Protective effect of the G-765C COX-2 polymorphism on subclinical atherosclerosis and inflammatory markers in asymptomatic subjects with cardiovascular risk factors

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Abstract

Background: Cyclooxygenase (COX)-2, a key regulatory enzyme in prostanoid synthesis, plays an important role in inflammatory processes. The $-765G>C$ COX-2 polymorphism has been associated with lower promoter activity in vitro and reduced levels of C-reactive protein (CRP) in atherosclerotic carriers of the C allele. However, its pathophysiological relevance in vivo has not been fully elucidated.

Methods and results: We assessed the $-765G>C$ polymorphism and COX-2 expression in 220 asymptomatic subjects free of cardiovascular disease, in relation to global vascular risk, carotid intima-media thickness (IMT), and inflammatory markers (fibrinogen, C-reactive protein [CRP], von Willebrand factor [vWF] and interleukin-6 [IL-6]). Genotype frequencies were: CC (7.7%), CG (34.5%), GG (57.7%). Among hypercholesterolemic subjects ($n=140$), C allele carriers had lower COX-2 expression ($p<0.05$), reduced carotid IMT ($p<0.01$) and diminished levels of inflammatory markers CRP, vWF and IL-6 ($p<0.05$), as compared to GG homozygous subjects. The association between carotid IMT and COX-2 polymorphism remained significant after adjusting for cardiovascular risk factors and inflammatory markers ($p=0.008$).

Conclusions: In asymptomatic hypercholesterolemic subjects the C allele of $-765G>C$ COX-2 polymorphism was associated with lower COX-2 expression, and reduced subclinical atherosclerosis and systemic inflammation compared with GG homozygous, thus conferring atherosclerosis protection in this cardiovascular risk population.

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Keywords: Atherosclerosis; COX-2 polymorphism; Hypercholesterolemia; Inflammation

1. Introduction

COX-1 and COX-2 catalyze the rate-limiting step in prostanoid synthesis, converting arachidonic acid into PGH_2, the precursor of a family of bioactive prostanoids, including thromboxane (TXA_2) and prostaglandins (PGs) [1,2]. COX-1 is a widely expressed constitutive enzyme that participates in tissue homeostasis. By contrast, COX-2, the inducible isoform, is expressed at low levels in most tissues but can be stimulated by LPS, growth factors and cytokines, such as TNF-α and interleukin-6 (IL-6) [3,4], being implicated in inflammatory processes, including atherosclerosis, rheumatoid diseases and carcinogenesis [5–7]. A direct role for COX-2 in atherosclerosis can be inferred from studies showing significant expression in human atherosclerotic lesions [8–10], as well as COX-2-derived PGE_2 increase in subclinical atherosclerosis [11,12]. Paradoxically, recent evidence points to a protective function of this enzyme in cardiomyocytes subjected to oxidative stress [13] and also in late preconditioning after ischemia/reperfusion injury [14]. Thus, it is likely that COX-2 exerts beneficial or detrimental effects depending on the rate of induction, the pathophysiological setting and the ability of specific cells to

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metabolize PGH_2 into cytoprotective or proinflammatory prostanoids [15].

Genetic polymorphisms in COX-2 might have an impact on COX-2 expression and prostanoid biosynthesis. Although several naturally occurring polymorphisms have been found in the COX-2 gene, their functional relevance and pathophysiological role remain to be elucidated [16,17]. Recently, Papafili et al. have described a common variant in the 5′-flanking region of the COX-2 gene −765G>C that is associated with lower promoter activity in vitro in the presence of the C allele, and reduced levels of C-reactive protein (CRP), a systemic marker of inflammation, in patients with clinical and subclinical atherosclerosis. This single nucleotide polymorphism (SNP) has also been associated with a reduction in the risk of future clinical cardiovascular events [17,18].

We therefore assessed COX-2 expression, subclinical atherosclerosis and inflammatory profile, in relation to this polymorphism, in subjects with cardiovascular risk factors but free of clinically overt atherosclerotic disease.

