Table 1. *Physiological results*

	pH	$PaCO_2$, mmHg	R_L, $cmH_2O \cdot ml^{-1} \cdot s^{-1}$
Saline, ip Saline, iv	7.492 ± 0.041	27.7 ± 3.7	0.412 ± 0.004
ATK, ip Saline, iv	7.455 ± 0.049	29.5 ± 3.3	0.418 ± 0.007
Saline, ip LPS/zymosan, iv	7.134 ± 0.050*	48.6 ± 4.6*	0.539 ± 0.020*
ATK, ip LPS/zymosan, iv	7.444 ± 0.041‡	32.2 ± 3.4‡	0.459 ± 0.014†‡

Values are means ± SE. R_L, lung resistance; ATK, arachidonyl trifluoromethyl ketone. *$P < 0.01$ vs. saline-treated group. †$P < 0.05$, ‡$P < 0.01$ vs. LPS/zymosan-treated group.

Table 2. *Total cell counts and cell fractions in BALF*

	Total cell counts × 10^5	Macrophages, %	PMNs, %	Lymphocytes, %
Saline, ip Saline, iv	1.12 ± 0.05	95.7 ± 0.3	0.4 ± 0.1	3.9 ± 0.3
Saline, ip LPS/zymosan, iv	3.56 ± 0.13*	93.4 ± 0.6	3.3 ± 0.5*	3.3 ± 0.5
ATK, ip LPS/zymosan, iv	2.28 ± 0.32†‡	94.7 ± 0.5	1.1 ± 0.1†‡	4.2 ± 0.4

Values are means ± SE. BALF, bronchoalveolar lavage fluid; PMN, polymorphonuclear neutrophil. *$P < 0.01$ vs. saline-treated group. †$P < 0.05$, ‡$P < 0.05$ vs. LPS/zymosan-treated group.

reflects physiological alterations in lung parenchyma. The administration of ATK significantly reduced LPS/zymosan-induced responses in E_L and R_L, whereas there were significant differences between saline-treated and ATK/LPS/zymosan-treated groups.

Administration of LPS/zymosan elicited respiratory failure, which was not observed in saline-treated groups. Hypoxemia was prominent in LPS/zymosan-treated mice, whereas ATK administration reduced LPS/zymosan-induced hypoxemia (Fig. 2). After LPS/zymosan treatment, increases in Pa_{CO_2} and decreases in pH were observed, although there were no differences in Pa_{CO_2} or pH levels between saline-treated and ATK/LPS/zymosan-treated groups. As shown, ATK had little effect on physiological data in saline-treated groups.

Analyses of BALF. Table 2 and Figs. 3 and 4 summarize the analyzed data of BALF. As shown, LPS/zymosan administration increased protein amount and number of PMN in BALF, indicating LPS/zymosan induced protein leakage and PMN infiltration. The protein leakage and PMN sequestration were significantly attenuated by the treatment of ATK. Meanwhile, there were significant differences in BALF protein amount and number of PMN between saline-treated and ATK/LPS/zymosan-treated groups.

TX and LT assay. To assess the biosynthesis of $cPLA_2$ products, we performed TXA_2 (measured as TXB_2), LTB_4, and $LTC_4/D_4/E_4$ assay of the BALF. Figures 5–7 summarize the results of BALF TXB_2, LTB_4, and $LTC_4/D_4/E_4$ assay in each experimental group. LPS/zymosan administration markedly increased TXB_2, LTB_4, and $LTC_4/D_4/E_4$ levels in BALF compared with the saline-treated group, whereas the levels of these eicosanoids were significantly reduced in the ATK/LPS/zymosan-treated group. However, there were significant differences in BALF TXB_2, LTB_4, and $LTC_4/D_4/E_4$ levels between saline-treated and ATK/LPS/zymosan-treated groups.

MPO activity assay. To assess the PMN infiltration in the lung, we performed MPO activity assay. Figure 8 shows the results of MPO activity in lung tissue. LPS/zymosan administration markedly increased MPO activity in lungs compared with the saline-treated group, whereas the MPO activity was significantly attenuated in the ATK/LPS/zymosan-treated group. However, no significant difference in lung MPO activity was observed between saline-treated and ATK/LPS/zymosan-treated groups.

Histological study. Figure 9 represents lung histology following LPS/zymosan administration. As shown, LPS/zymosan administration induced prominent lesions, as well as alveolar thickening, distortion, and cellular infiltration. In contrast, the alveolar architecture is well preserved and histological changes are minimal in ATK-treated animals.

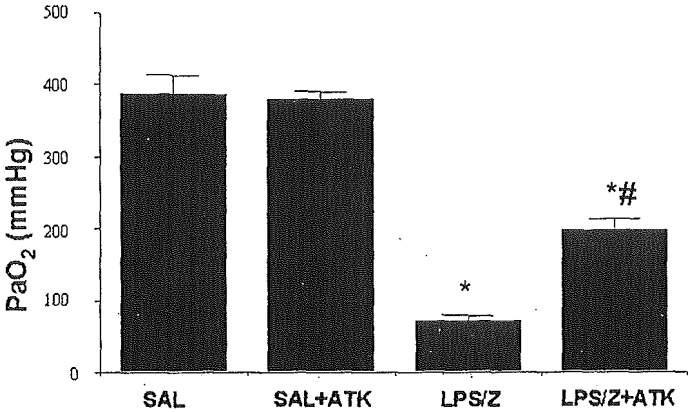

Fig. 2. Effects of cytosolic phospholipase A_2 ($cPLA_2$) inhibitor ATK in hypoxemia induced by LPS/zymosan treatment. *$P < 0.001$ vs. saline-treated group; #$P < 0.001$ vs. LPS/zymosan-treated group.

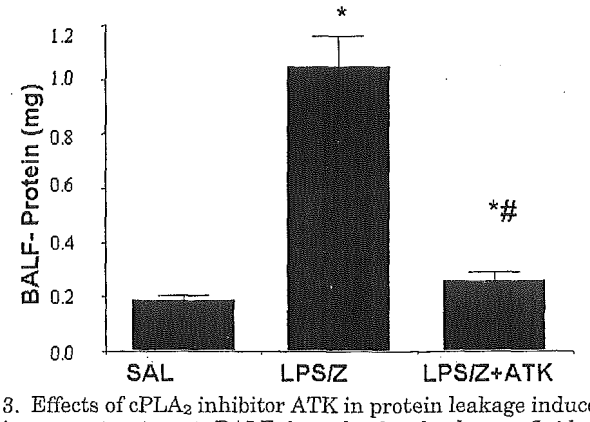

Fig. 3. Effects of $cPLA_2$ inhibitor ATK in protein leakage induced by LPS/zymosan treatment. BALF, bronchoalveolar lavage fluid. *$P < 0.01$ vs. saline-treated group; #$P < 0.01$ vs. LPS/zymosan-treated group.

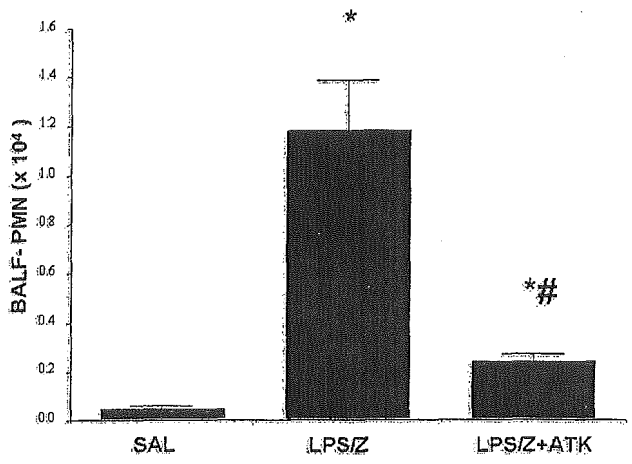

Fig. 4. Effects of $cPLA_2$ inhibitor ATK in neutrophil infiltration induced by LPS/zymosan treatment. PMN, polymorphonuclear neutrophil. *$P < 0.001$ vs. saline-treated group; #$P < 0.001$ vs. LPS/zymosan-treated group.

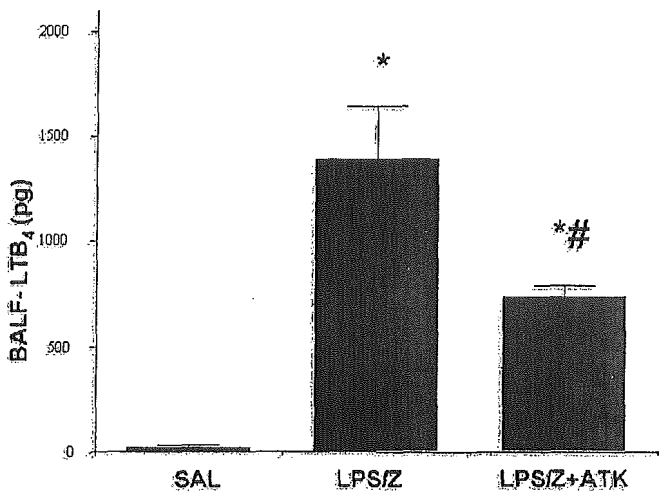

Fig. 6. Effects of $cPLA_2$ inhibitor ATK in LTB_4 production induced by LPS/zymosan treatment. *$P < 0.001$ vs. saline-treated group; #$P < 0.05$ vs. LPS/zymosan-treated group.

