS 26303 in the filtrates were measured using a neutral endopeptidase 24.11 inhibition assay. Lungs were blotted dry, weighed and prepared for determination of lung viral titres and extraction of ET. The trachea from each animal was carefully cleaned of adherent fat and connective tissue and prepared for autoradiography.

Lung virus titres

Lung tissues were homogenized in sterile saline with glass/glass tissue homogenizers, and the resulting suspension was clarified by centrifugation at $2,000 \times g$ for 5 min at 4°C. Infectious virus was assayed by allantosis-on-shell titration for infectivity as previously described [10]. Briefly, 6 × 6 mm pieces of allantois-on-shell from 11-day-old embryonated chicken eggs were incubated in sterile round bottom tubes containing 0.35 mL of Standard Medium (SM) and 25 μL aliquots of serial 10-fold dilutions of virus (10^{-2} to 10^{-6}) in SM. Five replicates were used at each dilution. Tubes were sealed and placed in a working horizontal shaker in a 35°C room for 48 h. The fluid from each tube was transferred to a haemagglutination tray and one drop of 10% washed goose red blood cells was added to each well. The trays were shaken and left to stand for 40 min. Positive haemagglutination indicated infection and the EID_{50} was calculated by the method of THOMSON [11]. The composition of the SM (pH 7.0) was (in mM): NaCl 137, KCl 8, $CaCl_2$ 7.2, $MgCl_2$ 0.52, glucose 1.7, acid-free gelatin 2.0 $g \cdot L^{-1}$, phenol red 2.5 $mg \cdot L^{-1}$, penicillin 5 UmL^{-1}, streptomycin 5 $\mu g \cdot mL^{-1}$ and amphotericin B 12.5 $ng \cdot mL^{-1}$.

Autoradiographic studies

Tracheal tubes were submerged in Macrodex (6% dextran 70 in 5% glucose) and frozen by immersion in isopentane, quenched with liquid nitrogen. Serial transverse sections (10 μm) were cut at -20°C and thaw-mounted onto gelatin/chromealum coated glass microscope slides. These sections were pre-incubated (2 × 10 min) at 22°C in a buffer (50 mM Tris-HCl, 100 mM NaCl, 0.25% bovine serum albumin, pH 7.4) containing the protease inhibitor phenylmethysulphonylfluoride (10 μM), and then in another buffer containing 0.2 nM ^{125}I-ET-1 alone for 2.5 h (total binding) or in the presence of BQ-123 (selective ET_A receptor ligand; 1 μM) or sarafotoxin S6c (selective ET_B receptor ligand; 100 nM). Nonspecific binding was determined in the combined presence of BQ-123 (1 μM) and sarafotoxin S6c (100 nM). After 2.5 h, tissue sections were washed twice for 10 min in buffer, rinsed in distilled water and dried under a stream of cold dry air. Emulsion-coated cover slips (Kodak NTB-2; Eastman Kodak Company, Rochester, NY, USA) were attached to one end of the glass slides with cyanoacrylate adhesive and incubated for 3 days at 4°C. The emulsion-coated coverslips were developed (Kodak Dektol, 1:1; Kodak Australasia Pty Ltd., Melbourne, Australia) for 3 min, rinsed for 15 s in dilute acetic acid (2%) containing hardener (Ilford Hypam; Ilford Imaging Australia, Melbourne, Australia) and fixer (Ilford Hypam, 1:4; Ilford Imaging Australia) for 2.75 min. Tissue sections were then stained for 30 s with Gill′s double strength haematoxylin, dehydrated in ethanol, cleared in xylene and mounted (DePeX; BDH Laboratory Supplies, Kilsyth, Australia) for light microscopy.

Autoradiographic grain densities were determined using a computer-assisted grain detection and counting system [12]. A total of twelve slides were assessed (4 × total binding, 3 × BQ-123, 3 × sarafotoxin S6c and 2 × BQ-123 and sarafotoxin S6c) and each slide contained one tracheal ring from each of 32 mice studied (*i.e.* eight mice in each of the four groups). Four estimates of grain density were made per tracheal ring; three over the tracheal smooth muscle band and one over a nontissue area in the airway lumen. Thus, a total of 1,536 fields were analysed (12 slides × 32 sections × 4 fields). Autoradiographic grain densities are expressed as grains·1000 μm^{-2} and presented as the mean grain density±SEM.

