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Induction of Vascular GTP-Cyclohydrolase I and Endogenous Tetrahydrobiopterin Synthesis Protect Against Inflammation-Induced Endothelial Dysfunction in Human Atherosclerosis

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Disclosures

None.

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Abstract

Background—The endothelial nitric oxide synthase cofactor tetrahydrobiopterin (BH4) is essential for maintenance of enzymatic function. We hypothesized that induction of BH4 synthesis might be an endothelial defense mechanism against inflammation in vascular disease states.

Methods and Results—In Study 1, 20 healthy individuals were randomized to receive *Salmonella typhi* vaccine (a model of acute inflammation) or placebo in a double-blind study. Vaccination increased circulating BH4 and interleukin 6 and induced endothelial dysfunction (as evaluated by brachial artery flow-mediated dilation) after 8 hours. In Study 2, a functional haplotype (X haplotype) in the *GCHI* gene, encoding GTP-cyclohydrolase I, the rate-limiting enzyme in bipterin biosynthesis, was associated with endothelial dysfunction in the presence of high-sensitivity C-reactive protein in 440 coronary artery disease patients. In Study 3, 10 patients with coronary artery disease homozygotes for the *GCHI* X haplotype (XX) and 40 without the haplotype (OO) underwent *S Typhi* vaccination. XX patients were unable to increase plasma BH4 and had a greater reduction of flow-mediated dilation than OO patients. In Study 4, vessel segments from 19 patients undergoing coronary bypass surgery were incubated with or without cytokines (interleukin-6/tumor necrosis factor- α /lipopolysaccharide) for 24 hours. Cytokine stimulation upregulated *GCHI* expression, increased vascular BH4, and improved vasorelaxation in response to acetylcholine, which was inhibited by the GTP-cyclohydrolase inhibitor 2,4-diamino-6-hydroxypyrimidine.

Conclusions—The ability to increase vascular *GCHI* expression and BH4 synthesis in response to inflammation preserves endothelial function in inflammatory states. These novel findings identify BH4 as a vascular defense mechanism against inflammation-induced endothelial dysfunction.

Keywords

tetrahydrobiopterin; inflammation; C-reactive protein; tetrahydrobiopterin; endothelial nitric oxide synthase; GTP-cyclohydrolase

Loss of normal endothelial function is an independent risk factor for atherosclerosis.¹ In human vascular disease states, systemic inflammation leads to impaired endothelial function through multiple mechanisms that involve increased vascular oxidative stress, activation of redox-sensitive transcriptional pathways, and reduced endothelial nitric oxide synthase (eNOS) function.^{2–4} Systemic inflammation is associated with increased generation of reactive oxygen species in the vascular wall⁵ and reduced endothelial function.⁶ However, the mechanisms that link endothelial dysfunction with inflammation in human vascular disease remain incompletely understood. In particular, it is not known whether endothelial function is an inevitable consequence of systemic inflammation or whether local mechanisms in the vascular wall can protect or modify the vascular response to systemic inflammation.

Endothelial nitric oxide (NO) generation by eNOS requires the enzymatic cofactor tetrahydrobiopterin (BH4). In vascular disease states, BH4 bioavailability is reduced, which leads to enzymatic uncoupling of eNOS, resulting in generation of O₂⁻ instead of NO.^{7,8} Vascular BH4 bioavailability depends on biosynthesis through a biochemical pathway in which GTP-cyclohydrolase (GTPCH) is the rate-limiting enzyme.^{9,10} The expression of *GCHI*, which encodes GTPCH, is induced by inflammation in cell culture models.^{11–13} Serum neopterin, a by-product of the same pathway, is considered to be an inflammatory marker with predictive value for the progression of coronary artery disease,¹⁴ and has been associated with increased cardiovascular risk.¹⁵ Previous studies in isolated cells demonstrated that haplotypes of the *GCHI* gene are associated with significant differences in *GCHI* expression and BH4 levels.¹⁶ The effect of this haplotype on *GCHI* expression in immortalized human mononuclear cells was revealed only after inflammatory stimulation,¹⁶ which suggests that this haplotype may affect the response of *GCHI* gene to proinflammatory stimulation.

Previous studies suggest a complex association between plasma biopterins, inflammation, and endothelial function in humans with coronary artery disease.⁷ Indeed, plasma BH4 is associated with high-sensitivity C-reactive protein (hsCRP) levels, but is inversely correlated with endothelial function.⁷ In contrast, vascular tissue BH4 is positively associated with endothelial function,⁷ and its oral supplementation may improve endothelial function.¹⁷ Thus, maintenance of vascular BH4 in response to inflammation may be an important protective factor in the endothelium, as recently proposed by Katusic et al.¹⁸ We sought to use controlled inflammatory stimuli and genetic differences in *GCHI* to investigate the relationships between tetrahydrobiopterin, endothelial function, and the vascular response to inflammation in both healthy subjects and patients with coronary artery disease.

Methods

Study Population and Protocol

We conducted 4 studies, as described below. All studies were approved by the Local Research Ethics Committees, and each subject gave written informed consent.

Study 1—To test the relationship between inflammation and BH4 levels, 20 healthy young individuals were randomly allocated to receive either vaccination with *Salmonella typhi*

capsular polysaccharide or placebo (normal saline) in a double-blind design. No subjects were receiving nonsteroidal antiinflammatory drugs, dietary supplements of folic acid, or antioxidant vitamins. Subjects had fasted for at least 12 hours, and had abstained from caffeine, ethanol, and flavonoid-containing beverages. At baseline, flow-mediated dilation (FMD) of the brachial artery was estimated, and blood samples were obtained. Next, all subjects received either *S typhi* capsular polysaccharide vaccine (0.025 mg Typhim Vi, Pasteur Merieux MSD; n=10) or placebo (normal saline, n=10) intramuscularly, as described previously.¹⁹ FMD and blood sampling were repeated at 8, 12, and 24 hours to define the kinetics of inflammatory responses.

Study 2—In this study, 1182 patients with coronary artery disease were genotyped for the *GCHI* haplotype. In this screening, 864 patients (73.1%) were OO, 287 (24.3%) were XO, and 31 (2.6%) were XX, in accordance with the Hardy-Weinberg distribution.

We then examined whether *GCHI* haplotype affected endothelial function of the brachial artery and plasma biopterin levels and whether the background low-grade inflammation had an impact on these associations. In this analysis, we included 440 patients from the prescreened patients, as shown in Table. Exclusion criteria from this study arm were any inflammatory, infective, liver, or renal disease; malignancy; acute coronary event during the previous 2 months; or clinically overt heart failure. Patients receiving nonsteroidal antiinflammatory drugs, dietary supplements of folic acid, or antioxidant vitamins also were excluded.

Patients underwent noninvasive evaluation of endothelial function in the brachial artery by estimation of FMD and endothelium-independent vasorelaxation to sublingual nitroglycerin. Blood samples were obtained for measurement of markers of systemic inflammation and oxidative stress and plasma biopterins.

Study 3—On the basis of the results from Study 1 and the observations from Study 2, we designed Study 3 to examine whether the *GCHI* haplotype had an impact on the inflammation-induced changes of plasma biopterins and arterial endothelial function in patients with coronary artery disease. For this study, we recruited 50 patients from the cohort included in Study 2, according to *GCHI* haplotype: 10 homozygotes for the *GCHI* X haplotype and 40 OO homozygotes. Patients were excluded if there was a history of previous infection with, or vaccination against, *S typhi*. Patients underwent evaluation of endothelial function by estimation of FMD and by blood sampling at baseline and 8 hours after vaccination with *S typhi* capsular polysaccharide vaccine.

Study 4—To further investigate the mechanisms relating acute inflammation to the regulation of vascular biopterins and NO bioavailability in the human vascular wall, we used an ex vivo model of human saphenous veins (SVs) and internal mammary arteries (IMAs). For these experiments, we recruited 19 patients undergoing coronary artery bypass grafting. During surgery, samples of SVs and IMAs were obtained and transferred to the laboratory within 30 minutes, as described previously.²⁰

Evaluation of Effects of Inflammation on *GCH1* Expression and Vascular

Biopterin Levels—Four sequential rings from the same SV and IMA were incubated for 24 hours ex vivo in sterile media (HBSS containing HEPES, 1% penicillin streptomycin, and 1% amphotericin B) in the absence (control) or presence of the GTPCH inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP; 1 mmol/L), in the presence of tumor necrosis factor- α (TNF- α ; 4 ng/mL) plus interleukin-6 (IL-6; 25 ng/mL) and lipopolysaccharide (LPS; 80 ng/mL) or TNF- α (4 ng/mL) plus IL-6 (25 ng/mL) plus LPS (80 ng/mL) plus DAHP. At the end of the incubation period, these vascular rings were frozen in -80°C for biopterin measurements and for gene expression studies.