DISCUSSION

The results of the current study show that $cPLA_2$ is important in the pathogenesis of acute lung injury. Inhibition of $cPLA_2$ significantly attenuated acute lung injury induced by endotoxemia. These observations indicate that pharmacological inhibition of $cPLA_2$ may be an effective treatment for acute lung injury, probably because it inhibits production of inflammatory mediators including TXs and LTs.

The sepsis syndrome is the most frequent cause of ARDS and is associated with 35–45% incidence of ARDS development (9, 10). It is postulated that both endotoxemia and phagocytosis of bacteria are involved in the pathogenesis of ARDS associated with septic syndrome (6). Therefore, we used the current model of acute lung injury induced by combined administration of LPS and zymosan (19). In this model, circulating LPS and phagocytosis of bacterial particles by LPS-primed PMN elicit acute lung injury, which may mimic sepsis-associated acute lung injury.

After LPS/zymosan administration, we observed increases in E_L, protein leakage, and PMN infiltration and severe exacerbation of gas exchange. PMN infiltration in the lung was confirmed by MPO activity assay and histology. Consistently, marked increases in TXs and LTs were detected in the BALF. These findings were significantly attenuated by the treatment of $cPLA_2$ inhibitor ATK. Potential mechanisms by which $cPLA_2$ mediates sepsis-induced acute lung injury include the release of proinflammatory mediators. The present results also suggest that the major mediator of PMN infiltration is a $cPLA_2$ product, most probably LTB_4 (38). Recent evidence using lung injury models overexpressing the LTB_4 receptor shows that LTB_4 is an important mediator of neutrophil-mediated lung injury (4). It is suggested that not only infiltration but also activation of PMN in lungs may be essential to induce the development of acute lung injury. The $cPLA_2$-initiated pathways may mediate both infiltration and activation of PMN triggered by septic syndrome, resulting in sepsis-associated ARDS. In human

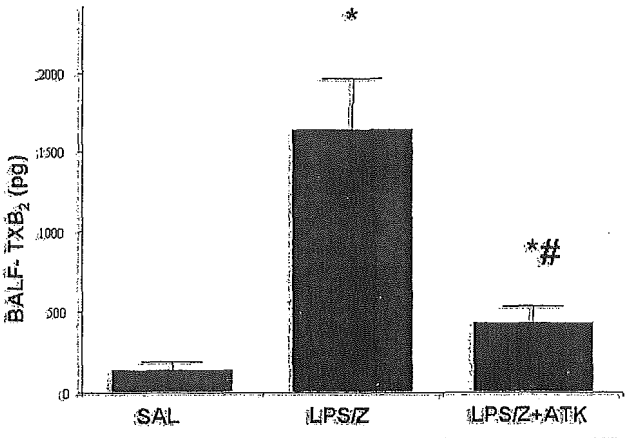

Fig. 5. Effects of $cPLA_2$ inhibitor ATK in thromboxane (TX) B_2 production induced by LPS/zymosan treatment. *$P < 0.05$ vs. saline-treated group; #$P < 0.01$ vs. LPS/zymosan-treated group.

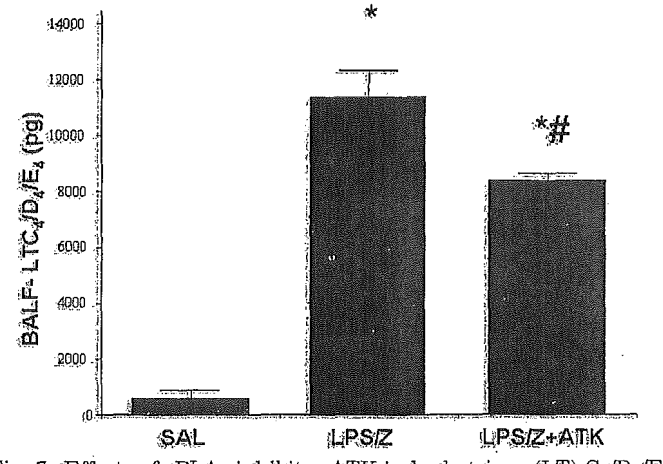

Fig. 7. Effects of $cPLA_2$ inhibitor ATK in leukotriene (LT) $C_4/D_4/E_4$ production induced by LPS/zymosan treatment. *$P < 0.001$ vs. saline-treated group; #$P < 0.05$ vs. LPS/zymosan-treated group.

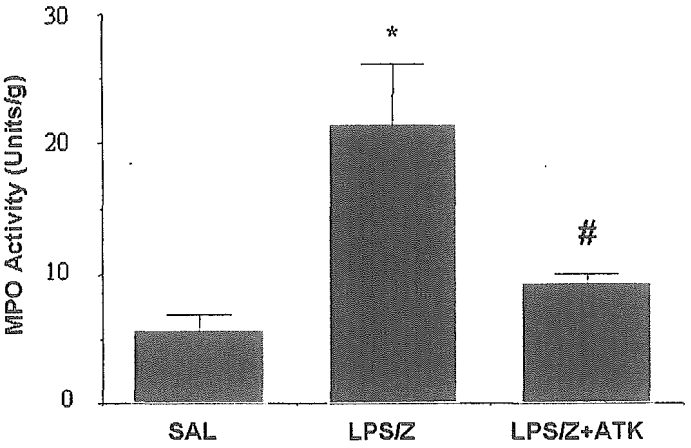

Fig. 8. Effects of $cPLA_2$ inhibitor ATK in lung myeloperoxidase (MPO) activity induced by LPS/zymosan treatment ($n = 4$ for each group). *$P < 0.05$ vs. saline-treated group; #$P < 0.05$ vs. LPS/zymosan-treated group.

neutrophils during sepsis, elevated $cPLA_2$ expression and activity have been recently reported, suggesting that $cPLA_2$ plays a major role in neutrophil function in septic syndrome (17).

Of note, it has been recently shown that acute lung injury induced by LPS/zymosan administration is attenuated in $cPLA_2$ gene-disrupted mice (30). It seems that the effects of ATK administration are similar to those of $cPLA_2$ gene disruption in terms of inhibiting lung injury. This observation may further confirm that the intervention of $cPLA_2$ could be an effective approach to treat acute lung injury. However, differences were also found between these two studies. In this study, we measured TXB_2, LTB_4, and cysteinyl LTs ($LTC_4/D_4/E_4$) in BALF to confirm the generation of $cPLA_2$ products. Although the ATK administration significantly attenuated LPS/zymosan-induced production of TXB_2, LTB_4, and cysteinyl LTs, the ATK administration reduced each eicosanoid by 73, 47, and 27%, respectively, compared with LPS/zymosan administration. In contrast, $cPLA_2$ gene disruption reduced each eicosanoid by >90% in this model, compared with LPS/zymosan administration in wild-type mice. This finding suggests that the present manner of ATK administration may still be insufficient to inhibit $cPLA_2$ completely. Because it is postulated that pharmacological intervention of $cPLA_2$ could be useful in the manage-

