Genetically Reduced Soluble Epoxide Hydrolase Activity and Risk of Stroke and Other Cardiovascular Disease

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Background and Purpose—The development of stroke has been linked to lowered levels of epoxyeicosatrienoic acids in the cerebral microvasculature. These substances are metabolized by the enzyme-soluble epoxide hydrolase encoded by the EPHX2 gene. We tested whether genetically reduced soluble epoxide hydrolase activity is associated with risk of ischemic stroke, myocardial infarction, and ischemic heart disease.

Methods—We genotyped participants from the Copenhagen City Heart Study (n = 10,352), the Copenhagen General Population Study (n = 26,042), the Copenhagen Carotid Stroke Study (n = 398 cases + 796 control subjects), and the Copenhagen Ischemic Heart Disease Study (n = 4,901 cases + 9,798 control subjects) for the R103C, R287Q, and Arg402-403ins variants in the EPHX2 gene and recorded hospital admissions due to ischemic stroke, myocardial infarction, and ischemic heart disease.

Results—The hazard/odds ratio for ischemic stroke did not differ from 1.0 for any of the EPHX2 genotypes or genotype combinations in the Copenhagen City Heart Study (P for trend = 0.15 to 0.76), in the Copenhagen General Population Study (P for trend = 0.75 to 0.95), and the Copenhagen Carotid Stroke Study (P for trend = 0.08 to 1.00). Similar results were obtained for myocardial infarction and ischemic heart disease in the 3 studies.

Conclusions—Our results show with significant power that genetically reduced soluble epoxide hydrolase activity is not a major risk factor for ischemic stroke, myocardial infarction, or ischemic heart disease in the Danish population. This suggests that the relationship between the EPHX2 gene and risk of ischemic stroke and other cardiovascular disease does not exist or its effect size is likely to be quite small. (Stroke. 2010;41:27-33.)

Key Words: epidemiology ■ EPHX2 ■ genetics of stroke

Stroke is a leading cause of morbidity and mortality worldwide. Various risk factors are known such as old age, high blood pressure, and smoking. Development of stroke also seems linked to the levels of epoxyeicosatrienoic acids in the cerebral microvasculature. These are involved in numerous aspects of the cerebrovascular system where they exert vasoactive and anti-inflammatory effects and are thought to protect against ischemic stroke (IS). The soluble epoxide hydrolase enzyme (sEH) represents a major pathway for removal of epoxyeicosatrienoic acids from the cerebral microvasculature. The minor alleles of the 3 investigated polymorphisms have been found to decrease enzyme activity considerably. Studies suggest that genetic or medical inhibition of sEH is protective against ischemic brain injury. sEH is involved in the metabolism of epoxy-lipids throughout the body as well as disposition of epoxides in plasma lipoprotein particles. Therefore, sEH may also influence risk of other cardiovascular disease by modifying blood pressure or plasma lipid levels and composition. Several studies have investigated the potential association of functional EPHX2 variants with the aforementioned end points; however, the number of subjects was mostly low (n = 48 to 1085 for human studies). Furthermore, findings have been inconsistent with some reporting a protective effect on stroke (OR = 0.33) and others reporting increased risk (OR = 1.46).

We hypothesized that 3 functional variants in the gene encoding the sEH enzyme, R103C, R287Q, and Arg402-403ins, are associated with decreased risk of IS, myocardial infarction, or ischemic heart disease (IHD). To test this hypothesis, we genotyped participants from the general population (the Copenhagen City Heart Study [CCHS, n = 10,352] and the Copenhagen General Population Study [CGPS, n = 26,042]) and from 2 case–control studies (the Copenhagen Carotid Stroke Study [CCSS, n = 398 cases + 796 control subjects] and the Copenhagen Ischemic Heart Disease Study [CIHDS, n = 4,901 cases + 9,798 control subjects]). In the case–control studies, cases were matched to 2 control...
subjects by age and gender. In the general population studies, cases were compared with all individuals with no event (the control subjects).

Methods
We studied 4 independent cohorts of white individuals of Danish descent. These were defined so that no individual appeared twice in any of the groups analyzed, thus permitting independent confirmation of findings in each group. Details about each study cohort have been given elsewhere.\(^{15-21}\) All subjects answered similar questionnaires; however, in the CCSS and the CIHDS, information on menopausal status and hormone replacement therapy was not available.

The studies were approved by Herlev Hospital and Danish ethical committees and were conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants.

