Monocyte cyclooxygenase-2 overactivity: a new marker of subclinical atherosclerosis in asymptomatic subjects with cardiovascular risk factors?

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Aims Cyclooxygenase-2 (COX-2)-mediated prostaglandin production by activated macrophages is associated with inflammation and atherosclerosis. We investigated the relationship between COX-2-mediated prostaglandin-E2 (PGE2) release, cardiovascular risk factors, and carotid atherosclerosis in apparently healthy subjects.

Methods and results PGE2 release by lipopolysaccharide-stimulated blood monocytes was measured by ELISA in 291 subjects (76.5% men, mean age 58) who underwent global vascular risk assessment and carotid ultrasonography. COX-2 expression (real-time RT–PCR) was analysed in a subgroup of 100 subjects (76% men, mean age 59). Inducible PGE2 production was associated with smoking and diabetes (P < 0.05), but not with arterial hypertension, dyslipidaemia, or obesity. Subjects in the highest tertile of PGE2 (>8.1 ng/mL) had significantly higher mean carotid intima–media thickness (IMT) than those in the lowest tertile (P < 0.01). No significant differences among tertiles were observed in the levels of inflammatory markers (C-reactive protein, fibrinogen, and von Willebrand factor). The association between PGE2 and carotid IMT remained statistically significant (P = 0.012) after adjustment for a number of cardiovascular and inflammatory risk factors. A correlation between COX-2 expression and PGE2 production was observed (P < 0.005).

Conclusions COX-2-mediated PGE2 overproduction by stimulated monocytes might provide a new marker of subclinical atherosclerosis in asymptomatic subjects exposed to cardiovascular risk factors.

Introduction

It is now well established that vascular inflammation plays a role in the pathogenesis of atherosclerosis.1,2 Activated macrophages are a key component not only in the development of atherosclerosis but also in plaque rupture leading to thrombosis.3 Prostaglandin (PG) biosynthesis has been implicated in the pathophysiology of cardiovascular processes and a variety of inflammatory diseases.3 The rate-limiting enzyme in the biosynthesis of PGS is prostaglandin-H2-synthase, or cyclooxygenase (COX). Two COX isoforms have been identified, referred to as COX-1 and COX-2.
In contrast to COX-1, a constitutively expressed enzyme, COX-2 is induced in response to growth factors, cytokines, phorbol esters, and lipopolysaccharide (LPS).\textsuperscript{4–6} Several studies have demonstrated the presence of COX-2 in atherosclerotic plaques, mainly co-localizing with macrophages.\textsuperscript{7–10} It has been proposed that COX-2-mediated PG production by activated macrophages may promote atherosclerosis through a number of mechanisms, including stimulated chemotaxis, induction of vascular permeability, propagation of the inflammatory cytokine cascade, stimulation of smooth muscle cell migration and proliferation, and synthesis of extracellular matrix.\textsuperscript{11} In addition, it has been shown that there is increased co-expression of COX-2 and matrix metalloproteinases (MMPs), enzymes that are involved in the destabilization of atherosclerotic plaques.\textsuperscript{12} Furthermore, selective inhibition of COX-2, with rofecoxib, resulted in a significant reduction in aortic atherosclerosis in low density lipoprotein (LDL-) receptor-deficient mice.\textsuperscript{13}

At present, clinical data evaluating the relationship of COX-2 and cardiovascular risk factors are lacking. Furthermore, no information is available on the potential association of COX-2 with subclinical atherosclerosis. We therefore investigated monocyte COX-2 activity in a cohort of adults free of clinically overt cardiovascular disease. Measurement of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) in LPS-stimulated whole blood monocytes was used to assess monocyte COX-2 activity.\textsuperscript{14} The carotid artery intima–media thickness (IMT) was used as a marker of subclinical atherosclerosis.\textsuperscript{15,16}

**Subjects and methods**

**Subjects**

The population studied consisted of 291 consecutive, apparently healthy, subjects (223 men, mean age 58 years, range 25–81 years) in whom global vascular risk assessment was performed in the course of a general health check-up by the Internal Medicine Department (University Clinic of Navarra, Spain). In all subjects, absence of history of coronary disease, stroke, or peripheral arterial disease was recorded; additional exclusion criteria were the presence of severely impaired renal function, arteritis, connective tissue diseases, alcohol abuse, or use of non-steroidal anti-inflammatory drugs in the 2 weeks prior to entering the study. By use of a random zero sphygmomanometer, supine blood pressure was measured at the right upper arm. By use of a random zero sphygmomanometer, supine blood pressure was measured at the right upper arm. Cigarette smoking (\textleq{1} cigarette a day), and diabetes (fasting glucose \textgeq{126 mg/dL} and/or use of pharmacological treatment).