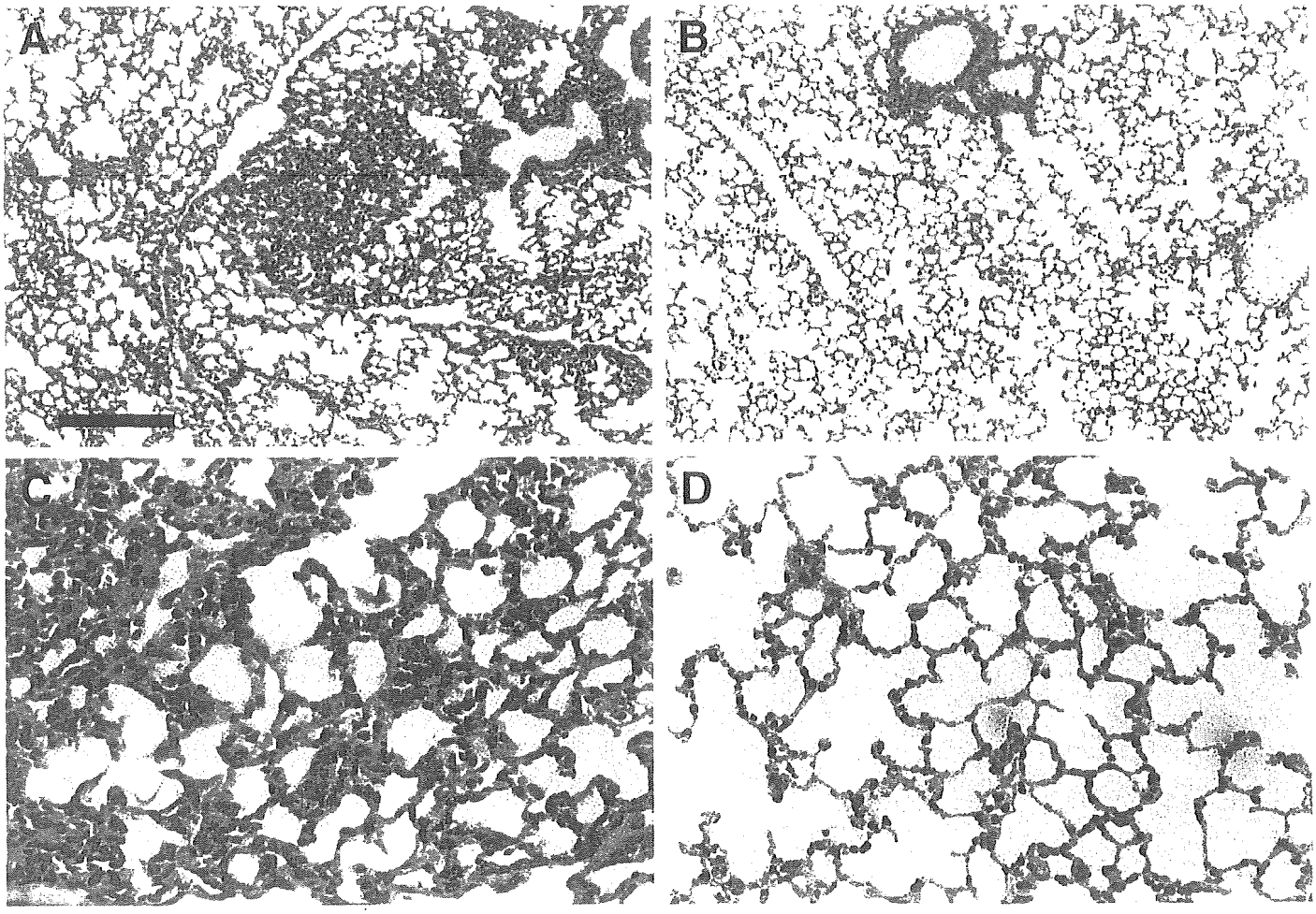

Fig. 9. Photomicrograph of lung tissues from LPS/zymosan-treated (*A*, *C*), and ATK/LPS/zymosan-treated (*B*, *D*) mice 4 h after LPS administration. Hematoxylin-eosin stain. Scale bar in *A* represents 200 μm in *A* and *B* and 50 μm in *C* and *D*.

ment of ARDS, the development of novel $cPLA_2$ inhibitors warrants future research.

In the present model of acute lung injury, we observed that the levels of Pa_{CO_2} and pH in the ATK/LPS/zymosan-treated group were the same as in saline-treated controls. However, LPS/zymosan-induced increases in E_L, severity of hypoxia, BALF protein, PMN, and eicosanoids were significantly attenuated but not eliminated by the treatment of ATK. These observations indicate that factors other than $cPLA_2$ may also play a role and contribute to physiological alteration. Recently, it has been demonstrated that secretory PLA_2 ($sPLA_2$), the other type of PLA_2, mediates LPS-induced lung injury and that the inhibition of $sPLA_2$ may also represent a therapeutic approach to acute lung injury (2). In addition, it has been suggested that oxygen radicals, adhesion molecules, and cytokines are also involved in this mechanism (8, 28). Recently, it was reported that $cPLA_2$ activation is essential for integrin-dependent adhesion of leukocytes (39). If one considers that there are as yet no pharmacological agents to reverse pulmonary edema and increase survival rates, these factors are potential targets to develop agents. The current study suggests that the intervention of $cPLA_2$ could be a promising clue to improve management of ARDS.

In summary, the inhibition of $cPLA_2$ significantly attenuated lung damage and respiratory failure induced by LPS/zymosan treatment. The current observations suggest that $cPLA_2$ products are involved in the pathogenesis of acute lung injury caused by septic syndrome. Inhibition of $cPLA_2$-initiated pathways might provide a novel and potential therapeutic approach to ARDS, to which no pharmaceutical agents are currently available.

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Effects of obstructive sleep apnea on circulating ICAM-1, IL-8, and MCP-1

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Ohga, Eijiro, Tetsuji Tomita, Hiroo Wada, Hiroshi Yamamoto, Takahide Nagase, and Yasuyoshi Ouchi. Effects of obstructive sleep apnea on circulating ICAM-1, IL-8, and MCP-1. *J Appl Physiol* 94: 179–184, 2003. First published September 27, 2002; 10.1152/japplphysiol.00177.2002.—Obstructive sleep apnea syndrome (OSAS) is one of the most important risk factors of cardiovascular disorders. In the treatment of OSAS, nasal continuous positive airway pressure (nCPAP) has been widely used and found to be effective. In the present study, we hypothesized that the hypoxic stress caused by obstructive sleep apnea would increase circulating intercellular adhesion molecule-1 (ICAM-1), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1) in untreated OSAS patients compared with an age-matched control group. In addition, we hypothesized that nCPAP may decrease OSAS-induced hypoxic stress and mediators. To examine these hypotheses, we measured circulating ICAM-1 and IL-8 before and after nCPAP therapy in OSAS patients. We observed that nCPAP decreased apnea, desaturation, and the circulating ICAM-1 and IL-8 levels in OSAS patients. The circulating levels of ICAM-1, IL-8, and MCP-1 in untreated OSAS patients were significantly greater than those in the controls. These observations suggest that nCPAP therapy could reduce OSAS-induced hypoxia and generation of inflammatory mediators. Treatment of OSAS using nCPAP can be, therefore, a potential approach to decrease risk of the progression of OSAS-associated disorders.

cytokines; cardiovascular disorders; ischemic heart disease; desaturation magnitude; hypoxic stress; intracellular adhesion molecule-1; monocyte chemoattractant protein-1; interleukin-8

RECENTLY, IT HAS BEEN SHOWN that obstructive sleep apnea syndrome (OSAS) is related to obesity, insulin resistance, and diabetes mellitus (17, 28, 33). Moreover, OSAS could be one of the most important risk factors of cardiovascular disorders, including hypertension, ischemic heart disease, and cerebrovascular events (12, 15, 23, 25), whereas hypoxic stress elicited by OSAS may be involved in the development of cardiovascular disorders. However, the exact mechanism remains to be elucidated.

One of the potential mechanisms is that OSAS-induced hypoxic stress increases circulating inflammatory mediators, leading to cardiovascular lesions. It has been recently suggested that atherosclerosis is related to inflammatory process induced by activation of proinflammatory mediators, including adhesion molecules (11) and cytokines (10, 31). To induce leukocyte migration to inflamed tissue, it is essential for leukocytes to adhere to microvascular endothelium (32). Potential mediators responsible for leukocyte attachment to endothelium include intercellular adhesion molecule-1 (ICAM-1), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1). It has been reported that ICAM-1, a member of the immunoglobulin superfamily, is required for leukocyte migration into inflamed area (3, 6, 35) and plays an important role in inflammatory disease, including bronchial asthma, lung injury, and ischemic heart disease (18–22). IL-8, a CXC chemokine that induces the migration and proliferation of endothelial cells and smooth muscle cells, is a potent angiogenic factor that may play a substantial role in atherosclerosis (4, 31). Increased expression of IL-8 has been reported in atherosclerotic lesions and circulating macrophages from patients with atherosclerosis (31). MCP-1 is upregulated in human atherosclerotic plaques, suggesting a role for MCP-1 in the development of early atherosclerotic lesions (5, 10).

Hypoxic stress increases the adherence of neutrophils to endothelial cells, and this increased adherence is mediated by proinflammatory mediators, including ICAM-1 (2) and IL-8 (13, 30). Furthermore, it has been reported that hypoxia induces the synthesis and expression of both ICAM-1 and IL-8 via the activation of nuclear transcription factor (NF)-κB (7, 36, 37).

In the treatment of OSAS, the efficacy of nasal continuous positive airway pressure (nCPAP) has been reported (8, 27). nCPAP improves sleepiness and quality of life in patients with OSAS, probably because nCPAP intervention removes sleeping upper airway collapse and decreases apnea episode (27). Although it is expected that nCPAP may ultimately improve the prognosis of various disorders associated with OSAS, its exact mechanism is not yet proven.

