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Evidence of Role in Acute-Phase Inflammatory Response**

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Common Promoter Variant in Cyclooxygenase-2 Represses Gene Expression

Evidence of Role in Acute-Phase Inflammatory Response

Anastasia Papafili,* Michael R. Hill,* David J. Brull, Robin J. McNulty, Richard P. Marshall, Steve E. Humphries, Geoffrey J. Laurent

Objective—Cyclooxygenase (COX)-2 is a key regulatory enzyme in the synthesis of prostanoids associated with trauma and inflammation. We investigated the COX-2 gene for functional variants that may influence susceptibility to disease.

Methods and Results—The promoter of COX-2 was screened for variants in healthy subjects by use of polymerase chain reaction–based methods. Promoter activity was investigated by using reporter expression experiments in human lung fibroblasts. Patients undergoing coronary artery bypass graft surgery, with measurements of plasma markers linked to COX-2 activity, were genotyped for association studies. A common COX-2 promoter variant, $-765G>C$, was found and shown to be carried by $>25\%$ of a group of healthy UK subjects. The $-765C$ allele had significantly lower promoter activity compared with $-765G$, basally ($28\pm 3\%$ lower, $P<0.005$) and in serum-stimulated cells ($31\pm 2\%$ lower, $P<0.005$). In patients subjected to coronary artery bypass graft surgery, the magnitude of rise in levels of C-reactive protein (CRP) was strongly genotype dependent. Compared with $-765G$ homozygotes, patients carrying the $-765C$ allele had significantly lower plasma CRP levels at 1 to 4 days after surgery (14% lower at the peak of CRP levels on day 3, $P<0.05$ for all time points).

Conclusions—For several acute and chronic inflammatory diseases, $-765G>C$ may influence the variability of response observed. (*Arterioscler Thromb Vasc Biol.* 2002;22:1631-1636.)

Key Words: cyclooxygenase-2 ■ promoter variant ■ coronary artery bypass graft surgery
■ C-reactive protein ■ inflammation

Cyclooxygenase (COX) is a key regulatory enzyme in eicosanoid metabolism, converting free arachidonic acid to prostaglandin (PG)₂, from which a number of prostanoids, including PGE₂, PGI₂, PGD₂, and thromboxane, are produced.¹ The prostanoids are important mediators in the control of normal tissue homeostasis and regulate inflammation in response to trauma or infection.² Two isoforms of COX have been identified, COX-1 and COX-2, which have common and specific roles.³ COX-1 is expressed constitutively in most cell types; however, COX-2 is inducible on cell activation and is mainly expressed at sites of inflammation. COX-2 expression is raised in several pathophysiological states, and the use of COX inhibitors to reduce COX-2 activity has proven beneficial in attenuating chronic inflammatory conditions, such as arthritis and inflammatory bowel disease.^{4,5} Several million people worldwide regularly use COX inhibitors. Regular use has been shown to decrease the relative risk of developing cardiovascular disease, stroke, and colorectal cancer.^{5,6}

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Although COX-2 is widely accepted as a proinflammatory agonist and is therefore a suitable target for treating chronic inflammatory disease, there is increasing evidence to suggest that COX-2 has other roles, including anti-inflammatory, antifibrotic, and antithrombotic properties.⁷⁻⁹ These alternative roles challenge the dogma that COX-2 is ubiquitously a foe, and indeed, there is evidence indicating that with certain tissue injuries, a limited expression of COX-2 can result in pathology, as in pulmonary fibrosis.^{8,10} It also appears that COX-2 has a dual role in inflammation: being a part of the onset of the inflammatory process and then later aiding in its resolution.⁷ The complexity of this is not fully understood; however, dysregulation of COX-2 expression may play a key role in COX-2–mediated pathology.