The local committee on human research approved the study, which was performed in accordance with the Declaration of Helsinki and all participants gave written informed consent.

**Laboratory studies**

Blood samples were collected in 0.1 M trisodium citrate and stored at \(-80\) \(^\circ\)C until analysis. Plasma fibrinogen activity was measured by a clotting assay (Clauss). High-sensitive C-reactive protein (hs-CRP) by an immunoassay system (Immulyte hs-CRP, Diagnostic Product Corporation, USA) and von Willebrand factor (vWF) (Aasserachrom von Willebrand Factor Factor, Diagnostica Stago, France) were determined using specific enzyme-linked immunosorbent assays (ELISAs). Inter- and intra-assay coefficients of variation for all ELISAs were \textless8%.

**Monocyte COX-2 activity and expression**

Monocyte COX-2 activity was assessed in peripheral blood samples (3 mL) incubated with 100 \(\mu\)M acetylsalicylic acid to inhibit COX-1 activity, without affecting PGE\textsubscript{2} production.\textsuperscript{14} Concentration of blood monocytes with bacterial endotoxin was ruled out before LPS incubation by Limulus test (BioWhitaker). Samples were then incubated in the presence of LPS (10 \(\mu\)g/mL) for 24 h at 37 \(^\circ\)C as previously described.\textsuperscript{14} PGE\textsubscript{2} levels were measured by ELISA (Cayman Chemical, USA), following the manufacturer’s instructions. Inter- and intra-assay coefficients of variation were \textless8%.

In a subgroup of 100 consecutive subjects, total RNA was extracted from unstimulated peripheral blood mononuclear cells with TriPure (Roche, Switzerland) and quantified by real time quantitative RT-PCR assay. Total RNA (<0.5 \(\mu\)g) was reverse transcribed with random primers with M-MLV reverse transcriptase, in the presence of RNase Out\textsuperscript{TM} (Invitrogen, USA).

One hundred nanograms reverse-transcribed RNA was primed with specific oligonucleotides for COX-2 (‘\textasciitilde’ACACGCTTAAA CTGGGCTTTT-3’ and 5’-CTCCTGCCCCACAGCAA-3’), \(\beta\)-actin (5’-AGGCTGAGCCTTTCCGA-3’ and 5’-CTGAGGCGCTCGGCG-3’), PCR was performed as previously described (95 \(^\circ\)C for 10 min and run for 40 cycles at 95 \(^\circ\)C for 15 s and 60 \(^\circ\)C for 1 min) with SYBR Green PCR Master Mix on the ABI PRISM 7000 Detection System (Applied Biosystems, USA).\textsuperscript{18} Potential genomic DNA contamination was excluded by using intron-encompassing primers. All samples were assayed in triplicate and normalized on the basis of their \(\beta\)-actin content.

**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. For 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.\textsuperscript{19}

Subjects were examined by the same two certified sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in 20 individuals who returned 2 weeks later for a second examination. The between-observer intra-class correlation coefficient was 0.76 (\(P<0.0001\)) and the between-subject repeatability was 0.82 (\(P<0.0001\)). The corresponding coefficients of variance were 5 and 10%, respectively.

**Statistical analysis**

Mean \(\pm\) SD is given for all continuous variables, and absolute numbers and percentages are given for qualitative variables.
The sample size was calculated on the basis of the association between PGE2 and carotid IMT (R = 0.20) observed in a pilot study of 86 subjects. The minimum sample size needed to set the alpha error at 0.05 and the beta error at 0.20 was 194 subjects. Univariate association of PGE2 release with clinical characteristics was performed by Student’s t-test and Pearson correlation test for continuous variables. Variables that were not normally distributed were logarithmically transformed when appropriate. For the linear regression model to be valid we checked for normality of residuals. Pearson correlation coefficients for continuous variables were also used to assess univariate correlations of carotid IMT with all variables.