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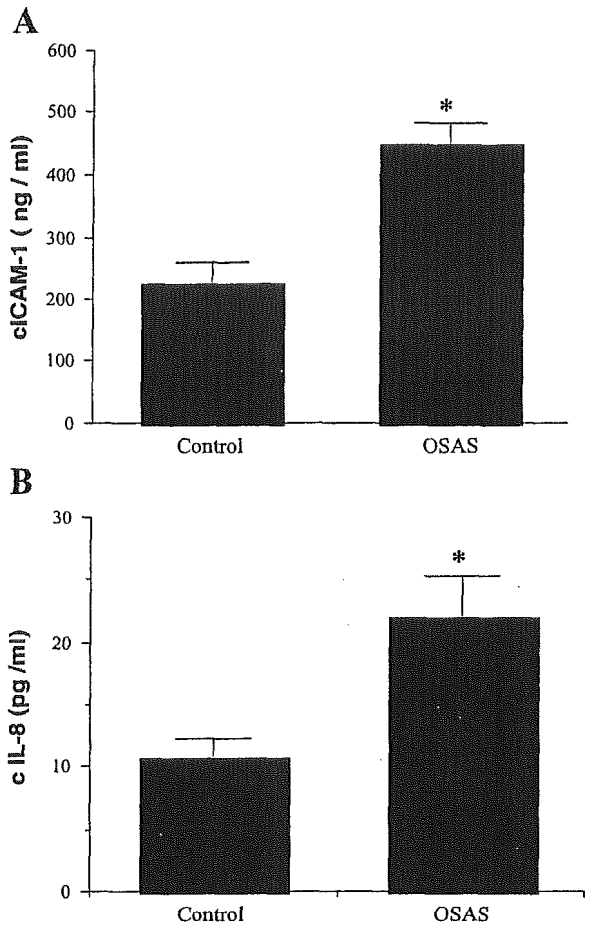

Fig. 1. Baseline levels of (*A*) circulating intercellular adhesion molecule-1 (cICAM-1) and (*B*) circulating interleukin-8 (cIL-8) in the control and obstructive sleep apnea syndrome (OSAS) groups. *$P <$ 0.05 vs. normal controls.

In the present study, we hypothesized that nCPAP may decrease OSAS-induced hypoxic stress and the generation of proinflammatory mediators. To examine this hypothesis, we measured circulating ICAM-1 and IL-8 before and after nCPAP therapy in OSAS patients.

METHODS

Subjects. Among patients diagnosed as OSAS in our department, 20 male subjects participated in the present study.

Table 1. *Characteristics of the subjects*

Group	*n*	Age, yr	Body Mass Index	Apnea Index
OSAS	20	47.8 ± 2.2	29.4 ± 1.4	38.9 ± 3.1*
Control	10	48.9 ± 2.9	28.4 ± 2.9	3.1 ± 0.4

Values are means ± SE; *n*, no. of subjects. OSAS; obstructive sleep apnea syndrome. *$P < 0.001$ vs. control group.

As age-matched controls, 10 male subjects were chosen and studied. No subjects had any history of cardiovascular, pulmonary, metabolic, or neuromuscular diseases. All subjects were in a stable condition for 1 mo before the study. The characteristics of the subjects in the OSAS and normal groups are shown in Table 1. There were no significant differences in age and body mass index (BMI) between the two groups, whereas apnea index (AI) in the OSAS group was markedly greater than that in the control.

After the polysomnography study, the patients with OSAS underwent therapeutic nCPAP treatment, and eight subjects continued to receive nCPAP successfully for 8–18 mo.

Polysomnography. The subjects underwent polysomnography for 2 consecutive nights. The polysomnography included an electroencephalogram, an electrooculogram, an electromyogram of the chin, and an electrocardiogram (DG Compact32, Medelec, Surrey, UK). We monitored ventilation and airflow using inductive plethysmography (Respitrace, Ambulatory Monitoring, Ardsley, NY) and thermistors (Fukuda-Sangyo, Chiba, Japan) placed at the nostril and mouth. Arterial oxygen saturation (Sa_{O_2}) was continuously measured via pulse oxymeter (Datex, Helsinki, Finland). Data acquisition was performed overnight from 9:00 PM to 6:00 AM the next morning.

Assessment of hypoxic episodes. To assess OSAS-induced hypoxia, we applied desaturation magnitude (DM) in this study. Desaturation episodes were defined as hypoxia of Sa_{O_2} <90%. We defined DM as

$$DM = \sum (90 - Sa_{O_2})t$$

where t is time of desaturation (in h). As shown in the equation, DM expresses the severity of hypoxic stress quantitatively.

Measurements of circulating ICAM-1, IL-8, and MCP-1. We obtained peripheral blood from the subjects at 9:00 AM before and after the nCPAP treatment. The blood samples were centrifuged at 250 *g* and 4°C for 10 min. The serum samples were then stored at −80°C until measurements. The concentrations of ICAM-1, IL-8 and MCP-1 in the serum were measured by ELISA method. The data are defined as

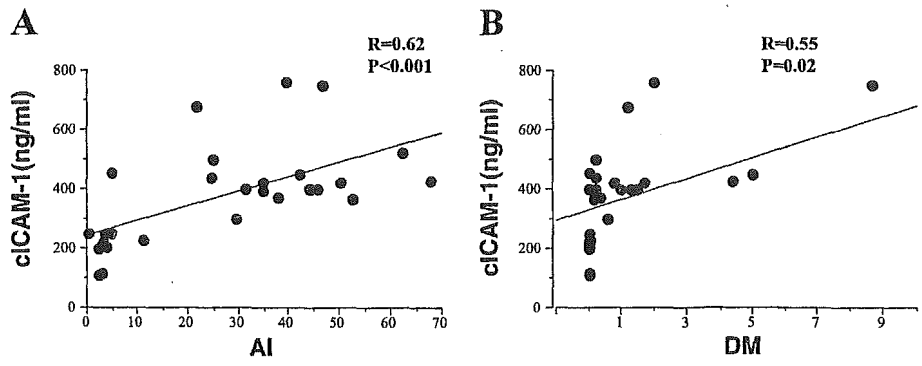

Fig. 2. Correlation between cICAM-1 and apnea-associated parameters, i.e., apnea index (AI; *A*) and desaturation magnitude (DM; *B*).

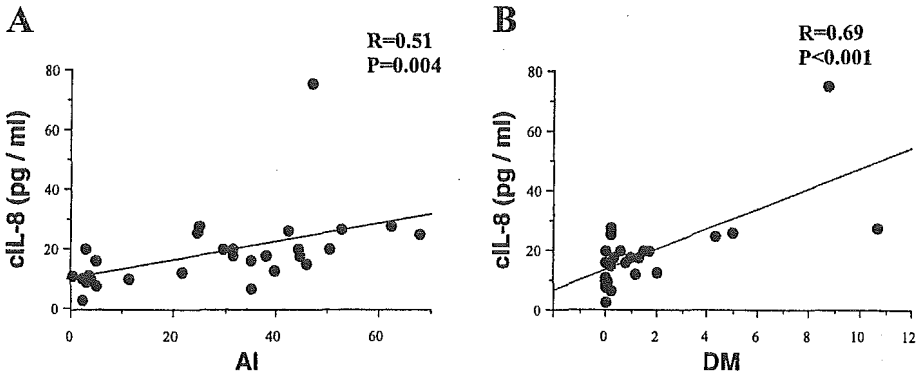

Fig. 3. Correlation between circulating IL-8 (cIL-8) and apnea-associated parameters, i.e., AI (*A*) and DM (*B*) desaturation magnitude (DM).

circulating ICAM-1 (cICAM-1), circulating IL-8 (cIL-8), and circulating MCP-1 (cMCP-1), respectively.

Data analysis. Comparisons of data between each experimental group were carried out with Student's *t*-test. Data are expressed as means ± SE. P values <0.05 were taken as significant.

RESULTS

Assessment of hypoxic episodes. There were significant differences in baseline DM between the OSAS and normal groups (2.01 ± 0.66 and 0.02 ± 0.01, respectively; $P < 0.001$), suggesting that the OSAS patients were exposed to significantly greater degree of hypoxia compared with the control subjects.

Baseline measurements of cICAM-1 and cIL-8. Figure 1 summarizes the cICAM-1 and cIL-8 levels in the baseline measurements. The levels of both cICAM-1 and cIL-8 in the OSAS group were significantly greater than those in the normal group.

Figure 2 demonstrates the relationships between cICAM-1 and AI and between cICAM-1 and DM. As shown, significant correlations are observed between cICAM-1 and apnea episodes. Similarly, significant correlation between cIL-8 and DM is detected, whereas the positive correlation is suggested between cIL-8 and AI (Fig. 3).

