

Short communication

Neuronal nitric oxide synthase gene polymorphism and IgE-mediated allergy in the Central European population

Background: Several findings suggest that nitric oxide (NO) plays a significant role in the regulation of the Th1/Th2 balance and contributes to the development of allergic diseases. Our study investigates a possible association of C/T transition located 276-bp downstream from the translation termination site in exon 29 of the human nitric oxide synthase type 1 (*NOS1*) gene with immunoglobulin E (IgE)-mediated allergic diseases in the Czech population.

Methods: The study included 688 subjects – 368 patients with clinically manifested allergic diseases and 320 unrelated controls with negative familial history of asthma/atopy. The *NOS1* genotypes were determined by polymerase chain reaction (PCR) and restriction analysis by *Eco72I*.

Results: No significant differences were found for allele or genotype frequencies of the 5266 C/T polymorphism in exon 29 of the *NOS1* gene between IgE-mediated allergic diseases (or asthma alone) and healthy subjects. However, this common polymorphism showed a significant association with signs of atopy, especially with total serum IgE levels [$\log_e$ IgE levels (mean $\pm$ SD): CC genotype = 4.34 ± 1.40 ; CT genotype = 4.58 ± 1.53 ; TT genotype = 5.01 ± 1.61 ; $P < 0.05$].

Conclusions: Our findings suggest that *NOS1* gene may participate in the pathogenesis of high total serum IgE levels in allergic diseases in our population. These findings provide support for *NOS1* as a candidate gene for IgE-mediated allergy.

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An increase in the prevalence of allergic diseases has been reported from different countries of the world during the last decades. Atopic diseases, such as allergic asthma, allergic rhinitis, and atopic eczema/dermatitis syndrome (AEDS), are considered to be multifactorial inflammatory disorders involving complex interactions between genetic and environmental factors (1, 2). Although the environmental factors play important roles in the development of atopy, there is a strong genetic predisposition in allergic subjects. A number of candidate genes have been proposed and many regions have been identified which show evidence for linkage to asthma and atopy, and a number of these have been replicated in independent populations (3–6). Among the variety of other loci, the gene for nitric oxide synthase type 1 or neuronal form NOS (*NOS1*) on chromosome 12q14–24.2 could be considered as a candidate gene (4–6).

The NOS belong to a group of enzymes, the NOS, that catalyse oxidation of L-arginine to form L-citrulline and NO. All three types are expressed in bronchial airways (7) or nasal mucosa (8) and levels of NO have been found to be elevated in expired gas of most asthmatics (9–10). The NO has several key effects on airways, i.e. it is important in host defence and has toxic effect on microorganisms

(bacteria, viruses etc.), it may increase mucus secretion and acts as potent vasodilator. Barnes and Liew (11) suggested that activation of the NOS system is the initiating step of allergic diseases via inhibition of Th1 cells by NO. In addition, significant correlations were found between exhaled NO levels and number of blood eosinophils (12). Production of NO by *NOS1* mediates nonadrenergic noncholinergic (NANC) neuronal inhibitory responses and acts as an inhibitory mechanism to cholinergic bronchoconstriction in airways (13).

Among the three known isoforms of NOS, namely NOS1 (neuronal NOS or nNOS), NOS2 (inducible NOS or iNOS) and NOS3 (endothelial NOS or eNOS), only NOS1 is encoded on chromosome 12 (14). The importance of NOS1 as an isoform involved in NO homeostasis was demonstrated in mice lacking a functional *nos1* gene. These mice had significantly lower fraction of exhaled NO levels than wild-type mice and were hyporesponsive to methacholine challenge (15).

The human *NOS1* gene is a single copy gene comprising 29 exons spanning 160 kb (14) and a number of polymorphisms were described in different parts of this gene. Dinucleotide (dC-dA)_n repeats were detected in the 5'-flanking region of exon 1, in the

intron 2, and within exon 29. One trinucleotide (AAT)_n repeat was found in intron 20, and three common single nucleotide polymorphisms were detected in exon 18, exon 22, and exon 29 (14, 16, 17). Several of these variants had been previously associated with diagnosis of asthma in a Caucasian population (16, 18–20) or with high levels of immunoglobulin E (IgE, 21), as well as with exhaled levels of NO in patients with asthma (22, 23).

In this study, we investigated a possible association of the single nucleotide polymorphism (SNP) with C/T transition located 276 bp downstream of the translation termination site (C5266T) in exon 29 of the human *NOS1* gene with IgE-mediated allergic diseases in the Czech population (homogenous Central European Caucasians). Therefore, the aim of our study was to determine whether the reported association with asthma in other populations occurs also in Czech allergic patients. Next, this study aimed to clarify whether the *NOS1* gene polymorphism is associated with intermediary phenotypes of allergy, such as serum IgE levels.