Extraction of endothelin from lung tissue

Lungs were homogenized with glass/glass tissue homogenizers in a buffer containing 1M acetic acid and 1 $\mu g \cdot mL^{-1}$ pepstatin A at a ratio of 10 mL buffer·g wet $weight^{-1}$ of tissue. Lung homogenates were then incubated in a boiling water bath for 10 min to inactivate proteolytic enzymes, cooled to 4°C and centrifuged at $100,000 \times g$ for 20 min. The resulting supernatants were frozen and stored at -85°C. ET was extracted from tissue supernatants as described previously [13]. Briefly, C18 Sep-Pak cartridges (Waters, Milford, MA, USA) were pretreated with 5 mL 90% acetonitrile (ACN) in 1% trifluoroacetic acid (TFA) followed by 5 mL 25% ACN in 1% TFA. Samples (100

μL of lung supernatant diluted with 100 μL 1% TFA and 200 μL 50% ACN) were then applied to pretreated C18 Sep-Pak cartridges. After sample application the cartridge was washed sequentially with 20 mL 25% ACN in 1% TFA, 10 mL H_2O and 10 mL 50% methanol. Ir-ET was eluted with 16 mL 50% methanol in 4% acetic acid. Eluted samples were then frozen at -85°C and dried under vacuum in a freeze drier unit (Model FD3; Dynavac, Melbourne, Australia). Dried samples were reconstituted in sample buffer supplied with the enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Co., Ann Arbor, MI, USA) and the assay performed in accordance with the manufacturer's instructions. The antibodies used in this assay cross-react with ET-1, ET-2 and ET-3, but not with big-ET.

Drugs

Substances used included: ^{125}I-ET-1, ET-1, BQ-123 (cyclo(D-Trp-D-Asp-L-Pro-D-Val-L-Leu)), sarafotoxin S6c (Auspep, Melbourne, Australia), CGS 26303 ((*S*)-2-biphenyl-4-yl-1-(1H-tetrazol-5-yl) ethylamino-methyl phosphoric acid) (Novartis Pharmaceuticals Corporation, Summit, NJ, USA), penicillin, streptomycin, amphotericin B, pepstatin A, phenylmethylsulphonylfluoride (Sigma Chemical Co., St Louis, MO, USA).

Statistical analysis

In the current study, four groups of mice were studied in a typical 2 × 2 factorial design. Thus, two-way analyses of variance (ANOVA) were used to determine virus- and CGS 26303-induced changes in ir-ET content and ET_B receptor density. The Bonferroni correction was used for multiple comparisons. A p-value ≤0.05 was considered statistically significant. Grouped data are presented as mean±SEM or mean (95% confidence interval (CI)).

Results

CGS 26303 plasma levels and infectious viral titres

Following administration by osmotic minipump (5 mg·kg^{-1}·day^{-1} for 6 days), the plasma levels of CGS 26303 in sham-inoculated mice (257 nM; 95% CI, 190–357 nM) were not significantly different from levels measured in virus-inoculated mice (162 nM; 95% CI, 105–251 nM; NS). Thus, the plasma levels of CGS 26303 achieved by osmotic pump administration were not affected by coincident viral infection. Similarly, the levels of infectious virus in the lungs of placebo-treated mice (7.46±0.32 log EID_{50} per lung) were not significantly different from levels measured in CGS 26303-treated mice (6.93±0.19 log EID_{50} per lung; NS). Thus, presence of CGS 26303 had no significant effect on the levels of infectious influenza A virus present in the lungs 4 days post-inoculation.