As indicated in Fig. 4, cICAM-1 is significantly correlated with cIL-8.

Effects of nCPAP on physiological parameters and circulating mediators. After nCPAP, the improvement in sleepiness was observed in all of the OSAS patients who successfully received therapeutic nCPAP. Consequently, nCPAP significantly decreased apnea and desaturation (Fig. 5).

Figure 6 summarizes the effects of long-term nCPAP on cICAM-1 and cIL-8 levels. As shown, nCPAP longer than 8 mo significantly decreased the levels of both cICAM-1 and cIL-8 in the treated OSAS group.

Figure 7 summarizes the cMCP-1 level in the OSAS group and normal group. The level of cMCP-1 in the

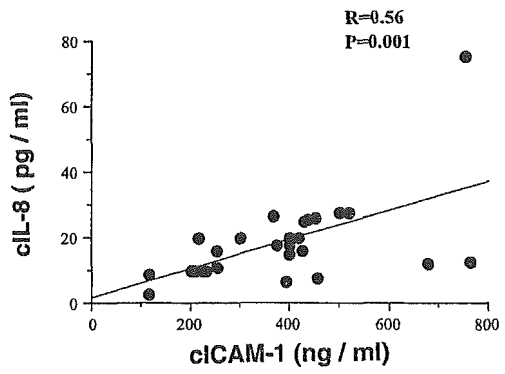

Fig. 4. Correlation between cICAM-1 and cIL-8.

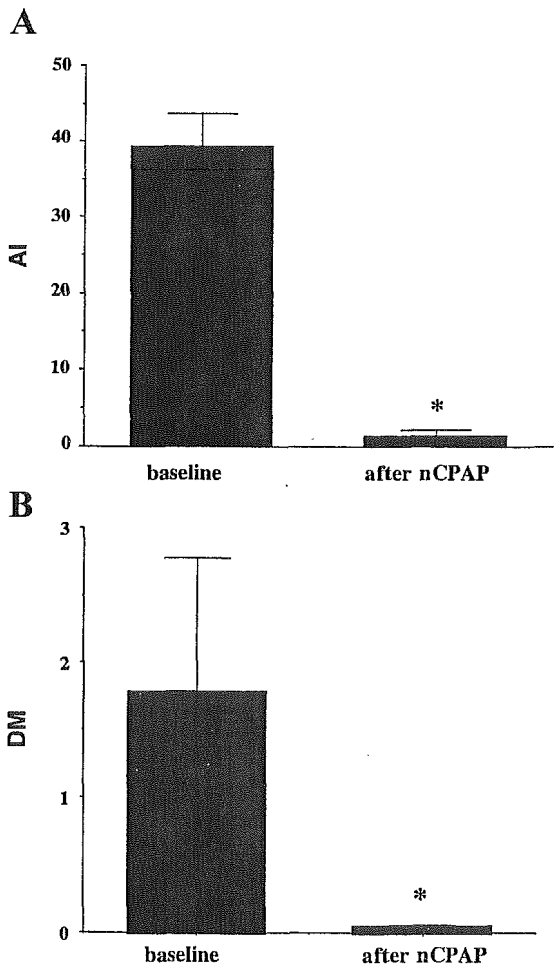

Fig. 5. Effects of nasal continuous positive airway pressure (nCPAP) on apnea-associated parameters, i.e., AI (*A*) apnea index and DM (*B*). *$P < 0.05$ vs. baseline.

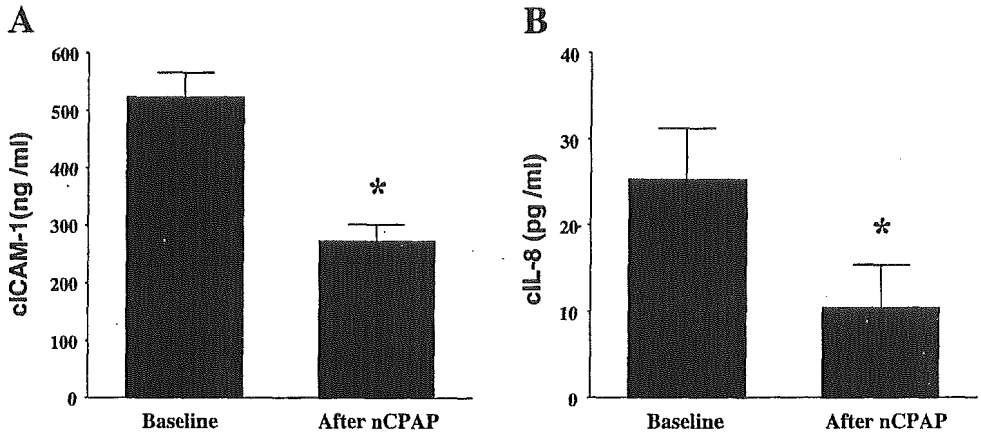

Fig. 6. Effects of nCPAP on cICAM-1 (*A*) and cIL-8 (*B*). *$P < 0.05$ vs. baseline.

OSAS group was significantly greater than that in the normal group.

DISCUSSION

The results of the present study demonstrate that nCPAP decreased apnea, desaturation, and the circulating ICAM-1 and IL-8 levels in the OSAS patients. In the baseline measurements, the levels of both ICAM-1 and IL-8 in the OSAS group were significantly greater than those in the control group. These observations suggest that nCPAP therapy could reduce OSAS-induced hypoxia and generation of inflammatory mediators, leading to the possible prevention of cardiovascular disorders.

Several issues warrant consideration before the results are discussed. First, we measured circulating ICAM-1 and IL-8 to assess the expression of cell-associated adhesion molecule and chemokine. Whereas this approach has been widely used (11, 20), it remains unclear whether the circulating levels of these mediators might precisely reflect the real expression of molecules attached to the endothelium or leukocytes. Second, the number of subjects in this study is relatively low, although the characteristics of the subjects were well matched. Increasing the number of subjects may be required to confirm the interpretation of the present results, and we should acknowledge this point.

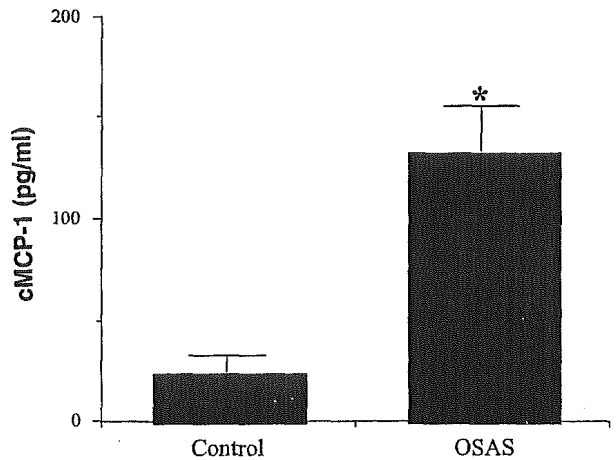

Fig. 7. Baseline levels of circulating monocyte chemoattractant protein-1 (cMCP-1) in the control and OSAS groups. *$P < 0.05$ vs. normal controls.

It has been recently postulated that inflammatory process has a crucial role in the pathogenesis of atherosclerosis, leading to the various cardiovascular disorders (1, 9). To promote migration of leukocytes from circulation to inflamed areas, it is essential for leukocytes to adhere to vascular endothelium via adhesion molecules (32). Especially, ICAM-1 has been reported to play important roles in leukocyte migration to inflamed area (2, 3, 29). ICAM-1 is an 80- to 110-kDa glycoprotein consisting of five immunoglobulin-like domains and a ligand for LFA-1α (18, 32). It has been demonstrated that the ICAM-1/LFA-1α pathway evolves to function in cell-cell adhesion (33) and mediates various inflammatory diseases (16, 19, 21, 22, 35). Recently, it has been reported that the circulating ICAM-1 levels are higher in patients with ischemic heart disease than those in controls (20). Moreover, the circulating ICAM-1 level may indicate a risk of future myocardial infarction, suggesting that antiadhesion therapies can be considered as a novel therapeutic means of cardiovascular disease (26). In the previous study, we have demonstrated that the circulating ICAM-1 level is significantly increased compared with the control group, suggesting that OSAS-induced hypoxia may induce the activation of ICAM-1 and the inflammation of endothelium in patients with OSAS (24). This observation may give rise to a hypothesis that the therapy for OSAS might be a potential approach to prevention of cardiovascular disorders via antiadhesion mechanism.

