

Long-term induction of β -CGRP mRNA in rat lungs by allergic inflammation

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Abstract

Calcitonin gene-related peptide (CGRP) is one of the major neuropeptides released from sensory nerve endings and neuroendocrine cells of the lung. Two CGRP isoforms, α - and β -CGRP, have been identified in rats and humans, but no studies have attempted to reveal direct evidence of differences in action or location of these

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isoforms in allergic inflammation (AI). We investigated mRNA expressions of α - and β -CGRP in lungs, nodose ganglia (NG), and dorsal root ganglia (DRG) of an animal model for AI of the airways, utilizing a model created by sensitizing Brown Norway (BN) rats with ovalbumin (OVA). By semiquantitative RT-PCR analysis, long-lasting enhanced expression of the β -CGRP mRNA was shown in the lungs of the AI rats (14.5-fold enhancement at 6 hr, 8.1-fold at 24 hr, and 3.7-fold at 120 hr after OVA-challenge compared to the level in the lungs of phosphate-buffered saline (PBS)-challenged control rats). In contrast, the mRNA expression of the α -CGRP in AI lungs showed only a transient increase after OVA-challenge (2.7-fold at 6 hr) followed by a lower level of expression (0.5-fold at 48 hr and 0.6-fold at 120 hr). The mRNA expressions of both isoforms in NG, but not in DRG, were transiently up-regulated at 6 hr after antigen challenge. In situ RT-PCR in combination with immunohistochemical analysis revealed that β -CGRP was expressed in neuroendocrine cells in clusters (termed neuroepithelial bodies [NEBs]) in AI lungs. These results indicate that the long-term induction of β -CGRP in NEBs may play an important role in pulmonary AI such as bronchial asthma.

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Keywords: Calcitonin gene-related peptide (CGRP); Allergic inflammation; Bronchial asthma; Neuroendocrine cells; Neuroepithelial bodies (NEBs); Rat

Introduction

A number of neuropeptides have been identified in the respiratory tracts of humans and animals (Solway and Leff, 1991). Tachykinins (substance P and neurokinin A) and calcitonin gene-related peptide (CGRP) are the major neuropeptides released from nerve endings. They are reported to cause a variety of responses, such as plasma extravasation and vasodilation, which are termed “neurogenic inflammation” (Barnes, 2001). The responses are enhanced through retrograde activation of the sensory nerves by an axon reflex, and neurogenic inflammation contributes to bronchial asthma. The actions of tachykinins, such as priming inflammatory cells (Tiberio et al., 2003) and adhesion molecules (Smith et al., 1993; Levite et al., 1998) have been studied, while CGRP is a potent vasodilator as well as bronchoconstrictor (Springer et al., 2003). Moreover, CGRP can induce a significant increase of eosinophils in the airways in vivo (Bellibas, 1996) and promote eosinophilic migration in vitro (Numao and Agrawal, 1992). These actions of CGRP are important for exacerbating allergic inflammation (AI) of the airways. On the other hand, several studies have shown that CGRP plays regulatory roles in excessive immune responses and a protective role against epithelial damage. These regulatory roles include (i) inhibition of the release of inflammatory mediators, such as leukotriene, and of their actions (Raud et al., 1991) (ii) suppression of antigen-presentation of several kinds of immune cells (Hosoi et al., 1993) and (iii) promotion of the migration and the proliferation of tracheal epithelial cells (White et al., 1993; Sanghavi et al., 1994). Therefore, CGRP appears to be multifunctional in AI.

Two CGRP isoforms generated from different genes (α - and β -CGRP) have been identified in rats and humans (Bennett and Amara, 1992). Comparison of the peptide sequences of α - and β -CGRP reveals a striking degree of sequence homology (human α -CGRP differs from β -CGRP in 3 of 37 amino acids, and rat α -CGRP differs from β -CGRP in 1 of 37 amino acids), and the two isoforms were considered to be almost equivalent in function. However, several other gene families of neuropeptides (e.g., the prolactin-growth hormone gene family, the opioid gene family) express homologous sequences and functionally different peptides (Russo et al., 1988). These findings led us to speculate that, although the

structural difference between α - and β -CGRP is small scale, it may also be important. In fact, up-regulation of α -CGRP and down-regulation of β -CGRP in spinal motoneurons after axotomy (Piehl et al., 1993) implies functional differences between the two isoforms in axonal regeneration. Although an increase of CGRP content in the lungs of rats and guinea pigs by allergic inflammation has been reported (Nilsson et al., 1990; Fischer et al., 1996), it remains unknown which isoform of CGRP is up-regulated and what cell type is the main source of the increased CGRP.

CGRP is thought to be mainly produced by two sources in the mammalian lung. One is sensory neurons that innervate the respiratory tracts. The perikarya of these neurons reside primarily in the vagal jugular and nodose ganglia, and the thoracic (Th_{1–6}) dorsal root ganglia (Springall et al., 1987). The other source is the pulmonary neuroendocrine cells, which occur in two forms: singly or in clusters termed “neuroepithelial bodies (NEBs)” (Cadieux et al., 1986). NEBs are presumed to be hypoxia-sensitive airway chemoreceptors (Youngson et al., 1993), and can produce CGRP (Springer et al., 2003). CGRP released from NEBs are thought to play a role in the regulation of regional ventilation and perfusion-blood-flow ratio, which leads to an adjustment of hypoxemia (Stevens et al., 1997). Interestingly, NEBs are also innervated by the vagal afferent (sensory) and efferent nerves, and the vagal sensory nerves and NEBs can interact with each other (Van Lommel et al., 1998). However, involvement of NEBs in AI has not been studied thoroughly in the context of neurogenic inflammation, and which CGRP isoform is expressed in NEBs still remains to be established.