Materials and methods

Study population

The DNA was obtained from Caucasian subjects ($n = 688$) of exclusively Czech nationality. All subjects were selected using detailed questionnaire modified according to the American Thoracic Society respiratory questionnaire (24) regarding lifetime symptoms suggestive of asthma, rhinoconjunctivitis and atopic eczema extended with additional questions on symptoms and therapy as well as on other diseases.

A total of 368 patients with clinically manifested IgE-mediated allergic diseases [IgE-mediated asthma and/or rhinitis and IgE-associated AEDS, 164 men and 204 women, aged 27.5 ± 11.7 years (mean $\pm$ SD)] were studied. The control group consisted of 320 unrelated age–gender selected subjects (163 men and 157 women), aged 37.9 ± 16.5 years without symptoms of any lung, skin, and cardiovascular disease. They had no clinical evidence of personal or familial allergic diseases.

Asthma was defined by using the questionnaire together with a physician's diagnosis of asthma, according to the Global Initiative on Asthma (GINA) criteria (25). The diagnosis of allergic rhinitis was based on the coordination between a typical history of allergic symptoms of either perennial or seasonal rhinitis and diagnostic test for IgE-mediated allergy (26). The AEDS was defined according to the diagnostic criteria for atopic eczema initially proposed by Hanifin and Rajka (27).

The IgE-mediated allergy was recognized by specific IgE (> 0.35 kU/l) and/or a positive skin prick test to one or more allergens as described previously (28–29).

All subjects gave written informed consent to participation in the study, which was approved by the Committee for the Ethics of Medical Experiment on Human Subjects, Medical Faculty, Masaryk University, Brno.

Genotype identification

The genomic DNA was isolated from peripheral blood leucocytes by a standard method using proteinase K digestion of cells.

Detection of the C/T transition located 276-bp downstream from the translation termination site (C5266T) in exon 29 of the human *NOS1* gene

The C/T polymorphism was detected using a modification of the method described previously (30). A 128-bp polymerase chain reaction (PCR) product was generated using primers 5'-ACTCCTTGA-GTTTTCTGCTGCGATG-3' and 5'-CCATGTTCCAGTGGTT-TCATGCACAC-3'. The authors introduced a mismatched base into one of the primers (underlined) in order to introduce a restriction site for *Eco72I* enzyme into the PCR product when the C allele was present. The PCR products were digested with *Eco72I* at 37°C and then electrophoresed on 3% agarose gels stained with ethidium bromide. The homozygous T allele of the *NOS1* gene appears as one 128-bp band and the homozygous C allele as 100 and 28-bp fragments. Heterozygotes exhibited all bands: 128, 100, and 28-bp.

Statistical analysis

Allele frequencies were calculated from the observed numbers of genotypes. The significance of differences in allelic frequencies between each two groups was determined by the Fisher's exact test. Chi-square analysis was used to test for a deviation of genotype distribution from Hardy–Weinberg equilibrium and for a comparison of differences in genotype combinations among groups. A nonparametric ANOVA (Kruskal–Wallis) was used to assess phenotypic differences in total IgE levels between the carriers of different genotypes of the *NOS1*. Contingency table analysis, odds ratio (OR), 95% confidence intervals (CI), and significance values were estimated with the use of the programme package Statistica v. 3.0 (StatSoft Inc., Tulsa, OK). Corrections for multiple comparisons (Bonferroni method) were performed and only the values of P_{corr} ($=P$ corrected) < 0.05 were considered to be significant.

Results

The *NOS1* genotype distributions and allele frequencies in the study groups are shown in Table 1. They were in Hardy–Weinberg equilibrium, both within each group and in the population as a whole. The distribution of alleles in the control group was similar to that previously reported in control subjects from other Caucasian population (16). We found no significant differences in the distribution of C/T alleles or genotypes among subjects both in the allergic diseases group (C/T: 73.1/26.9%) and in the IgE-mediated allergic asthmatic subgroup

Table 1. Genotype and allele frequencies of C/T in exon 29 of the *NOS1* gene in patients with immunoglobulin E (IgE)-mediated allergic diseases (or asthma alone) and controls

C/T polymorphism in exon 29	Genotypes*			Allele frequencies*	
	CC (%)	CT (%)	TT (%)	C	T
Control subjects ($n = 320$)	174 (54.38)	120 (37.50)	26 (8.13)	0.731	0.269
All allergic diseases ($n = 368$)	202 (54.89)	134 (36.41)	32 (8.70)	0.731	0.269
IgE-mediated asthma ($n = 150$)	83 (55.33)	56 (37.33)	11 (7.33)	0.740	0.260

* No significant differences were observed between the tested groups.

