Attenuation of antigen-induced airway hyperresponsiveness in CGRP-deficient mice

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Aoki-Nagase, Tomoko, Takahide Nagase, Yoshio Oh-hashi, Takayuki Shindo, Yukiko Kurihara, Yasuhiro Yamaguchi, Hiroshi Yamamoto, Tetsuji Tomita, Eijiiro Ohga, Ryozo Nagai, Hiroki Kurihara, and Yasuyoshi Ouchi. Attenuation of antigen-induced airway hyperresponsiveness in CGRP-deficient mice. Am J Physiol Lung Cell Mol Physiol 283: L963–L970, 2002. First published June 21, 2002; 10.1152/ajplung.00130.2002.—Bronchial hyperresponsiveness and eosinophilia are major characteristics of asthma. Calcitonin gene-related peptide (CGRP) is a neuropeptide that has various biological actions. In the present study, we questioned whether CGRP might have pathophysiological roles in airway hyperresponsiveness and eosinophilia in asthma. To determine the exact roles of endogenous CGRP in vivo, we chose to study antigen-induced airway responses using CGRP gene-disrupted mice. After ovalbumin sensitization and antigen challenge, we assessed airway responsiveness and measured proinflammatory mediators. In the sensitized CGRP gene-disrupted mice, antigen-induced bronchial hyperresponsiveness was significantly attenuated compared with the sensitized wild-type mice. Antigen challenge induced eosinophil infiltration in bronchoalveolar lavage fluid, whereas no differences were observed between the wild-type and CGRP-mutant mice. Antigen-induced increases in cysteinyl leukotriene production in the lung were significantly reduced in the CGRP-disrupted mice. These findings suggest that CGRP could be involved in the antigen-induced airway hyperresponsiveness, but not eosinophil infiltration, in mice. The CGRP-mutant mice may provide appropriate models to study molecular mechanisms underlying CGRP-related diseases.

BRONCHIAL HYPERRESPONSIVENESS and inflammation, including eosinophilia, are major characteristics of asthma (11, 12, 23). Recent studies have shown that various mediators, including cytokines, eicosanoids, and adhesion molecules, are involved in the development of asthma. Genetic features are also potentially associated with the etiology of asthma. On the basis of the inheritance pattern, a number of genes could have substantial roles in the pathogenesis of bronchial asthma (43). However, the exact molecular mechanisms of bronchial asthma remain to be elucidated.

Calcitonin gene-related peptide (CGRP), a 37-amino acid neuropeptide, has various biological actions, including responses to sensory stimuli, cardiovascular regulation, and vasodilation (2, 3, 19, 20). CGRP belongs to the calcitonin family of peptides, which includes calcitonin, amylin, and adrenomedullin. The calcitonin receptor-like receptor functions as a CGRP receptor in the presence of receptor activity-modifying protein 1 (RAMP1) (24). There are two CGRP isoforms: α-CGRP, which is present in the central and peripheral nervous system (42), and β-CGRP, which is expressed in specific neuronal sites (1). It has been shown that CGRP, a potent vasodilator (22), modulates hypoxic pulmonary vasoconstriction (17). Recent studies using genetically engineered mice have shown that CGRP-knockout mice exhibit increased blood pressure and overactivation of the sympathetic nervous system (38).

In the respiratory system, CGRP is synthesized by sensory C-fibers throughout the respiratory tree (47). CGRP is also found in neuroepithelial cells of the lung and coexists with tachykinins in many airway sensory nerves (20), and CGRP receptors have been found to densely populate lung vessels (17). In terms of its physiological role, it has been reported that CGRP potently constricts airway smooth muscle in humans (39) and guinea pigs (41). In addition, it has been shown that CGRP has a significant role in eosinophilia in allergic inflammation (7, 37). On the basis of these observations, it is assumed that CGRP might be involved in the pathogenesis of bronchial asthma.