Several genes induced in inflammation, such as tumor necrosis factor- α and interleukin (IL)-6, have nucleotide polymorphisms within the 5' flanking region of the gene; these nucleotide polymorphisms alter gene expression and are

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also reported to be associated with disease.^{11,12} The 5' flanking region of the human COX-2 gene, principally involved in regulating gene transcription, contains a canonical TATA box and several putative transcription-factor binding sites, including cAMP-responsive element, nuclear factor- κ B, nuclear factor-IL-6, glucocorticoid response element, polyomavirus enhancer activator 3, activator protein-2, CAAT box/enhancer binding protein, stimulatory protein-1 (Sp1), and a transforming growth factor- β response element, suggesting that a complex array of factors is involved in its regulation.^{13–16} Deletion and forced mutation experiments altering this sequence have identified critical regions and elements involved in inducing COX-2 gene transcription.^{15–17} Naturally occurring gene polymorphisms have been reported in COX-2; however, the functional significance of those found in the 5' flanking region is unclear.^{18,19}

We hypothesized that dysregulation of COX-2 expression has a role in COX-2-mediated pathology and that this, in part, may be due to functional changes in the 5' transcriptional regulatory promoter region of the gene. We report in the present study the presence of novel promoter variants in COX-2 and show that 1 of them, lying in a putative Sp1 binding site, affects the transcription rate of a reporter gene in transient transfection studies. To find a biological correlate for this functional promoter variant, we have examined an in vivo model of inflammation of patients undergoing coronary artery bypass graft surgery. Bypass surgery causes a significant acute-phase reaction, with a rise in the plasma levels of markers such as IL-6 and C-reactive protein (CRP),^{20–22} which are associated with COX-2 activity. IL-6 synthesis has been shown to be regulated by COX-2 via the production of prostaglandin (PG)_{E₂}.²³ Using this model, we demonstrate a phenotypic consequence of the functional promoter variant in an acute inflammatory response.

Methods

Subjects

Patients undergoing elective first-time coronary artery bypass graft surgery (n=173) were recruited from the Middlesex Hospital (London, UK) as part of the Coronary Artery Surgery Inflammation Study (CASIS), whose design has been previously described.²⁴ The study had approval of the hospital ethics committee, and written informed consent was obtained from all participants. The 454 white subjects used to estimate genotype frequencies in healthy UK subjects were a random subset of males who were taken from the Northwick Park Hospital Study II and who had baseline characteristics as follows (mean \pm SE): age 56.0 \pm 3.4 years, cholesterol 5.71 \pm 1.0 mmol/L, triglycerides 1.78 \pm 0.94 mmol/L, body mass index (BMI) 26.2 \pm 3.4 kg/m², and systolic blood pressure 136.7 \pm 18.6 mm Hg (see Humphries et al²⁵ for full details).

Measurement of IL-6, CRP, and Fibrinogen

Citrated blood samples (4.5 mL) were initially drawn before surgery and then again on the first 5 postoperative days. These were immediately centrifuged (3500g, 10 minutes), and plasma was separated and frozen at -20°C until analysis. IL-6 and fibrinogen concentrations were measured as previously described.^{24,26} CRP was measured on a BN Prospec (from Dade Behring). Interassay and intra-assay coefficients of variation were <4% and <2%, respectively, and assay sensitivity was 0.20 mg/L.

Patient Baseline Characteristics and Operative Details

Age, y	63.2 \pm 9.8
Sex, M/F	137/36
Current smokers	31 (18%)
Ex-/nonsmokers	142 (82%)
Treated hypercholesterolemia	123 (71%)
Treated hypertension	68 (40%)
Diabetes	34 (20%)
Family history of CAD	90 (52%)
Mean No. of grafts	2.8 \pm 0.7
Operation duration	193 \pm 39
CPB time, min	66 \pm 19
AoXC time, min	33 \pm 13
Length of ventilation, hours	10.2 \pm 4.6
Stay in intensive care, days	2.3 \pm 1.8
Postoperative stay, days	6.8 \pm 3.9

CAD indicates coronary artery disease; CPB, cardiopulmonary bypass; AoXC, aortic cross-clamp

SSCP and Genotyping

The proximal promoter region of the COX-2 gene was polymerase chain reaction (PCR)-amplified by using overlapping primer sets from normal healthy control subjects, and the products were examined by single-strand conformational polymorphism (SSCP); the methods used have previously been described.^{27,28} For PCR primers and their positions relative to the first transcribed nucleotide of the COX-2 gene, please see the expanded Methods section (available online at <http://atvb.ahajournals.org>). After electrophoresis, the product was visualized by SyBr Gold stain (Molecular Probes) on a Fuji FLA3000 imager. Within each primer set, PCR products with altered mobility patterns were sequenced with AmpliTaq DNA polymerase FS (Perkin-Elmer) fluorescently labeled dye-terminator chemistry and with the use of an Applied Biosystems 377 PRISM automated sequencer.