2. Methods

2.1. Subjects

A total of 220 apparently healthy subjects (80% males, median age 58 years), referred to the Internal Medicine Department of a single institution (University Clinic of Navarra) for global vascular risk assessment, were studied. Subjects were free from clinically apparent atherosclerotic disease based on (1) absence of history of coronary disease, stroke or peripheral arterial disease, and (2) normal ECG and chest-X-ray. Baseline clinical characteristics, cardiovascular risk factors and metabolic parameters in this population are summarized in Table 1. Exclusion criteria were the

Table 1
Biochemical parameters and cardiovascular risk factors in subjects classified according to the −765G>C polymorphism in the promoter of COX-2 gene

	CC (n=17)	CG (n=76)	GG (n=127)
Age (years)	57.1±9.8	56.5±10.1	59.4±10.7
Sex (male, %)	76.4	80.2	75.8
Glucose (mg/dL)	105.2±42.3	106.9±35.5	103.4±29.3
Total cholesterol (mg/dL)	218.5±48.1	229.4±35.4*	214.9±42.0
LDL-cholesterol (mg/dL)	147.3±38.8	157.1±31.7**	142.3±38.5
HDL-cholesterol (mg/dL)	49.1±14.2	49.9±13.1	48.4±13.1
Triglycerides (mg/dL)	111.0±45.1	116.4±65.1	121.0±71.5
Current smokers (%)	11.8	34.3	34.4
Hypertension (%)	41.2	55.2	51.9
Dyslipidemia (%)	81.2	81.5	78.1
Obesity (%)	18.7	35.5	33.1
Diabetes mellitus (%)	17.6	9.2	11.7
COX-2 expression[a]	0.12±0.05	0.13±0.1	0.14±0.1

*$p<0.05$, ** $p<0.01$ vs. GG.
[a] Normalized mRNA=target gene mRNA copies/β-actin mRNA copies.

presence of severely impaired renal function, arteritis, connective tissue diseases, alcohol abuse or use of nonsteroidal anti-inflammatory drugs in the 2 weeks before entering the study. Other conventional atherosclerotic risk factors recorded were: hypertension (systolic/diastolic blood pressure >139/89 mm Hg and/or use of antihypertensive drugs), obesity (body mass index ≥ 30 kg/m^2), smoking (≥ 1 cigarette a day), diabetes (fasting glucose ≥ 126 mg/dL and/or use of pharmacologic treatment), and family history of premature CHD (acute myocardial infarction before 60 years in a first degree relative). A subset of 140 hypercholesterolemic subjects was selected from the total population, on the basis of the following criteria: total cholesterol ≥ 220 mg/dL, LDL-cholesterol ≥ 130 mg/dL and/or statin treatment. Written informed consent was obtained before participation in the study, and the local committee on human research approved the protocol, which was performed in accordance with the principles of the Helsinki Declaration.

2.2. COX-2 genotyping procedure

Peripheral blood mononuclear cells were freshly obtained by centrifugation in a Ficoll gradient (Lymphoprep™). Total DNA and RNA were extracted from these cells using Tripure Isolation Reagent (Roche). To genotype the −765G>C variant, a fragment of 306 bp was amplified by PCR in the presence of 60 nM of the forward primer CF8 (5′-CCGCTTCCTTTGTCCATCAG-3′) and the reverse primer CR7 (5′-GGCTGTATATCTGCTCTA-TATGC-3′) as previously described [17]. Amplified product was digested with AciI (New England Biolabs) restriction endonuclease at 37 °C overnight and the resulting fragments were separated by electrophoresis in a 2% agarose gel, and visualized after ethidium bromide staining. Positive and negative digestion controls were included in all gels.

2.3. Quantitative real-time RT-PCR for COX-2 expression in peripheral blood mononuclear cells

Total RNA from mononuclear cells was reverse transcribed, and COX-2 expression was quantified by real-time quantitative PCR as previously described [12]. All samples were assayed in triplicate and values normalized on the basis of their β-actin content. Melt curve analysis was performed to ensure that only the specific product was amplified.