In the present study, we questioned whether α-CGRP might have pathophysiological roles in airway...
hyperresponsiveness and eosinophil infiltration, which are hallmarks of bronchial asthma. To determine the exact roles of α-CGRP in vivo, we chose to study the airway responsiveness and eosinophilia in α-CGRP gene-disrupted mice, which have been recently established (38). After sensitization and antigen challenge, we assessed airway responsiveness and measured proinflammatory mediators.

METHODS

Mice. α-CGRP-null mice were established as previously reported (38). Briefly, the mouse CT/α-CGRP genomic DNA was cloned from a BALB/c mouse genomic library in EMBL3 using synthetic oligonucleotide probes derived from the mouse CT/α-CGRP cDNA sequence. A 7.0-kb fragment containing exons 3–5 of the mouse CT/α-CGRP gene was subcloned into pBluescript (Stratagene). A targeting vector was constructed by replacing the 1.6-kb XbaI-XbaI fragment encompassing exon 5, which is specific for α-CGRP, with the neomycin resistance gene, and flanking the thymidine kinase gene. This plasmid was linearized with NotI and introduced into 129/Sv-derived SM-1 ES cells by electroporation; then the cells were selected in medium containing G418 and neomycin resistance gene, and germline transmission was achieved. The animals were then challenged with saline aerosol for 2 min. Aerosols were generated by an ultrasonic nebulizer and delivered through the inspiratory line into the trachea. Measurements of 10-s duration were sampled during tidal ventilation, 10 min after administration of saline aerosol. This representation of the baseline measurement. Then each dose of methacholine (Mch) aerosol was administered for 2 min in a dose-response manner (0.625–20 mg/ml). Airway responsiveness was assessed using the concentration of MCh required to increase RL to 200% of baseline values (30).

BAL fluid. BAL was performed (5 times with 1 ml of phosphate-buffered saline (PBS)) in each group. In each animal, 90% (4.5 ml) of the total injected volume was consistently recovered. After BAL fluid (BALF) was centrifuged at 450 g for 10 min, the total and differential cell counts of the BALF were determined from the cell fraction (29, 34, 35). The supernatant was stored at −70°C until assays were performed. The concentration of protein was measured by Lowry’s method, with bovine serum albumin as a standard.

Assay of IgE in BALF. IgE levels in the BALF were determined using ELISA kits (Amersham Pharmacia Biotech, Piscataway, NJ). The detection limit of the ELISA assays for IgE was 10 ng/ml.

Assay of thromboxane and leukotriene in BALF. Thromboxane (TX) A2 (TXA2, measured as TxB2) and leukotriene (LT) C4/D4/E4 were determined using enzyme immunoassay (EIA) kits (Amersham Pharmacia Biotech). The detection limits of the EIA assays for TxB2 and LTC4/D4/E4 were 3.6 and 10 pg/ml, respectively.

Assay of endothelin-1 in BALF. Endothelin-1 (ET-1) levels in the BALF were determined using ELISA kits (IBL, Fujioka, Japan). The detection limit of the EIA assays for ET-1 was 0.78 pg/ml.

Assessment of CGRP immunoreactivity. In each group, the lungs of the mouse were removed intact and fixed with 10% formalin. After fixation, the tissue blocks obtained from mid-sagittal slices of the lungs were embedded in paraffin. Blocks were cut 4 µm thick by using a microtome. The preparations were processed for immunostaining by means of the avidin-biotin-peroxidase complex method. The slides were deparaffinized in a xylene bath and dehydrated in ethanol. Endogenous peroxidase activities were blocked by treatment with 0.6% H2O2 in 100% methanol for 30 min at room temperature. The primary antibody, rabbit anti-CGRP (rat) IgG (Peninsula Laboratories, San Carlos, CA), was diluted 1:200 in 10% FCS-PBS and added to the preparations overnight in a cold room. After six washes with PBS, the preparations were exposed to biotin-bound goat antiserum against rabbit IgG as the second antibody for 60 min at room temperature (Histofine, Nichirei, Tokyo, Japan). After the slides were washed with PBS, the tissues were incubated in horseradish peroxi-

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dase-bound streptavidin for 45 min (Histofine). The peroxidase reaction was performed with 3,3'-diaminobenzidine tetrahydrochloride as a chromogen (Vector Laboratories, Burlingame, CA).