Study Cohorts
The CCHS\(^{15,16}\) is a prospective study of individuals randomly selected from the population of Copenhagen. Data were available on rates of ischemic cerebrovascular disease (including fatal or nonfatal IS, transient ischemic attack, or amaurosis fugax) and rates of IHD (including fatal or nonfatal MI or characteristic symptoms of angina pectoris, including revascularization procedures). From this study, we included 10 352 participants.

The CGPS\(^{19-21}\) is a cross-sectional study of individuals selected, like those in the CCHS, from the population of Copenhagen. Diagnoses of ischemic cerebrovascular disease and IHD were ascertained like in the CCHS. All 36 636 participants were included in the analyses of blood pressure and plasma lipids. For the analyses of clinical events, 26 042 participants were analyzed as a single cohort, 796 were used as control subjects for the CCSS, and 9798 were used as control subjects for the CIHDS (see subsequently).

The CCSS\(^{20}\) is a case–control study of 903 patients who were referred for carotid artery ultrasonography with documented evidence of ischemic cerebrovascular disease based on IS, transient ischemic attack, or amaurosis fugax together with a stenosis of at least 50% of the vessel diameter of a carotid artery, but not necessarily ipsilateral. Hemorrhage was excluded on CT. Of these, 398 patients diagnosed with IS were matched on gender and 1-year age strata with 796 control subjects without ischemic cerebrovascular disease from the CGPS. Control subjects did not undergo carotid artery ultrasonography.

The CIHDS\(^{17,19}\) is a case–control study of 4901 patients who were referred for coronary angiography with documented evidence of IHD based on characteristic symptoms of stable angina pectoris plus at least one of the following: atherosclerosis on coronary angiography, a previous MI, or a positive bicycle exercise electrocardiography test. These cases were matched on gender and 1-year age strata with 9798 control subjects with no history of IHD (MI or angina pectoris) from the CGPS. Of the 4901 patients with IHD, 2815 patients experienced an MI and were matched with 5628 control subjects without a history of MI or IHD and analyzed separately.

Genotyping and Biochemical Analyses
The ABI PRISM 7900HT Sequence Detection System (Applied Biosystems Inc, Foster City, Calif) was used to genotype 3 polymorphisms in the \(EPHX2\) gene (R103C, rs17057255; R287Q, rs751141; Arg402–403ins, rs2234887). Genotyping was verified by DNA sequencing. Total cholesterol, low-density lipoprotein cholesterol, and triglycerides were measured using standard hospital assays (Boehringer Mannheim, Mannheim, Germany, or Konelab, Helsinki, Finland). Low-density lipoprotein cholesterol was calculated using the Friedewald equation if triglycerides were <4 mmol/L (352 mg/dL) but measured directly at higher triglyceride levels.

Other Covariates
Participants filled out a questionnaire giving information about their use of medication and current and previous diseases. Women also stated menopausal status and whether they received hormone replacement therapy. All participants were divided into “smokers” (former and current) and “never-smokers.” Participants were also divided into 2 groups according to alcohol consumption: low to abstinent and frequent alcohol consumers (≥48 g alcohol weekly).

Body mass index was calculated as weight (kg) divided by height squared (m\(^2\)). In the CGPS, blood pressure was measured by trained technicians using an automated digital blood pressure monitor (Kivex) on the left arm after 5 minutes rest with the subject in the sitting position. The blood pressure cuff was 22×32 cm, but for subjects with an upper arm circumference of >46 cm, a cuff that measured 32×45 cm was used. Interobserver variation was tested and found to be statistically insignificant. Hypertension was defined as systolic blood pressure of ≥140 mm Hg, diastolic blood pressure of ≥90 mm Hg, and/or treatment with antihypertensive medication.

End Points
Information about IS, MI, and IHD was obtained by linking the participants to the national Danish Patient Registry and the national Danish Causes of Death Registry using each participant’s unique Central Person Register number.\(^{16-17,19}\) The diagnoses were defined according to World Health Organization International Classification of Diseases, 8th and 10th Revisions. IS was validated as described.\(^{20}\) IHD was International Classification of Diseases, 8th Revision 410 to 414 and International Classification of Diseases, 10th Revision I20 to I25. MI (International Classification of Diseases, 8th Revision 410 and International Classification of Diseases, 10th Revision I21 to I22) required at least 2 of the following 3: characteristic chest pain, elevated cardiac enzymes, or MI-specific electrocardiographic changes.