Recently, it has been demonstrated that IL-8 may play an important role in the development of atherosclerosis (4, 10, 31). Although monocytes contribute to the development of atherosclerotic lesions, IL-8 is a powerful trigger for firm adhesion of monocytes to vascular endothelium (10). It has been shown that hypoxia induces expression and/or generation of IL-8 (13, 30), indicating that OSAS-associated desaturation could lead to upregulatation of IL-8 expression. In addition, one could presume that the effective therapy

for OSAS may attenuate hypoxic stress, which may prevent the development of vascular lesions via the reduction of IL-8 production.

To treat patients with OSAS, nCPAP therapy is widely used, because nCPAP reduces excessive daytime sleepiness and improves quality of life (8, 27). Based on the recent studies, beneficial effects of nCPAP on the prognosis of OSAS-associated diseases are anticipated, but there exists little evidence to prove this notion. We therefore performed this study to address the question whether nCPAP could affect physiological phenomena and production of proinflammatory mediators. We observed that long-term nCPAP was effective to improve sleepiness, nocturnal apnea, and desaturation and that the levels of circulating mediators were reduced after nCPAP. One of the possible explanations is that nCPAP decreases hypoxic episodes, resulting in the reduction of hypoxia-induced inflammation and expression of ICAM-1 and IL-8. Considering the proinflammatory effects of ICAM-1 and IL-8, the attenuated production of these mediators elicited by nCPAP may suggest a novel approach to manage OSAS and prevent OSAS-associated inflammatory diseases.

To assess the severity of hypoxia induced by OSAS, we used DM. Possibly, this parameter may reflect OSAS-induced hypoxic stress more directly than AI. The usual way to assess the degree of OSAS includes the number of apnea episodes, but DM could reflect both decreases in Sa_{O_2} and time spent below 90%. However, to accurately analyze the hypoxic stress, exploring other indexes of hypoxic stress may be important and helpful.

We observed that there was a significant correlation between circulating ICAM-1 and IL-8 in the population studied. It has been demonstrated that nuclear transcription factor (NF)-κB regulates the synthesis and expression of both ICAM-1 and IL-8 (26, 27). In addition, NF-κB is upregulated by hypoxia, leading to the increased expression of both ICAM-1 and IL-8 (7, 37). These reports may explain the present findings that there were significant correlations between desaturation and mediators measured.

We further investigated the level of circulating MCP-1 in the normal and OSAS groups. Recently, it has been reported that the level of MCP-1 is increased in patients with coronary heart disease (14). In the present study, we observed that the level of MCP-1 in the OSAS group was increased compared with that of the normal group. Possibly, the increases in the circulating chemokines, including MCP-1, may play an important role in the pathogenesis in OSAS patients complicated with cardiovascular disease.

In summary, the circulating ICAM-1, IL-8, and MCP-1 levels increased in the OSAS patients compared with the normal subjects. After nCPAP therapy, significant decreases in the levels of ICAM-1 and IL-8 were observed in the OSAS group. Taken together, OSAS-induced hypoxia activates ICAM-1 and IL-8, resulting in the important risk factor of cardiovascular disorders. Treatment of OSAS with the use of nCPAP can be, therefore, a potential approach to decrease risk of the progression of OSAS-associated disorders.

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

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[1]*Departments of Geriatric Medicine and* [2]*Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655; and* [3]*Division of Integrative Cell Biology, Department of Embryogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto 862-0976, Japan*

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Aoki-Nagase, Tomoko, Takahide Nagase, Yoshio Ohhashi, Takayuki Shindo, Yukiko Kurihara, Yasuhiro Yamaguchi, Hiroshi Yamamoto, Tetsuji Tomita, Eijiro 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.

asthma; bronchial hyperreactivity; eosinophilia; leukotriene; knockout mouse

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

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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 ganciclovir. Homologous recombinants were identified by PCR and Southern blot analysis. Targeted ES cell clones were injected into C57BL/6 mouse blastocysts to generate chimeric mice. Male chimeras were then cross bred with C57BL/6 females, and germline transmission was achieved. Mice heterozygous for α-CGRP-mutant allele with the genetic background of the 129/Sv × C57BL/6 hybrid were mated. Offspring were genotyped at 4 wk of age. For genotyping, genomic DNAs were isolated from biopsied tail and subjected to PCR amplification. The animals were maintained on a 7:20-h light-dark cycle at 23°C. Mice were fed a standard laboratory diet and water ad libitum. Mutant mice (α-*CGRP*$^{-/-}$) and their littermate controls (α-*CGRP*$^{+/+}$) were used in the present study.

Sensitization and antigen challenge. Mice were sensitized with an intraperitoneal injection of 0.5 ml of a solution containing 0.1 mg of ovalbumin (OA) complexed with aluminum hydroxide (2 mg/ml). On *day 8*, the mice were boosted with the same mixture. On *day 12*, these sensitized mice were challenged for 30 min with 1% OA in saline aerosol generated with an ultrasonic nebulizer (Ultra-Neb100, DeVilbiss, Somerset, PA). Control mice received an intraperitoneal injection of saline and saline aerosols in the same manner. Three days after the aerosol challenge, we measured bronchial responsiveness or performed bronchoalveolar lavage (BAL).

Animal preparation. Animals were anesthetized with pentobarbital sodium (25 mg/kg ip) and ketamine hydrochloride (25 mg/kg ip) in combination and then paralyzed with pancuronium bromide (0.3 mg/kg ip). Anesthesia and paralysis were maintained by supplemental administration of 10% of the initial dose every hour. After tracheostomy, a metal endotracheal tube (1 mm ID, 8 mm long) was inserted in the trachea. Animals were mechanically ventilated (model 683, Harvard Apparatus, South Natick, MA) with tidal volumes of 10 ml/kg and frequencies of 2.5 Hz. The thorax was widely opened by means of a midline sternotomy, and a positive end-expiratory pressure of 2 cmH_2O was applied by placing the expired line under water. During the experiments, O_2 gas was continuously supplied to the ventilatory system. Under these ventilatory conditions, arterial pH, Po_2, and Pco_2 were 7.35–7.45, 100–180 mmHg, and 30–45 mmHg, respectively, at the end of experiments (Compact 3 blood gas analyzer, AVL Medical Systems). A heating pad was used to maintain the body temperature of animals.

Tracheal pressure was measured with a piezoresistive microtransducer (model 8510B-2, Endevco, San Juan Capistrano, CA) placed in the lateral port of the tracheal cannula. Tracheal flow was measured by means of a Fleisch pneumotachograph (Metabo, Lausanne, Switzerland). All signals were amplified, filtered at a cutoff frequency of 100 Hz, and converted from analog to digital with a converter (model DT2801-A, Data Translation, Marlborough, MA). The signals were sampled at a rate of 200 Hz and stored on an IBM-AT-compatible computer. Lung resistance (R_L) and elastance (E_L) were measured as previously described (27, 31, 32).

Airway responsiveness to methacholine administration. At the start of the protocol, two deep inhalations (3 times tidal volume) were delivered to standardize volume history. All 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 1 min after administration of saline aerosol. This represented 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 R_L 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) A_2 (TxA_2, measured as TxB_2) and leukotriene (LT) $C_4/D_4/E_4$ were determined using enzyme immunoassay (EIA) kits (Amersham Pharmacia Biotech). The detection limits of the EIA assays for TxB_2 and $LTC_4/D_4/E_4$ 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 EIA 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 midsagittal 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% H_2O_2 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-

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 (Scheffé'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 TxB_2 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 $LTC_4/D_4/E_4$ was significantly greater in OA-treated *CGRP*$^{+/+}$ mice than in any other group (Fig. 5).

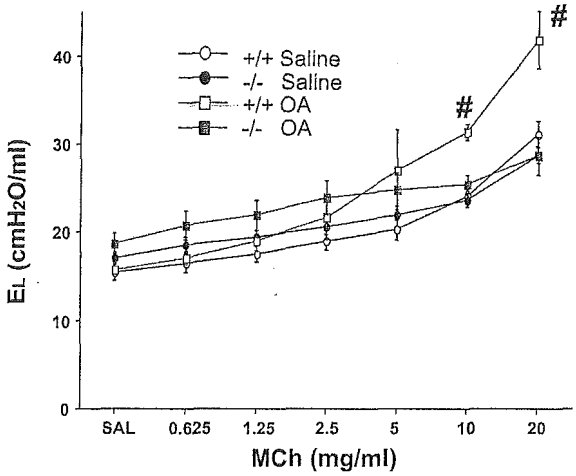

Fig. 2. MCh dose-response curves for lung elastance (EL) in wild-type and α-CGRP-deficient mice (n = 7–9). #$P < 0.05$ compared with other groups.