Evaluation of Effects of Inflammation on Endothelial Function Ex Vivo

—Four additional rings of the same SVs were incubated for 24 hours ex vivo in the absence (control) or presence of DAHP (1 mmol/L), with or without stimulation by TNF- α 4 ng/mL plus IL-6 25 ng/mL plus LPS 80 ng/mL. After incubation, vascular rings were transferred to an organ bath to evaluate endothelial function by quantification of vasomotor responses to acetylcholine and sodium nitroprusside.

Quantification of Endothelial Function by FMD

FMD and endothelium-independent vasodilation of the brachial artery were measured as described previously,²¹ with a linear-array transducer and automated offline analysis (Vascular Analyzer; Medical Imaging Applications LLC). In brief, brachial artery diameter was recorded before and 60 seconds after a 5-minute forearm blood flow occlusion. Another measurement was made 3 minutes after a sublingual spray of nitroglycerin was given (200 μg). FMD and endothelium-independent dilation of the brachial artery were recorded as the change in vessel diameter after forearm ischemia and sublingual nitroglycerin, respectively.²¹

DNA Extraction and Genotyping

Genomic DNA was extracted from whole blood by standard methods (QIAamp DNA blood Midi kit; Qiagen). The DNA samples were screened for that particular functional *GCH1* haplotype, identified as being associated with decreased *GCH1* expression and BH4 production in patients with coronary artery disease.²² The diagnosis of the particular *GCH1* haplotype of present interest is possible with 100% sensitivity and specificity by screening for 3 single-nucleotide polymorphisms in the *GCH1* gene that span the entire DNA range of the haplotype: c. -9610G \rightarrow A (dbSNP rs8007267G \rightarrow A) in the 5' untranslated region, c. 343 \rightarrow 8900A \rightarrow T (dbSNP rs3783641A \rightarrow T) in intron 1, and c.*4279 (dbSNP rs10483639C \rightarrow G) in the 3' untranslated region.²² Thus, for diagnostic purposes, the haplotype of interest reduces to rs8007267A/rs3783641T/rs10483639G. Genotyping assays for each of the 3 single-nucleotide polymorphisms above were custom designed and run with KASPar genotyping chemistry (KBioscience). Assays were run according to the manufacturer's conditions with 10 to 20 ng of genomic DNA in a final volume of 4 μL in a 384-well plate format, then run on an ABI 7900HT real-time PCR system (Applied Biosystems). Haplotypes were imputed from the genotyping data with PHASE v2 software, and diplotypes were assigned to each individual.

Blood Sampling and Biochemical Measurements

Venous blood samples were centrifuged at 2000g at 4°C for 15 minutes, and plasma or serum was collected and stored at -80°C until assayed.

Measurement of Plasma and Vascular Biopterins

Plasma and vascular BH4, BH2, and biopterin levels were each determined separately from the same sample by high-performance liquid chromatography followed by serial electrochemical and fluorescent detection, as described previously.⁷ Total biopterins were quantified by summing BH4, dihydrobiopterin, and biopterin. Biopterin levels were expressed as nanomoles per liter in plasma and picomoles per gram in vascular tissue.

Measurement of Systemic Inflammatory Markers

Plasma hsCRP was measured by particle-enhanced immunonephelometry (N Latex; Dade-Behring Marburg). Serum IL-6 was determined by enzyme-linked immunosorbent assay (ELISA kits by R&D Systems).

Measurements of Serum Lipid Levels

Serum total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides were measured by a chromatographic enzymatic method in a Technicon automatic analyzer RA-1000 (Dade Behring).

Vasomotor Studies

Endothelium-dependent and -independent dilatations were assessed with isometric tension studies, as we have described previously.²⁰ Vessel rings were equilibrated and passively pretensioned to 3 g, an optimal resting tension that was determined in baseline studies of contractile response to KCl. After precontraction with phenylephrine (3×10^{-6} mol/L), vasomotor responses to the endothelium-mediated agonist acetylcholine (10^{-9} to 10^{-5} mol/L) were quantified in the 4 equally sized segments from the same vessel, as described in the previous paragraph. Finally, relaxations to the NO donor sodium nitroprusside (10^{-10} to 10^{-6} mol/L) were evaluated in the presence of the NOS inhibitor *N*^G-nitro-L-arginine methyl ester (100 μmol/L).

Ribonucleic Acid Isolation and Quantitative Real-Time Polymerase Chain Reaction

Four serial rings of the same vessels (SVs and IMAs from 9 patients) from the ex vivo protocol were initially lysed in TRIzol reagent (Tri-Reagent, Sigma-Aldrich), followed by RNA purification from the aqueous phase with the RNeasy Micro kit (Qiagen). RNA was converted into cDNA (Superscript II reverse transcriptase; Invitrogen), then subjected to quantitative real-time polymerase chain reaction using the TaqMan system (Applied Biosystems; assay ID GCH Hs00609198-m1, assay ID GAPDH Hs02758991-g1, assay ID eNOS Hs01574659-m1, and assay ID iNOS Hs01075529-m1) and analyzed on an iCyclerIQ (Bio-Rad). Relative expression was calculated by the 2^{-CT} method (normalized to GAPDH).

Statistical Analysis

All continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Nonnormally distributed variables were log-transformed for analysis, and are presented as logs of the actual values. Normally distributed variables (raw or log-transformed) are presented as mean±SEM. Sample size calculations in studies 1 and 3 were based on pilot experiments and our previous work,^{7,22} and showed that with 10 patients per group, we would be able to detect a within-group change of log[BH4] of 0.438 nmol/L from baseline to postintervention levels, when the SD of the difference in the response was 0.38 nmol/L, with 90% power and $\alpha=0.05$. Similarly, in Study 1, with 10 patients per group, we would be able to detect a difference of the changes in log[BH4] between the 2 groups of 0.583 nmol/L when the SD of the mean change in the vaccinated group was 0.38 nmol/L, with 90% power and $\alpha=0.05$. For Study 3, with 10 patients in the XX group and 40 OO patients (controls), we would be able to detect a difference of the changes in log[BH4] between the 2 groups in response to vaccination of 0.445 nmol/L when the SD of the mean change in the XX group was 0.38nmol/L, with 90% power and $\alpha=0.05$. In study 2, we hypothesized that the proportion of OO versus XO+XX would be 3:1; therefore, with 75 OO and 25 XO+XX, we would be able to detect a difference in plasma log[BH4] of 0.389 nmol/L between the 2 groups when the SD of log[BH4] in the XX group was 0.320 nmol/L, with $\alpha=0.05$ and 90% power. However, we increased this number to 280 OO and 96 XO+XX to allow comparisons within each tertile of plasma hsCRP. For Study 4, sample size calculations based on our previous work^{20,23,24} showed that with n=9, we would be able to detect a 51% change in maximum vasorelaxation to acetylcholine when SD was 31%, with $\alpha=0.05$ and 90% power.

Baseline demographic characteristics (categorical variables) were compared between groups within each study arm by the χ^2 test. Continuous variables were compared between groups by unpaired *t* test or 1-way ANOVA for multiple comparisons followed by Bonferroni post hoc correction as appropriate. Comparisons of the responses to vaccination between groups were performed by 2-way ANOVA for repeated measures (with time-by-genotype interaction), as appropriate. Within-group comparisons (versus baseline) were performed by paired *t* test followed by Bonferroni post hoc correction. In Study 2, comparisons between the genotypes across the 3 tertiles of hsCRP were performed initially by 2 way ANOVA for independent samples, and then within each tertile comparisons were performed by using unpaired *t* test followed by Bonferroni post hoc correction. For the ex vivo incubations with proinflammatory stimuli, an overall repeated-measures test was performed, followed by individual comparisons after Bonferroni correction. For the vasomotor studies, vasorelaxation curves were compared by 2-way ANOVA for repeated measures (with concentration-by-group interaction).

