

# Localization of Inducible Nitric Oxide Synthase and Endothelial Constitutive Nitric Oxide Synthase in Airway Mucosa of Toluene Diisocyanate-Induced Asthma

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## ABSTRACT

Endogenous exhaled nitric oxide (NO) is increased in asthma patients, especially in the exacerbated state. To evaluate the role of NO in airway mucosa of toluene diisocyanate (TDI)-induced asthma in relation to clinical and immunologic findings, 20 TDI-induced asthma patients were enrolled and classified into two groups: 9 newly diagnosed patients (group I) and 11 patients having persistent asthma symptoms for >5 years despite work withdrawal (group II). Immunohistochemistry was applied to compare the expression of endothelial constitutive NOS (c-NOS) and induced NOS (i-NOS) in airway mucosa of both groups. They were observed in four areas: epithelium (EP), smooth muscle (SM), vascular endothelium (VE), and mucous gland (MG). The intensity of expression was graded on

a scale of one to four and mean values were presented from blind readings observed by two experts. Serum-specific immunoglobulin E (IgE) and immunoglobulin G (IgG) antibodies to TDI-human serum albumin conjugate were measured by enzyme-linked immunosorbent assay. e-NOS was found most frequently in VE (5/6 [83.3] versus 7/10 [70%]) followed by MG, EP, and SM in both groups. i-NOS was found most frequently in EP (7/9 [77.8%] versus 7/11 [63.6%]), followed by MG, VE, and SM in both groups. The intensities of i-NOS and c-NOS of MG were significantly higher in group I than in group II ( $p < 0.05$ ) with no significant differences in other areas ( $p > 0.05$ , respectively). There were no significant correlations between i-NOS or c-NOS expression and exposure or asthma symptom duration ( $p > 0.05$ ). No associations were found between i-NOS or c-NOS expression and the presence of specific IgE or IgG to TDI-human serum albumin conjugate ( $p > 0.05$ , respectively). In conclusion, i-NOS and c-NOS expressions were noted in EP, SM, VE, and MG in airway mucosa of TDI-induced asthma patients, which may contribute to airway inflammation. (Allergy and Asthma Proc 24:275–280, 2003)

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There is increasing evidence that endogenously produced nitric oxide (NO) may have both beneficial and detrimental effects on asthma.<sup>1</sup> These differential properties have been attributed to a dual physiological and pathological roles of NO depending on the enzyme responsible for its generation. NO is a highly reactive radical formed from the semiessential amino acid L-arginine by the action of

three isoforms of the enzyme NO synthase (NOS).<sup>2</sup> The constitutive isoforms (constitutive NOS [c-NOS]) are expressed in neurons (neuronal NOS), and endothelial cells (endothelial NOS [e-NOS]) of the airway<sup>3</sup> and are involved in the physiological regulation of the airway. The third isoform is induced after exposure to proinflammatory cytokines (induced NOS [i-NOS]) and is expressed in epithelial cells and inflammatory cells of the airway,<sup>4</sup> generating 1000-fold higher amounts of NO than c-NOS and may be responsible for the pathological effects of NO in asthma. In keeping with these findings, biopsy samples from patients with asthma showed increased i-NOS expression in epithelial cells<sup>5,6</sup> and raised levels of NO in exhaled air and sputum of asthmatic patients,<sup>7,8</sup> suggesting that inflammation, which is a key feature of asthma, is responsible for the increased expression of this enzyme. Furthermore, exhaled NO levels have been found to rise further during asthma exacerbations<sup>7</sup> and to become lower after treatment with corticosteroids.<sup>7,8</sup> Although there have been few studies to localize NOS expression in human airway mucosa, there has been no publication to observe i-NOS and c-NOS expression in airway mucosa of occupational asthma patients and to clarify the relationship with humoral immune response.