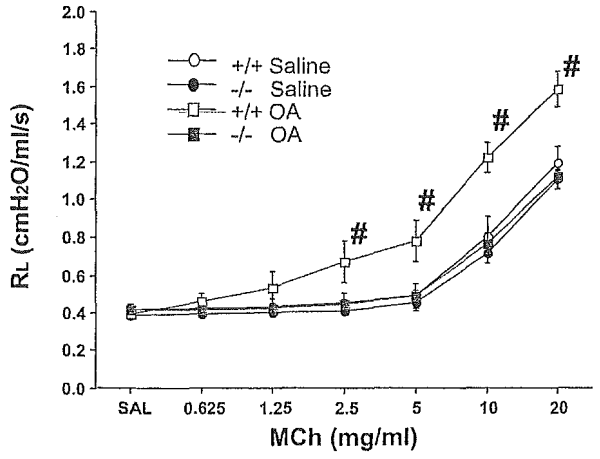

Fig. 1. Methacholine (MCh) dose-response curves for lung resistance (RL) in wild-type (+/+) mice and mice deficient in the α-isoform (−/−) of calcitonin gene-related peptide (α-CGRP) (n = 7–9). #$P <$ 0.05 compared with other groups. OA, ovalbumin.

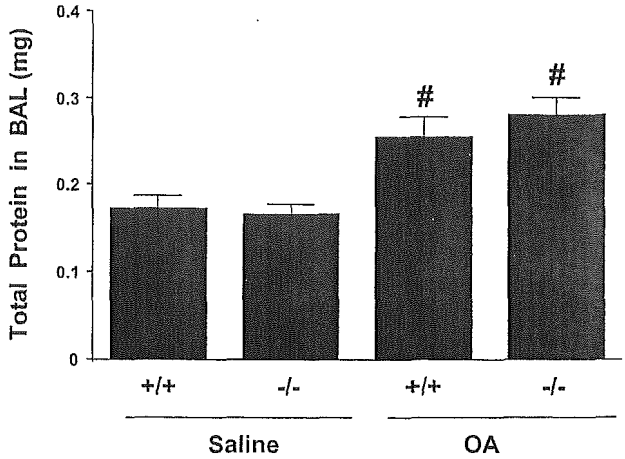

Fig. 3. Roles of CGRP in antigen-induced protein leakage. Protein leakage is assessed by total protein amount in bronchoalveolar lavage (BAL) fluid in wild-type and α-CGRP-deficient mice (n = 4–6). #$P < 0.05$ compared with saline groups.

Table 1. *Total cell counts and cell fractions in BALF*

	Total Cell Counts, $\times 10^5$	Macrophages, %	Lymphocytes, %	Eosinophils, %	Polymorphonuclear Neutrophils, %
Saline					
+/+	1.04 ± 0.05	94.5 ± 0.3	5.1 ± 0.4	0.1 ± 0.1	0.3 ± 0.3
−/−	1.01 ± 0.07	94.8 ± 0.3	4.9 ± 0.3	0.1 ± 0.1	0.3 ± 0.3
Ovalbumin					
+/+	5.83 ± 1.26*	64.3 ± 9.7 *	3.5 ± 0.4	31.5 ± 10.0*	0.8 ± 0.2
−/−	5.84 ± 0.61*	62.9 ± 10.7*	3.7 ± 0.8	32.7 ± 11.0*	0.8 ± 0.3

Values are means ± SE. BALF, bronchoalveolar lavage fluid. *$P < 0.05$ compared to saline groups.

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 mice.

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

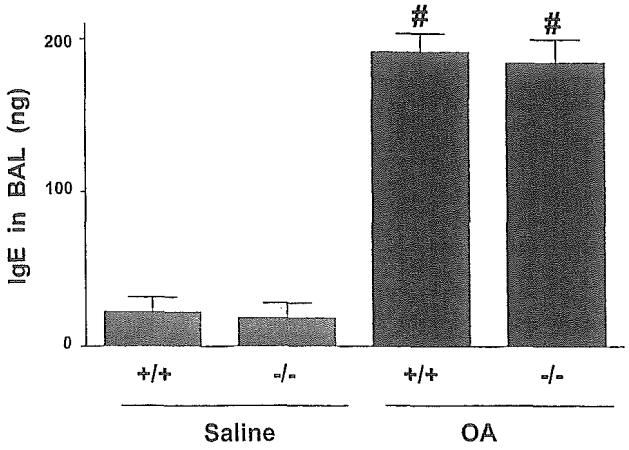

Fig. 4. IgE levels in BAL fluid in wild-type and α-CGRP-deficient mice (n = 4–6). #$P < 0.05$ compared with saline groups.

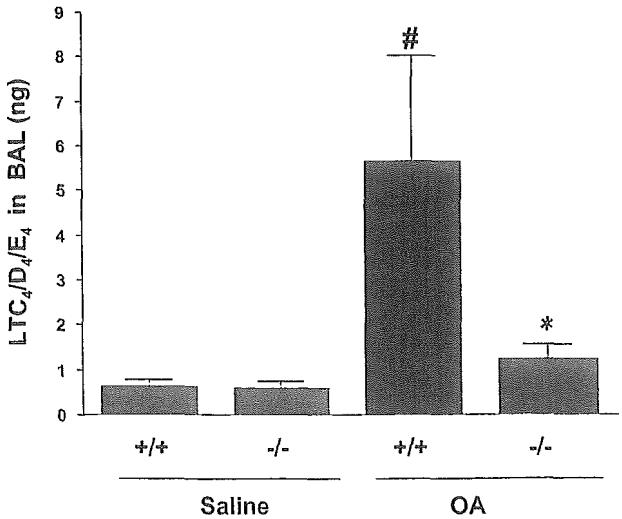

Fig. 5. Leukotriene (LT) $C_4/D_4/E_4$ levels in BAL fluid in wild-type and α-CGRP-deficient mice (n = 4–6). *$P < 0.05$ compared with sensitized wild-type mice. #$P < 0.05$ compared with saline groups.

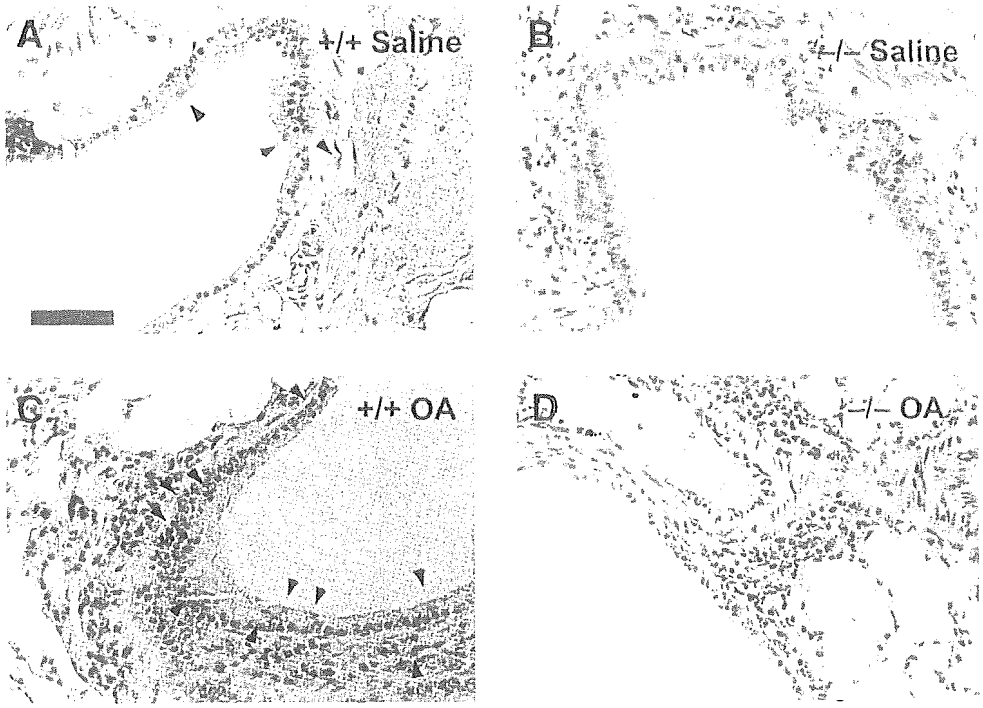

Fig. 6. Photomicrographs of CGRP immunohistochemical staining in large airways from saline-treated wild-type (*A*), saline-treated α-CGRP-deficient (*B*), OA antigen-treated wild-type (*C*), and OA antigen-treated α-CGRP-deficient (*D*) mice. Arrowheads, CGRP immunoreactivity. Immunoreactivity for CGRP was increased in antigen-treated wild-type mice (*C*) compared with unsensitized control (*A*). There was little immunostaining for CGRP in α-CGRP-deficient mice (*B* and *D*). Specimens were counterstained with hematoxylin. Scale bar, 50 μm.