Statistical Analyses
We used STATA/SE 10.1 for all analyses. From the 3 polymorphisms, we generated all possible genotype combinations and ranked the 5 most common according to their predicted sEH activity.\(^{9-11}\) For trend tests, the different groupings of subjects by genotype or genotype combination were coded 1, 2, 3, and so on according to predicted sEH activity. We also calculated the low/high hazard/odds ratios, which we have 90% power to exclude at 2-sided probability values <0.05.

We first analyzed the relationship of \(EPHX2\) genotype and genotype combination with blood pressure and plasma lipids in the CGPS, excluding those with hypertension or on lipid-lowering medication, respectively. Analyses were performed by analysis of variance.

Next, we analyzed the relationship between \(EPHX2\) genotype and risk of IS, MI, and IHD in the CCHS with the use of delayed entry from the 1976 to 1978 examination. Cox regression models with age as time scale and adjusted for age and gender were used to estimate hazard ratios for IS, MI, and IHD. This analysis was retested in the CGPS; conditional logistic regression analyses adjusted for age and gender were used to estimate odds ratios for IS, MI, and IHD.

Finally, another retesting of the relationship between \(EPHX2\) genotype and risk of IS, MI, and IHD was conducted using the CCSS for the IS end point and the CIHDS for the MI and IHD end points. For each of these analyses, cases were matched by age and gender with control subjects from the CGPS, and conditional logistic regression analyses based on the matching criteria of age and gender were used without adjusting for other covariates to estimate ORs for IS, MI, and IHD. Matching by the same criteria within the CCHS and the CGPS yielded similar results to those reported.

Results
Clinical characteristics of the participants in each of the 3 IS-related studies are presented in Supplemental Table I (available at http://stroke.ahajournals.org). As expected, indi-
individuals with IS were older and more likely male, except for the CCSS in which cases and control subjects were matched by age and gender. Individuals with IS had a higher frequency of diabetes, were more likely smokers, and more often took antihypertensive and lipid-lowering medication. Characteristics in relation to IHD are given in Supplemental Table II. Genotype frequencies were in accordance with the Hardy-Weinberg equilibrium in all of the 4 study populations (exact

Figure 1. Levels of systolic blood pressure and total plasma cholesterol according to EPHX2 genotype, allele, and genotype combination. None of the participants were homozygous carriers of the 103T allele, and this genotype was hence excluded from statistical analysis.
probability values are given in Supplemental Tables I and II). Allele frequencies accorded well with previous reports except for the 103T allele, which seems extraordinarily rare in the Danish population. Because we performed reruns twice, call rates were 99.97% for all 3 polymorphisms.

**Blood Pressure**

None of the individual EPHX2 genotypes or genotype combinations were associated with reduced systolic blood pressure in the CGPS (Figure 1, top panel). With or without exclusion of individuals on antihypertensive medication, probability values for trend did not reach statistical significance for any of the individual genotypes, alleles, or genotype combinations. Similar results were obtained for diastolic blood pressure and pulse pressure (data not shown). EPHX2 genotype was also not associated with hypertension (data not shown).

**Plasma Lipids**

None of the individual EPHX2 genotypes or genotype combinations were associated with decreased plasma cholesterol in the CGPS (Figure 1, lower panel). With or without exclusion of individuals on lipid-lowering medication, probability values for trend did not reach statistical significance for any of the individual genotypes, alleles, or genotype combinations. Similar results were obtained for plasma levels of high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides (data not shown). EPHX2 genotype was also not associated with use of lipid-lowering medication (data not shown).

**Risk of IS**

The hazard ratio for IS as a function of genotype did not differ from 1.0 for any of the individual EPHX2 genotypes, alleles, or genotype combinations in the CCHS ($P$ for trend $= 0.15$ to 0.76; Figure 2). This was confirmed by logistic regression in the CGPS ($P$ for trend $= 0.75$ to 0.95) and the CCSS ($P$ for trend $= 0.08$ to 1.00; Figure 2) in which ORs did not differ from 1.0.

**Risk of MI**

The hazard ratio for MI was increased for genotype combination 4 (1.29; 95% CI, 1.05 to 1.58; Figure 3) and the analyses by both genotype and allele showed a significant trend toward increased risk of MI for the 402-403ins allele ($P$ for trend $= 0.03$). However, these findings could not be replicated in the CGPS or the CIHDS. None of the risk estimates for the other genotypes, alleles, or genotype combinations differed significantly from 1.0 in any of the 3 studies ($P$ for trend $= 0.15$ to 0.76, 0.39 to 0.89, 0.20 to 0.52, respectively).

