

Functional effects and gender association of COX-2 gene polymorphism G₋₇₆₅C in bronchial asthma

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Background: Prostaglandins, generated via the COX pathways, are essential mediators of inflammation in bronchial asthma. The promoter polymorphism of COX-2 gene (G₋₇₆₅C), which might affect binding of transcription factors, has recently been described

Objective: To study distribution and function of the genetic COX-2 variant in patients with asthma compared with healthy controls.

Methods: Three groups of adults were studied: (1) patients with aspirin-induced asthma (AIA; n = 112), (2) asthmatic patients who tolerated aspirin (ATA; n = 198), and (3) a random population sample from city of Krakow (n = 547). The COX-2 promoter region was genotyped for the G₋₇₆₅C polymorphism. *Ex vivo* production of prostaglandin E₂ and prostaglandin D₂ by peripheral blood monocytes was measured.

Results: In the 2 asthmatic groups, the G₋₇₆₅C allele frequency was similar (AIA, 0.18; ATA, 0.19) and did not differ from that of controls (0.17). In asthmatic women, but not in men, CC homozygotes were overrepresented compared with controls (odds ratio, 3.08; 95% CI, 1.35-6.63; P = .01). There was no relationship between genotype and FEV₁, serum IgE, blood eosinophil count, or duration of the disease. In AIA but not in ATA patients, CC homozygosity was associated with more severe course of the disease, as reflected by need for oral corticotherapy. Production of 2 prostaglandins by monocytes was more than 10-fold higher in CC than in GG homozygotes, and the magnitude of this difference was not changed by LPS stimulation.

Conclusion: In asthma, the COX-2 ₋₇₆₅C homozygosity is associated with female sex. The CC homozygosity has functional effects resulting in increased capacity of monocytes to produce prostaglandins. (J Allergy Clin Immunol 2004;114:248-53.)

Key words: Cyclooxygenase, bronchial asthma, aspirin, prostaglandins, genetic polymorphism, aspirin-induced asthma, gender

Prostaglandin endoperoxide H synthase (PGHS), also named COX, catalyzes the conversion of arachidonic acid to the unstable intermediate prostaglandin H₂. This is the

Abbreviations used

AIA: Aspirin-induced asthma

ATA: Aspirin-tolerant asthma

NSAID: Nonsteroidal anti-inflammatory drug

PGD₂: Prostaglandin D₂

PGE₂: Prostaglandin E₂

PGHS: Prostaglandin endoperoxide H synthase

first step in the biosynthesis of a range of lipid mediators, termed *prostaglandins* and *thromboxanes*. PGHS has both COX and peroxidase activity.¹ The former is inhibited by aspirin and several other nonsteroidal anti-inflammatory drugs (NSAIDs).²

There are at least 2 COX genes coding the different enzymes: COX-1 and COX-2. Recently,³ more COX enzymes were discovered: COX-3 and 2 smaller forms of COX-1. All of these are derived from the COX-1 gene by alternative splicing of COX-1 messenger RNA. Little is known about their role in physiology and pathology.⁴ In contrast, COX-1 and COX-2 have been well characterized. COX-1, expressed constitutively in most tissues, is thought to release prostaglandins involved in housekeeping functions, such as the maintenance of gastrointestinal tract and vascular homeostasis. COX-2, undetectable in most tissues, can be upregulated by bacterial LPSs, cytokines, growth factors, and tumor promoters, suggesting its relevance to inflammation and cancer. However, distinguishing COX-1 as a constitutive enzyme and COX-2 as an inducible enzyme responsible for the formation of prostanoids in disease is an oversimplification of the biologic reality.⁵

A limited number of polymorphisms in the COX-2 promoter region have been identified.^{6,7} They were not located in any gene elements that could potentially affect transcription, activity of COX-2 enzyme, its susceptibility to NSAIDs, or the metabolite profile.⁸ Recently, a new variant in the COX-2 promoter, ₋₇₆₅G>C, was identified⁹; it locates within a putative binding site for Sp1, considered to be a positive activator of COX-2 transcription, acting through G-rich elements.