Multivariate linear regression analysis with step-wise selection was performed to assess the independent relationship between PGE2 and carotid IMT after adjusting for cardiovascular risk factors and inflammatory markers. All significant (P < 0.05) and near-significant (P < 0.1) variables in the univariate analysis were included in the model. The statistical analysis was performed with SPSS for Windows software package version 11.0. A two-tailed P < 0.05 was considered statistically significant.

Results

In the present study we have examined the monocytic production of PGE2, in response to LPS, in a series of 291 subjects free from clinically overt cardiovascular disease, in relation to the presence of traditional cardiovascular risk factors and to a non-invasive measurement of atherosclerosis (carotid ultrasound). Baseline clinical characteristics, cardiovascular risk factors, and levels of markers of inflammation and endothelial damage are shown in Table 1. Whereas in the absence of LPS, PGE2 was undetectable, mean LPS-inducible PGE2 levels were 7.7 ± 4.3 ng/mL (median 7.0 ng/mL, range 0.1–25.8 ng/mL) in the whole studied population.

As shown in Table 2, PGE2 levels were higher (P < 0.01) in males than in females (8.2 ± 4.4 vs. 5.9 ± 4.4 ng/mL) and its production was associated with smoking and the presence of diabetes (P < 0.05). No significant associations were found with arterial hypertension, dyslipidaemia, or obesity. As shown in Figure 1, inducible PGE2 increased with increasing number of cardiovascular risk factors (P trend < 0.005).

Table 3 shows the univariate associations of PGE2 production with clinical and analytical characteristics. PGE2 release was slightly correlated with glucose (R = 0.12; P = 0.036), total cholesterol (R = 0.12; P = 0.032) and carotid IMT (R = 0.13; P = 0.036), and negatively with HDL cholesterol (R = −0.18; P < 0.001).

In addition to its association with PGE2, carotid IMT was also directly correlated with age (P < 0.001), SBP (P < 0.001), glucose (P < 0.001), hs-CRP (P = 0.014), and fibrinogen (P < 0.001) (Table 4). Since both PGE2 and carotid IMT were related to some cardiovascular risk factors and markers of inflammation, a linear multivariable regression analysis was performed to assess the independent relationship between PGE2 and carotid IMT, after adjusting for these potential confounders (Table 5). The association between PGE2 and carotid IMT remained statistically significant (P = 0.012) after adjustment for age, sex, SBP, and fibrinogen, explaining 28% of the IMT variance.

Finally, a further analysis of COX-2 mRNA expression was performed in a subgroup of the first 100 consecutive subjects (76 men, mean age 59.3 years, range 27–78 years). The mean baseline COX-2/β-actin mRNA ratio was 0.15. Figure 2 shows the direct correlation.
between COX-2 mRNA expression and PGE₂ release ($R = 0.32$, $P < 0.005$).

**Discussion**

The main findings of the current study are as follows: first, PGE₂ production by stimulated monocytes was related to a clustering of cardiovascular risk factors in a population sample of adults free from clinically overt cardiovascular disease; second, PGE₂ production was significantly associated with carotid IMT, independently of other traditional and non-traditional cardiovascular risk factors; third, PGE₂ production was associated with monocyte COX-2 mRNA expression. Taken together, our results seem to suggest that COX-2-mediated PGE₂ release may be a marker of subclinical atherosclerosis.

PGE₂ levels increased with increasing number of cardiovascular risk factors, mainly smoking and diabetes. Elevated PGE₂ synthesis has been previously reported in smokers in comparison with non-smokers. Moreover, high glucose, via protein kinase C signalling, induces oxidative stress and up-regulation of COX-2 in human endothelial cells. The observation that PGE₂ levels were not associated with dyslipidaemia would agree with in vitro findings suggesting down-regulation of COX-2 in vascular endothelial cells accumulating cholesterol. The lack of correlation between PGE₂ release and other established markers of inflammation, such as fibrinogen and CRP, suggest either a different role in atherogenesis or independent regulatory pathways. It remains unclear, however, whether markers of systemic vascular inflammation track with one another in healthy individuals.