To examine whether the association between *GCHI* haplotype and plasma biopterins was independent of patients' demographic characteristics and background inflammation in Study 2, we performed linear regression in which log(total biopterins) or log(BH4) was included as a dependent variable, and as independent variables, we included log(hsCRP), the presence of the X haplotype, and those of the patients' demographic characteristics (age, sex, diabetes mellitus, hypertension, smoking, and dyslipidemia) that showed an association with the dependent variable in bivariate analysis at the level of 15%. A backward-elimination

procedure was applied to all models, with $P>0.1$ as a threshold to remove a variable from the model. All probability values were 2-tailed, and $P<0.05$ was considered statistically significant. All statistical analyses were performed with SPSS 18.0 (SPSS Inc).

Results

The characteristics of participants in the 4 studies are presented in Table 1.

Study 1: Effects of Acute Inflammation on Plasma Biopterins and Endothelial Function

After the initial observation that plasma biopterins are associated with inflammatory markers such as hsCRP in patients with coronary atherosclerosis,⁷ we first examined the direct relationship between inflammation and plasma biopterins. Twenty healthy volunteers were randomized to receive *S typhi* vaccine or placebo (normal saline) by intramuscular injection. This intervention was used as a model of acute inflammation, as we have described previously,¹⁹ to evaluate the effect of inflammation on circulating biopterins and endothelial function.

We observed that vaccination induced a rapid elevation of serum IL-6 by 3-fold at 8 hours ($P<0.01$ versus baseline), an effect that persisted through 12 hours ($P<0.01$ versus baseline) but that returned to baseline levels by 24 hours after vaccination ($P=NS$ versus baseline; Figure 1). Levels of hsCRP were elevated by 3-fold 24 hours after vaccination ($P<0.05$ versus baseline), but not at 8 hours or 12 hours ($P=NS$ versus baseline for both; Figure 1). Importantly, FMD in the brachial artery was reduced by approximately 50% at 8 hours ($P<0.01$ versus baseline) and remained reduced at 12 hours ($P<0.05$ versus baseline), following the same pattern as IL-6 (Figure 1).

We next tested the effects of vaccination on plasma biopterins. We observed that acute inflammation induced a striking elevation of all plasma biopterins by more than 2-fold at 8 hours ($P<0.01$ versus baseline; Figure 2). Plasma BH4 and total biopterins remained higher than baseline at 12 and 24 hours ($P<0.01$ for all; Figure 2). This striking effect supports the notion that the rapid elevation of plasma biopterins is a direct consequence of inflammation but is temporally associated with impairment of endothelial function after a systemic inflammatory stimulus.

Study 2: Impact of Low-Grade Inflammation on Regulation of Plasma Biopterins by the GCH1 Haplotype

Having demonstrated that an acute inflammatory stimulus induces an elevation of plasma biopterins in parallel with impairment of endothelial function, we next examined whether genetic variation in the *GCH1* gene is associated with altered response of plasma biopterins to chronic inflammation in patients with coronary artery disease. There was no significant effect of the *GCH1* haplotype on endothelial function in the population of Study 2 ($P=NS$ for all versus baseline; Figure 3). However, in subjects with plasma hsCRP levels in the upper tertile, indicating higher systemic inflammation, carriers of the *GCH1* X haplotype had significantly lower plasma BH4 and total biopterin levels than O homozygotes ($P<0.05$ for both; Figure 4). Furthermore, in these subjects, the *GCH1* X haplotype was associated with 40% lower FMD ($P<0.05$ versus O homozygotes; Figure 4). In contrast, there were no

associations between *GCHI* haplotype, plasma biopterins, and endothelial function in subjects in lower tertiles of hsCRP ($P=NS$ between genotypes for all; Figure 4). In multivariable analysis, the only independent predictors of log(plasma BH4) were log(hsCRP) (β [SE]: 0.112 [0.05], $P=0.029$) and the presence of the X haplotype (β [SE]: -0.181 [0.058], $P=0.002$). The only independent predictors of plasma log(total biopterins) were the presence of the X haplotype (β [SE]: -0.101 [0.05], $P=0.003$), smoking (β [SE]: 0.04 [0.02], $P=0.02$), and plasma log(hsCRP) (β [SE]: 0.056 [0.03], $P=0.06$).

Importantly, when the population was split according to the presence of the X haplotype, plasma log(hsCRP) was significantly correlated with plasma log(BH4) ($r=0.173$, $P=0.008$) and plasma log(total biopterins) ($r=0.164$, $P=0.013$) only among patients with the OO genotype, whereas there was no significant correlation between hsCRP and biopterin levels among carriers of the X haplotype [for log(BH4) $r=-0.018$, $P=0.863$ and for log(total biopterins) $r=-0.049$, $P=0.636$]. Total biopterins derive from the sum of BH4 and its oxidation products, dihydrobiopterin and biopterin, with BH4 accounting for >60% of total biopterins in the plasma. Therefore, because of the biological similarities between BH4 and total biopterins, no statistical correction for multiple testings was necessary for the above-mentioned simple correlations between these pterin species and hsCRP. These observations suggest that *GCHI* haplotype is an important determinant of the response of *GCHI* to proinflammatory stimulation. Accordingly, *GCHI* haplotype may be used to model endogenous variation of *GCHI* expression, and BH4 levels, in response to inflammation.

Study 3: *GCHI* Haplotype as a Model of Inability to Increase Plasma Biopterins in Response to Inflammation

Because we demonstrated that acute inflammation increases plasma biopterins (Study 1) and that the association between *GCHI* haplotype and plasma biopterins is observed only in the presence of high background inflammation (Study 2), we next used *GCHI* haplotype to investigate the hypothesis that the upregulation of *GCHI* expression and BH4 levels may modulate the response of the vascular wall to inflammation.

We recruited 50 patients with coronary artery disease, selected according to *GCHI* haplotype (40 OO and 10 XX homozygotes) identified by genotyping 1182 patients in Study 2. In Study 3, these patients underwent vaccination with *S typhi* vaccine, with reevaluation of plasma biopterins and endothelial function after 8 hours, as previously defined in Study 1. We observed that vaccination induced a similar elevation in serum IL-6 by 3-fold at 8 hours in both O and X homozygotes ($P<0.05$ versus baseline for both; Figure 5). As expected, hsCRP remained unchanged 8 hours after vaccination ($P=NS$ versus baseline; Figure 5). However, vaccination induced a significant elevation of plasma BH4 and total biopterins in O homozygotes ($P<0.01$ versus baseline for both) but had no effect on plasma biopterins in XX homozygotes ($P=NS$ versus baseline for both; Figure 6), which indicates that the *GCHI* haplotype is a key determinant of BH4 synthesis in response to inflammation. We next examined the impact of *GCHI* haplotype and the associated difference in BH4 synthesis on the change in endothelial function induced by acute inflammation. Vaccination induced a borderline decrease of FMD in O homozygotes ($P=NS$ versus baseline), whereas FMD was reduced by 60% in X homozygotes 8 hours after vaccination ($P<0.05$ versus baseline; Figure

6). Endothelium-independent vasodilation in response to nitroglycerin remained unchanged after vaccination in both OO and XX genotypes ($P=NS$ versus baseline for both; Figure 6). The change in FMD after vaccination was significantly different between the 2 *GCHI* genotypes ($P=0.03$), which suggests that differences in *GCHI* expression and BH4 synthesis are able to modify the vascular response to acute inflammation in humans.

Study 4: Vascular Upregulation of *GCHI* Gene in Response to Cytokines Stimulation and Endothelial Function

To further investigate the relationships between inflammation, *GCHI* expression, BH4 levels, and endothelial function, we next studied human vessels, obtained from patients undergoing coronary artery bypass grafting, to evaluate the effect of cytokine stimulation on *GCHI* expression, vascular BH4, and NO bioavailability. We observed that IL-6/TNF- α /LPS stimulation of these vessels for 24 hours induced a significant upregulation of the *GCHI* gene by 3-fold in SVs and by 2-fold in IMAs ($P<0.05$ versus control for both), which resulted in a 3- to 4-fold increase in vascular BH4 in both vessel types (Figure 7). The presence of the GTPCH inhibitor DAHP prevented the cytokine-induced elevation of BH4 ($P=NS$ versus control) despite the same upregulation of *GCHI* expression ($P<0.05$ versus control for both vessel types; Figure 7). Cytokine stimulation induced a significant elevation of eNOS gene expression in IMAs (from 0.02 ± 0.01 in control and 0.07 ± 0.03 in control +DAHP [$P=NS$ versus control] to 0.17 ± 0.07 Ct in cytokine-stimulated vessel rings [$P<0.05$ versus control]), an effect that was not affected by DAHP (0.17 ± 0.07 Ct, $P<0.05$ versus control). In contrast, iNOS (inducible NO synthase) gene expression was undetectable in most of the vascular segments (SVs or IMAs) used for the study, even after cytokine stimulation.