Both COX-1 and COX-2 are expressed in various airway cells in healthy subjects and patients with asthma.¹⁰⁻¹³ They contribute to prostaglandin synthesis, and their expression increases in bronchial asthma.¹⁴ COX

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products are important mediators of asthma,¹⁵ and they play a very special role in its distinct phenotype, called *aspirin-induced asthma* (AIA).¹⁶ In this clinical syndrome, which affects about 5% to 10% adult asthmatic patients, attacks of asthma are precipitated by COX-1 inhibitors^{17,18}; COX-2 expression is diminished in biopsies of nasal polyps^{19,20} but not in the bronchial biopsies.²¹ Different metabolisms of arachidonic acid via COX-2 pathway were reported in nasal epithelial cells²² and bronchial fibroblasts²³ isolated from aspirin-sensitive patients compared with those harvested from aspirin-tolerant patients. We wondered whether G_{-765C} COX-2 polymorphism could contribute to altered prostaglandin biosynthesis in bronchial asthma. Therefore, we studied its distribution in a large group of asthmatic patients that included well characterized aspirin-sensitive patients. Furthermore, we investigated prostaglandin production by blood monocytes *in vitro* as related to the genotype of the patients.

METHODS

Subjects

Polymorphism G_{-765C} of COX-2 gene was ascertained in 857 subjects from Polish population: 310 asthmatic patients and 547 healthy controls. Among asthmatic patients, 198 individuals reported usage of NSAIDs without adverse reactions (patients with aspirin-tolerant asthma [ATA]); in 75% of these patients, aspirin provocation tests²⁴ were performed and had negative results. In 112 patients, AIA was diagnosed on the basis of the positive history, and in 96%, it was confirmed by positive oral aspirin provocation tests.²⁴ The characteristics of the patients are given in Table I. There were no differences between AIA and ATA for age, sex, duration of asthma, atopy, serum IgE, and dose of steroids. AIA patients had higher eosinophil counts ($P = .01$) and lower FEV₁ values ($P = .04$). Healthy controls were Cracow inhabitants randomly sampled from the local administration registry. There were 235 men and 312 women in the control group, 16 to 85 years old (mean, 41.8 ± 14.4 years). The study was approved by the Jagiellonian University Ethics Committee, and all patients gave informed consent to participate.

Genotyping of G_{-765C} COX-2 polymorphism

Genomic DNA samples were obtained by standard methods from peripheral blood leukocytes of subjects studied. The promoter region of COX-2 was amplified with primers, 5'-ATTCTGGCCATCGC-CGCTTC-3' and 5'-CTCCTTGTCTTGAAAGAGACG-3', by using PCR. Reaction products were digested with *Bsh1236I* restriction endonuclease (Fermentas, Vilnius, Lithuania), which cut allele G. Genotypes were scored on 1.5% agarose gels (SFR Agarose; Amresco, Solon, Ohio) stained with 0.5 µg/mL ethidium bromide under an UV transilluminator.

Peripheral blood monocyte studies

Peripheral blood monocytes were isolated from 17 asthmatic women, all ATA, and selected to represent opposite genotypes of G_{-765C} polymorphism. Eight of them were CC and 9 were GG homozygotes. Briefly, venous blood (16 mL) was collected in a syringe with sodium citrate, and plasma was removed by centrifugation (400g, 10 minutes, room temperature). Packed cells were resuspended 1:1 in phosphate-buffered saline and layered onto 1.077 g/mL HistoPaque (Sigma, St. Louis, Mo). After centrifugation (400g, 30 minutes, room temperature), the interface containing mononuclear cells was collected, pelleted, and washed twice with

PBS. The monocytes suspension was adjusted to the cells' density of 10⁶ per milliliter and plated on a 6-well culture plate (2 × 10⁶ cells per well) in RPMI 1640 medium supplemented with 10% FBS, antibiotics (100 U penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B; Sigma), and L-glutamine. After 3 hours of incubation in a 37°C, 5% CO₂ atmosphere, the plates were washed 3 times with prewarmed culture medium to remove nonadherent cells. Adherent monocytes were left incubated in the culture medium overnight before stimulation experiments. The average viability of the cells, assessed by exclusion trypan blue staining, was 94%, and monocyte fraction in May-Grünwald-Giemsa stain preparations was greater than 90%. On the next day, the monocytes were incubated in a fresh medium with or without *Escherichia coli* LPS (Sigma) at a concentration 0.1 µg/mL for 4 hours. Thereafter, the medium was fortified in arachidonic acid (50 µg/mL) and after a further 30-minute incubation, medium was collected for prostaglandin studies. The monocytes were lysed with TRIzol reagent (LifeTechnologies, Gaithersburg, Md) for mRNA quantification.