Carotid IMT is a marker of subclinical atherosclerosis associated with cardiovascular risk factors. Here we report an association between PGE₂ production and carotid IMT. Interestingly, the association remained statistically significant after adjusting for traditional cardiovascular risk factors and other potential confounders. These previously unreported findings suggest that PGE₂ production might be an independent marker of subclinical atherosclerosis in asymptomatic subjects, but may also play a role in the mechanism of arterial intima–media thickening.

**Table 3** Correlation coefficients of mean PGE₂ release with clinical and laboratory parameters in the studied population

<table>
<thead>
<tr>
<th></th>
<th>$R$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>−0.05</td>
<td>0.317</td>
</tr>
<tr>
<td>BMI</td>
<td>0.11</td>
<td>0.059</td>
</tr>
<tr>
<td>SBP</td>
<td>0.01</td>
<td>0.789</td>
</tr>
<tr>
<td>DBP</td>
<td>−0.01</td>
<td>0.829</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.12</td>
<td>0.036</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.12</td>
<td>0.032</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>−0.18</td>
<td>0.001</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>−0.07</td>
<td>0.217</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>−0.01</td>
<td>0.811</td>
</tr>
<tr>
<td>CRP</td>
<td>−0.06</td>
<td>0.289</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>−0.11</td>
<td>0.056</td>
</tr>
<tr>
<td>vWF</td>
<td>−0.06</td>
<td>0.304</td>
</tr>
<tr>
<td>Carotid IMT</td>
<td>0.13</td>
<td>0.036</td>
</tr>
</tbody>
</table>

BMI, body mass index.

**Table 4** Correlation coefficients of mean carotid IMT with clinical and laboratory parameters in the studied population

<table>
<thead>
<tr>
<th></th>
<th>$R$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.34</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI</td>
<td>0.11</td>
<td>0.084</td>
</tr>
<tr>
<td>SBP</td>
<td>0.35</td>
<td>0.001</td>
</tr>
<tr>
<td>DBP</td>
<td>0.11</td>
<td>0.071</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.23</td>
<td>0.001</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.05</td>
<td>0.433</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.002</td>
<td>0.966</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.03</td>
<td>0.583</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.04</td>
<td>0.477</td>
</tr>
<tr>
<td>CRP</td>
<td>0.15</td>
<td>0.014</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.26</td>
<td>0.001</td>
</tr>
<tr>
<td>vWF</td>
<td>0.11</td>
<td>0.067</td>
</tr>
</tbody>
</table>

BMI, body mass index.

**Table 5** Stepwise multiple regression analysis for the association between mean carotid IMT (mm), cardiovascular risk factors, markers of inflammation, and inducible PGE₂ in the studied population

<table>
<thead>
<tr>
<th></th>
<th>$\beta$</th>
<th>SE($\beta$)</th>
<th>Partial $R$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>4.37 × 10⁻²</td>
<td>0.0010</td>
<td>0.28</td>
<td>0.001</td>
</tr>
<tr>
<td>Male sex</td>
<td>8.30 × 10⁻²</td>
<td>0.0200</td>
<td>0.21</td>
<td>0.001</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>2.03 × 10⁻¹</td>
<td>0.0001</td>
<td>0.27</td>
<td>0.001</td>
</tr>
<tr>
<td>CRP</td>
<td>4.32 × 10⁻⁴</td>
<td>0.0001</td>
<td>0.23</td>
<td>0.001</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>5.66 × 10⁻³</td>
<td>0.0020</td>
<td>0.16</td>
<td>0.012</td>
</tr>
</tbody>
</table>

BMI, body mass index.

Variables excluded were smoking, BMI, glucose levels, vWF, and CRP.

Adjusted $R^2 = 0.28$. 

