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# 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.


*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 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 $\geq$30 kg/m$^2$), smoking ($\geq$1 cigarette a day), diabetes (fasting glucose $\geq$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 $\geq$220 mg/dL, LDL-cholesterol $\geq$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.

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.

### 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′-GGCTGTATATCTGCTCTATATGC-3′) as previously described [17]. Amplified product was digested with *Aci*I (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-