The cytokine-induced upregulation of *GCHI* and the respective elevation of vascular BH4 in SV segments were associated with a striking improvement in NO bioavailability in these vessels, evaluated by the vasorelaxation in response to acetylcholine ($P<0.05$ for cytokine stimulation versus control; Figure 7). The effect of cytokine stimulation on vascular NO bioavailability was abolished in the presence of DAHP (Figure 7), which suggests that the effect was mediated through increased *GCHI* expression that led to increased BH4 biosynthesis. Cytokine stimulation had no impact on endothelium-independent vasodilation in response to sodium nitroprusside or on maximum vasoconstrictions in response to phenylephrine ($P=NS$ versus control for all; Figure 7).

Discussion

In the present study, we have demonstrated that plasma biopterins are increased in response to inflammation, and this inflammation-induced elevation of plasma biopterins is accompanied by a reduction of endothelial function. Using functional haplotypes of the *GCHI* gene as a model of altered *GCHI* induction in response to proinflammatory stimulation in vivo and ex vivo proinflammatory stimulation of human vessels, we have demonstrated the new concept that the ability to increase biopterin bioavailability is an endogenous defense mechanism of the vascular wall against chronic or acute inflammation.

BH4 is a critical cofactor of eNOS, necessary for the maintenance of its enzymatic coupling.^{8,25–28} Vascular BH4 deficiency leads to impaired endothelial function and increased vascular O₂⁻ generation in both experimental models^{28,29} and human vessels,⁷ whereas restoration of vascular BH4 bioavailability results in improved endothelial function.^{17,26,30,31} Because overexpression of GTPCH suppresses atherogenesis in animal models,^{32,33} this enzyme may be a therapeutic target in atherosclerosis.¹⁰ However, in cell culture models, GTPCH activity is upregulated when endothelial cells are exposed to a combination of IL-6, TNF- α , and LPS.^{11,34} Previous clinical studies, mainly in healthy subjects, suggested that increased plasma BH4 is associated with improved endothelial function,³⁵ but we have demonstrated that in patients with coronary atherosclerosis, plasma biopterins are positively correlated with C-reactive protein levels, which reflects systemic inflammation,⁷ in a manner previously described for neopterin, a metabolite produced by the same biochemical pathway.³⁶ Moreover, we observed that high plasma biopterins in these patients are associated with impaired endothelial function, whereas high vascular BH4 is a key determinant of improved endothelial function.⁷ These observations suggest complex associations between inflammation, plasma, and vascular biopterins and endothelial function that, until now, have lacked identification of cause and effect.

In the present study, we first used a model of acute inflammation in healthy individuals (vaccination with *S typhi* polysaccharide vaccine) to examine the impact of a specific inflammatory stimulus on plasma biopterins and endothelial function. We observed a striking elevation of plasma BH4 in parallel with the maximum elevation of IL-6 levels and the maximum impairment of endothelial function. Plasma BH4, IL-6, and endothelial function returned to baseline 24 hours after vaccination. Therefore, the present study demonstrates for the first time in humans that acute inflammation rapidly increases plasma BH4, but this is accompanied by endothelial dysfunction. Given the important beneficial role of BH4 in endothelial function, highlighted by previous animal studies, we then hypothesized that this close association of plasma BH4 with inflammation may represent a defense mechanism against inflammation.

A recently described haplotype in the *GCHI* gene is associated with differences in both plasma and vascular BH4 levels in patients with coronary atherosclerosis.^{16,22} Because this haplotype affects the response of the *GCHI* gene to inflammatory stimulation,¹⁶ we used *GCHI* genotype as a model system to test the hypothesis that the ability of vascular endothelium to increase BH4 biosynthesis is an endogenous defense mechanism against inflammation. Indeed, we first observed that in a large cohort of patients with coronary atherosclerosis, the biological effect of the *GCHI* X haplotype (which resulted in reduced expression of *GCHI* and impaired NO bioavailability in the vascular wall) was seen only in those patients with elevated background inflammation (at the highest tertile of circulating hsCRP). Because of the small number of XX genotypes identified in the present cohort, XX and XO genotypes were analyzed in a recessive model, as we have done in the past,²² and this is a limitation of the present study. We then screened a large cohort of coronary artery disease patients to identify homozygotes for the *GCHI* X haplotype and performed a clinical study to test the effects of inflammation induced by *S typhi* vaccination in *GCHI* XX and OO homozygotes. In subjects with the XX genotype, inflammation failed to increase plasma biopterins, and these patients developed a greater reduction of endothelial function than OO

patients. These findings imply that the ability to increase biopterins in response to cytokine stimulation may be a key defense mechanism of vascular endothelium against systemic inflammation.

Evidence from basic science suggests that *GCHI* expression is induced after stimulation of endothelial cells with cytokines such as TNF- α and IL-6,¹³ whereas LPS also upregulates *GCHI* expression in a rat model of endotoxic shock.¹² *S typhi* vaccine is currently the only model system of acute low-grade inflammation in humans, and it mimics atherosclerosis-related elevation of IL-6 and IL-1R without affecting the TNF or CD14 expression that is characteristic in sepsis or LPS-injection models.¹⁹

The impact of low-grade inflammation on biopterin biosynthesis in humans is totally unexplored. Acute inflammation leads to endothelial dysfunction in humans,^{19,37} whereas plasma biopterins are elevated in patients with coronary atherosclerosis and inversely associated with endothelial function in the presence of increased background inflammation.⁷ These apparently contradictory findings regarding the role of biopterins in atherosclerosis-associated inflammation need to be explored.

In the present study, we exposed human vessels to a combination of cytokines (IL-6, TNF- α) and LPS for 24 hours to examine the direct impact of cytokine stimulation on endothelial NO bioavailability and vascular biopterins. Surprisingly, ex vivo vasorelaxations in response to acetylcholine were significantly increased by 2- to 3-fold after cytokine stimulation in this model system, an effect that was prevented by the presence of DAHP, an inhibitor of GTPCH. Indeed, in those experiments, the cytokine-induced increase of vascular NO bioavailability was mediated by an upregulation of *GCHI* gene expression and a marked elevation of vascular BH4, whereas inhibition of vascular BH4 biosynthesis with DAHP suppressed that effect. Systemic inflammation, induced by vaccination in the clinical study, suppressed endothelial function, whereas in the ex vivo model of cytokine-induced stimulation of vascular *GCHI*, without any inflammatory cell infiltration of the vascular wall, direct cytokine/LPS stimulation increased vascular NO bioavailability through a BH4-mediated mechanism. Although the conclusion of the clinical studies and the ex vivo model is the same (ie, inability to increase vascular BH4 by inflammation results in endothelial dysfunction), the use of LPS in the proinflammatory cocktail used in the ex vivo experiments reflects sepsis-related inflammation that may be less directly implicated in atherogenesis, so this is a limitation of the study. Although iNOS mRNA was not detected in these vessels (even after stimulation), eNOS gene expression was upregulated in IMA segments after stimulation (an effect that was not affected by DAHP). This observation is consistent with the literature showing that cytokine stimulation of human veins in vivo acutely upregulates eNOS gene expression (but not iNOS).³⁸ Importantly, in the present study, the improved vasorelaxation in response to acetylcholine in SV segments after cytokine stimulation was prevented by DAHP, which suggests that vascular BH4 is the key regulator of endothelial function under conditions of acute inflammation. Finally, in the present study, we used SVs for the vasomotor studies (instead of IMA) because of the limited tissue availability of IMA and the technical difficulties in their routine use in this type of experiments. We have previously shown good correlation between FMD and

vasorelaxations of SV segments to acetylcholine,³⁹ and this is now considered to be a reliable model system in which to study endothelial function in humans.