We conducted the present study with two aims. The first aim was to explore which isoform of CGRP is more involved in AI, and the second was to identify the main source of CGRP in the airways at different times after allergen challenge. To address these problems, we investigated the gene expression and tissue distribution of α - and β -CGRP in the lungs, nodose ganglia (NG) and thoracic dorsal root ganglia (DRG) of Brown Norway (BN) rats after ovalbumin (OVA)-challenge. The responses for a particular antigen of these rats mimics those of human allergic asthma very closely, for instance, with high levels of allergen-specific IgE by the sensitization (Pauwels et al., 1979), in the early- and late-phase bronchoconstrictions which are associated with airway inflammation, and bronchial hyperresponsiveness after allergen challenge (Renzi et al., 1993).

Materials and methods

Animals

Male inbred BN rats weighing 120–150 g and 6–7 wk of age, purchased from a commercial source (Sankyo Labo Service Co., Tokyo, Japan), were housed in an air-filtered, pathogen-free environment in the Animal Research Facility at Yokohama City University. All experimental procedures in this study were approved by the Committee on Animal Research at Yokohama City University.

Sensitization and challenge

We used the sensitization protocol set out by Eidelman et al. (1988). Rats were actively sensitized by a single subcutaneous injection of a suspension containing 1 mg of OVA (grade V; Sigma, St. Louis, MO, USA) and 200 mg of Al(OH)₃ in sterile saline. Simultaneously, 0.5 ml of the adjuvant, *Bordetella pertussis* vaccine containing 1×10^{10} heat-killed organisms (Wako, Osaka, Japan), was administered

intraperitoneally. At 14 days after sensitization, the animals were placed in a Plexiglas chamber (20 liters) and challenged with a 2.0% solution of OVA for 30 min using an ultrasonic nebulizer (NE-12; Omron Ltd., Kyoto, Japan). At each of the following times after the OVA challenge, the animals were sacrificed, and then the lungs ($n = 5$; at 6, 24, 48, and 120 hr), NG ($n = 3$; at 6, 24, and 120 hr), and DRG (Th1-6) ($n = 3$; at 6, 24, and 120 hr) were removed. The control rats were treated with a single subcutaneous injection of the OVA/Al(OH)₃ solution and challenged with an aerosol of PBS ($n = 5$; at 24 hr).

OVA-specific IgE determination

To confirm the successful sensitization, the serum OVA-specific IgE values of the sensitized control and AI model rats were compared with those of non-sensitized rats ($n = 5$) by an ELISA method. Animal blood samples were collected by cardiac puncture when the animals were killed. Serum was obtained by centrifugation and frozen until use. We utilized the ELISA method as described by [Waserman et al. \(1992\)](#) to determine the serum OVA-specific IgE values. Ninety-six-well microtiter plates (Nalge Nunc International, Naperville, IL, USA) were coated overnight with mouse monoclonal antibody anti-rat IgE, (Zymed Laboratories Inc., San Francisco, CA, USA) diluted in carbonate/bicarbonate buffer (2 µg/ml). The plates were blocked with 0.5% casein in PBS, and successively treated with 100 µl of diluted serum samples (1:20) at 37 °C for 1 hr, 100 µl of biotinylated OVA (5 µg/ml) at 37 °C for 1 hr, and alkaline phosphatase-conjugated streptavidin (1:1000; Zymed) at 37 °C for 1 hr. Between steps, the wells were washed four times with PBS containing 0.05% Tween-20. After addition of disodium *p*-nitrophenyl phosphate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), plates were developed at room temperature and spectrophotometrically read at 405 nm with an ELISA plate reader (Model 3350; Bio-Rad, Hercules, CA, USA). Since rat OVA-specific IgE was not commercially available, results of the immunoglobulin assay were expressed in arbitrary units of absorbance. Plates were developed to give a reading of 1 unit of absorbance for a positive control sample from a sensitized BN rat.

Histopathology

Twenty-four hours after the OVA or PBS challenge, the lungs were removed and instilled with phosphate-buffered 10% formalin via a tracheal cannula at 20 cm H₂O pressure. Specimens from the upper lobes of right lungs were fixed for one day, embedded in paraffin, sectioned into 5-µm slices, and stained with May-Grünwald-Giemsa for the detection of eosinophils. A total of 5 animals in each group were used for the study.