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 Ohhashi 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 pathophysiologi-

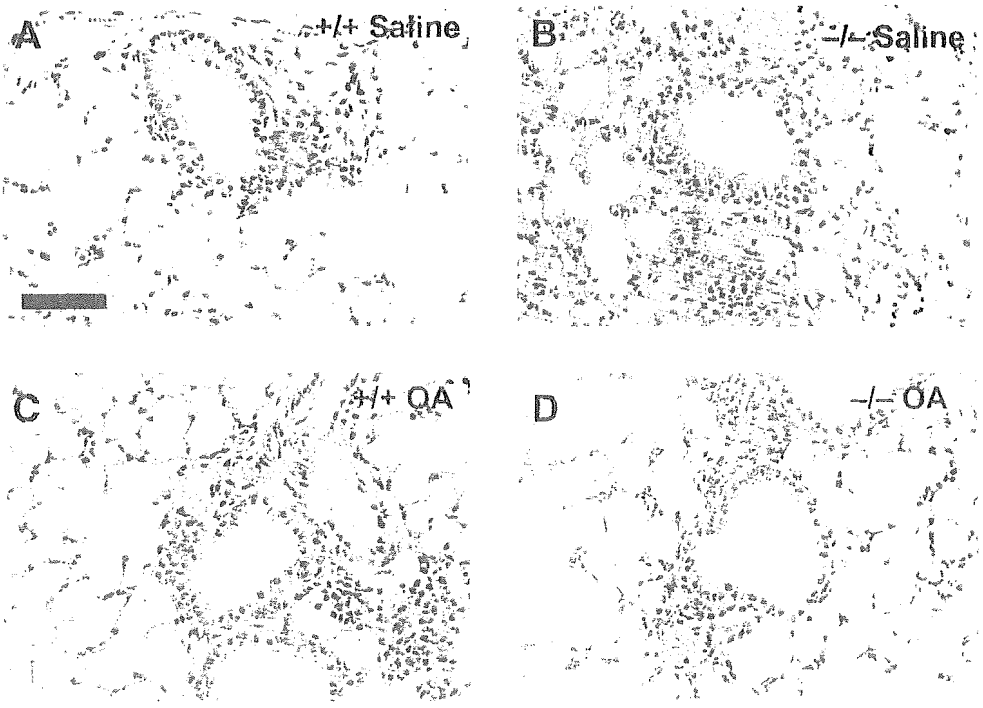

Fig. 7. Photomicrographs of CGRP immunohistochemical staining in small airways and lung parenchyma from saline-treated wild-type (*A*), saline-treated α-CGRP-deficient (*B*), OA antigen-treated wild-type (*C*), and OA antigen-treated α-CGRP-deficient (*D*) mice. There was little immunostaining for CGRP in each group. Specimens were counterstained with hematoxylin. Scale bar, 50 μm.

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

	Large Airways				
	Epithelium	Submucosa	Smooth muscle	Small Airways	Lung Parenchyma
Saline					
+/+	1.4 ± 0.2	1.4 ± 0.2	0.8 ± 0.1	0.4 ± 0.2	0.0 ± 0.0
−/−	0.2 ± 0.2*	0.2 ± 0.2*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0
Ovalbumin					
+/+	2.6 ± 0.2†	2.4 ± 0.2†	2.1 ± 0.1†	0.4 ± 0.2	0.2 ± 0.2
−/−	0.2 ± 0.2*	0.4 ± 0.2*	0.1 ± 0.1*	0.0 ± 0.0*	0.0 ± 0.0

Values are means ± SE. CGRP, calcitonin gene-related peptide. *$P < 0.05$ compared with wild-type (+/+) groups. †$P < 0.05$ compared with saline groups.

cal mechanisms underlying airway hyperresponsiveness were further examined using CGRP-mutant mice.

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, leukotriene, and ET-1 (28, 45, 49). These mediators are reported to be involved in airway hyperresponsiveness (28). Cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) 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 $CysLT_1$ receptor (9). In humans, it has been recently demonstrated that the $CysLT_1$ receptor is expressed in lung smooth muscle, lung macrophages, and peripheral blood leukocytes, while the identification of the $CysLT_1$ receptor is consistent with the anti-inflammatory actions of $CysLT_1$ 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

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 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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Identification of Multiple Novel Epididymis-Specific β-Defensin Isoforms in Humans and Mice[1]

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Defensins comprise a family of cationic antimicrobial peptides that are characterized by the presence of six conserved cysteine residues. We identified two novel human β-defensin (hBD) isoforms by mining the public human genomic sequences. The predicted peptides conserve the six-cysteine motif identical with hBD-4, termed hBD-5 and hBD-6. We also evaluated the characteristics of the mouse homologs of hBD-5, hBD-6, and HE2β1, termed mouse β-defensin (mBD)-12, mBD-11, and mouse EP2e (mEP2e). The mBD-12 synthetic peptide showed salt-dependent antimicrobial activity. We demonstrate the epididymis-specific expression pattern of hBD-5, hBD-6, mBD-11, mBD-12, and mEP2e. In situ hybridization revealed mBD-11, mBD-12, and mEP2e expression in the columnar epithelium of the caput epididymis, contrasting with the predominant expression of mBD-3 in the capsule or septum of the whole epididymis. In addition, the regional specificity of mBD-11, mBD-12, and mEP2e was somewhat overlapping, but not identical, in the caput epididymis, suggesting that specific regulation may work for each member of the β-defensin family. Our findings indicated that multiple β-defensin isoforms specifically and cooperatively contribute to the innate immunity of the urogenital system. *The Journal of Immunology,* **2002, 169: 2516–2523.**

Defensins are cationic antimicrobial peptides that include six specific cysteine residues and can be divided into the α- and β-defensin subfamilies. Three human α-defensins, human neutrophil peptides (HNP)[3]-1, -2, and -3, were isolated from human neutrophils and showed broad-spectrum microbicidal activity (1). The first mammalian β-defensin was discovered from the bovine respiratory tract, named tracheal antimicrobial peptide (2). Subsequently, lingual antimicrobial peptide was isolated from the bovine tongue (3).

Four human β-defensin (hBD) isoforms have been identified to date: hBD-1, -2, -3, and -4 (4–7). HE2β1, identified as one major splicing variant of the human EP2 gene, also contains the specific cysteine motif (8–11). All hBDs show potent antimicrobial activity, especially against Gram-negative bacteria, whereas the function of HE2β1 had not been confirmed (5–7, 12–14). In mice, mouse β-defensin (mBD)-1, -2, -3, -4, -5, -6, -7, -8, -9, -11, -13, and -35 have been identified at the National Center for Biotechnology Information (NCBI) gene bank, although the characteristics of mBD-5, mBD-9, mBD-11, mBD-13, and mBD-35 have not been published (15–21). mBD-1 and mBD-3 are regarded as mouse homologs of hBD-1 and hBD-2, respectively, and also showed antimicrobial activity (16, 18). hBD-1, hBD-2, and hBD-3 showed the widespread distribution in various organs like urogenital tissues, skin, respiratory tracts, intestinal tracts, testis, and placenta (22–26). Although the tissue distribution of mBD-5, mBD-7, mBD-8, mBD-9, mBD-11, mBD-13, and mBD-35 have not been evaluated in mice, the other known mBD isoforms also show the expression in multiple tissues, such as kidney, esophagus, tongue, trachea, and skeletal muscle (15–20).

Furthermore, the novel antimicrobial peptide Bin1b was identified in the rat epididymis and its putative amino acid sequence is included the conserved six-cysteine motif (27). Bin1b is partially homologous with HE2β1 and more homologous with the chimpanzee epididymal protein EP2E in its amino acid sequence (28). Interestingly, Bin1b showed no expression in the other major organs, such as the lung or kidney. Subsequently, hBD-4 cDNA was identified and its expression was also almost confined to the testis with much lower expression in the gastric antrum (7). These two isoforms are unique in their confined expression pattern.

Because the defensin genes comprise a large gene cluster in chromosome 8, the genomic sequence is useful to identify novel defensin genes (11, 29, 30). Recent reports indicate the presence of >25 human or mouse genes that could be encoding β-defensin peptide, although the characteristics of these genes have not been evaluated well (31). In this work we report the peculiar characteristics of multiple epididymis-specific β-defensin isoforms in humans and mice, including two novel hBD isoforms, named hBD-5 and hBD-6, and two novel mBD isoforms, named mBD-12 and mouse EP2e (mEP2e), respectively.

Materials and Methods

Cloning of hBD-5 and hBD-6 cDNA

We obtained the nucleotide sequence of the human genome around the β-defensin gene cluster in chromosome 8 from the NCBI public database (NT_019483). This sequence was translated in all six possible reading frames and was searched for the specific cysteine pattern; multiple possible

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[3] Abbreviations used in this paper: HNP, human neutrophil peptide; hBD, human β-defensin; mBD, mouse β-defensin; mEP2e, mouse EP2e; EST, expressed sequence tag.

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