Sections were screened and graded using the immunoreactivity score by two observers who were blind to the status of the specimen (5, 21). Visual assessments of the density of CGRP immunoreactivity were graded from none (score = 0) to abundant (score = 4). To examine the location of CGRP immunoreactivity, we analyzed large airways (airway diameter >0.2 mm), small airways (airway diameter <0.1 mm), and lung parenchyma. The scores represent the density of CGRP-immunoreactive cells and tissues that might include nerves, ganglia, and neuroepithelial bodies (NEBs) (5). In terms of the reproducibility of this assessment, inter- or intraobserver variances were not significant.

Materials and chemicals. Materials and chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified.

Data analysis. Comparisons of data among the experimental groups were carried out with ANOVA (Scheffe's test). Values are means ± SE. P < 0.05 was taken as significant.

RESULTS

Airway responsiveness to MCh administration. There were no significant differences in baseline RL and EL among each group. MCh dose-response curves for RL and EL are demonstrated in Figs. 1 and 2, respectively.

Airway responsiveness was assessed using the MCh concentration required to increase RL to 200% of baseline: 17.4 ± 1.8 and 17.6 ± 1.4 mg/ml for saline-treated α-CGRP+/+ and α-CGRP−/−, respectively, and 6.9 ± 1.3 and 16.5 ± 1.5 mg/ml for OA-treated α-CGRP+/+ and α-CGRP−/−, respectively (P < 0.05, OA-treated α-CGRP+/+ vs. other groups). Although bronchial hyperresponsiveness to MCh was observed in the OA-challenged wild-type mice, responses in the OA-challenged α-CGRP−/− mice were significantly reduced compared with the OA-challenged wild-type group.

Assessment of the BALF. Antigen exposure increased protein amount in BALF, although there was no difference between the wild-type and mutant mice (Fig. 3). Total cell counts and cell fractions in BALF are shown in Table 1, indicating the increases in the total cell number in the OA-sensitized groups. OA challenge induced eosinophil infiltration, whereas no differences in the fraction and number of BALF eosinophils were observed between the wild-type and α-CGRP−/− mice.

The IgE levels were significantly greater in OA- than in saline-treated mice. However, there were no significant differences between the wild-type and α-CGRP−/− mice (Fig. 4).

Measurements of thromboxane, leukotriene, and ET-1 in BALF. There were no significant differences in BALF TxB2 among the groups: 0.140 ± 0.047 and 0.135 ± 0.076 ng in saline-treated α-CGRP+/+ and α-CGRP−/−, respectively, and 0.423 ± 0.204 and 0.498 ± 0.262 ng in OA-treated α-CGRP+/+ and α-CGRP−/−, respectively.

BALF LTC4/D4/E4 was significantly greater in OA-treated α-CGRP+/+ mice than in any other group (Fig. 5).
Meanwhile, LTC_4/D_4/E_4 was reduced to the same level in antigen-treated CGRP^{−/−} mice and the saline-treated groups.

There were no differences in BALF ET-1 among the groups: 1.35 ± 0.32 and 1.01 ± 0.11 pg in saline-treated α-CGRP^{+/+} and α-CGRP^{−/−}, respectively, and 2.03 ± 0.38 and 2.70 ± 0.60 pg in OA-treated α-CGRP^{+/+} and α-CGRP^{−/−}, respectively.

**Assessment of CGRP immunoreactivity.** Figure 6 demonstrates the immunohistochemistry of CGRP in large airways. In the OA-sensitized wild-type mouse, significant immunoreactivity for CGRP was observed in the airway epithelium and submucosa, while the immunostaining was modest in the saline-treated wild-type animal. On the other hand, there was little CGRP immunoreactivity in saline- or OA-treated α-CGRP^{−/−} mice. In small airways and lung parenchyma, little CGRP immunoreactivity was observed in each experimental group (Fig. 7). Table 2 summarizes the visual assessment of CGRP immunoreactivity in each group. In airway epithelium, submucosa, and smooth muscle of large airways, the scores were significantly higher in OA-treated CGRP^{+/+} mice than in any other group, although there were marked differences between α-CGRP^{+/+} and α-CGRP^{−/−} mice. Meanwhile, the scores were much lower in peripheral airways and lung parenchyma than in large airways in the wild-type mouse.