Immunohistochemistry

Immunohistochemistry was performed as previously described ([Ito et al., 1998](#)). The left lungs from the control (24 hr after PBS challenge) and AI models of rats (6 hr, 24 hr and 120 hr after OVA challenge) were harvested and fixed in phosphate-buffered 10% formalin for 3 hr. After washing in 0.1 M phosphate-buffered solution (pH 7.3) and incubation with 20% sucrose in phosphate buffer, the lungs were embedded in OCT compound (Tissue-Tek, Sakura Finetechnical Co. Ltd, Tokyo, Japan), frozen with liquid nitrogen and stored at –80 °C until use. The frozen tissues were sectioned into 7-µm-thick slices, and two serial sections were used for immunohistochemistry; one was incubated with polyclonal

anti-CGRP antibody (Sigma; 1:1000 dilution), and the other was incubated with anti-PGP 9.5 antibody (UltraClone, Isle of Wight, UK; 1:40) to identify pulmonary NE cells. After the incubation with the primary antibody overnight at 4 °C, the sections were treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel, Durham, NC, USA; 1:200) and with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma) for 4 hr at room temperature. TRITC-conjugated phalloidin for F-actin was used to visualize the cytoskeletal structure of the lung (Nagahara et al., 1998). After rinsing, the sections were counterstained with 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim, Mannheim, Germany). Then they were mounted with a phenylenediamine and glycerol mixture and observed with an epifluorescent microscope and a confocal laser microscope equipped with argon ion and helium neon lasers (LSM GB200UV; Olympus, Tokyo, Japan).

Purification of RNA

Approximately 100 mg of lung tissue from each animal was put in 1 ml of TRIzol reagent (Invitrogen, Groningen, Netherlands) in a sterile polypropylene vial, and homogenized with a Polytron (Kinematica AG, Lucerne, Switzerland). NG and DRG from each animal were digested with 400 µg/ml proteinase K (Invitrogen) at 42 °C for 1 hr, and 1 ml of TRIzol reagent was added to the digested tissue. Homogenates of lung tissue and ganglia were incubated in 1.5-ml sterile tubes at room temperature for 10 min, 0.2 ml of chloroform was added, and the tubes were vortexed and centrifuged at 15,000 rpm for 15 min at 4 °C. The RNA layer was transferred to a fresh 1.5-ml tube containing 0.5 ml of chloroform, and centrifuged at 15,000 rpm for 10 min at 4 °C. The RNA pellet was then washed with 70% ethanol, air-dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water. The concentration of RNA was determined with a spectrophotometer (Gene Quant Pro; Amersham Biosciences Corp., Piscataway, NJ, USA).

RT-PCR

First-strand cDNA was synthesized from 4 µg of total RNA with 200 U of the Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) in the presence of 20 U of RNasin (Promega, Madison, WI, USA), 10 mM dithiothreitol (DTT) (Invitrogen), each dNTP at 1 mM, and 200 ng of pd(N)₆ random hexamer (Amersham Biosciences). Oligonucleotide sense and antisense primers for the PCR were as follows: α-CGRP, 5'-CCTGGTTGTCAGCATCTTGC-3' and 5'-CACATTGGTGGGCA-CAAAGT-3' (a 316-bp product); β-CGRP, 5'-CTGGCTCTCAGCAGCATGTG-3' and 5'-CACATTGGTGGGCACAAAGT-3' (a 333-bp product); the internal standard, glyceraldehyde phosphate dehydrogenase (GAPDH), 5'-TTCAACGGCACAGTCAAGG-3' and 5'-CATGGACTGTGGTCATGAG-3' (a 373-bp product). Although the gene encoding rat α-CGRP (*Calca*) is different from the gene encoding rat β-CGRP (*Calcb*), the sequence for the antisense primer is common to both genes (328–347 of α-CGRP mRNA, Genbank Accession No.M11597, and 346–365 of β-CGRP mRNA, Genbank Accession No.M11596). Since the sequences for the sense primers are specific to the respective genes, α-CGRP mRNA can be distinguished from β-CGRP mRNA by the PCR reactions with these 3 primers. PCR amplification was performed in a reaction volume of 25 µl containing 2 µl of diluted cDNA, 0.625 U of AmpliTaqGold™ DNA polymerase (Applied Biosystems, Foster City, CA, USA), each dNTP at 0.2 mM, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 1.5 mM MgCl₂ and sense and antisense primers each at 0.5 µM. After a “hot-start” at 95 °C for 9 min, 33 cycles (for lung cDNAs of

α - and β -CGRP), 30 cycles (for NG cDNA of β -CGRP), 27 cycles (for NG cDNA of α -CGRP and DRG cDNA of β -CGRP), 24 cycles (DRG cDNA of α -CGRP) and 20 cycles (for lung, NG, and DRG cDNA of GAPDH) of amplification were performed, with one cycle consisting of incubation at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min (Sango et al., 2002). The PCR products were resolved by electrophoresis in a 2% agarose gel at 100 V for 30 min. The intensity of the PCR fragments visualized by ethidium bromide staining was quantified with an Epi-Light UV system (EU-1150 II, Aisin Cosmos R and D Co., Ltd., Nagoya, Japan). The abundance of the respective mRNAs was expressed as the intensity of each CGRP cDNA fragment/intensity of the GAPDH cDNA fragment. For asthmatic model groups, this ratio was compared to the ratio of the control group using the following formula: ([intensity of α - or β -CGRP cDNA fragment/intensity of GAPDH cDNA fragment] of the sample of the asthmatic model group)/([intensity of equivalent type of CGRP cDNA fragment/intensity of GAPDH cDNA fragment] of the control group) = relative gene expression. RT-PCR experiments were repeated three times, and reproducibility was thereby confirmed. All PCR reactions were carried out under conditions in which amplification was linear by using the appropriate number of cycles.

