Heritability of Thromboxane A2 and Prostaglandin E2 Biosynthetic Machinery in a Spanish Population

Luis Vila, Angel Martinez-Perez, Mercedes Camacho, Alfonso Buil, Sonia Alcolea, Nuria Pujol-Moix, Marta Soler, Rosa Antón, Juan-Carlos Souto, Jordi Fontcuberta, José-Manuel Soria

Objective—Prostanoids play a critical role in clinical areas such as inflammation, thrombosis, immune response, and cancer. Although some studies suggest that there are genes that determine variability of some prostanoid-related phenotypes, the genetic influence on these traits has not been evaluated.

Methods and Results—The relative contributions of genetic and environmental influences to the prostanoid biosynthetic pathway-related phenotypes, cyclooxygenase isoenzymes, microsomal-PGE-synthase-1 and TxA-synthase expression, and thromboxane-A2 and prostaglandin-E2 production by stimulated whole blood, were assessed in a sample of 308 individuals in 15 extended families. The effects of measured covariates (such as sex, age, and smoking), genes, and environmental variables shared by members of a household were quantified. Heritabilities ranging from 0.406 to 0.634 for enzyme expression and from 0.283 to 0.751 for prostanoid production were found.

Conclusions—These results demonstrate clearly the importance of genetic factors in determining variation in phenotypes that are components of the prostanoid biosynthetic pathways. The presence of such strong genetic effects suggest that it will be possible to localize previously unknown genes that influence quantitative variation in these phenotypes, some of which affect multiple aspects of cell biology, with important clinical implications. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: atherosclerosis • gene expression • prostaglandins • thrombosis • thromboxanes • genomics

Prostanoids include prostaglandins (PG) and thromboxanes (Tx) and belong to a remarkable group of compounds involved in a variety of clinically important areas such as inflammation, thrombosis, allergic and immune responses, and cancer. Particularly, prostanoids are important mediators involved in vascular physiology and pathophysiology.1

In the biosynthetic pathways of prostanoid, the first step is conversion of arachidonic acid (AAc) into PGH2, which is catalyzed by cyclooxygenase (COX). Two isofoms of COX have been characterized in humans. COX-1 can be viewed as a constitutive enzyme and prostanoids formed through the action of COX-1 mediate the so-called “housekeeping” functions, such as the regulation of renal function and maintenance of the gastric mucosa integrity and hemostasis. COX-2 is expressed in some tissues and cell types such as endothelium or renal macula densa, but is induced in response to hormones, growth factors, proinflammatory cytokines, bacterial endotoxin, and tumor promoters. Also, COX-2 is typically overexpressed at inflammatory sites such as atherosclerotic lesions (reviewed in1–5).

PGE2, PGD2, PGF2, and TxA2 are formed from PGH2 in reactions catalyzed by specific synthases acting on PGH2.1 The Figure show a simplified scheme of prostanoid biosynthesis. TxA2, is one of the most potent prothrombotic agents released by activated platelets and macrophages3,4 that exhibits 2 major biological activities: the stimulation of platelet function and smooth muscle contraction, inducing platelet aggregation, vasoconstriction, and broncoconstriction.3 It is also an important proinflammatory and proatherogenic agent.7,8 The conversion of PGH2 into TxA2 is catalyzed by the TxA-synthase (TXAS).3,4 A membrane-bound hemoprotein that, according to their spectral characteristics, is a cytochrome P450 protein.9,10

PGE2 is a major prostanoid produced by many tissues and cell types including leukocytes and vascular smooth muscle cells (VSMCs) that mediates some of the cardinal features of inflammation, including pain, edema, and fever.11 It is important to note that PGE2 induces expression of matrix metalloproteinases,12 enzymes that are crucial in the degradation of extracellular matrix and plaque stability.13 Also, PGE2 inhibits the production of macromolecules of the extracellular matrix, further favoring plaque fragility.14 PGE-synthase (PGES) catalyzes conversion of PGH2 to PGE2. The first isozyme identified and characterized was the microsomal PGE-Synthase-1 (mPGES-1), which is inducible by
proinflammatory cytokines. There is convincing evidence of a pathophysiological role of mPGES-1 in cardiovascular pathology.15–19

Although there are a number of biochemical, functional, and pharmacological studies concerning PGE2 and TxA2, little is known about underlying factors involved in the determination of TxA2 and PGE2 biosynthetic machinery. To assess genetic and environmental correlations of proteins involved in prostanoids biosynthesis, we analyzed the phenotypic expression of the proteins related to TxA2 and PGE2 biosynthesis, using data from a set of extended Spanish families. Thus, the present work deals with the genetic and environmental factors that influence the biosynthetic pathways of these proinflammatory, prothrombotic and proatherogenic compounds.