Toluene diisocyanate (TDI) is the most common cause of occupational asthma in North America as well as in Korea.<sup>9</sup> Although considerable controversy remains regarding the pathogenesis of TDI-induced asthma, several groups of investigators<sup>9,10</sup> have suggested that it has a role as an immunologic mechanism. Indeed, serum-specific immunoglobulin E (IgE) and specific IgG antibodies have been identified in some sensitized workers. Long-term follow-up studies of subjects with isocyanate asthma have suggested that asthmatic symptoms may persist in some subjects for months or years after work withdrawal.<sup>11–13</sup> Our previous study<sup>14</sup> also showed that among 35 TDI-asthma patients, 51% of them had persistent asthmatic symptoms despite complete avoidance of exposure and adequate antiasthmatic medications. Although a previous investigation<sup>15</sup> showed that inhalation of TDI induced increased production of NO by rat bronchoalveolar lavage cells, to the best of our knowledge, there have been no data evaluating the role of NO in the development of asthma symptoms and the long-term prognosis of TDI-induced asthma patients.

In this study, to evaluate the role of NO in pathogenic mechanisms and long-term prognosis of TDI-induced asthma, we observed the two kinds (endothelial c-NOS and i-NOS) of NOS expression in the airway mucosa of TDI-induced asthma patients. The expressions of c-NOS and i-NOS were compared between two groups of patients: a newly diagnosed group and another group of TDI-induced asthma patients who had had persistent asthmatic symptoms for 5 years after work withdrawal requiring antiasthmatic medications.

## MATERIALS AND METHODS

### Subjects

Twenty subjects with TDI-induced asthma were enrolled. The subjects were classified into two groups; group I included 9 patients diagnosed as having TDI-induced asthma with bronchial biopsy done at initial diagnosis and group II included 11 patients complaining of persistent asthmatic symptoms moderate to severe persistent asthma, although they had been permanently removed from their work exposures to TDI and had received antiasthmatic medications according to symptom severity. Bronchial biopsy specimens were collected. The diagnosis of occupational asthma had been confirmed by positive responses to bronchoprovocation tests to TDI. For bronchoscopic biopsy, all subjects stopped using inhaled or oral steroids at least 4 weeks before the study. They underwent an interview, chest radiography, skin-prick tests with common inhaled allergens, lung function measurement, and inhalation challenge with both methacholine and TDI (80:20 = 2.4 form/2.6 form; Aldrich, Milwaukee, WI). All subjects gave their informed consent and the protocol was accepted by the Institutional Review Board of Ajou Medical Center, Suwon, Korea.

### Bronchial Challenge Test with Methacholine and TDI

The methacholine and TDI bronchial challenge tests were done according to the methods described previously.<sup>9,14</sup> Briefly, the subjects were exposed to pure TDI monomers (Aldrich) in a small enclosed room for 5–15 minutes until asthmatic symptoms were induced. The concentration of TDI measured by TLD-1, a toxic gas detector with Cheakey (HAD Scientific, Lincolnshire, IL), was 20 ppb. Forced expiratory volume in 1 second and mean forced expiratory flow 25–75% were measured with a spirometer (MultiSPIRO SX/PC; MultiSPIRO, Tempe, AZ) immediately before exposure and every hour for 8 hours after the exposure.

### Specific IgE and IgG Antibodies by Enzyme-Linked Immunosorbent Assay

The 2,4-TDI-human serum albumin (HAS) conjugates were prepared as described previously<sup>9</sup> by using a modification of Tse and Pesce's method. Serum-specific IgE and IgG levels were detected by enzyme-linked immunosorbent assay (ELISA) as described previously.<sup>9</sup> Briefly, 1  $\mu$ g of TDI-HSA conjugate or unconjugated HSA was dissolved in 0.1 mol/L of sodium bicarbonate buffer (pH 9.5) and coated in the ELISA plate individually (Costar Co., Cambridge, MA) at 48°C, washed with phosphate-buffered saline (PBS)-Tween 20, and blocked with 350  $\mu$ L of blocking buffer (PBS containing 5% bovine serum albumin and 0.1% Tween 20). Diluted serum from patient or control subjects at a concentration of 1:10 (for IgE) or 1:500 (for IgG) was incubated overnight at 37°C in both TDI-HSA and HSA-coated wells and washed three times. Biotin-labeled