2.4. Carotid ultrasonography

All subjects underwent ultrasonography of the common carotid arteries (CCAs). Ultrasonography was performed with a 5–12 MHz linear-array transducer (ATL 500 HDI). The measurement of IMT was made 1 cm proximal to the carotid bulb of each CCA at plaque-free sites. From each individual, the IMT was determined as the average of near-

and far-wall measurements of each CCA. Carotid artery IMT has been shown to be reproducible [12,19].

2.5. Systemic inflammatory markers

Plasma and serum samples were obtained from venous blood between 9 and 10 a.m. after overnight fast, and stored at -80 °C until analysis. CRP and IL-6 concentrations were analyzed by a high-sensitive immunoassays system (Immulyte hs-CRP, Diagnostic Product Corporation and Quantikine-HS IL-6, R&D systems respectively). Plasma fibrinogen activity was measured by a clotting assay (Clauss). von Willebrand factor (vWF) antigen was quantified by an enzyme-linked immunosorbent assay (Liatest vWF, Diagnostica Stago, France). vWF values were expressed as percentage in relation to a calibrated standard curve.

Inter and intra-assay coefficients of variation for all these assays were lower than 8%.

2.6. Biochemical parameters

Serum cholesterol, HDL and LDL-cholesterol, glucose and triglycerides were determined on fasting blood samples by standard enzymatic techniques.

2.7. Statistical analysis

Mean±SD (SE for logarithmically transformed variables) is given for all continuous variables, and absolute numbers and percentages for categorical variables. Differences among genotypes were assessed by ANOVA followed by Bonferroni post-hoc test. When no differences between CG and CC genotypes were found, they were pooled into the same group, and mean comparisons between genotypes were made by the Student's t test. Deviations of the Hardy–Weinberg equilibrium were assessed by a χ^2 test. Pearson test was performed for correlations of continuous variables. Univariate and multivariate lineal regression analysis were performed to assess the relationship between genotypes, inflammatory markers and subclinical atherosclerosis after adjustment for cardiovascular risk factors and other potential confounders. A two-tailed $p < 0.05$ was considered statistically significant.

The statistical analysis was performed with SPSS for Windows software package version 11.0.

3. Results

To examine the association of the $-765G{>}C$ polymorphism with COX-2 expression, 220 asymptomatic subjects with cardiovascular risk factors (mean age 58.1 ± 10.3 years, 77.7% men) were genotyped for this polymorphism by PCR analysis. Subjects carrying CC, CG and GG genotypes were 17 (7.7%), 76 (34.5%) and 127 (57.7%) respectively, and

allele frequencies 0.25 and 0.75 for C and G alleles respectively, consistent with the Hardy–Weinberg equilibrium. The 3 groups were comparable in relation to all clinical and biochemical parameters analyzed (Table 1), except for the levels of total and LDL-cholesterol, which were significantly augmented in CG (229.4 ± 43.1 mg/dL), as compared to either CC (218.5 ± 48.1 mg/dL) and GG (214.9 ± 42.0 mg/dL) genotypes. Since the expression of COX-2 is regulated by LDL-cholesterol at transcriptional level [20,21], and C allele was previously associated with less COX-2 expression [17], a homogeneous subgroup of subjects with similar cholesterol levels ($n = 140$) was further selected to assess the net effect of this SNP.

3.1. $-765G{>}C$ COX-2 polymorphism in the hypercholesterolemic population

The clinical characteristics and cardiovascular risk factors of the 140 asymptomatic hypercholesterolemic subjects (mean age 59.7 ± 9.4 years, 77.7% men) are shown in Table 2. Mean total cholesterol levels were 232.5 ± 38.7 mg/dL and LDL levels 158.0 ± 35.8 mg/dL. 28% out of the total population were receiving statins and 40% of hypertensives were taking antihypertensive drugs.

The genotype distribution was as follows: CC=12 (8.6%), CG=46 (32.8%), and GG=82 (58.6%); allele frequencies were 0.25 and 0.75 for C and G alleles respectively, consistent with the Hardy–Weinberg equilibrium. Clinical and biochemical parameters analyzed in relation to the COX-2 genotypes are also shown in Table 2. Demographic characteristics were similar in the three groups, and there were also no significant differences among genotypes in relation to analytical determinations and anti-atherosclerotic therapy.