Immunoreactive endothelin content

Ir-ET content in the lungs of virus-infected mice (202±39 pg·lung^{-1}) was 210% higher than that measured in sham-infected mice (66±18 pg·lung^{-1}, p<0.05) (fig. 1). However, pretreatment with CGS 26303, significantly attenuated this virus-induced increase in the levels of ir-ET (p<0.05, 2-way ANOVA), such that ir-ET levels were only 75% higher in virus-infected mice (109±14 pg·lung^{-1}) than in sham-infected mice (62±13 pg·lung^{-1}) (fig. 1).

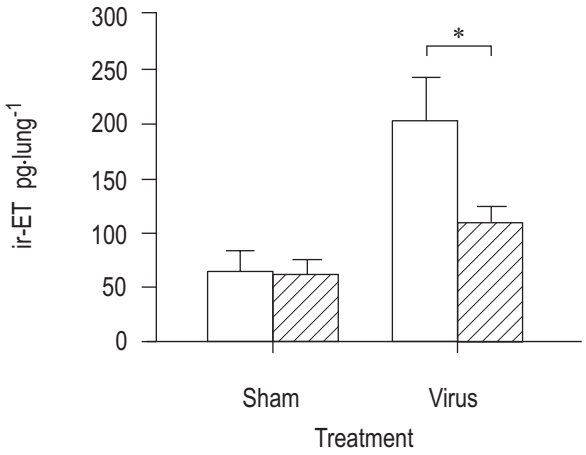

Fig. 1. – Levels of immunoreactive-endothelin (ir-ET) in the lungs of influenza A virus-infected (Virus) and sham-infected mice (Sham) during coincident administration of CGS 26303 (□) or placebo (▨). Data are presented as mean ± SEM (n=6–7). *: p<0.05.

Lung wet weights

On average, lung wet weight was 45±5% greater in virus-infected mice (160±5 mg) than in sham-infected mice (110±3 mg; p<0.05) (fig. 2). However, in CGS 26303-treated mice, lung wet weight was increased by only 17±5% during viral infection (135±6 mg in virus-infected lungs *versus* 116±2 mg in sham-infected lungs; fig. 2) (p=0.001, compared with placebo treatment).

Endothelin$_B$ receptor densities

The levels of specific ^{125}I-ET-1 binding on tracheal smooth muscle were similar in all four groups of mice (two-way ANOVA, table 1), although the relative proportions of ET_A and ET_B receptors differed between

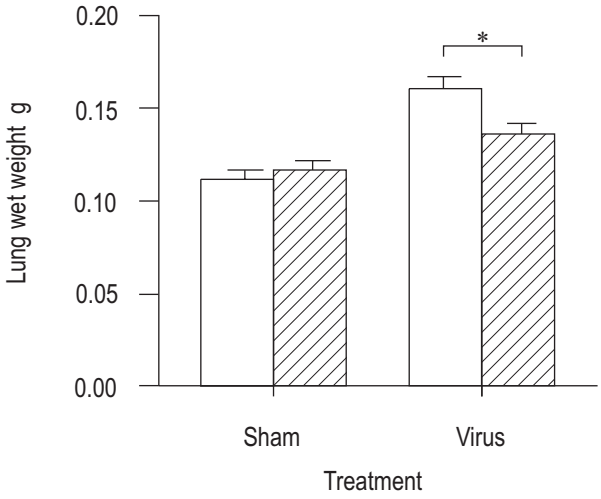

Fig. 2. – Lung wet weight of influenza A virus-infected (Virus) and sham-infected mice (Sham) during coincident administration of CGS 26303 (□) or placebo (▨). Data are presented as mean ± SEM (n=8). *: p<0.05.

Table 1. – Influence of CGS 26303 on virus-induced changes in the densities of endothelin $(ET)_A$ and ET_B receptors on murine tracheal smooth muscle

Treatment		ET receptor density		
CGS 26303	Virus	Total	ET_A	ET_B
No	No	95.8±7.6	28.2±6.2	67.6±12
Yes	No	91.1±7.9	34.8±5.2	56.3±4.7
No	Yes	99.7±8.7	68.0±10	31.7±5.3
Yes	Yes	105±10	50.6±5.7	54.3±8.0

Data are shown as mean±SEM (n=7–8). *: density of autoradiographic grains associated with specific ^{125}I-ET-1 binding.