TABLE I

## Comparison of Dermographic Data Between Group I and II Subjects

| Group       | Sex<br>(m/f) | Age (yrs)  | Atopy* | Exposure<br>Duration<br>to TDI<br>(mon) | FEV <sub>1</sub><br>(% predicted) | Methacholine<br>PC <sub>20</sub><br>(mg/mL) | Total IgE<br>(IU/mL) |
|-------------|--------------|------------|--------|-----------------------------------------|-----------------------------------|---------------------------------------------|----------------------|
| I (n = 9)   | 4/5          | 41.9 ± 1.3 | 2/7    | 90.5 ± 14.2                             | 76.8 ± 0.8                        | 2.69 ± 1.70                                 | 335.8 ± 143.0        |
| II (n = 11) | 6/5          | 44.6 ± 2.2 | 4/7    | 84.0 ± 0.20                             | 91.9 ± 6.5                        | 1.78 ± 1.55                                 | 245.7 ± 55.1         |
| p Value     | NS           | NS         | NS     | NS                                      | NS                                | NS                                          | NS                   |

All values are presented as means ± SEM.

NS = not significant; m = male; f = female; FEV<sub>1</sub> = forced expiratory volume in 1 second.

\*Atopy, presence/absence.

anti-IgE (Sigma Co.) or peroxidase-conjugated anti-IgG antibody (Vector Laboratory, Burlingame, CA) was added to each well and incubated for 1.5 hours at 37°C and washed with PBS-Tween 20, and then 100 µL of *O*-phenyl deamine or streptavidin-peroxidase was added as substrate. After 10 minutes of incubation at 37°C, reactions were stopped with 100 µL of 2N H<sub>2</sub>SO<sub>4</sub> and read using an ELISA reader (Molecular Devices Co., Los Angeles, CA) at 490 nm. The final absorbance value of each individual was decided after the subtraction of the HSA-coated value from that of the TDI-HSA-coated value. Positive cutoff values were determined as mean plus twofold SD of the absorbance values from 20 unexposed healthy controls.

### Bronchoscopy and Bronchial Biopsies

A fiberoptic bronchoscopy was performed after the bronchial challenge with TDI as described previously.<sup>15</sup> At least two endobronchial biopsy specimens were taken through a bronchoscope (Olympus BF, type 1T 10; Olympus Co., Tokyo, Japan) with sterile forceps (FB 15C; Olympus Co.) from the bronchial mucosa of the right lower lobar bronchus. The biopsy specimens were embedded in paraffin for immunohistology.

### Immunohistochemistry and Quantitation

The sections (4 µm thick) were obtained from biopsy specimens using a microtome (HM340E; Shandon, Elgany, France). NOS expressions were assessed by immunohistochemistry technique using monoclonal antibodies to i-NOS (Santa-Cruz, Santa Cruz, CA) and c-NOS (e-NOS; Santa-Cruz). Biotin-conjugated rabbit antimouse antibodies (1:200 in Tris-buffered saline; Sigma) were applied as the secondary antibodies and detected with the streptavidin-biotin complex using the LSAB kit (Dako, Carpinteria, CA) and aminoethylcabazole as chromogen. They were counterstained with Mayers' hematoxylin. The intensity of NOS expression of the same sections was observed by two experts and expressed as mean values evaluated in four fields per one tissue. For negative controls, the mouse IgG<sub>1</sub> anti-

bodies (1:200 v/v), were incubated with omission of primary antibodies.

### Statistical Analysis

Statistical analysis was done by Student's *t*-test and Mann-Whitney U test using the SPSS version 8.0 program (SPSS, Chicago, IL). Results were considered statistically significant if *p* < 0.05.

## RESULTS

### Clinical Characteristics of the Study Subjects

Table I describes the clinical features of the study subjects. There were no significant differences in the mean duration of exposure period, atopic findings, initial methacholine PC<sub>20</sub> level, initial forced expiratory volume in 1 second level, and serum total IgE level (*p* > 0.05, respectively).

### Localization of c-NOS Expression in Airway Mucosa

Figure 1 A shows the expression of c-NOS in group I subjects. Table II shows the comparison of c-NOS expression between group I and II subjects. In group I, c-NOS was found most frequently in the vascular endothelium (VE; 83.3%), followed by the mucous glands (MG; 66.6%), epithelium (EP; 50%), and smooth muscle (SM; 50%). In group II, c-NOS expression was found most frequently in the VE (70%), followed by the EP (18%), SM (10%), and MG (11.1%). c-NOS expression in the VE, EP, and SM tended to be higher in group I than in group II, with no statistical significance (*p* > 0.05, respectively). Although there were no statistical differences in the intensity of c-NOS expression in the EP, VE, and SM between groups I and II (*p* > 0.05), the intensity of c-NOS expression in the MG was significantly higher in group I than in group II, as shown in Fig. 2 A (*p* < 0.05).