**DISCUSSION**

The results of the present experiments show that antigen-induced bronchial hyperresponsiveness was significantly reduced in CGRP-deficient mice. Meanwhile, eosinophil infiltration elicited by antigen challenge was unaffected by disruption of the CGRP gene. Antigen-induced increases in BALF LTC_4/D_4/E_4 were significantly attenuated in α-CGRP-disrupted mice. These findings suggest that CGRP could be involved in the antigen-induced airway hyperresponsiveness, but not eosinophil infiltration, in mice.

CGRP has pleiotropic and pathophysiological effects on various cells and organs (3, 44). CGRP exerts trophic effects on skeletal muscle and vascular smooth muscle (3). CGRP also modulates some macrophage functions, including antigen presentation (13, 36). In the respiratory system, CGRP is synthesized by sensory C-fibers in the respiratory tree (47). However, the pathophysiological roles of CGRP in the lung have not been determined. Palmer et al. (39) demonstrated that CGRP potently constricts human airway smooth muscle. On the other hand, recent studies have reported that CGRP acts as a potent inhibitor of responses elicited by bronchoconstrictive stimuli (4, 33). Regarding eosinophil chemotaxis, Numao and Agrawal (37) reported that neuropeptides, including CGRP, may play a significant role in eosinophil infiltration by priming cells in allergic inflammation. Meanwhile, Teixeira et al. (46) demonstrated that CGRP has little effect on eosinophil accumulation in guinea pig skin. In the present study, we hypothesized that CGRP could

![Fig. 4. IgE levels in BAL fluid in wild-type and α-CGRP-deficient mice (n = 4–6). #P < 0.05 compared with saline groups.](Image 320x88 to 548x276)

![Fig. 5. Leukotriene (LT) C_4/D_4/E_4 levels in BAL fluid in wild-type and α-CGRP-deficient mice (n = 4–6). *P < 0.05 compared with sensitized wild-type mice.](Image 56x78 to 284x240)
play a significant role in the underlying mechanism of asthma. To test this hypothesis, we studied the allergic pulmonary responses using α-CGRP gene-disrupted mice, which have been recently established by Oh-hashi et al. (38).

Allergen-induced airway hyperresponsiveness was significantly attenuated in the α-CGRP-deficient mice, suggesting that the existence of CGRP per se might be associated with bronchial hyperresponsiveness, which is a major trait of asthma (11, 12, 23). To our knowledge, this is the first report to use mutant mice to study whether the CGRP gene and endogenous CGRP could be involved in the airway hyperresponsiveness. Recently, using a pharmacological approach, Dakhama et al. (6) found that exogenous administration of CGRP to sensitized and challenged mice results in the normalization of airway responsiveness. However, the exact mechanism to explain the involvement of CGRP in airway hyperresponsiveness remains to be clarified.

In the present study, the molecular and pathophysiolog-
CGRP gene expression might affect airway hyperresponsiveness. One of the possible mechanisms is that CGRP and CGRP gene expression might affect airway inflammation, including eosinophilia, after antigen challenge. Airway eosinophilia is one of the common features in asthmatic patients and could be involved in bronchial hyperresponsiveness (11, 23). In the present study, however, no significant difference in BALF eosinophil counts was observed between the wild-type and CGRP-deficient mice. These results suggest that disruption of the CGRP gene has little effect on antigen-induced airway eosinophilia in mice. Although the sequence of rodent CGRP contains the tetrapeptide eosinophil chemotactic factor, the effect of CGRP on airway eosinophil infiltration is not remarkable in the present model. Therefore, CGRP-dependent airway hyperresponsiveness might not be mediated by eosinophilia.