groups (fig. 3, table 1). Tracheal smooth muscle from placebo-treated, sham-infected mice contained a greater proportion of ET_B receptors (68±7% of specific ET receptors) than ET_A receptors (32±7% of specific ET receptors) (fig. 3). However, as previously described [7, 8], tracheal smooth muscle from virus-infected mice contained significantly lower densities of ET_B receptors (fig. 3). Administration of CGS 26303 had no significant effect on the density of ET_B receptors in sham-infected mice (62±4% of specific ET receptors), but markedly attenuated the virus-induced reduction in ET_B receptors. In mice that received CGS 26303, virus infection caused only a 17±6% reduction in ET_B receptor density compared with the 51±8% reduction observed in placebo-treated mice (p=0.01, fig. 3).

Discussion

Elevated levels of ir-ET within the airways and lung have been observed in many disorders of the lung, including respiratory tract viral infection [4]. In the current study, virus-induced increases in ir-ET levels in the lung were markedly attenuated by CGS 26303, an ECE inhibitor that has recently been shown to inhibit ET-1 synthesis in cultured guinea-pig tracheal epithelial cells [14]. The combination of these data indicate that virus-associated increases in ir-ET content were due, at least in part, to enhanced synthesis of ET by the airway epithelium, although recent studies indicate that tracheal smooth muscle cells can also express prepro-ET-1 and ECE-1 messenger ribonucleic acid (mRNA) [15]. Although the underlying mechanism of virus-induced increases in ET content is unknown, an increased expression of ET-1 mRNA is likely to be involved [6]. Cytokines produced during respiratory tract viral infection [16], stimulated prepro-ET-1 mRNA expression and ET-1 release [17] as well as ECE-1 mRNA expression [18] in human cultured bronchial epithelial cells. Although virus-induced increases in ECE levels have yet to be demonstrated, examination of the structure of the promoters for the ECE-1α and ECE-1β gene suggest that it is the latter isoform that is most likely to be expressed in pathological states [19], such as respiratory tract viral infection.

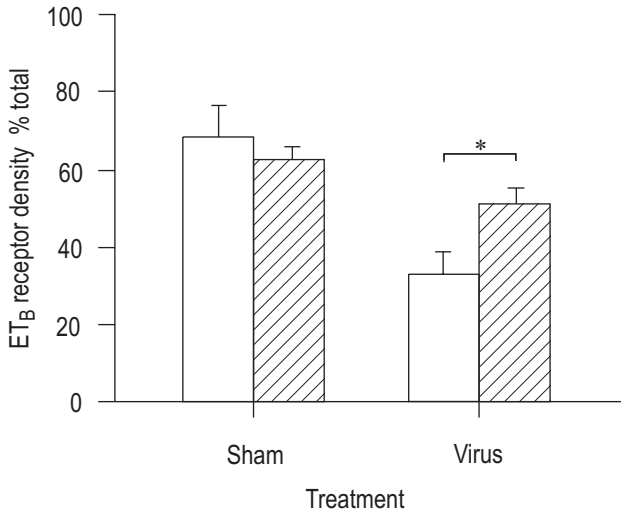

Fig. 3. – Endothelin $(ET)_B$ receptor density as a percentage of total ET receptor density in tracheal smooth muscle from influenza A virus-infected (Virus) and sham-infected mice (Sham) during coincident administration of CGS 26303 (□) or placebo (▨). ET_B receptor densities were determined in quantitative autoradiographic studies using ^{125}I-ET-1 and receptor-selective ligands. Data are presented as mean ± SEM (n=7–8). *: p=0.01.

Elevated levels of ET-1 are associated with reduced ET_B receptor densities in transplanted [20], congested [21] and virally-infected [4, 8] lungs. In the current study, CGS 26303 inhibited both the virus-induced increase in production of ir-ET and the reduction in ET_B receptor density. One explanation for these findings is that viral infection increased the production of ET which then induced ET_B receptor downregulation, a direct mechanism that is consistent with previously published studies showing that the ET_B receptor is readily susceptible to desensitization in airway preparations [7, 9, 22]. Whether this CGS 26303-induced attenuation of virus-induced loss of ET_B receptor density is translated into preserved ET_B receptor-mediated contractile responses in tracheal smooth muscle must await additional studies.