Figure 1 COX-2 activity, measured as PGE₂ release by LPS-stimulated monocytes, according to the number of cardiovascular risk factors (family history of premature coronary heart disease, smoking, obesity, arterial hypertension, dyslipidaemia, diabetes mellitus) in the overall population.
In vitro release of PGE2 from whole blood challenged mechanisms.27–29 For example, PGE2 has been shown to wall may promote atherosclerosis through a variety of PG production by activated macrophages in the artery pro- and anti-inflammatory effects of PGE2 and other activity,14,24 although other molecules may also partici-
pathway.30 Furthermore, PGs may mediate some of the responses related to atherogenesis.26 COX-2-mediated PG production by activated macrophages in the artery wall may promote atherosclerosis through a variety of mechanisms.27–29 For example, PGE2 has been shown to induce production of the inflammatory cytokine interleukin-6;4 in addition, LDL-stimulated chemotaxis of human monocytes and endothelial cells has been reported to be mediated by a COX-2-dependent pathway.30 Moreover, PGs may mediate some of the effects of the CD40-CD40L system through significant induction of COX-2 and PGE2 production.31 Finally, monocyte COX-2 expression and PGE2 synthesis have been shown to promote the release and activation of MMPs.12,32,33

Figure 2 Direct correlation (y = 1.75 + 0.31x) between COX-2 expression and PGE2 production in peripheral blood mononuclear cells from 100 asymptomatic subjects.

We found a slight but significant correlation between COX-2 mRNA expression and LPS-induced PGE2 levels, suggesting that COX-2 has a role in PGE2 production. In vitro release of PGE2 from whole blood challenged with LPS represents an index of monocyte COX-2 activity,14,24 although other molecules may also participate in PGE2 synthesis.25 There is also in vivo evidence that bacterial LPS may be involved in inflammatory responses related to atherogenesis.26 COX-2-mediated PG production by activated macrophages in the artery wall may promote atherosclerosis through a variety of mechanisms.27–29 For example, PGE2 has been shown to induce production of the inflammatory cytokine interleukin-6;4 in addition, LDL-stimulated chemotaxis of human monocytes and endothelial cells has been reported to be mediated by a COX-2-dependent pathway.30 Moreover, PGs may mediate some of the effects of the CD40-CD40L system through significant induction of COX-2 and PGE2 production.31 Finally, monocyte COX-2 expression and PGE2 synthesis have been shown to promote the release and activation of MMPs.12,32,33

Results provided herein suggest that assessment of PGE2, a major product of monocyte COX-2 activity, might be useful to identify asymptomatic subjects at high cardiovascular risk as well as to select those who would be likely to benefit from treatment with COX-2 inhibitors. However, since the activity of COX-2 also increases PGE2 biosynthesis,3,11 a balance between pro- and anti-inflammatory effects of PGE2 and other PGs in atherosclerotic lesion development cannot be ruled out. Experimental studies have demonstrated that selective inhibition of COX-2 reduces atherosclerosis in LDL-receptor-deficient mice,13 an effect also observed by non-selective COX-2 inhibition (e.g. statins).34,35 In addition, the COX-2 inhibitor celecoxib improved endothelial function, and reduced markers of inflammation and adverse outcomes in patients with coronary artery disease.36 However, recent data raise concerns regarding the thrombogenicity of coxibs, especially in cardiovascular risk patients.37,38

Some limitations of the present study must be recognized. In keeping with previous reports assessing the predictive value of inflammatory markers,39 our population sample was relatively small. In addition, the transverse design does not allow the opportunity to draw conclusions regarding the value of PGE2 measurement in the prediction of cardiovascular events. Further prospective studies are required to examine the relationship between COX-2-derived prostanoids and subclinical atherosclerosis in larger cohorts. Finally, the effect of COX-2 gene promoter variants on PGE2 levels needs to be investigated.40,41

In summary, we describe for the first time that COX-2-dependent PGE2 release by blood monocytes is related to sub-clinical atherosclerosis in apparently healthy subjects exposed to cardiovascular risk factors. Our findings extend previous data showing that an inflammatory profile can be identified in subjects at risk of atherosclerosis, and set the stage for clinical studies aimed at proving the potential of monocyte COX-2 activity measurement as both a marker of inflammation-induced atherosclerosis and a target for specifically addressed therapeutic interventions.

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References