Possibly, immunization provoked by antigen challenge might be affected by modulation of the CGRP gene. However, increased IgE levels after antigen challenge were observed in both groups, whereas there were no significant differences in measured IgE levels between the wild-type and CGRP-deficient groups. Alveolar protein leakage or airway mucus secretion assessed by BALF protein was consistent with the results of IgE measurement in this study. These findings indicate that modulation of the CGRP gene might not affect the mechanism of IgE production.

Recently, it has been shown that bronchial asthma is related to the generation of various potent mediators, including thromboxane, leukotrienes, and ET-1 (28, 45, 49). These mediators are reported to be involved in airway hyperresponsiveness (28). Cysteinyl leukotrienes (LTC4, LTD4, and LTE4) are reported to be among the most important targets for treating bronchial asthma. It has been shown that administration of cysteinyl leukotriene antagonist reduces antigen-induced airway hyperresponsiveness (10, 49) and the increases in airway smooth muscle after antigen exposure (49). Irvin et al. (14) demonstrated that antigen-induced airway hyperresponsiveness is significantly decreased in 5-lipoxygenase-deficient mice, suggesting the important role of leukotrienes in development of airway hyperresponsiveness. The potential sources of cysteinyl leukotrienes in the lung include alveolar macrophages, eosinophils, basophils, mast cells, and platelets (40). The proinflammatory activities of cysteinyl leukotrienes, including bronchoconstriction, mucus secretion, and plasma exudation, are mediated via the interaction with its receptor, the CysLT1 receptor (9). In humans, it has been recently demonstrated that the CysLT1 receptor is expressed in lung smooth muscle, lung macrophages, and peripheral blood leukocytes, while the identification of the CysLT1 receptor is consistent with the anti-inflammatory actions of CysLT1 receptor antagonists (9).

Potentially, genetic disruption of the CGRP gene may modulate the production levels of various potent mediators. We therefore measured possible mediators in the BALF and found that the production of cysteinyl leukotrienes was enhanced in the sensitized wild-type mice. In contrast, the level of cysteinyl leukotrienes was significantly reduced in the sensitized CGRP-deficient mice. There were no significant differences in thromboxane or ET-1 in each group. These observations indicate that CGRP gene disruption might inhibit the production of cysteinyl leukotrienes, which could be associated with reduced airway hyperresponsiveness. Meanwhile, after antigen challenge of wild-type and CGRP-deficient mice, there were no significant differences in the number of alveolar macrophages or eosinophils, i.e., potential sources of cysteinyl leukotrienes. One of the possible mechanisms to explain this observation is that CGRP might be involved in activation of the 5-lipoxygenase pathway.

In the present study, we used mutant mice deficient in α-CGRP but not β-CGRP. Therefore, these mutant mice should express β-CGRP. Because the α-CGRP antibody used in this study cross-reacts with β-CGRP (79%), the CGRP immunoreactivity represents α-CGRP and, similarly, β-CGRP. The very small amounts of CGRP immunoreactivity in the mutant mice may indicate β-CGRP expression in the lung. It has been previously reported that α-CGRP concentrations are approximately four times greater than β-CGRP concentrations in the rat lung, whereas in the intestine, β-CGRP concentrations are up to seven times greater than α-CGRP concentrations (26). Presumably, it seems that β-CGRP expression in the lung might not be affected by disruption of the α-CGRP gene in mice.

In the wild-type mice, we observed substantial CGRP immunoreactivity in the epithelium and submucosal tissues in large airways but not in small airways or parenchyma. Presumably, CGRP-immunoreactive