DNA was extracted from peripheral blood leukocytes by standard phenol/chloroform extraction techniques or a salting-out method.^{29,30} The $-765\text{G}>\text{C}$ variant was genotyped by *AciI* (NEB) restriction endonuclease digest of the PCR product generated by the use of primers CF8 and CR7 (Table I, available online at <http://atvb.ahajournals.org>). Digested products were separated in an 8% MADGE gel,³¹ and genotype was determined by 2 independent observers blinded to clinical details. The primers CF8 to CR7 gave a 306-bp band that in the presence of -765G was digested by *AciI* into 2 fragments of 188 and 118 bp. Positive and negative controls were included in all gels.

Reporter Gene Constructs

PCR primers CF11 and CR11 (Table I) containing recognition sites for *NheI* and *HindIII*, respectively, were used to generate a 1933-bp product of the COX-2 gene ($-1811+108$) from clone 973 M2 from the library RPCI5 (kindly supplied by the Sanger Center Clone Resource Group, Cambridge, UK). A commercially available kit (Advantage Genomic PCR, Clontech) was used for PCR, and the product was purified by using a QIAquick PCR purification kit (Qiagen). After restriction enzyme digestion, the product was directly ligated into pGL3 Basic Luciferase vector (Promega), linearized by digestion with *NheI* and *HindIII*, and transformed into XL1-Blue supercompetent cells (Stratagene). The sequence of the plasmid was confirmed by automated sequencing. Clone 973 M2 has a G residue at position -765 upstream from the COX-2 transcription start site. To generate a plasmid with a C residue at position -765 , a QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used, along with the -765G plasmid DNA as a template and the following oligonucleotide and its reverse complement sequence, GAATTTAC-

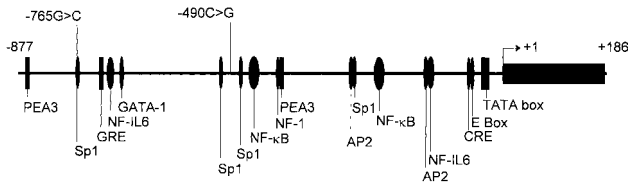


Figure 1. COX-2 gene 5' flanking region and exon 1. Schematic representation of putative transcription factor response elements (based on Tazawa et al¹⁴ and Yang et al¹⁵) and the relative positions of the novel sequence variants, $-490\text{C}>\text{G}$ and $-765\text{G}>\text{C}$, are shown. The arrow and +1 denote the transcriptional start site.

CTTCCCCCTCTCTTCC. The sequence of the -765C plasmid was confirmed by automated sequencing. Two separate preparations of each plasmid DNA were generated for transfection by using the EndoFree Plasmid Maxi Kit (Qiagen).

Transfections

Human fetal lung fibroblast (HFL1, purchased from American Type Culture Collection) cells were seeded into a 12-well plate and grown to confluence in DMEM, supplemented with 10% heat-inactivated FCS and penicillin (100 U/mL)/streptomycin (100 $\mu\text{g}/\text{mL}$), all obtained from Life Technologies. The HFL1 cells were cotransfected with 1 μg reporter plasmid and 0.05 μg control plasmid pRL-TK containing the *Renilla* luciferase gene by a synthetic integrin-targeted nonviral vector.³² Negative controls included transfection with an empty pGL3 Basic vector. After transfection, cells were rescued in 10% serum for 1 hour, made quiescent for 16 hours in serum-free medium, and then stimulated with 2% FCS or left untreated and harvested 8 hours later. *Firefly* and *Renilla* luciferases were measured sequentially by using the Dual-Luciferase Reporter Assay System (Promega) in a Tropix TR717 microplate luminometer (PE Applied Biosystems). Each transfection experiment was carried out in quadruplicate, and experiments were performed twice, each with separate preparations of plasmid DNA.