3.2. COX-2 expression in monocytes in relation to the $-765G{>}C$ polymorphism

Results of COX-2 gene expression in blood peripheral mononuclear cells in relation to $-765G{>}C$ polymorphism are shown in Table 3. The lowest baseline COX-2 expression was found in hypercholesterolemic subjects with CC genotype (0.10 ± 0.04) compared with the GC (0.13 ± 0.02) and GG (0.14 ± 0.01) genotypes. When assembling carriers of the $-765C$ allele, mean COX-2 expression was significantly lower (0.11 ± 0.06) than in GG homozygous subjects ($p = 0.04$).

3.3. Carotid IMT in relation to the $-765G{>}C$ polymorphism

In order to investigate the association of the COX-2 polymorphism with atherosclerotic plaque development, the carotid IMT was measured as a function of the $-765GC$ genotype. As shown in Fig. 1, subjects homozygous for the C allele had significantly reduced carotid IMT (0.60 ± 0.08

Table 2
Biochemical parameters and cardiovascular risk factors in asymptomatic hypercholesterolemic subjects classified according to the −765G>C COX-2 polymorphism

	Total ($n=140$)	CC ($n=12$)	CG ($n=46$)	GG ($n=82$)
Age (years)	59.7±9.4	60.1±8.1	58.2±9.3	60.6±9.5
Sex (male, %)	77.7	83.3	76.1	76.5
Current smokers (%)	29.7	16.7	28.3	32.5
Hypertension (%)	52.9	41.7	56.5	52.5
Obesity (%)	35.8	27.3	41.3	33.8
Diabetes mellitus (%)	11.5	8.3	8.7	13.6
SBP (mm Hg)	133.2±21.7	128.4±18.6	134.3±25.1	133.3±20.2
DBP (mm Hg)	83.4±9.6	81.8±7.4	83.7±11.0	83.5±9.1
Total cholesterol (mg/dL)	232.5±38.7	232.7±46.3	237.6±32.9	229.5±40.7
LDL-cholesterol (mg/dL)	158.0±35.8	159.2±36.2	164.3±30.5	154.3±38.4
HDL-cholesterol (mg/dL)	49.6±13.5	48.3±14.6	49.7±13.9	49.7±13.2
Triglycerides (mg/dL)	126.1±67.2	126.1±41.2	125.7±64.4	126.3±72.3
Glucose (mg/dL)	103±29.3	109.0±49.8	105.9±35.7	101.9±20.3
Statin treatment (N/Y)	101/39	10/2	33/13	58/24
Antihypertensives (N/Y)	110/30	9/3	30/16	71/11

SBP=systolic blood pressure, DBP=diastolic blood pressure.

mm) compared with either GC (0.67±0.14 mm, $p<0.05$) and GG (0.73±0.16 mm, $p<0.01$) genotypes.

3.4. Systemic markers of inflammation in relation to the −765G>C polymorphism

To further assess whether the −765G>C COX-2 promoter variant would influence the systemic levels of inflammatory and endothelial activation markers, CRP, IL-6, fibrinogen and vWF concentrations were compared in relation to the different genotypes. As shown in Table 3, subjects homozygous for the C allele exhibited significantly lower concentrations of CRP (0.23±0.03 mg/dL vs. 0.37±0.03 mg/dL, $p<0.01$) and vWF (79.0±23.2% vs. 108.5±45.6%, $p<0.01$), whereas differences in fibrinogen levels did not reach statistical significance. Likewise, carriers of the C allele also showed significantly lower IL-6 levels as compared to GG homozygous (1.6±0.4 pg/mL vs. 2.1±1.6 pg/mL, $p<0.05$).