Materials and Methods
In this study we have included 308 individuals belonging to 15 extended families. The depth and complexity of these pedigrees is illustrated in the supplemented Table I (available online at http://atvb.ahajournals.org). For a detailed description of the methods, see the supplemental materials.

Results
Characterization of the Population and Samples
Table 1 shows the characteristics of the sample and the values of the phenotypes analyzed. The ages ranged from 5 to 93 years, and the number of male and female subjects was similar with a similar mean age and range.

Because TxA2 is produced mainly by platelets, in agreement with a statistical correlation found in this study between TxAS levels and platelet number (r=0.12, P=0.015) the production of TxA2 after A23187 challenge was normalized by the number of platelets and by the total platelet volume. Similarly, leukocytes, and particularly monocytes, were probably the main contributors to PGE2 formation. This is consistent with the fact that COX-2 expression correlated with the total number of leukocytes (r=0.16, P=0.003). Therefore, data concerning PGE2 formation in response to A23187 and LPS was normalized by the total number of leukocytes and number of monocytes.

PGE2 Production
Significant lower levels of PGE2 production were observed in females, irrespective of the absolute or relative expression of this parameter. No statistically significant differences were observed between males and females in any of the other phenotypes analyzed. Age effects were significant only for parameters concerning PGE2 production by whole blood in response to LPS stimulus. A significant negative dependence on age was found for absolute production of PGE2 (r=0.0001), relative to the number of leukocytes (r=0.0349), and relative to the number of monocytes (r=0.0003). In addition, the absolute production of PGE2 in response to LPS was significantly higher in smoking subjects (P=0.003), although it was not statistically significant when the parameter was expressed in relative terms, either as leukocyte or monocyte number. Also, smokers tended to express more TxAS (P=0.037) than nonsmokers.

Contribution of Inheritance to the Variability in Prostanoids Biosynthesis
The components of variance are shown in Table 2 based on the most parsimonious model (ie, the model that best fits the observed data and exhibits the minimum complexity) for each phenotype, including only significant sources of variation.
The remaining variance not accounted for in Table 2 is attributable to individual-specific random environmental influences and random error. The levels of expression of COX-1, COX-2, mPGES-1, and TxA2 showed highly significant heritabilities, ranging from 0.406 to 0.634 after correcting for covariate effects. The proportion of the residual phenotypic variability accounted for shared household effects tended to be considerably smaller than that accounted for genetic effects. This indicates that genes are important in determining the expression of these traits.

Both parameters of platelet function (PFA-collagen/epinephrine and PFA-collagen/ADP) showed significant heritabilities. This indicates that genes are important in determining the expression of these traits.

Between Traits

Phenotypic and Genetic Correlation

The expression value of all of the enzymes (COX-1, COX-2, mPGES-1, and TxA2) showed significant positive phenotype correlation among all of them (Table 3). Nevertheless, when phenotypic correlations were partitioned using a bivariate variance component model in terms of genetic and environmental correlations, the scenario was different. Indeed, no genetic correlation was observed between the COX-1, COX-2, mPGES-1, and TxA2.
expression of COX-1 and the expression of any other enzyme analyzed. In contrast, it is important to note that a significant genetic correlation was observed between COX-2 expression and mPGES-1 (P = 0.02). Surprisingly, a strong genetic correlation was found between COX-2 expression and TxA2 (P = 9.4 × 10^{-10}). Both COX-1 and COX-2 expression exhibited positive significant environmental correlation only with TxA2 expression. TxA2 and mPGES-1 also exhibited a significant genetic positive correlation (P = 3.2 × 10^{-10}).

Table 4 presents the significant probability values of correlations between the expression of enzymes and LPS-induced PGE2 production. No significant correlation between any enzyme and prostanoid production by whole blood was found, with the exception of COX-2 expression and LPS-induced PGE2 production, which showed a significant phenotypic correlation. It is noteworthy that this phenotypic correlation was mainly genetic in nature, because no environmental correlation was found in the partitioned analysis of the variance. Table 5 shows the regression coefficients and probability values of significant correlations between production of prostanoids by whole blood. Production of PGE2 strongly correlated with TxA2, irrespective of the parameters that were expressed in absolute or relative terms. The probability values from the bivariate variance component model indicated that environmental correlations were stronger than genetic ones in all cases.