Statistical Analysis

Allele frequencies were estimated by gene counting. A χ^2 test was used to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium. A *t* test was used to compare levels of luciferase between constructs. Statistical significance was set at $P<0.05$. IL-6 and CRP values were not normally distributed; thus, data were logarithmically transformed before analysis. Fibrinogen values at all time points were normally distributed. Geometric mean \pm SE values have been quoted where data are shown. Differences between genotypes were assessed by ANOVA and by the Student *t* test for unpaired data. One-way ANCOVA was performed as previously described.²⁶

Results

Screening of 5' Flanking Region of COX-2 Gene

Screening of the COX-2 gene by PCR-SSCP, covering the region from -1122 to 54 bp, revealed variation at positions -490 and -765 from the transcription start site. Sequencing identified that in each case this involved a single nucleotide substitution. A C residue was replaced by G at position -490 ($-490\text{C}>\text{G}$), and a G residue was replaced by C at position -765 ($-765\text{G}>\text{C}$); see Figure 1. The $-490\text{C}>\text{G}$ and $-765\text{G}>\text{C}$ variants are identified by *Bsi* EI and *Aci* I restriction endonucleases, respectively. These enzymes were used to genotype the variants in 454 white healthy UK men to assess allele frequencies. The $-490\text{C}>\text{G}$ variant was rare; it was present as a heterozygote genotype only in the single individual for whom PCR-SSCP identified a change. For

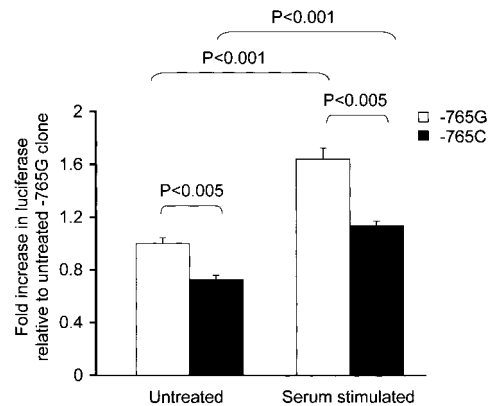


Figure 2. Firefly luciferase activity generated from $-1821+108$ region of the COX-2 promoter in human lung fibroblasts, measured as (mean \pm SE) fold increase in light emission relative to the untreated $-765\text{G}>\text{C}$ construct. Results are from 1 representative experiment but were comparable across 2 separate preparations of plasmid DNA, and they show the mean of 4 transfections, corrected for transfection efficiency by *Renilla* luciferase cotransfection.

$-765\text{G}>\text{C}$, the genotype distribution was GG=339, GC=109, CC=6, and the rare allele frequency was 0.13 (95% CI 0.11 to 0.16).

Functional Studies

The $-490\text{C}>\text{G}$ variant is not located within any known transcription factor binding site; however, the $-765\text{G}>\text{C}$ is located within a putative Sp1 binding site (Figure 1). Transfection of $-765\text{G}>\text{C}-1811+108$ constructs into HFL1 cells revealed the -765C allele to have significantly lower expression than the -765G allele in untreated ($28\pm 3\%$ lower, $P<0.005$) and serum-stimulated ($31\pm 2\%$ lower, $P<0.005$) cells (Figure 2). Treatment of HFL1 cells with serum resulted in a 1.64 ± 0.08 -fold induction ($P<0.001$) of luciferase expression in the presence of the -765G allele and a 1.57 ± 0.05 -fold induction ($P<0.001$) in the presence of the -765C allele, relative to their corrected untreated alleles (Figure 2).

