Epoxyeicosatrienoic Acids are Mediated by EPHX2 Variants and may be a Predictor of Early Neurological Deterioration in Acute Minor Ischemic Stroke

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Aim: To investigate the association of plasma epoxyeicosatrienoic acids (EETs) with early neurological deterioration (END), and whether EETs are mediated by EPHX2 variants in patients with minor ischemic stroke (MIS).

Method: This was a prospective, multi-center observational study in patients with acute MIS in the Chinese population. Plasma EETs levels were measured on admission. Single nucleotide polymorphisms (SNPs) of EPHX2 rs751141 were genotyped using mass spectrometry. The primary outcome was END within 10 days after admission. END was defined as an increase in NIHSS of 2 or more points. The degree of disability was assessed using the modified Rankin Scale (mRS) at 3 months after admission.

Results: A total of 322 patients were enrolled, of which 85 patients (26.4%) experienced END. The mean EETs level was 64.1 ± 7.5 nmol/L. EETs levels were significantly lower in patients with END compared to patients without END. Frequency of EPHX2 rs751141 GG was higher in patients with END than in patients without END, and EPHX2 rs751141 GG genotype was associated with lower EETs levels. Low level (<64.4 nmol/L) of EETs was an independent predictor of END (first and second quartiles) in multivariate analyses. END was associated with a higher risk of poor outcome (mRS scores 3–6) at 3 months.

Conclusion: END is fairly common and associated with poor outcomes in acute MIS. EPHX2 variants may mediate EETs levels, and low levels of EETs may be a predictor for END in acute MIS.

Key words: Early neurological deterioration, Epoxyeicosatrienoic acids, Minor ischemic stroke, Outcome, EPHX2 variants

Introduction

One third of patients with acute ischemic stroke develop early neurological deterioration (END), which in turn is associated with increased mortality and long-term functional disability1,2). END is a common occurrence in patients with minor ischemic stroke (MIS)3,4). Studies have shown that 21% to 50% of MIS patients suffer from END during the 7–10 days after stroke onset4,5), and approximately 15% to 30% of these patients were dead or disabled at the time of 3-month follow-up, despite mild symptoms at presentation6). The underlying mechanisms of this association are not completely understood, although biochemical factors associated with END have been reported7,8).

Arachidonic acid (AA) is a major membrane fatty acid that can be metabolized by cytochrome P450 (CYP) epoxygenases into four epoxyeicosatrienoic acids (EETs), which can then be metabolized by soluble epoxide hydrolase (sEH) to yield less biologically-active dihydroxyeicosatrienoic acids (DiHETEs)9). EETs play an important role in cerebral blood flow regulation and neuroprotection after brain injury10,11). Previous
and June 2015. All enrolled patients underwent computed tomographic angiography or magnetic resonance angiography of the brain, as well as color duplex ultrasound investigation of the carotid arteries. Common electrocardiogram (ECG), 24-h Holter electrocardiogram (ECG) and echocardiogram were performed to reveal any possible cardio-embolic stroke. The inclusion criteria were: (1) age ≥ 40 years old; (2) diagnosis of MIS with National Institutes of Health Stroke Scale (NIHSS) score ≤ 3 points at admission 18); (3) no history of carotid endarterectomy or carotid stent therapy; (4) the etiology of stroke in MIS was due to atherothrombotic or small artery disease, according to the Trial of Org 10172 in Acute Stroke Treatment criteria 19); and (5) no history of clopidogrel or aspirin treatment for at least seven days prior to admission. The exclusion criteria were: (1) allergy to clopidogrel and aspirin; (2) patients who declined participation in the study; (3) cardiac or any other etiology of stroke (determined or undetermined) 19); (4) cerebellar infarction or multiple infarction; (5) previous myocardial infarction (MI); (6) usage of warfarin or heparin in the preceding 2 weeks or within 10 days after admission; (7) blood platelet count < 100 × 10^9/L or > 450 × 10^9/L; (8) fever, hypoxia, or hemodynamic compensation at admission; (9) other conditions such as asthma or severe cardiovascular, liver, or renal disease. All patients received standard therapy based on the practice guidelines 20). Detailed background information on the patients was described in our previous article 21).

Table 1. EETs levels and EPHX2rs751141 genotype distribution in patients with or without END

| Variables | Patients with END \((n = 85)\) | Patients without END \((n = 237)\) | \(P\) value |
|-----------|-------------------------------|---------------------------------|-----------|
| EETs (nmol/L) | 60.3 ± 7.3 | 68.4 ± 8.1 | <0.001 |
| DiHETEs (nmol/L) | 84.1 ± 7.5 | 75.3 ± 7.2 | <0.001 |
| EPHX2rs751141 | | | |
| GG, n (%) | 66 (77.6) | 123 (51.9) | <0.001 |
| AG, n (%) | 17 (20.0) | 100 (42.2) | |
| AA, n (%) | 2 (2.4) | 14 (5.9) | |

END, early neurological deterioration; EET, epoxyeicosatrienoic acids; DiHETEs, dihydroxyeicosatrienoic acids.

studies from our lab have shown that low plasma EETs levels were associated with plaque stability or carotid stenosis in ischemic stroke patients, and independently associated with high risk of ischemic stroke 12-14). However, whether EETs are a risk factor for END after acute MIS has not been well studied.