Direct sequencing of these PCR products (Sawady Technology Co., Ltd., Tokyo, Japan) confirmed that they were authentic α -CGRP, β -CGRP and GAPDH mRNA, respectively.

In situ RT-PCR

The lungs were removed from AI model groups (6 hr and 24 hr after OVA-challenge) for *in situ* RT-PCR. We followed the Nuovo's protocol (Nuovo, 1994), in which extreme precautions against RNase contamination were not necessary until the end of protease digestion. Sections of 6- μ m thickness were cut on a cryostat and mounted onto silane-coated glass slides; we wore gloves and used dry-heat sterilized slides during the preparation of cryosections. The specimens were immersed in proteinase K (1 μ g/ml) at room temperature for 10 min and then treated with RNase-free DNase I (Invitrogen) overnight at 37 °C. The slides were incubated at 42 °C for 30 min with 50 μ l of RT-reaction solution containing 1 μ M antisense primer and MMLV-RT (Takara, Otsu, Japan). PCR reaction was carried out in a solution containing 4.5 mM MgCl₂, each dNTP at 200 μ M, 10 μ M digoxigenin-11-dUTP (Boehringer Mannheim), sense and antisense primers each at 0.8 μ M and 5 U of Taq DNA polymerase (Takara) in a final volume of 50 μ l per sample. The PCR reaction was performed with an initial denaturing step at 94 °C for 3 min, followed by 30 cycles of annealing and extension at 56 °C for 2 min, denaturation at 94 °C for 3 min, and final denaturation at 94 °C for 1 min. The digoxigenin-labeled PCR products were detected after incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500) for 1 hr and developed in a nitroblue tetrazolium chloride and 5'-bromo-4-chloro-3-indolphosphate (Boehringer Mannheim) for 30 min. DNase I treatment was omitted for a positive control and the RT step was omitted for a negative control.

Some of the serial sections prepared for immunohistochemistry (described as above) were used for *in situ* RT-PCR and immunohistochemistry to confirm that CGRP mRNA-positive cells were also CGRP-immunoreactive.

Statistical analysis

Results for individual groups were expressed as mean $\pm$ SEM. Statistical comparisons between groups were performed with the unpaired, two-tailed Student's *t* test.

Results

Antigen-specific IgE production

The serum OVA-specific IgE values (absorbance units, means $\pm$ SEM calculated from 5 animals) were 0.90 ± 0.31 in the OVA-sensitized control rats (24 hr after PBS challenge), 1.12 ± 0.19 in the AI model rats (24 hr after OVA challenge), and 0.02 ± 0.02 in the non-sensitized rats. The values in the control and AI rats were significantly ($p < 0.001$) higher than those in the non-sensitized rats. Similarly, the levels of OVA-specific IgE in the AI rats at 6 hr (0.99 ± 0.41), 48 hr (1.06 ± 0.34), and 120 hr (0.84 ± 0.36) after the OVA challenge were significantly ($p < 0.001$) higher than those in the non-sensitized rats.

Histopathologic analysis

Histologic examination of lung tissues from the AI rats at 24 hr after OVA challenge demonstrated a marked infiltration of eosinophils and mononuclear cells in the peribronchial, perivascular and alveolar

