Cytochrome P450 metabolites of arachidonic acid are elevated in stroke patients compared with healthy controls

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ABSTRACT

CYP450AAM [arachidonic acid metabolites of the CYP450 (cytochrome P450) enzyme system] have a range of biological functions. CYP450AAM are involved in the pathogenesis of hypertension, renal function and vascular function, yet their role in stroke has not been clarified. We aimed at determining the levels of circulating CYP450 metabolites in patients with acute ischaemic stroke (<96 h) compared with healthy age- and gender-matched controls. This was a retrospective case-controlled study of 44 acute ischaemic stroke patients and 44 matched controls. A subset of acute ischaemic stroke patients was available for follow-up. Acute ischaemic stroke patients had elevated plasma CYP450AAM, including 20-HETE (20-hydroxyeicosatetraenoic acid) (1921 ± 170 compared with 1108 ± 170 pmol/l, \(P < 0.001\)), EETs (epoxyeicosatrienoic acids) (77.88 ± 3.34 compared with 35.35 ± 3.34 nmol/l, \(P < 0.0001\)) and DiHETEs (dihydroxyeicosatetraenoic acids) (92.87 ± 4.61 compared with 68.17 ± 4.61 nmol/l, \(P < 0.0001\)), as well as increased plasma \(F_2\)-isoprostane levels (3754 ± 538 compared with 1947 ± 538 pmol/l, \(P < 0.02\)), the latter a marker of oxidative stress, compared with controls. In a subset analysis of the stroke patients, plasma 20-HETE, EETs and \(F_2\)-isoprostanes were attenuated 30 days after the stroke. Baseline 20-HETE levels were also associated with lesion size and functional indices within the stroke patients. The present study highlights the elevation in CYP450AAM and oxidative stress in acute ischaemic stroke patients. Further investigation of the effect this has on long-term clinical outcome or whether this can be modified by treatment is warranted.

INTRODUCTION

Ischaemic brain injury is thought to result from a cascade of events that includes inflammation, free radical formation and cell death [1]. Studies have shown significant increases in brain concentrations of NEFA (non-esterified ‘free’ fatty acids), including arachidonic acid, following ischaemia and their accumulation may

Key words: acute ischaemic stroke, arachidonic acid, cytochrome P450 (CYP450), dihydroxyeicosatetraenoic acid (DiHETE), epoxyeicosatrienoic acid (EET), 20-hydroxyeicosatetraenoic acid (20-HETE), oxidative stress.

Abbreviations: BHT, butylated hydroxytoluene; BP, blood pressure; CBF, cerebral blood flow; CYP450, cytochrome P450; CYP450AAM, arachidonic acid metabolites of the CYP450 enzyme system; DiHETE, dihydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; MBI, modified Barthel index; MRS, modified Rankin scale; MMSE, Mini Mental Status Examination; NIHSS, National Institutes of Health Stroke Scale; ROI, region of interest; sEH, soluble epoxide hydrolase.

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contribute to inflammation, oxidative damage and increases in intracellular calcium [2]. Arachidonic acid is a major membrane fatty acid that can be metabolized by the CYP450 (cytochrome P450) enzymes to a range of bioactive compounds [3,4] including EETs (epoxyeicosatrienoic acids) and HETEs (hydroxyeicosatetraenoic acids), the major form of which is 20-HETE [5,6]. Arachidonic acid can also undergo free radical attack to form stable secondary metabolites such as F3-isoprostanes [7].

Little is known about the role of 20-HETE in acute ischaemic stroke in humans. Production of 20-HETE has been demonstrated in the cerebral vasculature of the stroke-prone spontaneously hypertensive rat and contributes to stroke severity [8]. Pharmacological inhibition of 20-HETE synthesis reduces infarct size in rats following transient occlusion of the middle cerebral artery [9,10]. In humans, there is limited data available, due to the lack of specific inhibitors of 20-HETE synthesis. Studies investigating polymorphisms in the CYP4F2 (CYP450, family 4, subfamily F, polypeptide 2) gene have shown associations with stroke risk, although this is dependent on the population studied. In a male Swedish population, the V433M variant, a functional polymorphism that reduces 20-HETE production in vitro, is associated with increased BP (blood pressure) and a higher risk of incident ischemic stroke [11]. In contrast the wild-type GG genotype and G allele associated with ischemic stroke in Chinese men [12] and cerebral infarction in Japanese men [13]. However, none of these studies examined circulating levels of 20-HETE. We have shown previously that the functional V433M variant was associated with increased urinary 20-HETE excretion and systolic BP in a hypertensive and normotensive population [14].