SEH is a key enzyme in the metabolic conversion and degradation of EETs 9). Increasing EETs levels, by inhibiting the sEH enzyme, decreases cerebral damage following stroke. This improved following cerebral ischemia is a consequence of improving cerebral vascular structure or function and protecting neurons from cell death 10, 11). Thus, sEH is a potential novel therapeutic target for cardiovascular diseases and ischemic stroke 11). Genetic variations in the sEH gene — EPHX2 — are associated with ischemic stroke risk 15). In experimental studies, sEH inhibition and gene deletion reduced infarct size after focal cerebral ischemia in mice 16, 17). However, the relationship between EPHX2 variant, EETs levels, and risk of END in patients with acute MIS has not been well investigated. Clarifying this relationship is critical for understanding the mechanisms of END, and for preventing and treating END within the context of stroke. Therefore, the aim of the present study was to evaluate the potential associations between EETs levels, EPHX2 variants, and END in Chinese patients with MIS.

**Materials and Methods**

**Study Population**

This prospective multi-center study of MIS was conducted in the People’s Hospital of Deyang City, the Second and Third Affiliated Hospital of Wenzhou Medical University. The study protocol was approved by the Ethics Committees at the participating hospitals. Written informed consent was obtained from each patient prior to study enrollment. We consecutively enrolled 322 patients with MIS who had their first strokes and were admitted to the participating hospitals within 24 h of stroke onset between March 2013 and June 2015.
following criteria: (i) SNPs with minor allele frequency >0.05; (ii) SNPs that have been assessed in our previous studies\textsuperscript{12-15}; (iii) SNPs leading to amino acid changes.

Genotypes of the \textit{EPHX2} rs751141 were performed using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method, according to our previous study\textsuperscript{12-15}. Genotype calling was performed in real time with MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer software version 3.4 (Sequenom Inc., San Diego, CA).
Assessment of Clinical Outcomes

The primary outcome of this study was END, which was defined as an increase in NIHSS score of ≥2 points within 10 days after admission, excluding for hemorrhagic transformation (HT) of an infarct or a new infarct in another vascular territory (recurrent ischemic stroke, RIS)\(^4,21\). For each patient, an NIHSS assessment was performed by a member of the stroke team upon presentation to the emergency department, and subsequently on a daily basis throughout the period of hospitalization. An additional NIHSS assessment was performed whenever a patient’s condition deteriorated. Upon notification of deterioration, a stroke team member reassessed the patient and performed the additional NIHSS evaluation. The secondary outcome was a composite of HT, RIS, MI and death during the first 10 days after admission. RIS was defined as a new focal neurologic deficit of vascular origin lasting at least 24 h, diffusion weighted imaging (DWI)-positive lesion(s) which corresponded to their clinical symptom(s) and was proven to be non-hemorrhagic. Death was defined as vascular mortality due to MI, ischemic stroke, or other vascular causes. The degree of disability was measured using the modified Rankin Scale (mRS) at 3 months after admission by a certified stroke team member. A good outcome was defined as mRS ≤ 2 points, while mRS > 2 points was considered a poor outcome. During the 3-month treatment, interviews were conducted every month by investigators who were blinded to EETs levels of the patients.

Statistical Analysis

Previous studies have reported the prevalence of END to be approximately 21%–50% in patients with MIs\(^4,5\). Using this estimate, we calculated a minimum sample-size requirement of 310 patients for determining the true incidence rate within ±15% with 95% confidence.

We examined total EETs by quartiles of decreasing levels to evaluate for possible threshold effects. Baseline and clinical characteristics were compared using \( \chi^2 \) test or Fisher exact test (categorical variables) and the Student t-test (continuous variables). Deviation of Hardy-Weinberg equilibrium for genotype frequencies was also analyzed by \( \chi^2 \)-test. Difference of genotype frequencies between patients with or without END was compared by \( \chi^2 \)-test, while plasma EETs and DiHETEs levels were compared between patients with or without END using Student’s t-test. Differences of plasma EETs and DiHETEs levels among EPHX2 genotypes were compared using analysis of variance. Variables that showed a significant associa-
tion \((p<0.1)\) with END on univariate analysis were included in the multivariate logistic regression model for evaluation of possible contributing factors for END. A Cox proportional hazard model was used to assess the probability of END according to EETs levels. The association of EETs levels with mRS score at 3 months was analyzed using a Spearman rank-order correlation. All tests were two-sided, and the threshold of \(p<0.05\) was used to denote statistical significance. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