**Discussion**

Identifying the genetic and environmental factors that influence susceptibility to complex human traits has been challenging, owing to the complexity of both genetic and environmental factors. In this context, the prostanoid biosynthetic pathway has been implicated in a variety of clinically
important areas (complex traits), mainly because of its proinflammatory and proatherogenic role. To our knowledge, this is the first report that evaluates the relative contributions of genetic and environmental influences to the prostanoid biosynthetic pathway-related phenotypes.

One of the most important components of prostanoid-pathways is the TxA2, where its production showed a high heritability in response to calcium ionophore and particularly when the phenotype is expressed in relative terms to platelet number or volume assuming that platelets are the major cells involved in the production of TxA2 in the whole blood test. This was consistent with the high heritabilities that were found for the phenotypes concerning the enzymes involved in the production of TxA2 in the whole blood test. This was also consistent with data reported by other authors. Bray et al estimated heritabilities of several parameters related with platelet function in white and black families with premature coronary artery disease. They found that aggregation in response to epinephrine and ADP was significant in both populations, whereas in response to collagen platelet aggregation was only highly heritable in the black population. In our study, based on a white population, we found that PFA-collagen/epinephrine is strongly determined by genes, at least in white populations.

In addition, the same group found high significant heritabilities of TxA2 production, both by whole blood stimulated with collagen and after low dose aspirin administration. Interestingly, we observed that TxAS and mPGES-1 also exhibited a significant positive genetic correlation, indicating that both enzymes might be regulated by a common set of genes in certain cell type, probably monocytes. Unlike platelets, monocytes are able to express COX-2 and mPGES-1 in addition to COX-1 and TxAS. This could account for the strong genetic correlation between COX-2 and TxAS expression that we found. Interestingly, we observed that TxAS and mPGES-1 also exhibited a significant positive genetic correlation, indicating that both enzymes might be regulated by a common set of genes in certain cell type, probably monocytes. In our study, enzyme expression was determined in terms of mRNA. Hence, unlike TxA2 production, the contribution of monocytes was likely the most relevant regarding enzyme expression. In addition, basal levels of mRNA encoding COX-2, mPGES-1 and TxAS, showed highly significant heritabilities.

In addition to the cardinal features of inflammation, including pain, edema, and fever, PGE2 induces expression of matrix metalloproteinases, enzymes considered crucial in the degradation of plaque stability. It is also known that PGE2 inhibits the production of macromolecules in the extracellular matrix, further favoring plaque fragility. The first PGES isoenzyme identified and characterized was a 16-kDa protein with glutathione-dependent PGES activity, now called “mPGES-1.” This enzyme was inducible by

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>COX-2-Expression (Genetic)</th>
<th>COX-2-Expression (Environmental)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE2-LPS</td>
<td>0.20 (0.018)</td>
<td>0.35 (0.081)</td>
</tr>
<tr>
<td>PGE2-LPS/10⁶ leu.</td>
<td>0.18 (0.038)</td>
<td>0.37 (0.043)</td>
</tr>
<tr>
<td>PGE2-LPS/10⁶ mon.</td>
<td>0.19 (0.030)</td>
<td>0.36 (0.041)</td>
</tr>
</tbody>
</table>

Phenotypical correlations were decomposed using a bivariate variance component model in terms of genetic and environmental correlations. All significant correlations were positive and are highlighted.
proinflammatory cytokines. Functional coupling of mPGES-1 with COX-2 was reported earlier, although now this cannot be generalized. Conversely, a cytosolic-PGES (cPGES) seems to act functionally coupled with COX-1, even though this cannot be generalized also. The latter enzyme is ubiquitously expressed and identical to p23, a protein somewhat related to steroid hormone receptor-mediated signal transduction. Another type of microsomal-PGES called “mPGES-2” was further characterized and was reported to use PGH2 generated by both COX-1 and COX-2 activities.