An alternate, or perhaps additional, mechanism that should be considered is that the increased production of ET during viral infection promoted the release of inflammatory cell cytokines and mediators, which in turn caused a reduction in ET_B receptor density. Consistent with this latter indirect mechanism, ET-1 has demonstrable pro-inflammatory effects in the airways, including the influx of eosinophils in a murine model of allergic inflammation [23]. In addition, various inflammatory cell cytokines, including interleukin (IL)-1 and tumour necrosis factor (TNF)-α, have been reported to modulate ET receptor levels [24, 25]. In this regard, it is interesting to note that CGS 26303 inhibited the virus-induced increase in lung wet weight, perhaps by blunting the pro-inflammatory effects of ET-1. However, the CGS 26303-induced reduction in virus-induced lung weight may be otherwise explained by considering haemodynamic influences, such as a reduction in ET-1-induced venoconstriction and the associated pulmonary congestion.

The levels of infectious virus measured in the lungs of mice on day four post-inoculation were not significantly influenced by concomitant administration of CGS 26303 and, similarly, the plasma levels of CGS 26303 were not significantly affected by respiratory tract viral infection. Thus, it is unlikely that the observed changes in the levels of ir-ET and ET_B receptor density in virus-infected mice treated with CGS 26303 (compared with placebo) were due to impaired growth of virus in the airways of CGS 26303-treated mice. Similarly, the differential effect of

CGS 26303 on ir-ET and ET_B receptor levels in virus- and sham-infected mice cannot be explained on the basis of differences in the plasma levels of CGS 26303.

CGS 26303 is a potent inhibitor of neutral endopeptidase (NEP) 24.11 as well as ECE and thus due consideration must be given to the possibility that the effects observed in the current study may, at least in part, have resulted from NEP inhibition. NEP is present in the airway epithelium of several animal species, including humans [26, 27], and is thought to play a role in the catabolism of ET-1 [14, 28]. Thus, in the present study, CGS 26303-induced inhibition of NEP may have had several effects. Firstly, NEP may well act as an "endothelinase" and thus inhibition of NEP by CGS 26303 might be expected to reduce the breakdown of ET-1 and thus increase its levels. However, compared to placebo-treated mice, the levels of ir-ET in CGS 26303-treated mice were either unchanged (in sham-infected mice) or significantly reduced (virus-infected mice), indicating that inhibition of the production of ET rather than prevention of its breakdown was the predominant functional effect of CGS 26303 in the current study. Secondly, inhibition of NEP by CGS 26303 may elevate the levels of other bioactive peptides, with unknown effects on the ET system. However, although NEP plays a role in the regulation of lung growth and maturation in foetal mice [29], the levels of NEP in the upper airways of adult mice are very low (G. Colasurdo, personal communication) in comparison to several other animal species. Indeed, although hitherto untested differences in the activity of NEP in mouse and rat trachea may explain, at least in part, the recently reported observation that parainfluenza-1 virus significantly attenuates ET_B receptor density and function in mouse, but not in rat trachea [30]. The higher activities of NEP in rat trachea may lead to greater catabolism of ET-1 and thereby protect the ET_B receptor from downregulation. In summary, although further definitive studies are required, it appears that within the murine trachea the major influence of CGS 26303 is on ECE rather than NEP.

The major finding of the current study was that CGS 26303 inhibited the virus-induced increase in immunoreactive endothelin content as well as the decrease in $endothelin_B$ receptor density. These findings, together with other data demonstrating that $endothelin_B$ receptors are readily downregulated, support the argument that the elevated levels of endothelin-1 present in virally infected mice caused $endothelin_B$ receptor downregulation. Furthermore, the results of the current study using an endothelin-converting enzyme inhibitor will be of strategic importance in future studies aimed at determining the mediator role of endothelin-1 in virus-induced airway hyperresponsiveness.

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