**Results**

A total of 322 patients (201 men; mean age, 68.3 years) were enrolled in this study, the average duration of hospital stay was 12.4 days. No patients were discharged within 10 days. Based on the defined criteria, 85 patients (26.4%) experienced END within 10 days after admission. The median (interquartile range [IQR]) time to deterioration from first NIHSS score collected was 22 (5.3–29) hours. The median (IQR) increase in total NIHSS score was 3 (2–6) at the time of deterioration. Baseline characteristics of patients with and without END were described in our previous article. Univariate analyses revealed that old age, hypertension, and mRS score at 3 months. However, there was no significant association between EETs levels and secondary outcome (Table 2). There was no significant difference in rate of tissue plasminogen activator treatment between the low EETs and high EETs groups (Table 2). There was also no significant difference of EETs levels between patients with atherothrombotic and those with small artery disease (63.7 ± 7.6 nmol/L vs. 64.5 ± 7.8 nmol/L, \(p=0.322\)).

| Genotype | EETs (nmol/L) | DiHETEs (nmol/L) |
|----------|---------------|-----------------|
| GG \((n=189)\) | 59.6 ± 7.8 | 83.9 ± 9.1 |
| AG \((n=117)\) | 67.9 ± 8.2 | 75.2 ± 7.3 |
| AA \((n=16)\) | 68.8 ± 3.2 | 74.8 ± 4.4 |
| \(p^*\) | <0.001 | <0.001 |

*Statistical significance was based on analysis of variance, compared among genotypes.

EETs, epoxyeicosatrienoic acids; DiHETEs, dihydroxyeicosatrienoic acid.

Table 4. Association of *EPHX2* genotypes with EETs and DiHETEs levels

After multiple logistic regression analyses, 3 factors (EETs, Diabetes mellitus, and *EPHX2* rs751141 GG) emerged as independent predictors of END. The odds ratio for END increased with decreasing quartile of EETs levels, using the highest quartile (fourth quartile) as the reference value. The first and second quartiles of EETs levels were identified as independent pre-
Outcomes in patients with aneurysmal subarachnoid hemorrhage. However, the relationship between EETs levels and END after acute MIS has not been well investigated. The present study is the first to identify a positive relationship between EETs levels and END in acute MIS. The mechanisms underlying these associations are not yet understood. EETs exert vascular relaxation effects, and have diverse protective roles in the cardiovascular system against stroke, including vasodilation, neuroprotection, promotion of angiogenesis and suppression of platelet aggregation, oxidative stress and post-ischemic inflammation. EETs can be metabolized by sEH to yield less biologically active DiHETEs. Pharmacological inhibition or genetic deletion of sEH has been shown to increase EETs levels, reduce infarct size after focal cerebral ischemia, and protect from stroke-induced brain injury. This protective effect of EETs has also been demonstrated in an animal model. All these findings suggest a potential molecular mechanism that links low EETs with risk of END.

Our data revealed that EPHX2 rs751141 GG was independently associated with END and EETs levels. One study showed that patients with at least one copy of the variant EPHX2 had lower mean EETs levels, and this is consistent with our current findings. Przybyle-Zawislak and colleagues reported that the EPHX2 variant increases sEH enzyme activity and results in reduction in EETs levels. They also found that EPHX2 rs751141 GG genotype is associated with EETs levels in Black, Asian, and White healthy populations. Our previous study showed that the mean EETs level was 61.76 ± 4.52 nmol/L in ischemic stroke patients and 73.68 ± 4.88 nmol/L in healthy controls. The EETs levels associated with poor outcome (mRS scores 3–6) at 3 months.

Table 5. Logistic Regression Model of Independent Predictors of END and Odds Ratio according to EETs Quartiles

| Factor                                    | OR*   | 95% CI       | P value |
|-------------------------------------------|-------|--------------|---------|
| EETs Quartile, pmol/L                     |       |              |         |
| fourth quartile (reference)               |       |              |         |
| third quartile                            | 1.36  | 0.57–3.67    | 0.413   |
| second quartile                           | 2.46  | 1.06–6.89    | 0.028   |
| first quartile                            | 2.96  | 1.18–8.77    | 0.018   |
| Age                                       | 0.87  | 0.79–1.28    | 0.694   |
| Hypertension                              | 1.28  | 0.83–1.96    | 0.185   |
| Diabetes mellitus                         | 1.42  | 1.04–2.13    | 0.031   |
| Intracranial artery or carotid stenosis   | 1.38  | 0.98–2.01    | 0.072   |
| DiHETEs                                   | 1.22  | 0.77–1.75    | 0.264   |
| EPHX2rs751141 GG                          | 2.12  | 1.03–6.24    | 0.022   |

END, early neurological deterioration; EET, epoxyeicosatrienoic acids; DiHETE, dihydroxyeicosatrienoic acid; CI, confidence intervals; OR, odds ratio.