Table 3
COX-2 expression and inflammatory profile in relation to the COX-2 −765G>C polymorphism in hypercholesterolemic subjects

	CC	CG	GG	P
	($n=12$)	($n=46$)	($n=82$)	C allele vs. GG
COX-2 expression				
Baseline mRNA[a]	0.10±0.04	0.13±0.02	0.14±0.01	0.040
Inflammatory markers				
CRP (mg/dL)[b]	0.23±0.03††	0.29±0.02	0.37±0.03	0.022
IL-6 (pg/mL)	1.7±0.7	1.5±0.8††	2.1±1.6	0.022
Fibrinogen (mg/dL)	291.7±85.3	299.4±63.6	304.7±87.4	ns
VWF (%)	79.0±23.2††	118.9±42.4‡	108.9±45.6	ns

‡$p<0.01$ vs. CC.
[a] Normalized mRNA=target gene mRNA copies/β-actin mRNA copies.
[b] Logarithmically transformed (mean±SE).

3.5. Multiple regression analysis

Taking into account the possible associations between −765G>C and carotid IMT with cardiovascular risk factors and inflammatory markers, further multiple regression analysis was performed to assess whether the reduction in carotid IMT observed in C allele carriers was independent of potential confounders. As shown in Table 4, the association between both parameters remained statistically significant after adjusting for traditional cardiovascular risk factors and inflammatory markers ($p=0.008$), with a reduction in
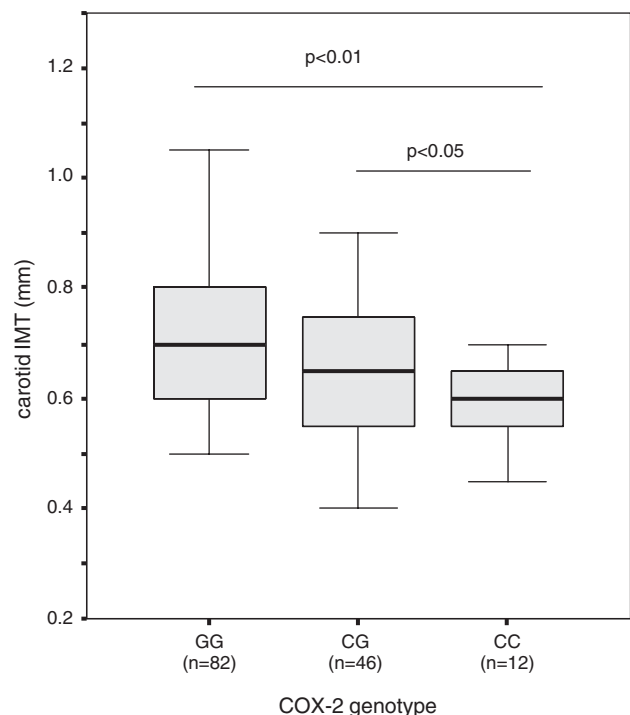

Fig. 1. Box-plot showing the carotid IMT in relation to the −765G>C COX-2 polymorphism. Median and percentile 75th is reported.

Table 4
Multiple linear regression analysis for the association of COX-2 −765G>C polymorphism with carotid IMT in hypercholesterolemic subjects

Genotypes	R (95% CI)	p	R[a] (95% CI)	p a
GG	Referent	–		–
GC	−0.18 (−0.34 to −0.02)	ns	−0.25 (−0.40 to −0.09)	0.021
CC	−0.22 (−0.37 to −0.06)	0.016	−0.29 (−0.43 to −0.13)	0.008

[a] Adjusted for age, gender, smoking, hypertension, obesity and diabetes, IL-6, vWF, CRP and fibrinogen.

carotid IMT of 8.3% in homozygous CC compared to subjects with GG genotype.

4. Discussion

In a population sample of hypercholesterolemic subjects without clinically overt atherosclerotic disease, we show that the common variant −765G>C in the COX-2 gene promoter can play a protective role on subclinical atherosclerosis and systemic inflammation. Subjects carrying the C allele had significantly lower COX-2 expression and reduced carotid IMT, as well as diminished levels of the inflammatory markers CRP, IL-6 and vWF, compared with GG homozygous subjects. Taken together, our results indicate that the C allele might offer some protection against clinical events related to atherosclerosis development.