Our previous results indicate that mPGES-1 is the main isoenzyme involved in PGE2 biosynthesis under inflammatory conditions. It is widely accepted that during inflammation there is an increased production of PGE2 attributable to the action of COX-2/mPGES-1. COX-2 is detectable after vascular damage and is highly expressed in atherosclerotic lesions. In addition, expression of COX-2 in VSMCs has contributed to abdominal aortic aneurism (AAA) in mice. Suppression of mPGES-1 increases PGI2 biosynthesis in VSMCs, when COX activity is not the limiting step. This is consistent with the fact that suppression of mPGES-1 depresses systemic PGE2 biosynthesis. Upregulation of mPGES-1 has been found in symptomatic atherosclerotic carotid plaques, associated with elevation of metalloproteinase-2 and -9. Increased mPGES-1 levels were found in atherosclerotic plaques in diabetic patients when compared to nondiabetics. Recently it has been reported that mPGES-1 deletion suppresses experimental AAA in mice. Hence the data point to a pathophysiological role of COX-2/mPGES-1 pathway in cardiovascular disease. Our data showed a significant phenotypic correlation between basal expression of COX-2 and LPS-induced PGE2 production. It is clear that this phenotypic correlation was mainly genetic in nature. Unfortunately, we could not determine mRNA levels of any enzyme after LPS stimulation of whole blood, because mRNA was highly degraded during incubation. Nevertheless, our results suggest that initial levels of COX-2 were essential for further LPS-induced PGE2 biosynthesis.

Our results document the importance of genetic factors that influence prostanoid-related phenotypes in this Spanish population. Genes appear to be the largest identifiable determinant of quantitative variation for all of the traits. The use of quantitative trait locus (QTL) mapping, accession numbers, early trivial names of enzymes, and nomenclature.

We are indebted to all of the families who participated in the GAIT Project.

Sources of Funding
This study was supported partially by grants No. 2 R01 HL070751-05 from the U.S. National Institutes of Health, PI-08/0420, PI-08/0756, SAF2008/01859 and RECAVA-RD06/0014. J.M.S. was supported by “Programa d’Establització d’Investigadors de la Direcció d’Estratègia i Coordinació del Departament de Salut” (Generalitat de Catalunya).

Disclosures
None.

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Arterioscler Thromb Vasc Biol. published online October 22, 2009;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplement Material

Material and Methods.

Enrollment of Family Members and Blood Collection. The Spanish families available for our studies were a new set of extended families from the GAIT (Genetic Analysis of Idiopathic Thrombophilia) Project [1,2]. This new set of families included 308 individuals belonging to 15 extended families. The criteria for inclusion in the study have been described previously [1,2]. Briefly, recruitment of family members was based in Barcelona. To be included, a family had to have at least 10 living individuals in three or more generations. Families were selected through a proband with idiopathic thrombophilia, which was defined as multiple thrombotic events (at least one spontaneous), a single spontaneous episode of thrombosis with a first-degree relative also affected, or onset of thrombosis before age 45. The proband’s thrombophilia was considered idiopathic because all known biological causes (e.g., antithrombin deficiency, Protein S and C deficiencies, activated protein C resistance, plasminogen deficiency, heparin cofactor II deficiency, Factor V Leiden, dysfibrogenemia, lupus anticoagulant, and antiphospholipid antibodies) of thrombophilia were excluded. These thrombophilic factors were also absent in all affected relatives. The subjects were interviewed by a physician to determine their health and reproductive history, current medications, alcohol consumption, physical activity, and use of sex hormones (oral contraceptives or hormonal replacement therapy). They were questioned also about previous episodes of venous or arterial thrombosis and the age at which these events occurred, as well as potentially correlated disorders such as diabetes and lipid disease. All procedures were reviewed by the Institutional Review Board of the Hospital de Sant Pau (Barcelona). Adult subjects gave informed consent for themselves and for their minor children. The depth and complexity of these pedigrees is illustrated by the number of relative pairs contained therein (supplement Table I).

From a total of 331 individuals, those that took steroids, were excluded from the enzyme expression and prostanoid levels study. In addition those that took platelet anti-aggregation drugs and/or non-steroid anti-inflammatory drugs within the 15 days previous to the blood
extraction were also excluded from prostanoid level study. Thus, the remaining 308 individuals were included in the analysis of enzyme expression and 255 of them for prostanoid biosynthesis.

A sample of 10 mL of peripheral venous blood was collected from all participants in heparin-containing tubes and aliquoted for mRNA analysis and for the whole blood assays. Total mRNA extraction and storage were performed according to standard protocols. Standard EDTA and citrate samples were obtained for performing whole blood cell counts and platelet function analyses.

**Whole blood cell counts:** were determined in the standard hematology analyzer Sysmex XE-2100® (Roche Diagnostics). The results obtained included: total leukocyte count, absolute monocyte count, platelet count, and plateletcrit or total platelet volume.