Coronary Artery Bypass Graft Study

Baseline characteristics of the 173 bypass patients are shown in the Table. A total of 123 (71%) subjects were receiving statin therapy, and 82 (47%) were receiving β -blockers. There was no difference in the genotype distribution of the bypass patients (GG=115, GC=56, and CC=2) compared with the 454 white healthy subjects ($P=0.10$). The rare allele frequency (0.17, 95% CI 0.13 to 0.21) was slightly but not significantly higher than that found in the healthy subjects ($P=0.19$). However, the frequency of carriage of the rare allele (GC+CC combined) was higher in the bypass group compared with the healthy control group (33.5% versus 25.3%, respectively; odds ratio 1.49 [95% CI 1.02 to 2.17]; $P=0.04$). Carriers of 1 or more -765C allele were combined to assess IL-6, fibrinogen, and CRP levels by genotype, because there were only 2 individuals who had the genotype -765CC . IL-6 and fibrinogen levels were not significantly different by $-765\text{G}>\text{C}$ genotype at baseline or at any time after bypass surgery (Figure 3A, data not shown for fibrino-

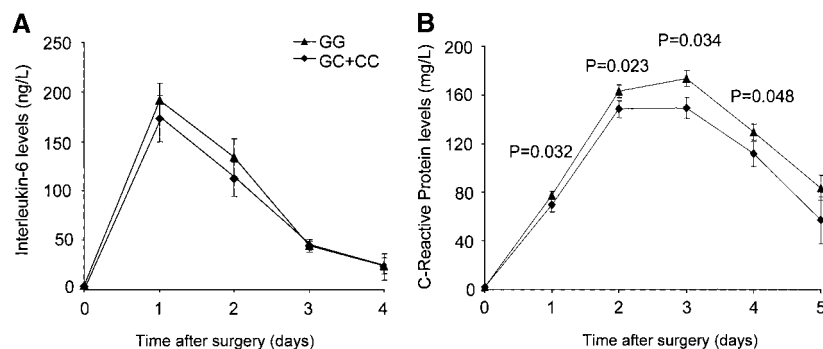


Figure 3. Circulating plasma levels of IL-6 and CRP by $-765G>C$ genotype. IL-6 levels were not significantly influenced by $-765G>C$ genotype (A). For CRP, the magnitude of this rise was strongly genotype dependent (B). Only significant probability values are shown.

gen). At baseline, mean CRP levels were lower in carriers of 1 or more $-765C$ allele (2.1 ± 0.2 for GG versus 1.8 ± 0.3 for GC+CC), although this difference was not statistically significant ($P=0.37$). As expected, BMI was positively correlated with basal CRP levels ($r=0.21$, $P=0.02$), but after adjustment for this confounder, levels were not significantly associated with the $-765G>C$ genotype ($P=0.18$). After bypass surgery, CRP levels were significantly higher at all subsequent postoperative time points compared with baseline ($P<0.005$ for all comparisons), with peak CRP levels recorded on the third postoperative day (mean 166 ± 5 mg/L). The magnitude of this rise was strongly genotype dependent (Figure 3B). Mean CRP values were lower for carriers of ≥ 1 rare $-765C$ allele at all times after surgery. This difference remained significant after multivariate analysis for all CRP values recorded for days 2 to 4 (by ANCOVA, $P=0.024$, $P=0.013$, and $P=0.026$ at 2, 3, and 4 days after surgery, respectively). Data were adjusted for age, sex, smoking, cholesterol levels, diabetes, BMI, statin therapy, duration of surgery, and bypass and aortic cross-clamp time. No single parameter was associated with a significant CRP-lowering effect after surgery (BMI and operation duration showed significant positive correlations with peak CRP values). At the peak of CRP levels on day 3, mean CRP levels in carriers of $-765C$ (149.57 ± 8.58 mg/L) were $14 \pm 0.05\%$ ($P<0.05$) lower than patients homozygous for $-765G$ (173.64 ± 6.30 mg/L).