In a control experiment, by using the same methodologic set-up, except for the addition of a COX-2 inhibitor rofecoxib (0.2, 2.0, and 20.0 µmol/L) 30 minutes before arachidonic acid activation of monocytes,²⁵ we observed a dose-dependent inhibition of LPS-stimulated prostaglandin E₂ (PGE₂) production (inhibitory concentration 50% = 0.58 µmol/L; n = 3). Thus, inhibition of PGE₂ production by a highly selective COX-2 inhibitor added additional evidence that COX-2 activity was the source of PGE₂ variance in our experiments.

Real-time analysis of total cellular mRNA

Total cellular RNA, extracted from monocytes according to the manufacturer's instructions, were reverse-transcribed with human β-actin reverse primer 5'-GGGTACATGGTGGTGCCG by using avian myeloblastosis virus reverse transcriptase (Amresco, Solon, Ohio). Two microliters of the product was amplified in the presence of the forward primer 5'-AGCGGGAAATCGTGCCTG in the iCycler Real Time PCR (Biorad, Hercules, Calif) by using SybrGreen (Molecular Probes, Eugene, Ore). The relative abundance of specific human β-actin cDNA template was referred as a cycle threshold of fluorescence and used for comparison between the groups to standardize the monocytes number.

PGE₂ and prostaglandin D₂ measurements

Prostaglandin E₂ and prostaglandin D₂ (PGD₂) concentrations in collected culture media were measured after immediate addition of deuterium-labeled prostaglandins ([²H₄]-PGD₂ and [²H₄]-PGE₂; Cayman Chemicals, Ann Arbor, Mich) as internal standards. Sequential steps of organic phase extraction, derivatization with pentafluorobenzyl ester, thin-layer chromatography purification, and derivatization with methoxamine and trimethylsilylation preceded gas chromatography–negative chemical ionization–mass spectrometry (Hewlett Packard 5989B; Palo Alto, Calif). Two ions were recorded for quantification, 524 mass-to-charge ratio for prostaglandins and 528 mass-to-charge ratio for deuterated standards.²³ Concentration of prostaglandins was expressed in pg/0.5 mL collected culture media and was standardized for the amount of β-actin transcripts in the cultured cells.

Determination of atopic status

Subjects were defined as atopic if they had a consistent positive history of allergy caused by allergen exposure and at least 1 positive skin prick test to a panel of 7 common aeroallergens.

Statistical analysis

Statistical evaluation was performed by using personal computer and Statistica 5.5 software (StatSoft; Tulsa, Okla). Summary statistics

TABLE I. Clinical and laboratory profile of the asthmatic patients*

| | AIA + ATA | AIA | ATA |
|---|---------------|---------------|---------------|
| No. patients | 308 | 112 | 198 |
| Age (y) | 44.5 ± 13.4 | 46.7 ± 11.5 | 43.1 ± 14.3 |
| Sex (F/M ratio) | 203/105 | 71/41 | 132/66 |
| Duration of asthma (y) | 13.5 ± 10.1 | 14.6 ± 8.8 | 12.9 ± 10.8 |
| Atopy (yes/no) | 138/126 | 42/53 | 96/73 |
| Blood eosinophil count (per mm ³) | 352.5 ± 357.0 | 421.3 ± 374.5 | 304.0 ± 337.3 |
| Serum IgE (IU/mL) | 206.7 ± 538.5 | 158.8 ± 221.3 | 234.6 ± 655.1 |
| FEV ₁ (% of predicted value) | 84.8 ± 19.0 | 81.6 ± 16.8 | 86.5 ± 19.9 |
| Patients on inhaled corticosteroids | 210 | 76 | 134 |
| Dose of inhaled corticosteroids† | 819 ± 439 | 827 ± 438 | 815 ± 441 |
| Patients on oral corticosteroids | 119 | 53 | 66 |
| Dose of oral corticosteroids‡ | 9.2 ± 6.5 | 8.5 ± 6.3 | 9.7 ± 6.6 |

*Values are expressed as means ± SDs.