### Table 2. Visual assessment of airway CGRP immunoreactivity using immunoreactivity scores

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<tr>
<th></th>
<th>Epithelium</th>
<th>Submucosa</th>
<th>Smooth muscle</th>
<th>Small Airways</th>
<th>Lung Parenchyma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline +/-</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>-/-</td>
<td>0.2 ± 0.2a</td>
<td>0.2 ± 0.2a</td>
<td>0.0 ± 0.0a</td>
<td>0.0 ± 0.0a</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Ovalbumin +/-</td>
<td>2.6 ± 0.2†</td>
<td>2.4 ± 0.2†</td>
<td>2.1 ± 0.1†</td>
<td>0.4 ± 0.2</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>-/-</td>
<td>0.2 ± 0.2a</td>
<td>0.4 ± 0.2a</td>
<td>0.1 ± 0.1a</td>
<td>0.0 ± 0.0a</td>
<td>0.0 ± 0.0</td>
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Values are means ± SE. CGRP, calcitonin gene-related peptide. aP < 0.05 compared with wild-type (+/+) groups. †P < 0.05 compared with saline groups.
cells and tissues include nerve fibers in submucosal tissues, whereas airway epithelium contains nerves and NEBs. Terada et al. (47) reported that nerve plexuses of CGRP-immunoreactive fibers are located in the basal part of the rat tracheal epithelium. These CGRP-immunoreactive intraepithelial nerves lack myelin and Schwann sheaths and run through the bases of the epithelial cells (47). In this study, the CGRP immunoreactivity in large airway epithelium was remarkable, and it was enhanced by antigen challenge. These observations suggest that the epithelium of central airways, including nerves and NEBs, may have a significant role in antigen-induced airway hyperresponsiveness. Meanwhile, it is assumed that the contribution of peripheral airways and parenchyma to CGRP-related airway physiology is small.

Recently, Dakhama et al. (6) showed that CGRP expression was diminished in airway epithelium and submucosal nerve plexuses only after the third OA challenge, although CGRP depletion did not occur after the single antigen exposure. In our study, however, the single antigen challenge enhanced CGRP immunoreactivity in large airways in the wild-type mice, whereas little CGRP immunoreactivity was observed in CGRP-deficient mice in the absence or presence of antigen challenge. The present findings suggest that endogenous CGRP per se may be related to the development of antigen-induced airway hyperresponsiveness.

Genetic features, including single-nucleotide polymorphism, are potentially associated with the etiology of asthma. On the basis of the inheritance pattern, a number of genes could have substantial roles in the pathogenesis of bronchial asthma (43). Murine models of asthma have been recently used to investigate individual genes associated with airway hyperresponsiveness (8, 15, 16, 18, 31, 48). Because CGRP is one of the genes regulating the function of CGRP, potent mediators possibly involved in bronchial asthma (39), genes regulating the function of CGRP, calcitonin receptor-like receptor, and RAMP1 could be targets to study the pathogenesis of asthma. Consistently, the present observations suggest that α-CGRP and the α-CGRP gene play significant roles in the molecular mechanism underlying bronchial asthma, indicating that the α-CGRP gene could be a target for single-nucleotide polymorphism research. The α-CGRP-mutant mice used in this study may contribute to the study of the genetic roles of α-CGRP in bronchial asthma and may provide novel insights into the pathophysiological roles of α-CGRP and the α-CGRP gene in vivo.

In summary, reduction of antigen-induced airway hyperresponsiveness was detected in α-CGRP-deficient mice. Meanwhile, eosinophilic infiltration associated with antigen exposure was not altered by disruption of the α-CGRP gene. Antigen-induced increases in cysteinyl leukotriene production were significantly reduced in α-CGRP-disrupted mice. Disruption of the α-CGRP gene might inhibit production of cysteinyl leukotrienes, which could be associated with reduced airway hyperresponsiveness. Antigen challenge enhanced CGRP immunoreactivity in the wild-type mice, whereas little CGRP immunoreactivity in epithelium or submucosa was observed in α-CGRP-deficient mice. These findings suggest that endogenous CGRP may be involved in development of antigen-induced airway hyperresponsiveness. Taken together, CGRP and CGRP gene expression might be involved in the pathogenesis of bronchial asthma by acting as a mediator. The CGRP-mutant mice may provide appropriate models to study molecular and pathophysiological mechanisms underlying diseases related to CGRP.

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AIRWAY RESPONSIVENESS IN CGRP-DEFICIENT MICE