Discussion

COX-2 has a major regulatory role in the production of prostanoids associated with trauma and inflammation. In the present study, we describe a functional COX-2 promoter polymorphism, $-765G>C$, which is carried by $>25\%$ of a healthy UK white population. In vitro studies in untreated human lung fibroblast cells revealed a significantly lower reporter expression from $-765C$ compared with $-765G$ of $\approx 30\%$, and this difference was maintained, although not enhanced, after stimulation with serum. We also report on in vivo observations of patients undergoing coronary artery bypass graft surgery and show that patients carrying $-765C$ have significantly lower plasma CRP levels compared with levels in patients homozygous for $-765G$.

The mechanism through which $-765G>C$ reduces promoter activity has yet to be shown. However, it is located within a putative Sp1 site in the promoter of COX-2 between -766 to -761 bp upstream from the transcriptional start site

and may thus alter Sp1 binding to this region. Sp1 is considered to be a positive activator of transcription, and it can bind and act through G-rich elements, such as the GC box.³³ However, it is also possible that $-765G>C$ may alter the binding ability of other DNA binding elements to this region, including Sp3. Sp3 will compete for the same binding site as Sp1 and has been shown to be a repressor of Sp1-mediated transcription in promoters containing multiple binding sites.^{33,34} The proximal COX-2 promoter contains multiple putative Sp1 binding sites^{13,15} and has previously been shown to specifically bind Sp1 and Sp3.¹⁷ We are currently investigating protein/DNA binding to this region with $-765C$ and $-765G$.

Our in vivo observations of patients undergoing bypass surgery, in which patients with ≥ 1 copy of the $-765C$ allele were found to have significantly lower plasma CRP levels after surgery compared with patients homozygous for $-765G$, are completely consistent with the in vitro data, which show $-765C$ with lower promoter activity than $-765G$ and demonstrate a phenotypic consequence of $-765G>C$ in an inflammatory state. The mean CRP levels at baseline (2.0 ± 0.2 mg/L) were also lower in carriers of 1 or more $-765C$ allele, although this difference was not statistically significant. In this case, the influence of $-765G>C$ on CRP measurements is likely to be confounded by other factors when CRP levels are so low. Bypass surgery is a well-characterized inflammatory stimulus^{20–22} that causes a significant acute-phase reaction with induction of inflammatory stimuli associated with COX-2. The effect of $-765G>C$ on in vivo proinflammatory and prothrombotic arachidonate derivatives such as PGE₂ and thromboxane is not known; however, the association of $-765G>C$ with plasma CRP levels suggests that COX-2 and CRP may be coordinately or sequentially regulated. Indirect evidence to support this includes several studies reporting a concomitant rise in plasma levels of acute-phase proteins linked to prostaglandin activity.^{35–37} NSAIDs lowering COX activity have also been shown to modify acute-phase responses.³⁸ There is evidence suggesting that IL-6 regulates CRP levels²⁵; however, whether the influence of $-765G>C$ on CRP levels is through IL-6 is unclear. The CRP promoter contains several IL-6 response elements, and the functional IL-6 promoter variant, $-174G>C$, is reported to affect levels of CRP.^{25,39} Induction of COX-2 precedes IL-6 gene expression and, via the production of PGE₂, has been shown to regulate IL-6 synthesis in macrophages.²³ We did not find a significant association

between $-765G>C$ and plasma IL-6 levels after surgery. Possibly, after such an acute severe injury, other mechanisms and stimulatory responses may compensate for any effect of $-765G>C$ on the production of IL-6. Similarly, no association was observed between $-765G>C$ and fibrinogen levels. However, it is likely that $-765G>C$ has a direct effect on CRP expression and that CRP is more sensitive to small changes in COX-2 levels than IL-6 or fibrinogen.