†Daily dose equivalent to fluticasone propionate (µg).

‡Daily dose equivalent to methylprednisolone (mg).

were expressed as means and SDs or 95% CIs. The general linear model, including ANOVA and analysis of covariance, was used to test for significant differences between means. The post hoc Tukey procedure was used for multiple comparisons.

The χ^2 approach for testing Hardy-Weinberg equilibrium was used for genotype frequency analysis. The level of significance was set at $P < .05$.

RESULTS

Genotype distribution and its relationship to asthma phenotype

The genotype prevalence and allele frequencies are shown in Table II. In controls, the observed genotypes distribution did not deviate from that expected under Hardy-Weinberg equilibrium. However, in the whole group of asthmatic subjects, the deviation was significant because of the high number of CC homozygotes (Table II). The CC homozygotes were markedly overrepresented in the subgroup of asthmatic female subjects compared with control female subjects ($P = .01$; odds ratio [OR], 3.08), but not in the subgroup of asthmatic male subjects compared with control male subjects ($P > .05$). The frequency of CC homozygotes was equally high in female subjects with AIA ($q = 0.085$; OR, 3.16) as in subjects with ATA ($q = 0.083$; OR, 3.04; Table III). Statistical analysis revealed no relationship between the genotype and serum IgE levels, FEV₁, blood eosinophil count, or duration of the disease. Atopy was more frequent in CC homozygotes compared with patients with CG + GG genotypes ($P = .02$). The atopy distribution was similar in AIA and ATA CC homozygotes ($P > .05$). The clinical characteristics of CC homozygotes are presented in Table IV.

There was a nonsignificant tendency ($P = .08$) to treat more AIA patients with oral corticosteroids than ATA patients. Analysis of oral steroids use according to *COX-2* genotype and asthma phenotype revealed that AIA CC homozygotes received a higher steroid dose than the rest of the AIA patients ($P = .02$) or ATA CC homozygotes ($P = .01$). The duration of chronic oral corticotherapy was

significantly longer ($P = .02$) in AIA CC homozygotes compared with ATA CC homozygotes (Table IV).

Monocyte PGE₂ and PGD₂ biosynthesis capacity

Prostaglandin production was measured in the supernatants of the monocyte cultures collected from the ATA women patients. Two groups were studied: CC ($n = 8$) and GG ($n = 9$) subjects. The groups did not differ with respect to duration of asthma, atopy, serum IgE, blood eosinophil count, and dose of inhaled and oral steroids. A third of patients in each group were on oral steroids (5.6 ± 2.1 mg methylprednisolone/24 h). The baseline PGE₂ production was strikingly higher in the CC homozygotes compared with the GG genotype ($P < .001$; Table V). Similar difference in the production of PGD₂ was observed between the groups ($P < .001$). A magnitude of these differences remained similar after LPS stimulation despite a 2-fold increase of prostaglandins produced (Table V). In the raw data before standardization for β -actin mRNA (not shown), differences were likewise significant.

DISCUSSION

COX-2 is a highly inducible enzyme regulated at the transcriptional and posttranscriptional level. Papafili et al⁹ reported on a common promoter polymorphism of *COX-2* gene (*PGHS2*), which caused an approximately 30% difference of expression in cells transfected with plasmids carrying promoter variants. This polymorphism consisted on G/C transversion 765 nucleotides upstream from the transcription start. In our study, we confirmed presence of this nucleotide variant and documented allelic frequencies of G₋₇₆₅C in the Polish population similar to those originally reported in British subjects.⁹ Moreover, we report on an intriguing genetic association between the less frequent ₋₇₆₅C allele and asthma. Homozygotes for this allelic variant were overrepresented among patients

TABLE II. Genotype frequencies in the studied groups

| | CC | CG | GG | C frequency | H-W equilibrium (P) |
|--------------------|----|-----|-----|-------------|---------------------|
| AIA + ATA, n = 310 | 17 | 81 | 212 | 0.185 | .02 |
| AIA, n = 111 | 6 | 28 | 77 | 0.18 | .07 |
| ATA, n = 199 | 11 | 53 | 135 | 0.195 | .12 |
| Controls, n = 547 | 13 | 158 | 376 | 0.168 | .45 |