EETs are endogenous brain mediators, produced by astrocytes and endothelium and involved in the regulation of CBF (cerebral blood flow) [15,16]. The EETs are known to mediate vasodilation, reduce apoptosis, fever and platelet aggregation and promote angiogenesis [15]. The vascular actions of EETs are moderated by their metabolism to the inactive DiHETEs (dihydroxyeicosatetraenoic acids) by the sEH (soluble epoxide hydrolase) [3].

To date, no studies have examined the circulating levels of 20-HETE, EETs and DiHETEs in acute ischaemic stroke patients. Our aim was to investigate plasma levels of CYP450 metabolites and oxidative stress in acute ischaemic stroke patients compared with age- and gender-matched controls. A secondary aim was to investigate plasma levels of CYP450AAM (arachidonic acid metabolites of the CYP450 enzyme system) and oxidative stress at 30 days post-stroke in a subset of patients available to us.

MATERIALS AND METHODS

Study protocol

This was a retrospective analysis of 44 stroke patients and 44 age- and gender-matched controls taking part in studies carried out within our department. The stroke patients were recruited from Western Australian tertiary hospitals (Royal Perth Hospital and Sir Charles Gairdner) and the aetiology of the ischaemic stroke was classified according to the TOAST criteria [17]. Patients suffering a transient ischaemic attack or haemorrhagic stroke were not included. Stroke patients were studied within 96 h of presenting with a stroke. The 44 unmedicated healthy controls were selected from historical databases held in the University of Western Australia, School of Medicine and Pharmacology Unit at Royal Perth Hospital. They had previously been recruited on the basis of having no known disease nor were they taking any prescription medication or routine non-prescription medication.

Baseline measures

Medical history and medication use were obtained through questionnaires. Within the stroke population, these included the NIHSS (National Institutes of Health Stroke Scale), a brief clinical scale rating neurological impairment where a score of 0 is considered normal with an increasing number indicative of more deficiencies. The MBI (modified Barthel index), a widely used activities of daily living scale used to determine disability, where the greater the number the more independence the patient is likely to have. The MRS (modified Rankin scale), a simple scale to assess handicap where a score of 0 is associated with no symptoms, a poor outcome is generally >3 with 6 representing death. The MMSE (Mini Mental Status Examination) where a score out of 30 used to assess cognitive function (<9 is severe, 10–20 is moderate, 21–24 is mild and >25 is normal). All individuals underwent 24 h ambulatory BP monitoring. A blood sample was collected into EDTA/BHT (butylated hydroxytoluene)/glutathione, plasma was isolated following centrifugation and samples were stored at −80 °C until analysis. Stroketool-CT (DIS-Digital Image Solutions, Cologne, Germany) was used for post processing of DICOM files for analysis of CBF in acute ischaemic stroke patients. When multiple slices were available, the slice with greatest lesion area evident on the plain CT (computed tomography) was chosen for analysis. A CBF threshold of 100 ml per 100 ml of tissue/min was applied to CBF maps to minimize the effect of vascular pixels. Pixels with zero value were then replaced with the mean CBF value. This was done to minimize the effect of variation in placement of the ROI (region of interest). An ROI was placed to encompass the affected hemisphere, and then contralaterally to encompass the control hemisphere. Reading was completed by one observer.
blinded to treatment allocation. The interpreter reliability (established by repeat blinded evaluation of 32 scans [18%]) was high (intraclass correlation of 0.98).

All participants provided written informed consent and the studies were approved by The Royal Perth Hospital or The University of Western Australia Human Ethics Committees.

**Follow-up**

A subset of stroke patients was available for follow-up at 30 days post-stroke where a second blood sample was collected into EDTA/BHT/glutathione. Plasma was isolated following centrifugation and stored at −80°C until analysis. No stroke patients who were available for follow-up were excluded, however, logistical reasons associated with blood collection and storage prevented all available patients from being included.

**Analysis of plasma CYP450 metabolites and markers of oxidative stress**

Plasma 20-HETE was analysed using stable isotope dilution GC/MS as described previously [18]. Ions monitored were m/z 433 and 439 for endogenous 20-HETE and the d20-20-HETE (internal standard) respectively. Total plasma EETs and DiHETEs were measured using stable isotope dilution GC/MS following base hydrolysis and separation on HPLC. Ions monitored were m/z 319 and 330 for endogenous EET and d11-14,15-EET (internal standard) respectively and m/z 481 and 492 for endogenous DiHETE and d11-11,12-DiHETE (internal standard) respectively.

Plasma F2-isoprostane concentrations were analysed using stable isotope dilution GC/MS as described previously [18].

**Statistical analysis**

Data were analysed using SPSS version 17.0. Previous studies within our Department [18] have indicated that 30 subjects per group provides 80% power to detect a 20% difference in 20-HETE. Comparisons between groups were made using ANOVA. Paired Student’s t tests were used for follow-up analysis, and Spearman correlation for lesion and CBF data. Results are presented as means ± S.E.M. Univariate regression models were adjusted for age and gender.