Taken together, the findings of the present study show that inflammation upregulates *GCHI* expression in the vascular wall, which leads to increased BH4 bioavailability that acts as a defense mechanism of the vascular endothelium against systemic inflammation. Plasma BH4 appears to be a marker of systemic inflammation and changes rapidly in response to acute inflammation. Inability to increase vascular GTPCH activity and BH4 biosynthesis in response to inflammation leads to a significant impairment of endothelial function. These novel findings demonstrate that plasma BH4 is a marker of systemic inflammation, whereas elevation of vascular BH4 is an endogenous defense mechanism that contributes to maintenance of endothelial function in response to systemic inflammation.

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Clinical Perspective

Vascular tetrahydrobiopterin (BH4) is an essential cofactor of endothelial nitric oxide synthase (eNOS), and its deficiency induces “uncoupling” of this enzyme, which leads to production of superoxide (O_2^-) instead of nitric oxide (NO). We hypothesized that BH4 synthesis is stimulated by inflammation, and this may serve as an endogenous defense mechanism of the vascular wall against systemic inflammation. To address this hypothesis, we performed 4 sequential studies. In Study 1, acute inflammation (induced by vaccination with *Salmonella typhi* vaccine) rapidly increased circulating BH4 in parallel with systemic inflammatory markers and impaired endothelial function in healthy individuals. In Study 2, we observed that a functional haplotype (X haplotype) in the *GCHI* gene, encoding GTP-cyclohydrolase I, the rate-limiting enzyme in BH4 biosynthesis, was associated with endothelial dysfunction in subjects with increased background inflammation. In Study 3, we screened a large cohort of patients with coronary atherosclerosis and recruited patients on the basis of their *GCHI* genotype to receive proinflammatory stimulation with *S typhi* vaccine. We observed that those with XX genotype were unable to increase plasma biopterins after vaccination, and these patients had a greater reduction of FMD than those with OO genotype. In Study 4, we used ex vivo models of human arteries and veins to demonstrate that the ability of these vessels to increase *GCHI* gene expression and improve BH4 bioavailability in response to cytokine stimulation preserves vascular endothelial function. These novel findings suggest that vascular BH4 may constitute a novel therapeutic target for the treatment of endothelial dysfunction in inflammatory states such as human atherosclerosis.

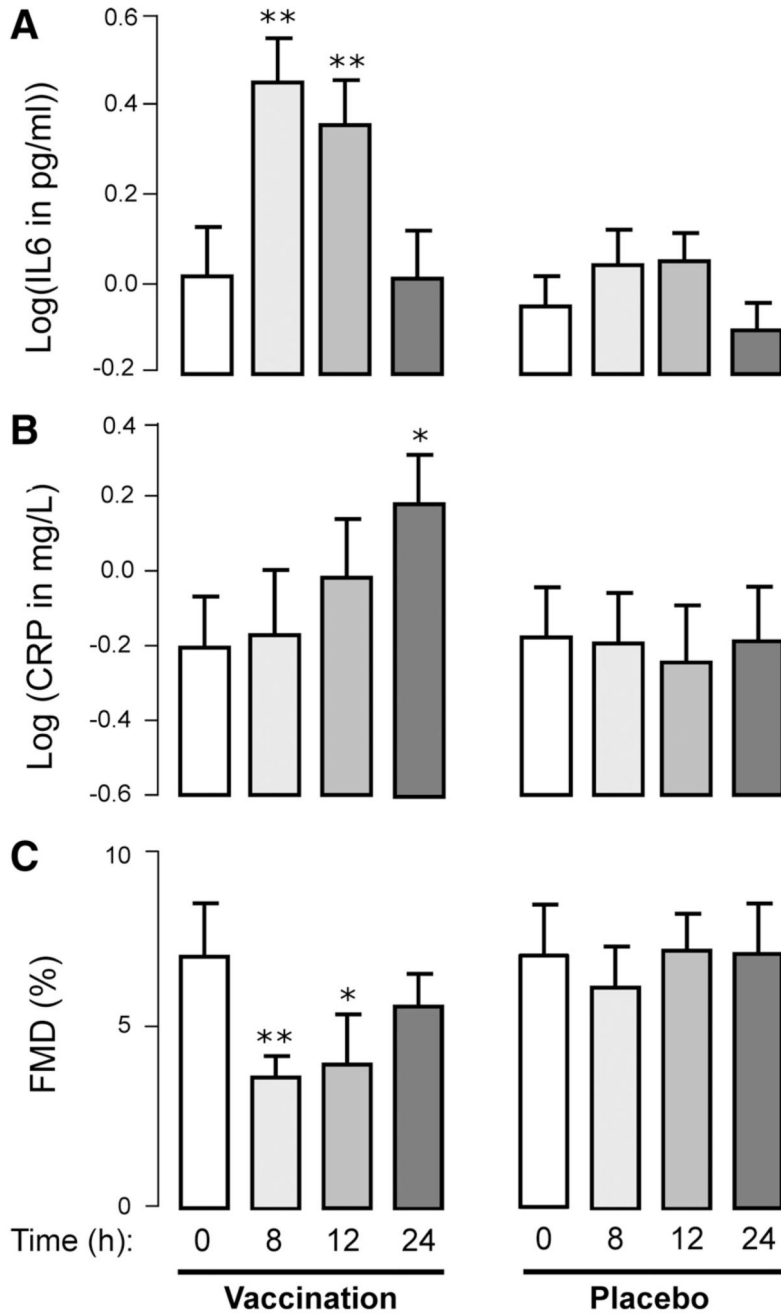


Figure 1. Vaccination with *Salmonella typhi* vaccine (n=10) induced a significant elevation of interleukin-6 (IL-6) at 8 and 12 hours, whereas IL-6 levels returned to baseline after 24 hours (A). Conversely, high-sensitivity C-reactive protein (CRP) levels were significantly increased only 24 hours after vaccination (B). Flow-mediated dilation (FMD) followed a similar pattern to that of IL-6, with a significant reduction at 8 and 12 hours after vaccination and a return to baseline at 24 hours (C). Infusion of placebo (n=10) had no significant effect on IL-6, CRP, or FMD over 24 hours. Values are expressed as mean±SEM. Two-way

repeated-measures ANOVA revealed a significant time-by-group interaction for IL-6 ($P<0.01$), CRP ($P<0.05$), and FMD ($P<0.01$). * $P<0.05$, ** $P<0.01$ vs baseline by paired t test followed by Bonferroni post hoc correction for 3 tests.