**Risk of IHD**

The hazard ratio for IHD as a function of genotype was increased for 402-403ins heterozygotes (1.20; 95% CI, 1.05 to 1.38; Figure 4) and for rare allele carriers in the allelic analysis (1.19; 1.04 to 1.35) as well as for genotype combination 4 (1.20; 1.04 to 1.39). None of these findings could be replicated in the CGPS or the CIHDS. None of the risk estimates for the other individual genotypes or genotype combinations differed significantly from 1.0 in any of the 3 studies ($P$ for trend $= 0.40$ to 0.74, 0.39 to 0.89, 0.61 to 0.88, respectively).

**Discussion**

The sEH enzyme is regarded a novel therapeutic target for cardiovascular disease. This is because sEH is involved in the regulation of blood pressure and has been associated with risk of cardiovascular disease. In our study of 4 large samples from the Danish population, we find that genetically
reduced sEH activity is not associated with blood pressure or risk of IS, MI, or IHD.

Ischemic Stroke

An association of genetically reduced sEH activity with IS is reported in humans of various ethnicities and in animal models of human disease. Most studies claim a protective effect of decreased sEH activity on IS,2,5,6,24 whereas another study showed an association of the 287Q allele with increased risk of IS.14 A study by Fornage et al suggests that certain genotype combinations rather than individual genotypes are responsible for the lowered enzyme activity in vivo.6 In our study, which includes 12 times more individuals than all previous studies combined,2,5,6,14,24 we found no association between genetically reduced sEH activity and risk of IS.

MI and IHD

Several reports exist linking EPHX2 polymorphisms to altered risk of IHD.23,25 Fornage et al found that the R287Q polymorphism was a predictor of coronary artery calcifica-

Figure 3. Risk of MI according to EPHX2 genotype, allele, and genotype combinations. None of the participants were homozygous carriers of the 103T allele, and this genotype was hence excluded from statistical analysis.

Figure 4. Risk of IHD according to EPHX2 genotype, allele, and genotype combinations. None of the participants were homozygous carriers of the 103T allele, and this genotype was hence excluded from statistical analysis.
tion in blacks but not in white subjects in the Coronary Artery Risk Development in Young Adults (CARDIA) study.23 This is in contrast to the findings of Lee et al who report that genetically increased, not lowered, sEH activity is associated with coronary heart disease in whites in the Atherosclerosis Risk In Communities (ARIC) study.25 Polymorphisms of the EPHX2 gene have also been linked to subclinical heart disease.24,26 By the nature of our study, we are unable to rule out such an association. However, in our study, we found no association between genetically reduced sEH activity and risk of MI or IHD.

Blood Pressure and Plasma Lipids
sEH is abundant in the cerebral and renal microvasculature where it regulates hydrolysis of vasoactive epoxycisatrienoic acids.27 Studies involving animal models of hypertension have shown that sEH inhibition has a beneficial effect on hypertension, but it does not affect blood pressure in normotensive individuals.4 In humans, genetically lowered sEH activity does not appear associated with risk of hypertension.28

Several reports suggest that sEH plays a hyperlipidemic role in normal metabolism and that genetically lowered enzyme activity and stability may attenuate this effect.13 Data published by Sato et al suggest that EPHX2 is a modifier gene affecting phenotype in individuals with familial hypercholesterolemia who carry the LDLR mutation. They found that carriers of the 287R allele present with more severe hyperlipidemia than noncarriers.12 No such effect was seen in individuals without the LDLR mutation. In our study, we did not genotype for the LDLR mutation. However, our findings do not support an association of EPHX2 genotype with plasma lipid levels.

Limitations
Although our 4 studies had limitations and potential biases based on their different study designs (eg, they defined cases somewhat differently), the results were congruent. All participants in this study are Danish whites, and although this eliminates any blurring due to ethnic heterogeneity of the study population, our results may apply to whites only. The 103T allele seems very rare in the Danish population, and any effect of this allele might elude detection in our study.

Summary
Reports indicate that polymorphisms in the EPHX2 gene are associated with altered risk of stroke and other cardiovascular disease. Although we have studied 3 of the polymorphisms with the highest reported effect on sEH activity in humans, we cannot exclude a relationship between variants in the noncoding region of the EPHX2 gene and expression levels. However, our results show with significant power that genetically reduced sEH activity is not a major risk factor for IS, MI, and IHD in the Danish population. This suggests that the relationship between the EPHX2 gene and risk of IS and other cardiovascular disease does not exist or its effect size is likely to be quite small.

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Disclosures
None.

References
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