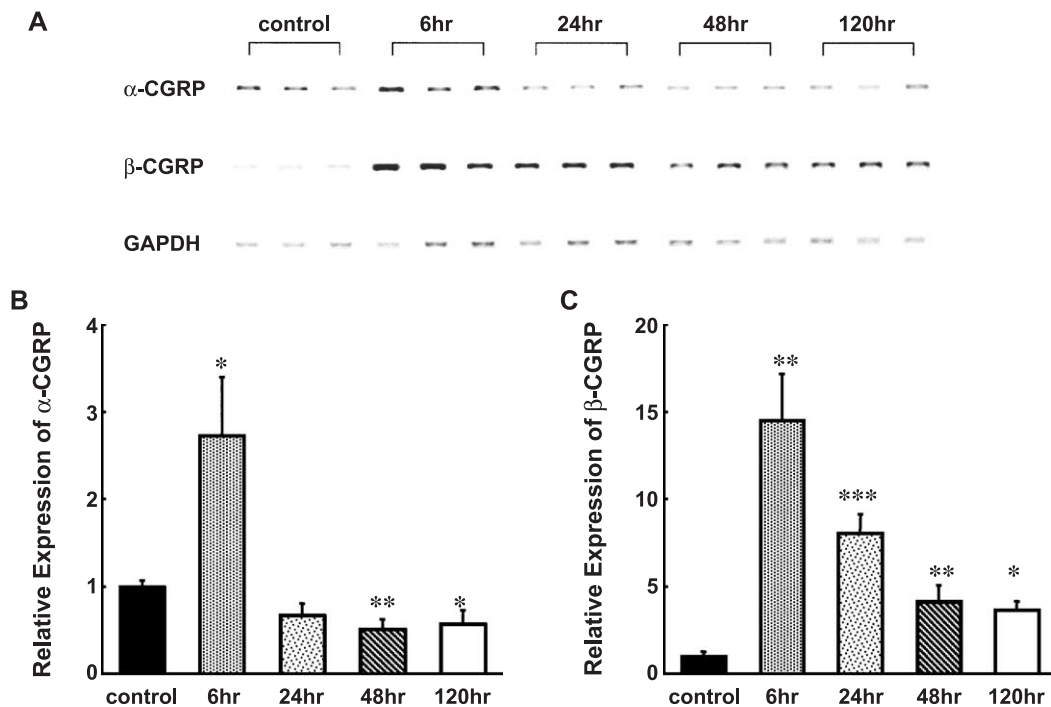


Fig. 1. Relative mRNA expressions of α - and β -CGRP in the lung determined by semiquantitative RT-PCR. (A) The pictures of gel electrophoresis showing the PCR products of mRNA for α -CGRP, β -CGRP and GAPDH in the control group and the allergic inflammation (AI) model group (AI) at 6 hr, 24 hr, 48 hr, and 120 hr after OVA-challenge. The results shown are from 3 independent experiments. (B) The expression of α -CGRP in the AI group (6 hr, 24 hr, 48 hr, and 120 hr after OVA challenge) relative to the expression of α -CGRP in the control group. (C) The expression of β -CGRP in the lungs of AI group relative to β -CGRP expression in the control group at various time points. Values in B and C represent the mean $\pm$ SEM of 5 experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as compared with the control group.

regions. In contrast, lung tissues from control rats showed only a small number of infiltrated eosinophils around bronchioles (not shown).

Semiquantitative analysis of mRNA expression of α - and β -CGRP in lungs, NG and DRG

Our preliminary RT-PCR analysis has shown higher intensity of α -CGRP fragments than that of β -CGRP fragments in the lungs (33 cycles of the reaction), NG (30 cycles of the reaction) and DRG (27 cycles of the reaction) of the non-sensitized rats (data not shown). The predominant gene expression of α -CGRP as compared with β -CGRP was also observed in these tissues of the control rats 24 hr after PBS challenge (Fig. 1A and Fig. 2A). To analyze mRNA expressions for the two isoforms of CGRP in the AI rats, the amounts of the PCR fragments of α - or β -CGRP obtained at various time points after OVA challenge were expressed relative to the values of PCR fragments in the lungs (Fig. 1B and C) and NG (Fig. 2B and C) of the control rats. In lung tissues, enhanced (2.73 ± 0.71 -fold for α -[$p < 0.05$] and 14.54 ± 2.77 -fold for β -CGRP [$p < 0.002$]) expression of

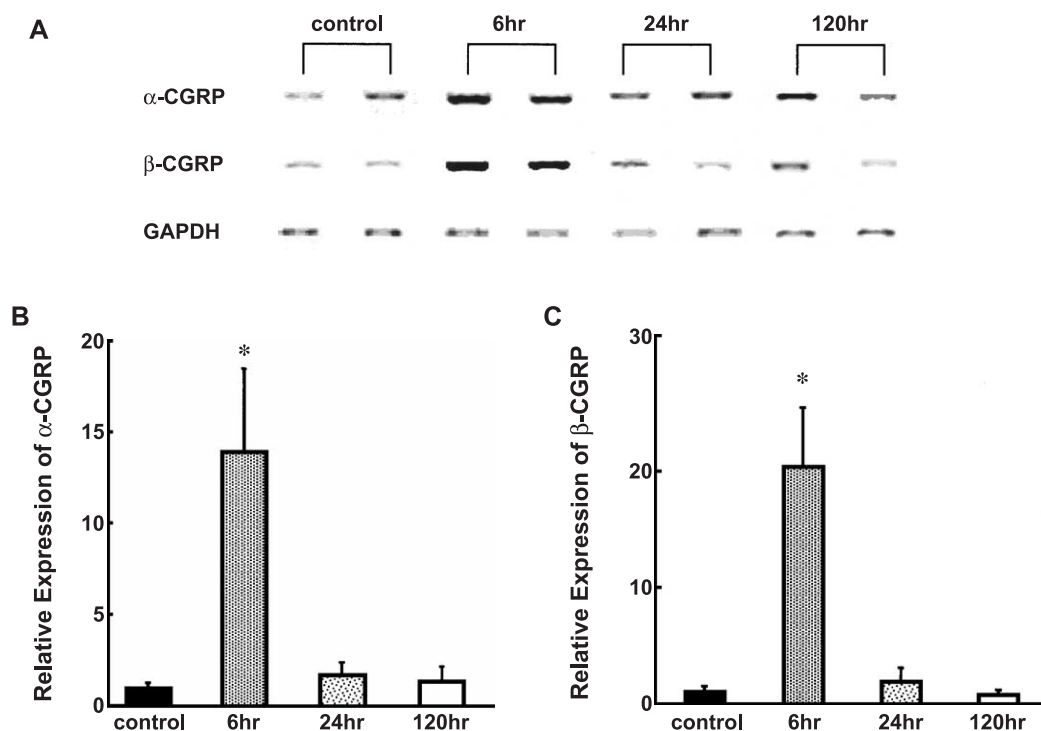


Fig. 2. Relative mRNA expressions of α - and β -CGRP in the NG determined by semiquantitative RT-PCR. (A) The pictures of gel electrophoresis showing the PCR products of mRNA for α -CGRP, β -CGRP and GAPDH in the control group and the AI group at 6 hr, 24 hr, 48 hr, and 120 hr after OVA-challenge. The results shown are from 2 independent experiments. (B) The expression of α -CGRP in the NG of the AI group (6 hr, 24 hr, and 120 hr after OVA challenge) relative to the expression of α -CGRP in the control group. (C) The expression of β -CGRP in the NG of the AI group at various timepoints relative to the expression of β -CGRP in the control group of rats. Values in B and C represent the mean $\pm$ SEM of 3 experiments. * $p < 0.05$ as compared with the control group.