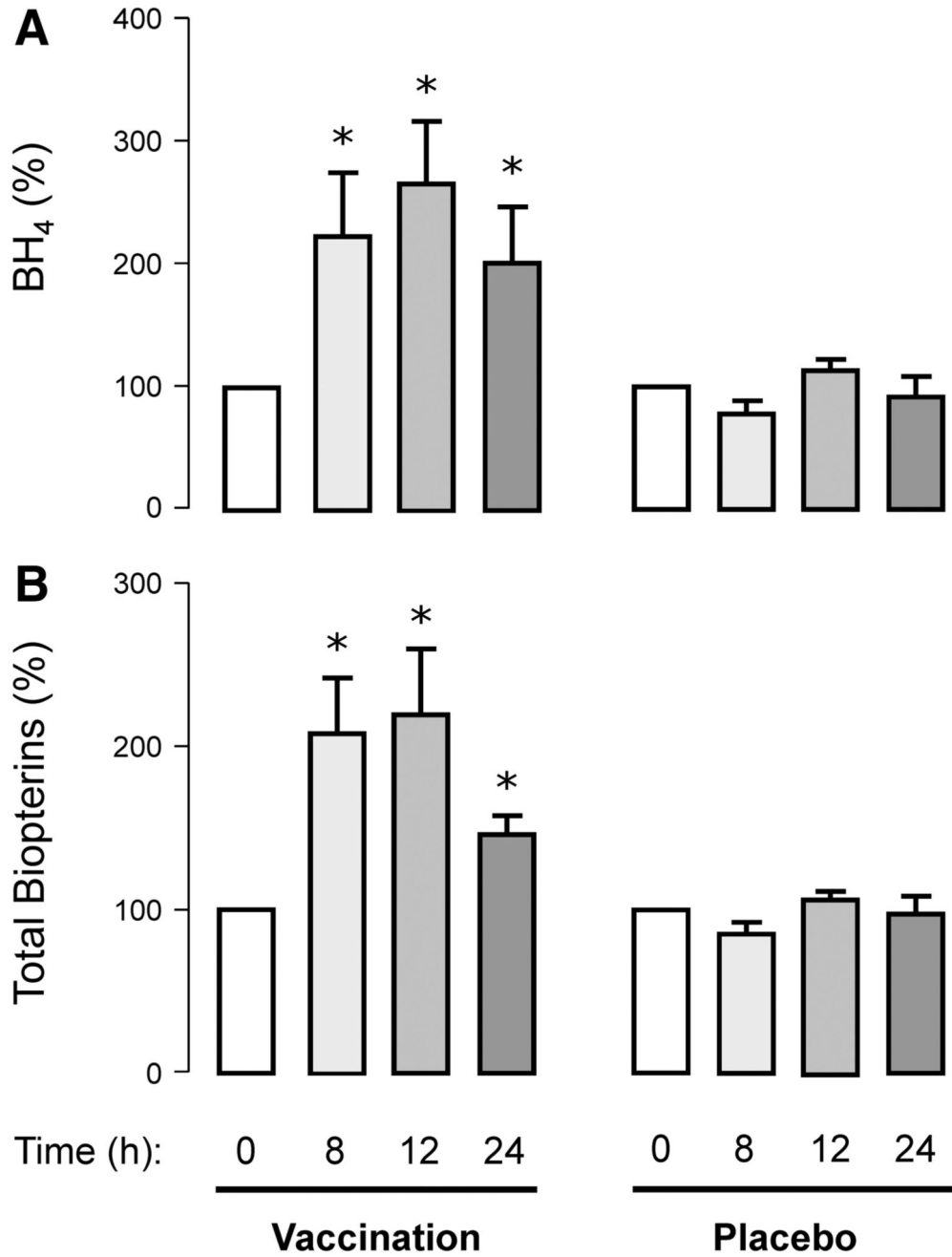


Figure 2. Vaccination with *Salmonella typhi* vaccine (n=10) induced a significant elevation of tetrahydrobiopterin (BH₄; **A**) and total biopterins (**B**), with their peak elevation between 8 and 12 hours. Infusion of placebo (n=10) had no significant effect on interleukin-6, C-reactive protein, or flow-mediated dilation over 24 hours. Postintervention values are presented as percent of baseline (each value indexed to their 0-hour value) and are expressed as mean±SEM. Two-way repeated-measures ANOVA revealed a significant time-by-group

interaction for both BH4 and total biopterins ($P < 0.01$ for both). * $P < 0.01$ vs baseline by paired t test followed by Bonferroni post hoc correction for 3 tests.

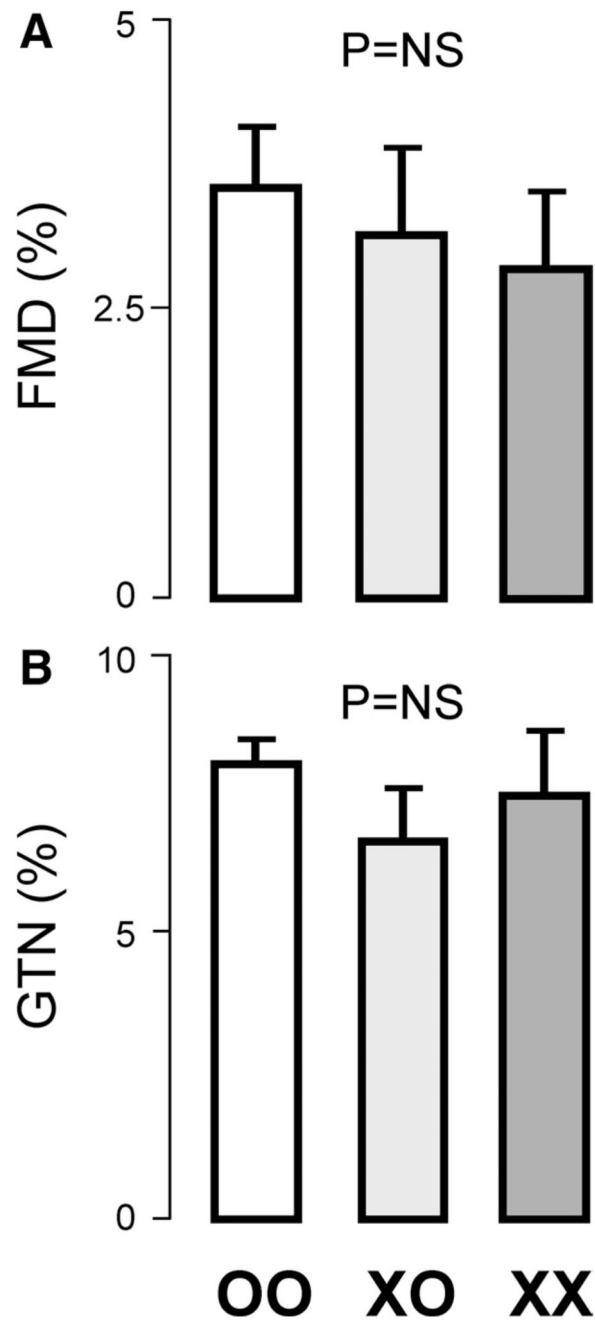


Figure 3.

In patients with coronary artery disease, the *GCHI* haplotype (40 OO and 10 XX) had no significant effect on flow-mediated dilation (FMD; **A**) or endothelium-independent vasodilation in response to nitrates (GTN; **B**). Values are expressed as mean \pm SEM.

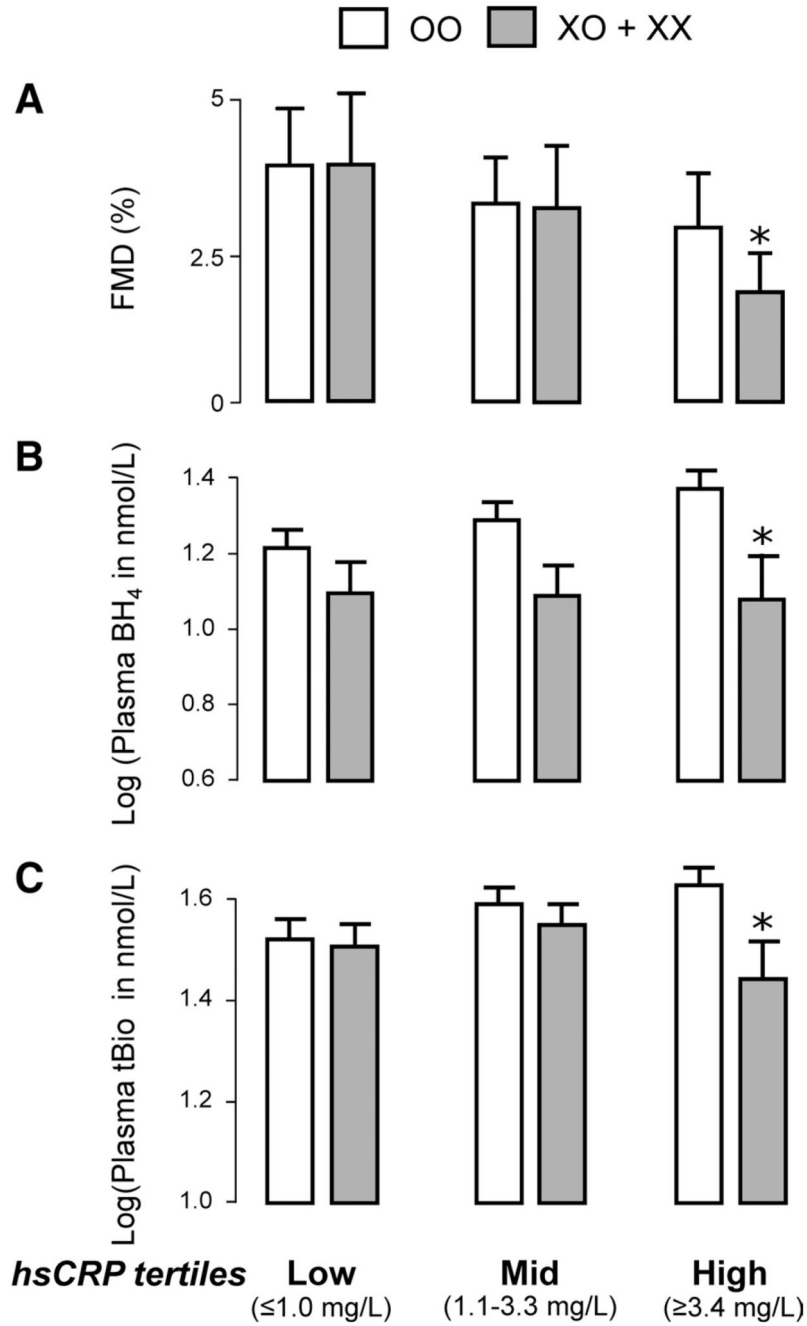


Figure 4.