OR for Age, and DiHETEs mean per 1-Standard Deviation increases.

Discussion

In this prospective, multi-center observational study, the incidence of END was 26.4% in acute MIS. EETs levels were significantly lower in patients with END than patients without END. EPHX2 rs751141 GG genotype was independently associated with lower EETs levels. Low levels (<64.4 nmol/L) of EETs was associated with higher risk of poor outcome (mRS scores 3–6) at 3 months.

Previous research has suggested an association between EETs levels and stroke risk. Our previous work also showed that EETs levels were associated with plaque stability or carotid stenosis, and independently associated with a high risk of ischemic stroke. EETs have been shown to play an important role in the regulation of cerebrovascular tone; they protect against cerebral ischemia, and are associated with outcomes in patients with aneurysmal subarachnoid hemorrhage. However, the relationship between EETs levels and END after acute MIS has not been well investigated. The present study is the first to identify a positive relationship between EETs levels and END in acute MIS. The mechanisms underlying these associations are not yet understood. EETs exert vascular relaxation effects, and have diverse protective roles in the cardiovascular system against stroke, including vasodilation, neuroprotection, promotion of angiogenesis and suppression of platelet aggregation, oxidative stress and post-ischemic inflammation. EETs can be metabolized by sEH to yield less biologically active DiHETEs. Pharmacological inhibition or genetic deletion of sEH has been shown to increase EETs levels, reduce infarct size after focal cerebral ischemia, and protect from stroke-induced brain injury. This protective effect of EETs has also been demonstrated in an animal model. All these findings suggest a potential molecular mechanism that links low EETs with risk of END.
The findings of this study should be noted. First, some studies have shown the association of biomarkers such as high-sensitive C reaction protein, inflammatory cytokines and brain natriuretic peptide with END. However, these biomarkers were not measured in this study and we did not account for the effect of these biomarkers on END. Second, the time interval from onset to admission was variable. Therefore, patients who had already deteriorated within that time prior to enrollment may not have been recognized as END. This could have underestimated the incidence of END. Third, although this study examined the association of EPHX2 variant with EETs levels and END, some functional genetic variants in the EET metabolic pathway, such as CYP2C8 were not genotyped, we did not eliminate effects of other genetic variants on EETs levels and END. Thus, future studies involving a larger set of genetic variants could be conducted. Fourth, EETs levels may change dynamically after ischemic stroke, and maybe affected by ischemia itself. In this study, plasma EETs levels were measured on admission. We did not investigate effects of acute ischemia itself on EETs levels. Further studies are needed to evaluate the association between dynamic changes in EETs levels and END. Finally, the lack of a control group in the present study is a limitation. Thus, well-designed studies are needed to validate our findings in future.

In some studies, the degree of carotid stenosis as well as middle cerebral artery occlusion have been shown to be associated with END. In the present study, we did not observe such an association. Our results were consistent with one recent study in the literature. However, these findings must be validated in larger, multi-center studies in the future.

In spite of these novel findings, several limitations of this study should be noted. First, some studies have shown the association of biomarkers such as high-sensitive C reaction protein, inflammatory cytokines and brain natriuretic peptide with END. However, these biomarkers were not measured in this study and we did not account for the effect of these biomarkers on END. Second, the time interval from onset to admission was variable. Therefore, patients who had already deteriorated within that time prior to enrollment may not have been recognized as END. This could have underestimated the incidence of END. Third, although this study examined the association of EPHX2 variant with EETs levels and END, some functional genetic variants in the EET metabolic pathway, such as CYP2C8 were not genotyped, we did not eliminate effects of other genetic variants on EETs levels and END. Thus, future studies involving a larger set of genetic variants could be conducted. Fourth, EETs levels may change dynamically after ischemic stroke, and maybe affected by ischemia itself. In this study, plasma EETs levels were measured on admission. We did not investigate effects of acute ischemia itself on EETs levels. Further studies are needed to evaluate the association between dynamic changes in EETs levels and END. Finally, the lack of a control group in the present study is a limitation. Thus, well-designed studies are needed to validate our findings in future.
In conclusion, END is fairly common in acute MIS in the Chinese population, and is associated with poor outcomes. Decreased EETs levels and EPHX2 rs751141 GG were significantly associated with END. EPHX2 rs751141 GG genotype may mediate EETs levels. Low level of EETs may be a predictor for END in acute MIS. A detailed understanding of the basic mechanisms could provide valuable insights into potential prevention and treatment of END.

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Conflict of Interest

The authors declare no conflicts of interest.

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