both CGRP isoforms was observed at 6 hr after the allergen challenge. Significant enhancement of mRNA expression for β -CGRP continued throughout the observation period (8.05 ± 1.16 -fold at 24 hr [$p < 0.001$], 4.14 ± 1.01 -fold at 48 hr [$p < 0.02$] and 3.65 ± 0.54 -fold at 120 hr [$p < 0.003$]). In contrast, the enhancement of mRNA expression for α -CGRP was transient, and the expression was decreased after 24 hr (0.68 ± 0.14 -fold at 24 hr [$p = 0.066$], 0.52 ± 0.12 -fold at 48 hr [$p < 0.01$] and 0.58 ± 0.16 -fold at 120 hr [$p < 0.04$]) (Fig. 1).

In NG, mRNA expressions for both CGRP isoforms were transiently up-regulated at 6 hr after the challenge (14.04 ± 4.58 -fold for α -[$p < 0.05$] and 19.43 ± 4.83 -fold for β -CGRP [$p < 0.02$]), while the expressions of both genes had decreased by 24 hr and were thereafter not significantly different from those in the controls (Fig. 2).

There were abundant mRNA expressions for both CGRP isoforms in DRG in the control rats and AI rats at any time (6 hr, 24 hr, and 120 hr) after the challenge. We observed no significant differences in the relative expression of α -or β -CGRP between the AI rats (6 hr, 24 hr, or 120 hr) and the controls (Fig. 3).

Localization of CGRP in AI rat lungs by *in situ* RT-PCR analysis and immunohistochemistry

By using RT-PCR analyses, we showed enhanced mRNA expressions of α -and β -CGRP in AI rat lungs at 6 hr after OVA challenge. To clarify cellular localization of the amplified products in

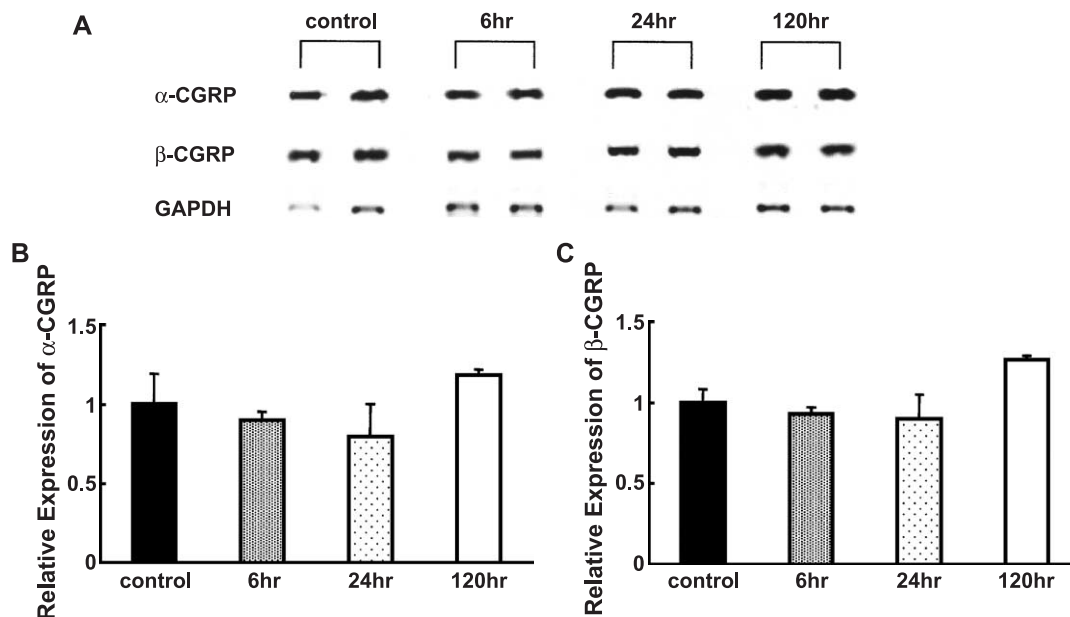


Fig. 3. Relative mRNA expressions of α -and β -CGRP in the DRG determined by semiquantitative RT-PCR. (A) The pictures of gel electrophoresis showing the PCR products of mRNA for α -CGRP, β -CGRP and GAPDH in the control group and the AI group at 6 hr, 24 hr, 48 hr, and 120 hr after OVA-challenge. The results shown are from 2 independent experiments. (B) The expression of α -CGRP in the DRG of the AI group (6 hr, 24 hr, and 120 hr after OVA challenge) relative to the expression of α -CGRP in the control group. (C) The expression of β -CGRP in the DRG of the AI group at various timepoints relative to the expression of β -CGRP in the control group of rats. Values in B and C represent the mean + SEM of 3 experiments.