The functional differences between the $-765G$ and $-765C$ alleles, although statistically significant, are relatively small, and it remains to be seen whether the variant will have clinical relevance. It is possible that the influence of $-765G>C$ on CRP levels may have implications for various disease and inflammatory conditions. CRP is one of the earliest of the acute-phase proteins to be elevated and is increased in plasma in response to a wide range of disorders, including infection, trauma, surgery, and cancer.⁴⁰ It is also associated with chronic inflammatory diseases, such as rheumatoid arthritis and cardiovascular disease.^{41,42} CRP has proinflammatory and anti-inflammatory functions. The $-765C$ variant may be protective in cardiovascular disease, inasmuch as raised CRP levels predict cardiovascular events.^{43,44} In acute lung injury, however, the $-765C$ variant may increase risk, inasmuch as CRP acts as a major anti-inflammatory agent, inhibiting neutrophil function.^{45,46} The $-765C$ variant may also facilitate infection, inasmuch as a major function of CRP is to activate complement and bind to foreign pathogens in innate immunity.⁴⁰

The presence of other naturally occurring polymorphisms in the promoter of COX-2 has been reported; however, none have so far been shown to be functional or to be associated with inflammatory disease.^{18,19} None of these changes are located within recognized transcription factor binding sites. We did not identify these changes in the present study; however, this may be due to low allele frequencies and the ethnic group studied.^{18,19}

The effect of $-765G>C$ and other COX-2 gene variants may have implications in the use of NSAIDs. Regular use of NSAIDs has proven effective in reducing the risk of developing diseases, in particular, cardiovascular disease and bowel cancer.^{5,6} The role of $-765G>C$ may be potentially important, inasmuch as prospective studies have shown that subjects with elevated CRP levels are most likely to benefit from NSAIDs as a preventative measure for myocardial infarction and stroke.⁶

In summary, we have described a common promoter variant in the COX-2 gene, $-765G>C$, and we have shown in reporter expression studies that the $-765C$ allele, compared with $-765G$ allele, reduces promoter activity. We demonstrate a phenotypic consequence of $-765C$ in an inflammatory state by showing that it is associated with lower CRP levels compared with the levels associated with $-765G$. Thus, the presence of $-765G>C$ may have implications for a number of chronic and acute inflammatory states associated with disease. Furthermore, genotyping $-765G>C$ in patients using NSAIDs might be of predictive value in terms of their response.

Acknowledgments

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Table I. Primer sets used to amplify the 5' flanking region of the cyclooxygenase-2 gene.

Primer set	Label	Sequence	Product size (bp)	Annealing Site
1	CF1 CR1	GCGGAAAGAAACAGTCAT CTGAGGAGTTCCTGGACGT	131	-77 - -60 +54 - +36
2	CF2 CR2	CAGCTTCCTGGGTTTCCGAT GACAATTGGTCGCTAACCGA	201	-193 - -174 +8 - +12
3	CF3 CR3	TCGGGCAAAGACTGCGAAG ATGACTGTTTCTTTCCGC	264	-323 - -305 -60 - -77
4	CF4 CR4	TCAGGGCCGCTCAGATTCCT ATCGGAAACCCAGGAAGCTG	251	-405 - -424 -174 - -193
5	CF5 CR5	GCATATAGAGCAGATATACAGCC TGGACTGATCGCCTTGGATG	258	-601 - -579 -344 - -363
6	CF6 CR6	ACAGCTATGTACTACTGAAGG CGTGTCTGGTCTGTACGTCT	212	-677 - -658 -466 - -485
7	CF7 CR7	GCCTCTCTTTCCAAGAACAAG GGCTGTATATCTGCTCTATATGC	187	-765 - -744 -579 - -601
8	CF8 CR8	CCGCTTCCTTTGTCCATCAG GCTACCTTCAGTGTACATAGC	231	-884 - -865 -654 - -674
9	CF9 CR9	CAGTATCTCCTATGAAGGGCC GGTCCTAAGCAGTTACCCTG	201	-994 - -975 -794 - -813
10	CF10 CR10	CCCAATAAGCCCAGGCAACTG GGCATTCTCTCCCTGATGC	188	-1122 - -1102 -935 - -955
11§	CF11 CR11	acgattgctagCTCTCTCACAGTATGGATTC tatGGGGTAaGCTTTGCTGTCTGAG	1933	-1811 - -1792 +108 - +87

§CF11 and CR11 include altered sequence (lowercase) that introduce *Nhe* I and *Hind* III sites, respectively.