TABLE III. Genotype distribution across asthmatic patients according to sex

| | | No. patients | CC | CG | GG |
|-------|--------------------|--------------|----|-----|-----|
| Women | All subjects | 515 | 26 | 140 | 349 |
| | Asthmatic subjects | 203 | 17 | 56 | 130 |
| | Controls | 312 | 9 | 84 | 219 |
| Men | All subjects | 342 | 4 | 99 | 239 |
| | Asthmatic subjects | 107 | 0 | 25 | 82 |
| | Controls | 235 | 4 | 74 | 157 |

TABLE IV. Clinical characteristics of CC homozygote patients with asthma*

| | AIA (n = 6) | ATA (n = 11) | AIA + ATA (n = 17) |
|---|---------------|---------------|--------------------|
| Age (y) | 47.5 ± 6.4 | 32.4 ± 8.6 | 37.7 ± 10.7 |
| Sex (F/M ratio) | 6/0 | 11/0 | 17/0 |
| Duration of asthma (y) | 17.2 ± 7.8 | 13.3 ± 10.9 | 14.7 ± 9.8 |
| Atopy (yes/no) | 4/1 | 9/2 | 11/5 |
| Blood eosinophil count (per mm ³) | 664.0 ± 472.0 | 231.8 ± 217.3 | 357.9 ± 372.1 |
| Serum IgE (UI/mL) | 393.4 ± 653.7 | 242.7 ± 339.2 | 292.9 ± 448.9 |
| FEV ₁ (% of predicted value) | 77.7 ± 10.5 | 94.7 ± 13.1 | 88.8 ± 14.6 |
| Inhaled steroids (yes/no) | 5/1 | 10/1 | 15/2 |
| Dose of inhaled steroids† | 725.0 ± 256.2 | 670.0 ± 505.6 | 688.3 ± 428.6 |
| Patients on oral steroids | 6/0 | 2/9 | 8/9 |
| Dose of oral steroids‡ | 16.0 ± 13.1 | 6.0 ± 2.8 | 13.5 ± 12.1 |
| Duration of oral corticotherapy | 10.4 ± 1.7 | 0.4 ± 0.9 | 3.5 ± 4.9 |

*Values are expressed as means ± SDs.

†Daily dose equivalent to fluticasone propionate (µg).

‡Daily dose equivalent to methylprednisolone (mg).

with asthma, and this genotype seemed to interact with sex, found only in affected women.

Asthma is more common in boys than girls during early childhood. The prevalence equalizes between sexes during adolescence and then switches to female predominance in adulthood.^{26,27} This difference is seen especially in AIA, in which female subjects outnumber male subjects by about 2.5:1, and the onset of symptoms occurs earlier than in male subjects.¹⁶ Peripheral blood monocytes obtained from CC homozygous women produced increased quantities of prostaglandins. It is therefore interesting to note that these cells are responsive to stimulation, with both estrogen and progesterone²⁸ producing more PGE₂. Moreover, the promoter region of COX-2 has 2 distinct regulatory motifs responsible for sex-dependent expression of the gene: progesterone receptor binding motif located at 709-727 nucleotides, and possibly SRY (testis-determining factor) responsive element at 1206-1222 nucleotides upstream from the transcription start (Transcription Factor Database, <http://www.gene-regulation.com>²⁹). In women, local regulation of COX-2 expression in granulosa-lutein cells remains under hormonal control during the ovulatory cycle. Chronic inflammatory disorders are also more common in postpubertal women. In a prospective study of menopausal women, the risk of asthma was positively associated with estrogen replacement therapy.³⁰

COX-2 gene promoter is particularly rich in nucleotide motifs binding proteins activating transcription. Nuclear

TABLE V. Prostaglandin production by monocyte cultures from opposite homozygotic asthmatic patients*

| Genotype | LPS | PGE ₂ | | PGD ₂ | |
|----------|-----|------------------|---------------|------------------|------------|
| | | Mean | 95% CI | Mean | 95% CI |
| CC | - | 702.7 | 575.9-857.5 | 39.5 | 24.9-62.2 |
| CC | + | 1546.9 | 1247.6-1916.0 | 79.3 | 48.7-128.7 |
| GG | - | 61.8 | 50.4-75.8 | 2.3 | 0-4.1 |
| GG | + | 123.3 | 100.5-151.1 | 9.2 | 5.5-15.0 |

-, Not stimulated with LPS; +, stimulated with LPS.