Table 2. Association between C/T polymorphism in the exon 29 of the *NOS1* gene and total serum immunoglobulin E (IgE) levels in allergic patients

Serum IgE levels (IU/ml)	C/T polymorphism in exon 29 of the <i>NOS1</i> gene		
	Genotypes		
	CC (%)	CT (%)	TT (%)
Total IgE < 151	129 (59.72)	73 (33.80)	14 (6.48)
Total IgE > 150	71 (47.65)	61 (40.94)	17 (11.41)
	T allele OR = 2.08 (95% CI: 1.52–2.85) P < 0.01		

OR, odds ratio; CI, confidence interval.

(C/T: 74.0/26.0%) compared with healthy controls (C/T: 73.1/26.9%).

However, this common polymorphism showed a significant association with signs of atopy, especially with total serum IgE levels. The effect of this polymorphism on total serum IgE level was highest in TT homozygotes [$\log_e$ IgE levels (mean $\pm$ SD): 5.01 ± 1.61], intermediate in heterozygotes (4.58 ± 1.53), and lowest in wild-type homozygotes (4.34 ± 1.40) with significance $P < 0.05$.

In further analysis, when atopic persons exhibiting total serum IgE levels below 150 IU/ml were compared with those having levels above 150 IU/ml, the T allele (TT and CT genotypes) was significantly associated with high total serum IgE levels (OR = 2.08, 95% CI: 1.52–2.85, $P < 0.01$; Table 2).

Finally, there were no significant associations among the *NOS1* alleles and/or genotypes and other quantitative traits investigated in our study, including pulmonary function tests [forced expiratory volume in first second (FEV₁)/functional vital capacity (FVC); % FEV₁/FVC, the Tiffenau test] or selected specific IgE levels (data not shown).

Conclusions

Asthma and allergy have been described as complex traits for which genetic predisposition is essential in disease aetiology, but not fully understood, largely because of the myriad potential phenotypes (e.g. IgE levels, specific allergic sensitization, disease severity). Genome-wide screening for susceptibility to allergy has highlighted several candidate regions so far, including chromosome 12q15–24.2 (4–6). Evidence of linkage to asthma has been established in distinct Caucasian populations (6, 31), in Hispanic (31), and in an Afro-Caribbean population (5). This region has also been linked to total serum IgE in the Amish (5), in a Caucasian population distinct from those noted above for linkage in asthma (32), and in Afro-Caribbeans (5).

A number of association studies over the last several years have assessed the effect of the *NOS1* gene polymorphisms in allergic diseases (16, 18–23). In one of them, Grasemann et al. (16) suggest that more frequent *NOS1* 5266C allele was associated with asthma in a

Caucasian population. In contrast to their findings, we observed no difference of the *NOS1* 5266C/T genotypes between asthmatic (or allergic) patients and control subjects in our Czech Caucasian population. Conversely, in our population *NOS1* 5266 T allele was associated with high total serum IgE levels. Our observation indicating the involvement of the *NOS1* gene in intermediary phenotype of the IgE-mediated allergic diseases is consistent with the report of Immervoll and coworkers (21), who have found an association of a C5266C/T polymorphism *NOS1* on 12q24 with total serum IgE levels in a German population. For assessing polymorphism the question concerning its possible functional significance is highly essential. This variant, however, is located in the 3'-untranslated region (3'-UTR) of exon 29, and does not result in amino acid substitution. Generally, it is assumed that a noncoding alteration may affect splicing, transcription, the efficiency of translation, as well as mRNA transcript generation, stability, processing or subcellular targeting. Previously it has been shown that 3'-UTR of exon 29 affects the size of *NOS1* mRNA (14, 33) and thus this polymorphism could affect the function of *NOS1* gene via *NOS1* mRNA diversity (34). Alternatively, this polymorphism might be in linkage disequilibrium with other truly causative allele elsewhere. Genetic susceptibility to asthma and other IgE-mediated allergic diseases is complex and heterogeneous and it probably involves several genes, perhaps each contributing with a small increment. Inconsistencies between the genotype-phenotype association studies may be attributable to the presence of several confounding factors, such as phenotypic differences between cases, differences in genetic backgrounds or environmental exposures.