A polymorphism in the 5′-flanking region of the gene (−765G>C) has been associated with functional changes in COX-2 promoter activity in vitro [17]. Subjects with the C allele might be protected from systemic inflammation, since lower levels of CRP, an established marker of inflammation and cardiovascular risk, were observed in patients with clinical atherosclerosis. However, there is little information available regarding the possible influence of this polymorphism on COX-2 expression and atherosclerosis development in vivo as well as its possible association with reduced risk of cardiovascular events [17]. Recently, C allele has been shown to be associated with reduced risk of myocardial infarction, stroke [18] and a decreased risk of Alzheimer's disease [22]. Moreover, our group has shown that COX-2-dependent PGE$_2$ release by blood monocytes is related to subclinical atherosclerosis in apparently healthy subjects exposed to cardiovascular risk factors [11,12]. COX-2 expression and PGE$_2$ synthesis have been shown to promote the release and activation of MMPs likely involved in plaque growth and rupture [10,23]. These data prompted us to assess the functional relevance of that polymorphism in relation to inflammation and subclinical atherosclerosis, in a population of subjects free from clinically apparent cardiovascular disease.

We found, for the first time, that in hypercholesterolemic subjects the C allele was independently associated with reduced carotid IMT, an established surrogate of atherosclerosis [24], after adjusting for traditional cardiovascular risk factors. The model explained an 8.3% reduction in carotid IMT in CC homozygous compared with the remaining genotypes. These data would suggest that this polymorphism may offer some protection against clinical events related to atherosclerosis plaque development [25]. Since lower COX-2 expression could be demonstrated in this subgroup, an inflammation-related effect can be suggested on the mechanism or carotid arterial thickening [26]. In contrast, Cipollone et al. failed to demonstrate any effect of this SNP on lesion size in the coronary artery assessed by angiography in a population with clinical cardiovascular disease [18].

The fact that lower COX-2 expression was found in C allele carriers in the present study could partially account for a reduced COX-2-mediated inflammation-dependent plaque growth. Supporting these observations, we also found that the COX-2 polymorphism was associated with a reduction in systemic inflammation, C allele carriers showing significantly lower levels of CRP, IL-6 and vWF, an effect not due to statin treatment, since no differences in the proportion of patients using lipid-lowering agents were found. Whereas there are several evidences linking COX-2 expression and inflammation, the precise mechanism remains unclear [27–29]. An association between COX-2 and IL-6 has been established in macrophages via PGE$_2$ [30]; furthermore, the magnitude of rise in CRP levels in cardiovascular patients was also found to be strongly dependent on the −765G>C polymorphism [17]; since IL-6 is known to regulate CRP [31,32], it can be speculated that this polymorphism influences CRP levels via IL-6, which is also supported by our present findings. Subjects homozygous for the C allele also showed lower vWF levels, suggesting reduced endothelial damage [33] as compared with the remaining genotypes. Whereas there are no reports of COX-2-mediated induction of vWF, immunohistochemical studies have shown their colocalization in endothelial cells [34].

Taken together, we found hypercholesterolemic subjects carrying the C allele of the −765G<C COX-2 polymorphism exhibited a more favourable phenotypic response consisting of reduced carotid IMT and systemic inflammation. A main difference between genetic and pharmacological inhibition of COX-2 to protect against cardiovascular events [28], would be in the preserved endothelial function and reduced systemic inflammation by the SNP.

Some limitations of the present study must be recognized. Our population sample was relatively small with high male prevalence. In addition, the cross-sectional design does not allow us to draw conclusions regarding the value of COX-2 polymorphism in the prediction of cardiovascular events. In the multivariate analysis we tried to adjust for most of the possible confounders, but other genetic,

metabolic and environmental factors not included in the present analysis could also influence the IMT. Finally, the effect of this polymorphism cannot be extrapolated to other populations at atherosclerotic risk.

In conclusion, we documented a functional effect of $-765G > C$ polymorphism in the COX-2 promoter, causing lower COX-2 gene expression, associated with reduced inflammation and subclinical atherosclerosis in middle-aged asymptomatic hypercholesterolemic subjects. Further studies in larger populations are warranted in order to validate the clinical significance of this polymorphism for atherosclerotic protection in cardiovascular risk patients.

Acknowledgements

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