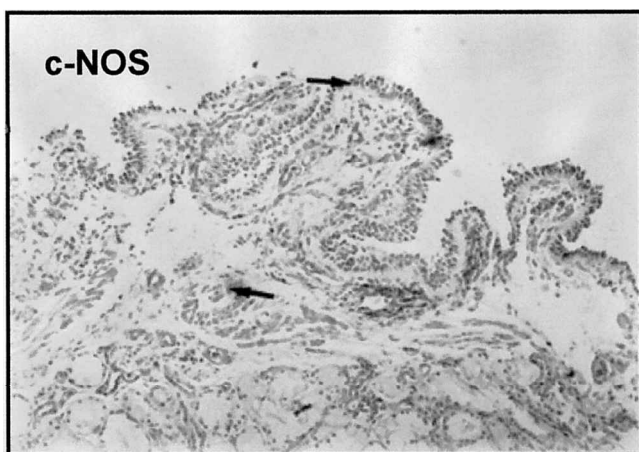
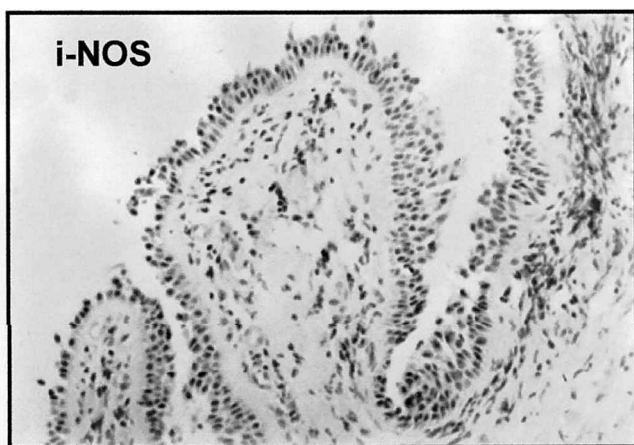
**A****B**

**Figure 1.** Expression of (A) c-NOS and (B) i-NOS in airway mucosa of group I patients. Both i-NOS and c-NOS expressions were noted in the EP, SM, VE, and MG.

#### Localization of i-NOS Expression in Airway Mucosa

**F**igure 1 B shows i-NOS expression in group I subjects. Table II summarizes the comparison of i-NOS expression in airway mucosa between the two study groups. In group I, i-NOS expression was found most frequently in the EP (77.8%), followed by the MG (57.5%), VE (33.3%), and SM (11.1%). In group II, i-NOS was found most frequently in the EP (63.6%), followed by the MG (60%), VE (45.6%), and SM (45.6%). Although there was no statistical significance in the intensity of i-NOS expression in the EP, SM, and VE between groups I and II ( $p > 0.05$ , respectively), the intensity of i-NOS in the MG was significantly higher in group I than in group II ( $p = 0.05$ ), as shown in Fig. 2 B.

#### Relationship Between i-NOS and c-NOS Expression and Clinical Parameters

**T**here were no significant correlations between i-NOS or c-NOS expression and exposure or asthma symptom duration ( $p > 0.05$ , respectively). There also were no sig-

nificant associations between the presence of serum-specific IgE or serum-specific IgG antibodies to TDI-HSA conjugate and i-NOS or c-NOS expression ( $p > 0.05$ , respectively, data not shown).

#### DISCUSSION

**T**his study showed that two kinds of NOS (i-NOS and c-NOS) were expressed in airway mucosa of TDI-induced asthma patients, which suggests a possible involvement of NO in airway inflammation of TDI-induced asthma. NO may have proinflammatory effects and the high amounts of NO formed by i-NOS may be deleterious in asthma.<sup>17</sup> Under circumstances of increased airway inflammation such as clinical exacerbations and late responses after allergen challenge, exhaled NO levels tend to become elevated, and these elevated NO levels can be modulated by steroids.<sup>18</sup> Previous studies have shown that there is a correlation between exhaled NO level and log PC<sub>20</sub><sup>N</sup> and a correlation between the peak flow variability and exhaled NO levels, suggesting higher NO levels in patients can indicate more severe asthma. Because NO is noninvasive and easy to measure, it is considered an indication of airway inflammation. It is hoped that NO also will reflect inflammation during asthma management.