the tissue, in situ RT-PCR histochemistry is the most suitable method. However, this method is technically more complicated than classical in situ hybridization, and the previous studies with in situ hybridization showed α -CGRP mRNA in rat lungs (Keith et al., 1991; Springall and Polak, 1993). Radioisotope (RI)-labeled probes used in these studies are thought to be more sensitive than digoxigenin (DIG)-labeled probes, but RI-labeled probes were not available in our laboratory. In our preliminary in situ hybridization study with a DIG-labeled probe, only very weak signals could be detected. Then we carried out in situ RT-PCR with a DIG-labeling system, which was found to be more sensitive than in situ hybridization with a DIG system. The visible signal for α -CGRP mRNA was detected in some of single epithelial cells in AI lungs 6 hr after challenge (Fig. 4A), while β -CGRP mRNA expression was observed in clustered epithelial cells of AI rat lungs at 6 hr and 24 hr after OVA challenge (Fig. 4B). At a higher magnification, positive immunohistochemical staining for CGRP and PGP 9.5 in the serial sections revealed that these clusters were NEBs (not shown). The positive controls for in situ RT-PCR demonstrated intense nuclear staining (Fig. 4C), and the negative controls showed the absence of staining (Fig. 4D). We could detect no visible signals for either α - or β -CGRP mRNA in the lungs of control rats (not shown).

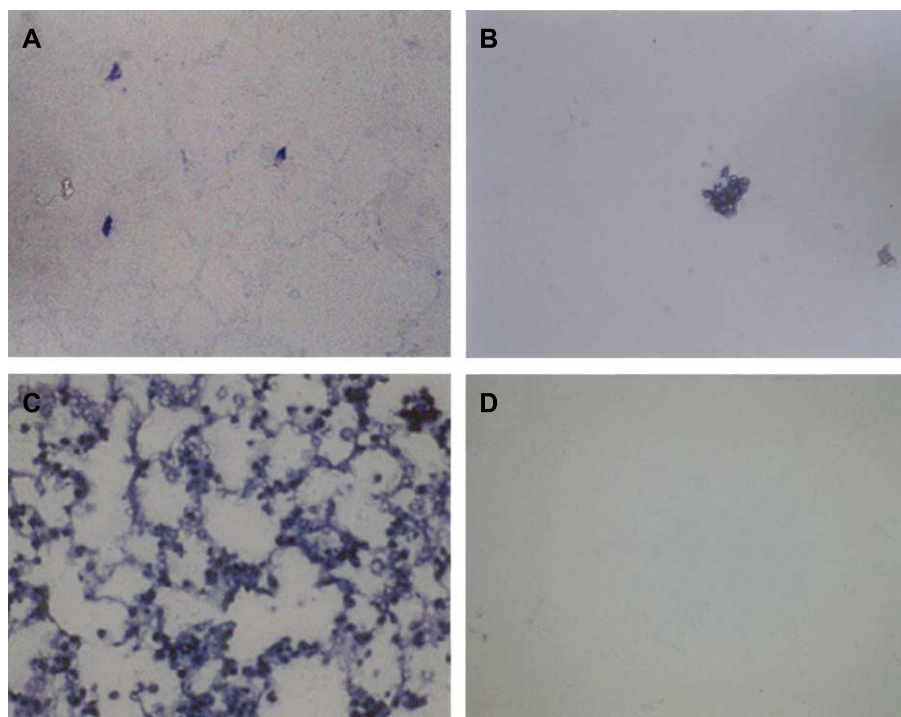


Fig. 4. In situ RT-PCR analysis of mRNA expressions for α - and β -CGRP in the lungs of AI rats. (A) α -CGRP mRNA was detected in some of single epithelial cells in AI lungs at 6 hr after OVA-challenge ($\times 100$). (B) β -CGRP mRNA expression was mainly observed in clustered epithelial cells in AI lungs at 24 hr after OVA-challenge ($\times 100$). C and D are positive and negative controls for β -CGRP, respectively ($\times 100$). (The controls for α -CGRP were not shown).

Discussion

The present study with allergic inflammation (AI) model rats resulted in the following findings;

- (i) transient up-regulation of α -CGRP mRNA in the lungs of AI rats, long-lasting up-regulation of β -CGRP mRNA and localization of this isoform in NEBs in the lungs of AI rats,
- (ii) abundance of α - and β -CGRP mRNA in the thoracic DRG of control and AI rats, and
- (iii) transient up-regulation of α - and β -CGRP mRNA in the NG of AI rats.

The results of our study about the different modulations of the gene expressions of the CGRP isoforms may indicate that different regulatory pathways exist not only regarding the CGRP isoforms, but also regarding the cells producing CGRP.