*Prostaglandin values are expressed in pg/0.5 mL culture medium.

factor κB is regarded as the main transcription factor responsible for induction of COX-2 during inflammation. It was suggested⁹ that localization of G₋₇₆₅C polymorphism within a putative Sp1 transcription factor motif could explain its functional effects. However, the nucleotide change of G₋₇₆₅C also creates E2F transcription factor binding element tTCGCGc, with a perfect match of the core and matrix nucleotides (Transcription Factor Database²⁹). E2F transcription factor, known also as *adenovirus-induced*, is a cyclin-dependent regulator of expression of several genes.

In our experimental set-up, we unequivocally demonstrated that peripheral blood monocytes from female asthmatic subjects with opposite homozygous genotypes differ in their prostaglandin biosynthesis capacity. In contrast with the results of Papafili et al,⁹ whose

engineered cell line had only 30% lower luciferase activity for allele $-765C$ construct, biosynthesis of prostaglandins in monocyte cultures was more than 10-fold higher in patients homozygous for allele C . This difference was evident at basal conditions and remained unchanged after stimulation of monocytes with bacterial LPS. We suggest that the allelic variant of *COX-2* gene, characterized by $-765C$ polymorphism, has a functional consequence resulting in enhanced biosynthesis of prostaglandins by monocytes. It cannot be excluded, however, that in our monocyte preparation, despite overnight quiescence, the cells were already at some level of stimulation. Thus, the polymorphism may manifest mostly in proinflammatory conditions, like the presence of bacterial endotoxins. In the experimental lung pathology, both isoforms of cyclooxygenases were attributed with some protective properties. In *COX-1* or *COX-2* knockout mice, allergic lung responses were augmented compared with responses in control animals.³¹ *COX-1* seemed more important in lung protection, probably because of more efficient coupling with constitutive cytosolic synthase producing PGE₂.

In human airway disease, PGE₂ plays both a beneficial and a deleterious role. It suppresses some inflammatory reactions and protects against acute allergen or aspirin provocation,^{16,32} but it can also promote T_H2-type responses, stimulate angiogenesis, and enhance survival of the inflammatory cells.³³ Our observations do not bear on aspirin-precipitated asthmatic attacks, because such attacks are associated with inhibition of *COX-1* but not *COX-2*. Furthermore, we did not observe a relationship between aspirin sensitivity and *COX-2* genotype distribution. The frequency of CC homozygotes was similar among patients with AIA and ATA. However, only in the former phenotype was the homozygosity associated with more severe course of the disease, as reflected by need for higher dosing of oral corticosteroids and longer duration of this therapy. Increased PGD₂ and PGE₂ biosynthesis by AIA homozygotes would fit with these clinical findings. In AIA, a continuous activation of mast cells and macrophages leading to steady release of PGD₂ was described.³⁴ Enhanced PGD₂ biosynthesis might affect the clinical presentation of asthma through its bronchoconstrictive action and potent chemoattractive properties for T_H2 lymphocytes, basophils, and eosinophils. Furthermore, AIA is characterized by hypereosinophilia,¹⁶ and the patients we studied were not an exception to this rule. The antiapoptotic effects of PGE₂³⁵ would lead to enhanced survival of eosinophils and, in consequence, to extended airway inflammation. Aspirin hypersensitivity is a marker of severity of asthma,^{16,36} and at least half of the patients require chronic oral corticotherapy to control their disease. The results presented here indicate that CC homozygosity might be 1, though certainly not the only, factor contributing to the severe AIA course.

In conclusion, we document a functional effect of G_{-765C} polymorphism within the *COX-2* gene promoter, which causes an order of magnitude enhanced biosynthesis of prostaglandins in human monocytes *in vitro*. The effect seems dependent on sex, and in our association

study has a significant effect on risk and phenotype of asthma in women. The asthma phenotype related to G_{-765C} polymorphism within *COX-2* is characterized by female sex, atopy, and an increased requirement for steroid therapy, especially in aspirin-sensitive patients.

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