Recent attention has focused on the role of NO in the respiratory tract and, in particular, the capacity for respiratory airway EP to express NOS. i-NOS localization was reported in bronchial EP of bronchogenic cancer and bronchiectasis patients using immunohistochemistry and *in situ* hybridization techniques as well as i-NOS expression in EP of asthmatic airway.<sup>19</sup> This is the first study to localize both i-NOS and c-NOS expressions in relation to prevalence of serum-specific antibodies in airway mucosa of TDI-induced asthma patients, which is the most common type of occupational asthma.

In a previous investigation<sup>6</sup> using immunohistochemical methodology in human lung tissue of uninvolved areas of surgical tumor resections, c-NOS expression was observed in nerve element, large vessel endothelium with no bindings of airway or alveolar macrophages, and occasionally in the endothelium and EP. However, in this study, c-NOS expression was noted in VE, SM, and MG, as well as EP in both groups of patients, which was different from the results of Hamid *et al.*<sup>5</sup> i-NOS expression was found most frequently in EP and also was noted in MG, SM, and VE. Because EP is the first barrier to TDI exposure, i-NOS expression noted in the epithelial layer of TDI-asthma patients may induce airway inflammation caused by TDI. The presence of i-NOS and c-NOS in various areas of airway mucosa of TDI-induced asthma suggests that NO may be of importance in the development and persistence of asthma symptoms in TDI-induced asthma, because NO may induce vasodilation<sup>19</sup> and regulation of eosinophils<sup>20</sup> and neutrophils<sup>21</sup> and favor the formation of Th2 cell phenotype.<sup>22</sup> These findings suggest that NO may contribute to the development of

TABLE II

## Localization of NOS Expression in Airway Mucosa of TDI-Induced Asthma Patients

| Patient                   | i-NOS       |             |             |            | c-NOS       |           |            |            |
|---------------------------|-------------|-------------|-------------|------------|-------------|-----------|------------|------------|
|                           | EP          | SM          | VE          | MG         | EP          | SM        | VE         | MG         |
| <b>Group I</b>            |             |             |             |            |             |           |            |            |
| (1)                       | 3           | 0           | 0           | 4          | 0           | 2         | 4          | 5          |
| (2)                       | 0           | 0           | 0           | 0          | 2           | 2         | 4          | 1          |
| (3)                       | 2           | 0           | 0           | 2          | 0           | 0         | 4          | UI         |
| (4)                       | 2           | 0           | 3           | 4          | UI          | UI        | UI         | UI         |
| (5)                       | 2           | 0           | 3           | 4          | 4           | 0         | 4          | 0          |
| (6)                       | 2           | UI          | UI          | UI         | UI          | UI        | UI         | UI         |
| (7)                       | 0           | 0           | 0           | 2          | UI          | UI        | UI         | UI         |
| (8)                       | 2           | 2           | 0           | 4          | 0           | 0         | 0          | 2          |
| (9)                       | 3           | 0           | 2           | 4          | 2           | 2         | 4          | 5          |
| No. of patients expressed | 7/9 (77.8)  | 1/9 (11.1)  | 3/9 (33.3)  | 7/8 (87.5) | 3/6 (50)    | 3/6 (50)  | 5/6 (83.3) | 4/6 (66.6) |
| <b>Group II</b>           |             |             |             |            |             |           |            |            |
| (1)                       | 3           | 4           | 4           | 4          | 0           | UI        | UI         | UI         |
| (2)                       | 3           | 3           | 4           | 4          | 0           | 0         | 4          | 0          |
| (3)                       | 3           | 0           | 2           | 1          | 0           | 0         | 2          | 0          |
| (4)                       | 0           | 0           | 0           | 1          | 0           | 0         | 4          | 0          |
| (5)                       | 2           | 1           | 4           | UI         | 0           | 0         | 4          | UI         |
| (6)                       | 0           | 0           | 0           | 0          | 2           | 0         | 4          | 3          |
| (7)                       | 0           | 0           | 0           | 0          | 0           | 0         | 0          | 0          |
| (8)                       | 2           | 1           | 2           | 0          | 0           | 0         | 4          | 0          |
| (9)                       | 0           | 0           | 0           | 0          | 0           | 0         | 0          | 0          |
| (10)                      | 1           | 0           | 0           | 2          | 2           | 2         | 4          | 0          |
| (11)                      | 2           | 1           | 0           | 2          | 0           | 0         | 0          | 0          |
| No. of patients expressed | 7/11 (63.6) | 5/11 (45.6) | 5/11 (45.6) | 6/10 (60)  | 2/11 (18.2) | 1/10 (10) | 7/10 (70)  | 1/9 (11.1) |