When the population of patients with coronary artery disease was divided according to tertiles of plasma high-sensitivity C-reactive protein (hsCRP, as a marker of their background inflammatory status), carriage of the X haplotype was associated with significantly lower flow-mediated dilation (FMD; **A**), higher plasma tetrahydrobiopterin (BH₄; **B**), and higher plasma total biopterins (tBio; **C**) than O homozygotes only in those patients at the highest tertile of plasma hsCRP. Values are expressed as mean \pm SEM. Two-way ANOVA for independent samples revealed significant hsCRP tertile-by-genotype

interactions for FMD ($P<0.05$), plasma BH4 ($P<0.01$), and total biopterins ($P<0.05$).

* $P<0.05$ vs OO in individual comparisons using unpaired t test after Bonferroni correction for 3 tests.

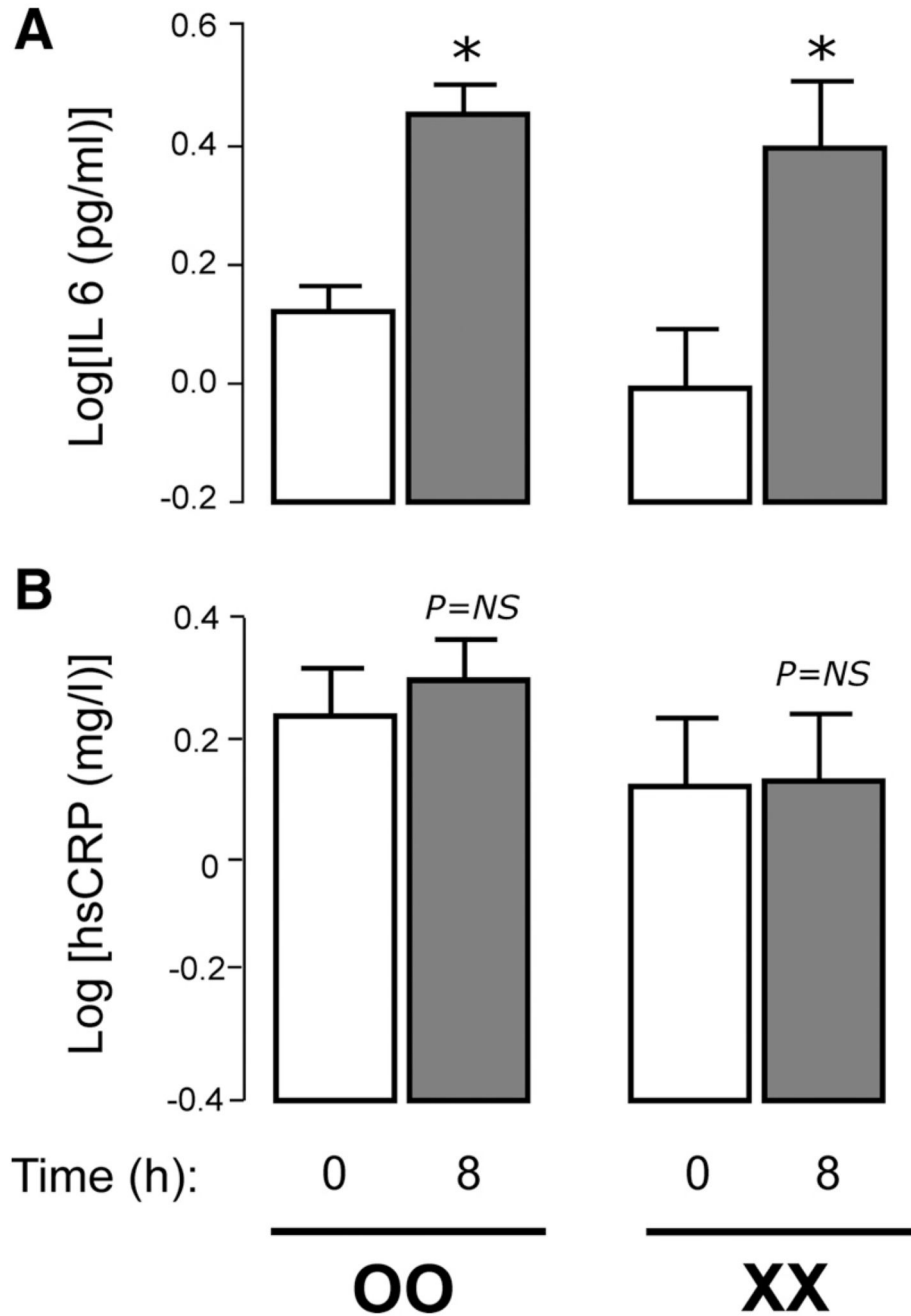


Figure 5.

When patients with coronary artery disease were recruited by *GCHI* genotype (40 OO and 10 XX) to undergo *Salmonella typhi* vaccination, we observed that vaccination induced a similar elevation of interleukin-6 (IL-6) at 8 hours in both genotypes (A), whereas C-reactive protein (hsCRP) levels remained unchanged at 8 hours in both genotypes (B). Values are expressed as mean \pm SEM. Two-way repeated-measures ANOVA revealed no significant time-by-genotype interaction. Within-groups comparisons (8 vs 0 hours) were performed with an unpaired *t* test. **P*<0.01 vs Oh.

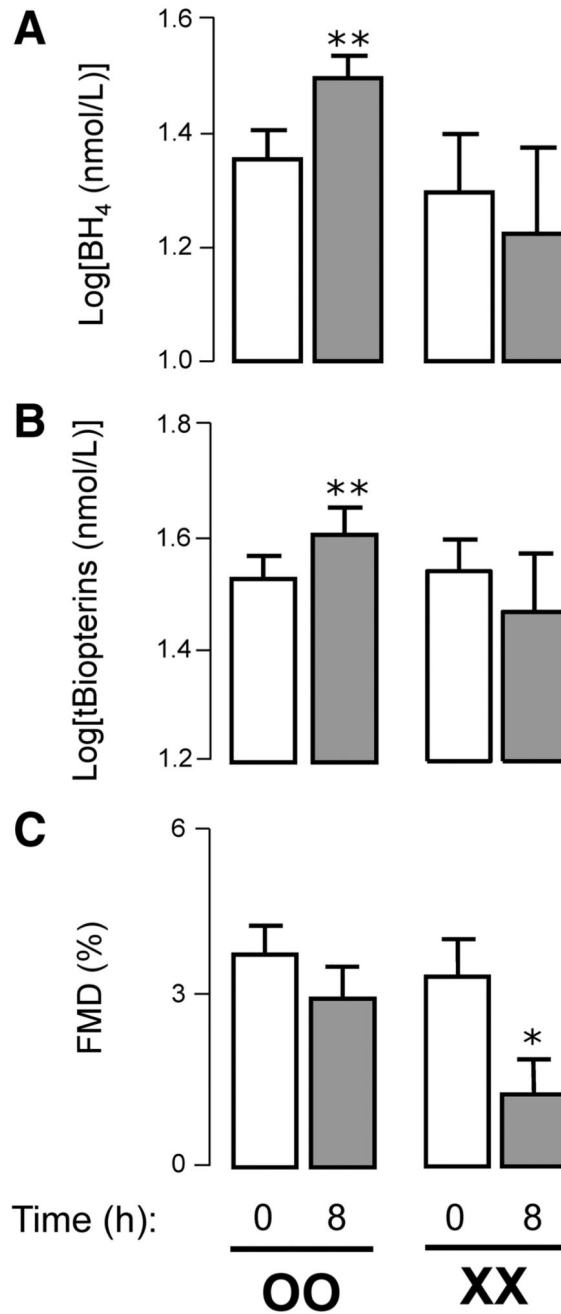


Figure 6.

When patients with coronary artery disease were recruited by *GCHI* genotype (40 OO and 10 XX) to undergo *Salmonella typhi* vaccination, we observed that vaccination induced a significant elevation of tetrahydrobiopterin (BH₄; **A**) and total biopterins (tBiopterins; **B**) at 8 hours only in OO but not in XX genotypes. However, flow-mediated dilation (FMD) was reduced significantly only in the presence of XX haplotype (**C**). Values are expressed as mean±SEM. Two-way repeated-measures ANOVA revealed significant time-by-genotype

interaction for BH4, total bipterins, and FMD ($P < 0.05$ for all). * $P < 0.05$, ** $P < 0.01$ vs 0 hours by unpaired t test.