**RESULTS**

**Patient characteristics**

The majority of individuals were male (66%) and the average age was 56 ± 1.3 and 57 ± 1.3 years (values are means ± S.E.M) for control and stroke subjects respectively. As expected ambulatory 24 h systolic and diastolic BPs were elevated in the stroke patients when compared with controls (Table 1). The cause of the ischaemic stroke was ascertained in 44 stroke patients as large-vessel atherosclerosis (n = 10), small vessel occlusion (n = 11), cardioembolism (n = 13), other cause (n = 2) and unknown cause (n = 8). Day 0 functional scores were collected from 44 stroke patients and are presented in Table 2. Day 0 lesion area and CBF data were available in 24 of the acute ischaemic stroke patients. The lesion area range was 38–3037 mm2, mean 826 ± 974 mm2; CBF in the affected hemisphere was 17.7–47.5 ml/100 ml/min, mean 34.2 ± 7.3 ml/100 ml/min; and CBF in the control hemisphere was 17.7–46.10 ml/100 ml/min, mean 34.3 ± 7.1 ml/100 ml/min.

**Plasma CYP450 metabolites**

Stroke patients had significantly higher plasma 20-HETE (1921 ± 170 compared with 1108 ± 170 pmol/l; P = 0.001), EETs (77.88 ± 3.34 compared with 35.35 ± 3.34 nmol/ml; P < 0.0001) and DiHETEs (92.87 ± 4.61 compared with 68.17 ± 4.61 nmol/ml; P < 0.0001) compared with controls, before and after adjustment for age and gender (Figure 1). Adjustment for systolic or diastolic BP did not alter results for plasma 20-HETE or EETs. Plasma DiHETEs were no longer significantly different between groups (P = 0.08) following adjustment for systolic BP. Baseline blood collection time (0–96 h) did not affect CYP450AAM in stroke patients and there was no significant correlation between collection time and plasma 20-HETE (r = −0.219, P = 0.153), EETs (r = 0.04, P = 0.795) or DiHETEs (r = 0.227, P = 0.138). Smoking status did not affect CYP450AAM concentrations and there were no significant differences between non-smokers, ex-smokers and current smokers (results not shown). None of the control group was a smoker or regular user of aspirin. Within the stroke population, those receiving anti-platelet therapy had significantly lower plasma EETs (21.41 ± 1.61 compared with 27.31 ± 1.98 nmol/l; P < 0.05) whereas plasma 20-HETE and DiHETEs were not different.

In a subset analysis (n = 13) of stroke patients at 30 days post-stroke, plasma 20-HETE and EETs concentrations were attenuated (Table 3). Plasma DiHETE concentrations were not able to be analysed due to an insufficient number of samples available. There were no significant differences in baseline levels of plasma 20-HETE and EETs between stroke patients who were followed up and those who were not (results not shown).

Twenty-four patients had baseline information on lesion size and CBF. Within this subset, there was a significant positive correlation between lesion size and plasma 20-HETE (r = 0.44, P < 0.05), but not EETs (r = −0.318, P = 0.13) or DiHETEs (r = −0.137, P = 0.522). There were no associations between CBF and any of the CYP450AAM. Circulating 20-HETE was also associated with greater neurological impairment (NIHSS) (b = 0.716, P < 0.001), a reduced MBI (b = −0.493,
Table 1  Patient characteristics

Values are means ± S.E.M. or numbers (%). Rx, medication.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n = 44)</th>
<th>Stroke patients (n = 44)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.0 ± 1.3</td>
<td>57.0 ± 1.3</td>
<td>0.578</td>
</tr>
<tr>
<td>Gender (n) (male/female)</td>
<td>29/15</td>
<td>29/15</td>
<td>0.589</td>
</tr>
<tr>
<td>24 h systolic BP (mmHg)</td>
<td>118.3 ± 1.4</td>
<td>139.6 ± 3.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>24 h diastolic BP (mmHg)</td>
<td>71.9 ± 1.1</td>
<td>82.1 ± 1.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hypercholesterolaemia (n)</td>
<td>—</td>
<td>8 (18%)</td>
<td></td>
</tr>
<tr>
<td>Diabetes (n)</td>
<td>—</td>
<td>8 (18%)</td>
<td></td>
</tr>
<tr>
<td>Atrial fibrillation (n)</td>
<td>—</td>
<td>4 (9%)</td>
<td></td>
</tr>
<tr>
<td>Peripheral vascular disease (n)</td>
<td>—</td>
<td>2 (5%)</td>
<td></td>
</tr>
<tr>
<td>Other cardiovascular disease (n)</td>
<td>—</td>
<td>13 (30%)</td>
<td></td>
</tr>
<tr>
<td>Anti-hypertensive Rx (n)</td>
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<td>23 (52%)</td>
<td></td>
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<tr>
<td>Lipid-lowering Rx (n)</td>
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<td>13 (30%)</td>
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<tr>
<td>Anti-platelet Rx (n)</td>
<td>—</td>
<td>18 (41%)</td>
<td></td>
</tr>
<tr>
<td>Anti-diabetic Rx (n)</td>
<td>—</td>
<td>4 (9%)</td>
<td></td>
</tr>
<tr>
<td>Tobacco use (n)</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>—</td>
<td>25 (57%)</td>
<td></td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>—</td>
<td>10 (23%)</td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>—</td>
<td>9 (20%)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1  Plasma CYP450AAM