Transient up-regulation of α -CGRP mRNA vs. long-term induction of β -CGRP mRNA in AI rat lungs

The predominance of α -CGRP expression in the sensitized control lungs and a transient up-regulation of α -CGRP mRNA in AI lungs in the present study suggest that α -CGRP may play a role in the proinflammatory condition and in the early stage of the inflammatory process. The amino acid residues of rat α -CGRP from positions 32 to 35 are Val³²-Gly-Ser-Glu³⁵. This tetrapeptide was identified as the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Davies et al., 1992). The eosinophil chemotactic activity of α -CGRP is dramatically enhanced by degradation of this isoform to produce ECF-A with neutral endopeptidase 24.11 (NEP) (Davies et al., 1992), an enzyme that is abundant in the airways (Davies et al., 1992; Sales et al., 1991). In addition, it has recently been reported that AI-induced bronchial hyperresponsiveness and production of leukotriene were attenuated in α -CGRP-knockout mice (Aoki-Nagase et al., 2002). These findings indicate a contribution of α -CGRP to the progression of allergic airway inflammation. In contrast to the rapid decline of α -CGRP mRNA expressions from 6 hr to 24 hr after OVA-challenge, increase of β -CGRP mRNA expression in the AI lung lasted until the end of the observation period (120 hr after OVA-challenge). The prolonged up-regulation of β -CGRP expression suggests a functional role of this isoform in both early and late stages of allergic airway inflammation. β -CGRP differs from α -CGRP by one amino acid, substitution of a lysine residue (Lys) for glutamate (Glu) at position 35 in rats, which results in a lack of ECF-A production after degradation of this isoform with NEP. Therefore, it appears unlikely that β -CGRP up-regulated in the AI rat lungs induces eosinophilic infiltration. Rather, β -CGRP might play a regulatory role in allergic inflammation. CGRP possesses strong immunosuppressive activity on some essential molecules for antigen-presentation, such as major histocompatibility complex (MHC) class II molecules (Nong et al., 1989) and co-stimulatory molecules of the B7 family (Asahina et al., 1995; Fox et al., 1997; Carucci et al., 2000). It is believed that B7-1 (CD 80) and B7-2 (CD 86) induce helper T cell type 1 (Th1) and type 2 (Th2) responses, respectively (Kuchroo et al., 1995), and that Th2 responses are important for the progression of AI (Romagnani, 1992). CGRP can specifically inhibit B7-2 expression on some kinds of antigen presenting cells (Asahina et al., 1995; Fox et al., 1997), thereby suppressing Th2 responses. It has also been demonstrated that CGRP inhibits T cell proliferation through an enhancement of the apoptosis of thymocytes (Wang et al., 2002; Millet et al., 2000). We speculate that β -isoform might be responsible for the inhibitory actions of CGRP on the immune system. Although further studies are needed to elucidate the functional differences between the two isoforms in the allergic inflammation, our

speculation seems to be supported by findings, in which immune tolerance is induced by repeated exposure to a particular antigen in AI-induced BN rat lungs (McMenamin et al., 1995).

A recent study by Xing et al. (Xing et al., 2000) showed synthesis and secretion of β -CGRP from T cell in rats. This finding raises the possibility that β -CGRP is produced by T cells as well as NEBs in allergic airway inflammation. In the present study, however, no significant expression of β -CGRP in either mRNA or peptide levels has been observed in the lymphocytes in AI rat lungs. We believe that NEBs are main source of β -CGRP in AI rat lungs.

Abundant mRNA expressions of both CGRP isoforms in control and AI rat DRG

The present study showed abundant mRNA expressions of α - and β -CGRP in DRG, but not in NG, in the control rats. This finding confirms the previous study by Sternini and Anderson (1992): both CGRP isoforms were expressed in a vast population of neurons in DRG, while only a minor component of the afferent CGRP innervation derived from neurons located in NG of the normal (non-sensitized) rat. Consistently, the retrograde axonal tracing study (Springall et al., 1987) showed that most CGRP-immunoreactive nerves of rat trachea and lungs originated from vagal jugular ganglia (instead of NG) and thoracic DRG. We also observed abundant mRNA expressions of both CGRP isoforms in AI rat DRG. Although the OVA-challenge induced no significant up-regulation of CGRP mRNA in DRG, we cannot deny the possibility that CGRP released from the axon terminals of DRG neurons into the airways participate in the allergic inflammation.

Transient up-regulation of mRNA for both CGRP isoforms in AI rat NG

In contrast to the different expression patterns between the two CGRP isoforms in AI rat lungs, mRNA of both isoforms in NG were transiently up-regulated at 6 hr after OVA-challenge and then steeply declined to the control level at 24 hr. This finding is consistent with the previous study by Fischer et al. (1996), which showed transient increases in the ratio of substance P- and CGRP-immunoreactive neurons in NG of the guinea pig 24 hr after OVA-challenge. A recent study by Myers et al. (2002) demonstrated AI-induced production of substance P/CGRP in large-diameter neurons in NG of guinea pigs. The large-diameter NG neurons are unresponsive to noxious or nociceptive stimuli, and project fast-conducting, low-threshold mechanosensitive A δ fibers to the airways. Thus, stimulation of low-threshold mechanosensors may induce substance P/ CGRP synthesis in NG neurons during allergic airway inflammation. It is plausible that both CGRP isoforms up-regulated in NG are transported to the vagal sensory nerve endings and released into the airways, where they may be involved in the process of allergic reactions.

Conclusion

The major findings in the present study, taken together with the findings of other immunological research, indicate that β -CGRP may play important roles in the regulation of allergic responses, and that NEBs may participate in pulmonary allergic inflammation. Considering that immune tolerance can be induced by repeated exposure to a particular antigen in AI-induced BN rat lungs, future studies on β -

CGRP and immune tolerance may lead to advances in the understanding and treatment of allergic diseases such as bronchial asthma.

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