Bronchial biopsy was done at initial diagnosis.

UI = unidentifiable.

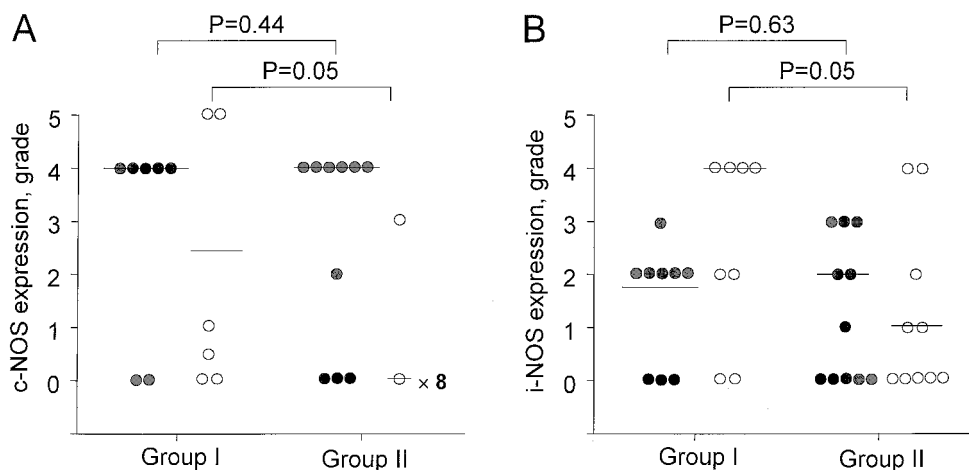


**Figure 2.** Comparison of (A) c-NOS and (B) i-NOS expression in the MG (○) and epithelial layer (●) between groups I and II. Significant difference was noted in the MG between two groups ( $p = 0.05$ ).

TDI-induced asthma and chronic airway inflammation with patients complaining of persistent asthmatic symptoms for >5 years. Additional studies will be needed to elucidate the regulatory role of NO in airway inflammation of TDI-induced asthma.

Our previous study on the outcome of 41 TDI-induced asthma patients for 2 years showed that >50% had complained of persistent asthma symptoms despite work withdrawal and antiasthmatic medications. In this study, we compared both types of NOS expression in airway mucosa

of the two groups of TDI-induced asthma: group I of newly diagnosed patients and group II who had persistent asthmatic symptoms for >5 years. Both c-NOS and i-NOS were observed in airway mucosa of group II as well as in group I. Their expression patterns were similar and no significant differences were found in intensity of c-NOS and i-NOS expression except in the MG between the two groups. Although it is possible that expressed i-NOS and c-NOS may represent responses to airway inflammation induced by TDI, these findings suggest that both i-NOS and c-NOS may contribute to persistent asthmatic symptoms.

The cellular and molecular mechanisms leading to increased NO are still unknown. Although multiple mechanisms that function coordinately may be involved, increased production of NOS synthesis in an *in vitro* study<sup>16,17</sup> showed that i-NOS expression in EP was regulated predominantly at a transcriptional level by inflammatory stimuli such as tumor necrosis factor  $\alpha$ , interferon  $\gamma$ , and interleukin- $1\beta$ ,<sup>16,17</sup> which are released by various inflammatory cells. In this study, we could not find any significant relations with clinical parameters and prevalences of specific IgE and IgG antibodies. Additional investigations will be needed to elucidate the regulatory mechanisms of NO related with inflammatory response airway mucosa.

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