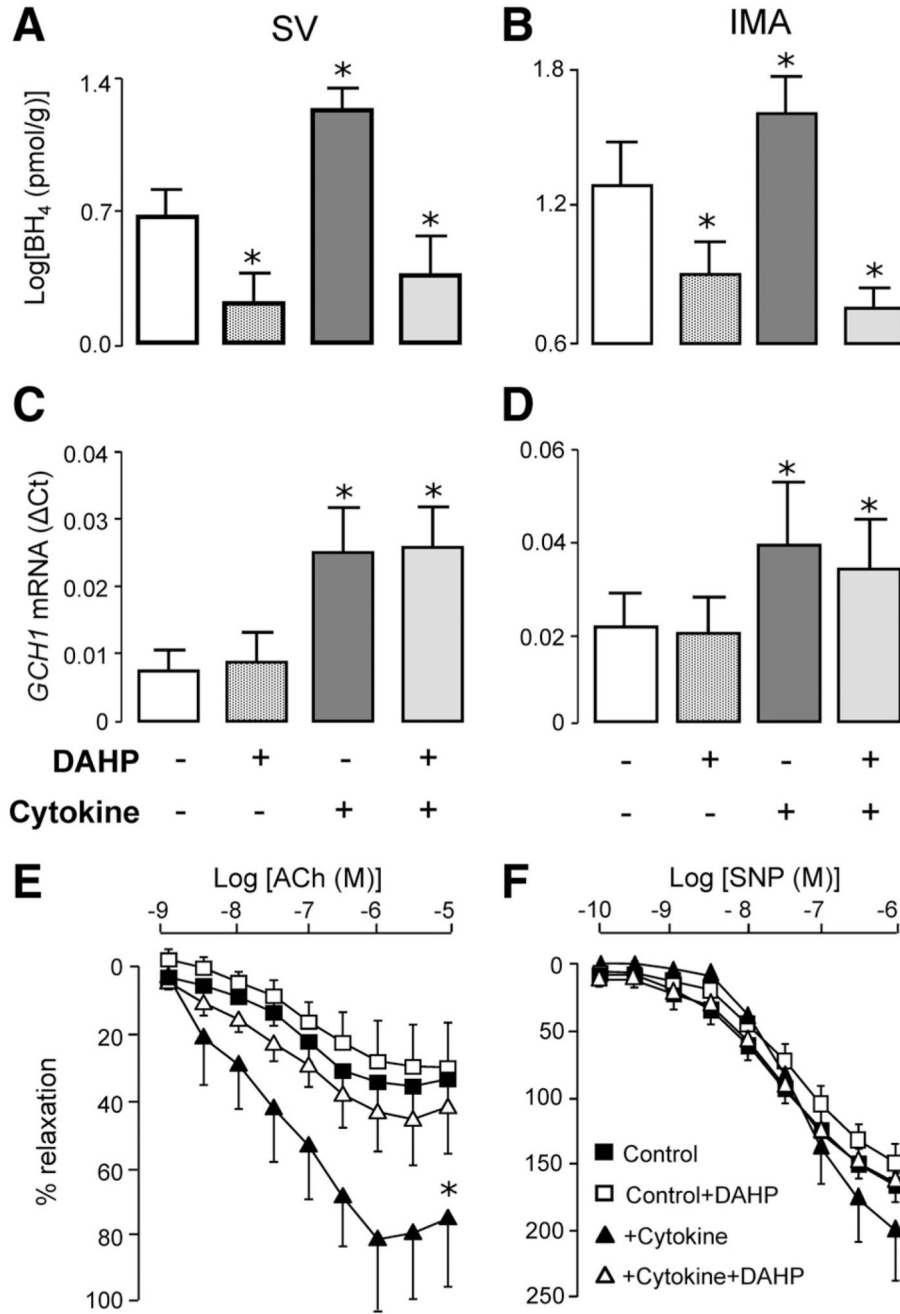


Figure 7. Incubation of saphenous vein (SV; **A**) and internal mammary artery (IMA; **B**) segments for 24 hours with a combination of cytokines (tumor necrosis factor- α 4 ng/mL plus interleukin-6 10 nmol/L) and lipopolysaccharide (80 ng/mL) induced a significant elevation of vascular tetrahydrobiopterin (BH₄), an effect that was prevented by the GTPCH inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP; 1 mmol/L). Similarly, cytokine stimulation for 24 hours upregulated *GCH1* gene expression in both SV (**C**) and IMA (**D**) segments, effects that were not modified by DAHP. The same combination of cytokines also increased

vasorelaxation of SV segments in response to acetylcholine (ACh), an effect that was prevented by DAHP (E), whereas it had no effect on vasorelaxation in response to sodium nitroprusside (SNP; F). There were no significant differences in phenylephrine precontractions between the groups in either the ACh curve (E; Control: 1.54 ± 0.72 g [$79.6 \pm 7.1\%$ of KCl contraction]; DAHP: 1.84 ± 0.64 g [$64.0 \pm 8.6\%$ of KCl contraction]; cytokines: 0.85 ± 0.31 g [$86.6 \pm 3.7\%$ of KCl contraction]; cytokines+DAHP: 1.02 ± 0.26 g [$85.7 \pm 6.3\%$ of KCl contraction]) or the SNP curve (F; Control: 1.14 ± 0.14 g [$79.5 \pm 6.1\%$ of KCl contraction]; DAHP: 2.21 ± 0.66 g [$62.4 \pm 10.5\%$ of KCl contraction]; cytokines: 1.1 ± 0.68 g [$66.9 \pm 10.5\%$ of KCl contraction]; cytokines+DAHP: 1.17 ± 0.42 g [$78.9 \pm 15.6\%$ of KCl contraction]). * $P < 0.01$ vs control (no cytokines, no DAHP).

Table
Demographic Characteristics of the Participants

	Study 1 (Vaccination of Healthy Young Individuals)		Study 2 (Patients With CAD, by <i>GCHI</i> Haplotype)			Study 3 (Vaccination of Patients With CAD, by <i>GCHI</i> Haplotype)		Study 4 (Ex Vivo Study)
	Vaccine	Placebo	00	X0	XX	00	XX	
No. of subjects (No. of males)	10 (9)	10 (8)	280 (244)	85 (73)	11 (6)	40 (33)	10 (7)	19 (5)
Risk factors								
Age, y	29±1	31±3	65±1	67±1	67±2	60±2	66±2	68±2
DM, %	0	0	29	26	23	23	20	10
Hypertension, %	0	0	68	78	76	72	80	68
Smokers, %	30	40	40	33	38	51	48	26
Dyslipidemia, %	0	0	71	73	71	51	50	47
Glucose, mg/dL	87±4	87±4	130±4	124±7	109±5	122±9	108±6	104±16
BMI, kg/m ²	24.8±0.9	25.3±1.5	27.8±0.2	27.8±0.5	27.5±0.9	28.7±0.6	26.84±1.1	27.8±0.8
Serum lipid levels, mg/dL								
Cholesterol	170±15	206±19	167±3	177±5	175±8	165±7	182±7	172±12
TG	104±18	129±19	143±7	149±13	130±12	152±33	125±20	147±26
HDL	49±6	49±4	40±1	40±1	45±3	42±2	49±6	42±5
Medication, %								
ACEI	0	0	55	54	46	41	40	57
ARBs	0	0	13	13	21	33	20	15
CCBs	0	0	27	32	25	13	20	21
β-blockers	0	0	77	75	65	76	70	73
Aspirin	0	0	77	80	85	80	80	63
Clopidogrel	0	0	36	30	37	51	40	31
Diuretics	0	0	24	33	30	26	30	36
Statins	0	0	82	82	80	80	90	78

CAD indicates coronary artery disease; 00, patients without the *GCHI* X haplotype; XX, homozygotes for the *GCHI* X haplotype; DM, diabetes mellitus; BMI, body mass index; TG, triglycerides; HDL, high-density lipoprotein; ACEI, angiotensin-converting enzyme inhibitors; ARBs, angiotensin receptor blockers; and CCBs, calcium channel blockers.

There was no significant difference between groups within each study. Continuous variables are expressed as mean±SEM.