The results of this study and of our previous association studies (35, 36) for asthma/atopy-susceptibility loci suggest that genes coding NO synthases could be involved in aetiopathogenesis of allergic diseases such as asthma and/or their intermediary phenotypes in the Czech population. In summary, although the results of several previous studies indicated that specific variants of the *NOS1* gene might be associated with asthma (16, 18–20), the result of our study suggests that the C/T polymorphism in exon 29 of the *NOS1* gene is likely to contribute to intermediary phenotype of allergic diseases in the Czech population.

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References

- JOHANSSON SG, HOURIHANE JO, BOUSQUET J, BRUIJZEEL-KOOMEN C, DREBORG S, HAAHTELA T et al. A revise nomenclature for allergy: an EAACI position statement from the EAACI nomenclature task force. *Allergy* 2001;**56**:813–824.
- HOLGATE ST. Genetic and environmental interaction in allergy and asthma. *J Allergy Clin Immunol* 1999;**104**:1139–1146.
- KOPPELMAN GH, STINE OC, XU J, HOWARD TD, ZHENG SL, KAUFFMANN HF et al. Genome-wide search for atopy susceptibility genes in Dutch families with asthma. *J Allergy Clin Immunol* 2002;**109**:498–506.
- DIZIER MH, BESSE-SCHMITTLER C, GUILLOU-DATAILLE M, ANNESI-MAESANO I, BOUSSAHA M, BOUSQUET J et al. Genome screen for asthma and related phenotypes in the French EGEA study. *Am J Respir Crit Care Med* 2000;**162**:1812–1818.
- BARNES KC, NEELY JD, DUFFY DL, FREIDHOFF LR, BREAZEALE DR, SCHOU C et al. Linkage of asthma and serum IgE concentration to markers on chromosome 12q: evidence from Afro-Caribbean and Caucasian populations. *Genomics* 1996;**37**:41–50.
- THOMAS NS, WILKINSON J, HOLGATE ST. The candidate region approach to the genetics of asthma and allergy. *Am J Respir Crit Care Med* 1997;**156**:S144–S151.
- BARNES PJ. NO or no NO in asthma? *Torax* 1996;**51**:218–220.
- HESS A, BLOCH W, ROCKER J, PETERS S, STENNERT E, ADDICKS K et al. Detection of nitric oxide synthases in physiological and pathophysiological processes of the nasal mucosa. *HNO* 2000;**48**:489–495.
- MASSARO AF, MEHTA S, LILLY CM, KOBZIK L, REILLY JJ, DRAZEN JM. Elevated nitric oxide concentrations in isolated lower airway gas of asthmatic subjects. *Am J Respir Crit Care Med* 1996;**153**:1510–1514.
- KHARITONOV SA, YATES D, ROBBINS RA, LOGAN-SINCLAIR R, SHINEBOURNE EA, BARNES PJ. Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* 1994;**343**:133–135.
- BARNES PJ, LIEW FY. Nitric oxide and asthmatic inflammation. *Immunol Today* 1995;**6**:128–130.
- SILVESTRI M, SPALLAROSSA D, FRANGOVA YOURUKOVA V, BATTISTINI E, FREGONESE B, ROSSI GA. Orally exhaled nitric oxide levels are related to the degree of blood eosinophilia in atopic children with mild-intermittent asthma. *Eur Respir J* 1999;**13**:321–326.
- BELVISI MG, WARD JK, MITCHELL JA, BARNES PJ. Nitric oxide as a neurotransmitter in human airways. *Arch Int Pharmacodyn* 1995;**329**:97–110.
- HALL AV, ANTONIOU H, WANG Y, CHEUNG AH, ARUBS AM, OLSON SL et al. Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J Biol Chem* 1994;**269**:33082–33090.
- DE SANCTIS GT, MACLEAN JA, HAMADA K, MEHTA S, SCOTT J, JIAO A et al. Contribution of nitric oxide synthases 1, 2, and 3 to airway hyper-responsiveness and inflammation in a murine model of asthma. *J Exp Med* 1999;**189**:1621–1630.
- GRASEMANN H, YANDAVA CH, DRAZEN JM. Neuronal NO synthase (NOS1) is a major candidate gene for asthma. *Clin Exp Allergy* 1999;**29**:39–41.
- GRASEMANN H, DRAZEN JM, DEYKIN A, ISRAEL E, DE SANCTIS GT, PILLARI A et al. Simple tandem repeat polymorphisms in the neuronal nitric oxide synthase gene in different ethnic population. *Hum Hered* 1999;**49**:139–141.