**COX-1, COX-2, TxA5 and mPGES-1 mRNA analysis.** 3.5 mL of anticoagulated peripheral venous blood was centrifuged at 1300xg for 10 min at room temperature. The pellet was washed with PBS buffer at pH 7.4. Lysis of erythrocytes was achieved by adding 50 ml of Tris-HCl 20 mM, 5 mM MgCl$_2$, 6 H$_2$O pH 7.5 to the pellet and incubating for 10 min in an ice-water bath. After centrifugation at 1600xg for 15 minutes, the pellet was washed with Tris-HCl. The cell pellet was collected in 1 ml of Ultraspec (Biotecx Laboratories, Inc, Houston, Texas, USA) and stored at –80°C.

For mRNA analysis, total RNA was extracted from the cell pellet according to the manufacturer's instructions. Reverse transcription was performed with 1 μg of RNA per 20 μL reaction as previously described [3]. The mRNA expression of COX-1, COX-2, TxA5 and mPGES-1 genes was determined by Real-Time-PCR in a ABI Prism 7000 using pre-designed validated assays (TaqMan Gene Expression Assays; Applied Biosystems) and universal thermal cycling parameters. Relative mRNA expression was expressed as transcript/β-actin ratios.
Whole Blood Assays. An aliquot of 1.5 mL of heparinized whole blood was warmed at 37°C prior addition of an ethanolic solution of calcium ionophore A23187 (Sigma) to yield a final concentration of 25 μmoles/L. Blood was then incubated at 37°C for 10 min. Afterwards, the blood was immediately centrifuged at 4°C and the supernatant was stored at –80°C until analyzed. TxA₂ production was estimated by the difference of TxB₂ levels after and before stimulation with A23187. TxB₂ before stimulation was measured in the plasma obtained as described above and, in general, no levels were detectable.

Bacterial lipopolysaccharide (LPS, from Escherichia coli Serotype 055:B5, Sigma) to yield a final concentration of 10 μg/mL was added to another aliquot of 3.5 mL of heparinized whole blood and was then incubated at 37°C for 24 hours. Afterwards the blood was immediately centrifuged at 1300xg for 10 min at room temperature and the supernatant stored at –80°C until analyzed.

Prostanoid Analysis. PGE₂ and TXB₂ were analyzed by specific enzyme immunoassays (EIA, PGE₂ GE Healthcare and TXB₂ Caymam Chemical) following the manufacturer’s instructions.

Platelet function analysis: Platelet adhesion and aggregation under high shear rate was tested with the platelet functional analyzer PFA® (Dade Behring) using cartridges containing membranes coated with collagen-epinephrine (COL-EPI) and coated with collagen-ADP (COL-ADP). The results were the closure times (CT) of the pores included in the membranes, expressed in seconds.

Statistical Methods. The statistical methods used in our study have been described previously [4,5]. Briefly, the phenotypic covariance among relatives was used to estimate the additive genetic and shared environmental components of variance. Fixed effects included female sex, age, smoking and depending on traits, use of non-steroid anti-inflammatory drugs and number of leukocytes and platelets. Discrete covariate (female sex) was scaled so that the regression coefficients represented the effect of the covariate versus its absence.
Maximum likelihood methods were used to estimate simultaneously the means and variances as well as the effects of covariates, heredity, and household effect, using the computer package SOLAR [6]. The significance of covariate effects as well as the significance of genetic and household effects was assessed by means of the likelihood ratio test.

To investigate the correlation between quantitative variations of phenotypes, in the pedigree-based, maximum-likelihood method were used to analyze both quantitative phenotypes jointly using a continuous trait variance component technique [7] and the SOLAR computer package. This analysis allowed the phenotypic correlations among these traits to be partitioned into factors due to common genetic influences and common environmental influences [6]. Because the pedigrees were ascertained through a thrombophilic proband, all analyses included an ascertainment correction to allow unbiased estimation of parameters relevant to the general population. To achieve this, the likelihood for each family ascertained through a thrombophilic proband was conditioned on the phenotype of the proband.

References


**Supplement Table I.** Description of the family members relationship based on the number of relative pairs.

<table>
<thead>
<tr>
<th>N</th>
<th>Relation</th>
<th>Degree of relation</th>
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<td>Self</td>
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<tr>
<td>310</td>
<td>Parent-offspring</td>
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</tr>
<tr>
<td>154</td>
<td>Siblings</td>
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<td>127</td>
<td>Grandparent-grandchild</td>
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<tr>
<td>367</td>
<td>Avuncular</td>
<td>2</td>
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<tr>
<td>6</td>
<td>Great grandparent-grandchild</td>
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<tr>
<td>103</td>
<td>Grand avuncular</td>
<td>3</td>
</tr>
<tr>
<td>317</td>
<td>1st cousins</td>
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<td>269</td>
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