- GAO PS, KAWADA H, KASAMATSU T, MAO XQ, ROBERTS MH, MIYAMOTO Y et al. Variants of NOS1, NOS2, and NOS3 genes in asthmatics. *Biochem Biophys Res Commun* 2000;**267**:761–763.
- GRASEMANN H, YANDAVA CN, VAN'S GRAVESANDE KS, DEYKIN A, PILLARI A, MA J et al. A neuronal NO synthase (NOS1) gene polymorphism is associated with asthma. *Biochem Biophys Res Commun* 2000;**272**:391–394.
- GRASEMANN H. Die Genetik der neuronalen NO-synthase (NOS1) in der Etiologie des Asthma bronchiale. *Pneumologie* 2001;**55**:390–395.
- IMMERVOLL T, LOESGEN S, DUTSCH G, GOHLKE H, HERBON N, KLUGBAUER S et al. Fine mapping and single nucleotide polymorphism association results of candidate genes for asthma and related phenotypes. *Hum Mutat* 2001;**18**:327–336.
- WECHSLER ME, GRASEMANN H, DEYKIN A, SILVERMAN EK, YANDAVA CHN, ISRAEL E et al. Exhaled nitric oxide in patients with asthma. *Am J Respir Crit Care Med* 2000;**162**:2043–2047.
- GRASEMANN H, KNAUER N, BÜSCHER R, HÜBNER K, DRAZEN JM, RATEJEN F. Airway nitric oxide levels in cystic fibrosis patients are related to a polymorphism in the neuronal nitric oxide synthase gene. *Am J Respir Crit Care Med* 2000;**162**:2172–2176.
- American Thoracic Society. Definition and classification of chronic bronchitis, asthma and pulmonary emphysema. *Am Rev Respir Dis* 1988;**136**:225–244.
- Global Strategy for Asthma Management and Prevention. Global initiative for asthma. NIH Publication No. 01-3659, NHLBI/WHO 2002.
- BOUSQUET J, VAN CAUWENBERGE Pn, KHALTAEV N. Allergic rhinitis and its impact on asthma: ARIA workshop report. *J Allergy Clin Immunol* 2001;**108**:S148–S153.
- HANIFIN JM, RAJKA G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol (Stockh)* 1980;**92**:44–47.
- IZAKOVIČOVÁ HOLLÁ L, VAŠKŮ A, IZAKOVIČ V, ZNOJIL V. The interaction of the polymorphisms in transporter of antigen peptides (TAP) and lymphotoxin α (LT- α) genes and atopic diseases in the Czech population. *Clin Exp Allergy* 2001;**31**:1418–1423.
- BUCKOVA D, HOLLA LI, VASKU A, ZNOJIL V, VACHA J. Lack of association between atopic asthma and the tumor necrosis factor alpha-308 gene polymorphism in a Czech population. *J Invest Allergol Clin Immunol* 2002;**12**:192–197.
- SHINKAI T, OHMORI O, HORI H, NAKANUTA J. Allelic association of the neuronal nitric oxide synthase (NOS1) gene with schizophrenia. *Mol Psychiatry* 2002;**7**:560–563.
- The Collaborative Study on the Genetics of Asthma (CSGA). A genome-wide search for asthma susceptibility loci in ethnically diverse populations. *Nat Genet* 1997;**15**:389–392.
- NICKEL R, WAHN U, HIZAWA N, MAESTRI N, DUFFY DL, BARNES KC et al. Evidence for linkage of chromosome 12q15-q24.1 markers to high total serum IgE concentrations in children of the German multicenter allergy study. *Genomics* 1997;**46**:159–162.
- WANG Y, NEWTON DC, MARSDEN PA. Neuronal NOS: gene structure, mRNA diversity, and functional relevance. *Crit Rev Neurobiol* 1999;**13**:21–43.

34. SHINKAI T, OHMORI O, HORI H, NAKAMURA J. Allelic association of the neuronal nitric oxide synthase (NOS1) gene with schizophrenia. *Mol Psychiatry* 2002;**7**:560–563.
35. IZAKOVIČOVÁ HOLLÁ L, BUČKOVÁ D, KUHOVÁ V, STEJSKALOVÁ A, FRANCOVÁ H, ZNOJIL V et al. Prevalence of endothelial nitric oxide synthase gene polymorphisms in patients with atopic asthma. *Clin Exp Allergy* 2002;**32**:1193–1198.
36. SCHÜLLER M, ŠTELCL M, RYBNÍČEK O, BUČKOVÁ D, HOLLÁ LI. The insertion/deletion polymorphism of the ecNOS gene in Czech children with IgE-mediated allergic diseases. *Allergy* 2002;**57**:369–370.