(a) 20-HETE, (b) EETs, (c) DiHETEs and (d) F2-isoprostanes. # P < 0.001, * P < 0.0001 and † P = 0.02 as determined following adjustment for age and gender by ANOVA.

P = 0.02) and reduced cognitive function (MMSE) (b = −0.844, P < 0.0001). There was no association between these functional indices and plasma EETs or DiHETEs.

Because gender is known to influence CYP450 metabolites, we investigated differences between males and females. Within the whole group and also in the stroke patients, there was no significant difference between men and women in plasma CYP450 metabolites. Within the control subjects, there were also no significant differences except for plasma EETs, which were significantly higher in women compared with men (P = 0.0002).

Plasma F2-isoprostanes

Plasma F2-isoprostanes were significantly elevated in stroke patients compared with controls (3754 ± 538 compared with 1947 ± 538 pmol/l, P = 0.02) before and after adjustment for age and gender (Figure 1). Plasma
F₂-isoprostane levels were not affected by smoking status and there were no significant differences between non-smokers, ex-smokers and current smokers (results not shown). At 30 days post-stroke, in a subset of stroke patients \( \bar{n} = 28 \) plasma F₂-isoprostane levels were attenuated (Table 3).

## DISCUSSION

The major finding from the present study is that patients suffering from an acute ischaemic stroke have elevated CYP450-derived plasma 20-HETE, EETs and DiHETEs concentrations, as well as increased oxidative stress as evidenced by increased plasma F₂-isoprostanes, compared with age- and gender-matched controls. At 30 days post-stroke, the levels of 20-HETE, EETs and F₂-isoprostanes were attenuated relative to when patients first presented. Within a subset of the stroke patients, 20-HETE was associated with lesion size and reduced functional outcome. These findings raise the possibility of a role for CYP450AAM in both the pathogenesis of acute ischaemic stroke and a worse clinical outcome.

20-HETE is a potent vasoconstrictor [3] although less is known about its role in the brain. In rats, 20-HETE synthesis inhibition with TS-011 (a pharmacological inhibitor) reduced both cerebral 20-HETE levels and total infarction volume following transient occlusion of the middle cerebral artery [9,10]. There was no effect on CBF, and EET and DiHETE levels were not detected [9]. We have previously shown in humans that 20-HETE excretion is associated with both hypertension [14] and endothelial dysfunction [19]. A recent study in Singaporeans observed elevated levels of total plasma and urinary HETEs in ischaemic stroke patients compared with controls, which then decreased with recovery [20]. Although that study is in agreement with our present findings, it is harder to interpret as several isomers of HETEs were analysed together. The biological activity of each individual isomer is not completely understood, and several of these are actually produced via non-enzymatic free radical-mediated peroxidation of arachidonic acid [20]. The 20-HETE isomer is a known vasoconstrictor, however, and as ischaemic stroke results from vascular occlusion, it is possible and supported by the present findings that 20-HETE may play a role in the pathogenesis of acute ischaemic stroke. Interestingly, at 30 days post-stroke, plasma levels of 20-HETE were reduced. This may be due to acute activation of the CYP450 enzyme immediately following stroke or a subsequent reduction of arachidonic acid substrate in the days following an acute ischaemic stroke. Although we observed no relationship between 20-HETE and CBF, the positive correlation we observed between 20-HETE and lesion size supports animal studies that have found reduced infarct size following inhibition of 20-HETE synthesis [9]. The associations between 20-HETE and functional outcomes also strengthen the hypothesis that 20-HETE plays a role in the pathophysiology of acute ischaemic stroke and subsequent clinical outcome.

In contrast with the vasoconstrictor actions of 20-HETE, EETs are vasodilators that activate K⁺ channels to hyperpolarize VSMCs (vascular smooth muscle cells) [6]. They have been proposed as the endothelium-derived hyperpolarizing factor [21]. The vascular actions of EETs are moderated by their metabolism to the inactive DiHETEs by the sEH enzyme [3]. In a mouse model of stroke, inhibiting sEH results in reduced ischaemic

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Day 0</th>
<th>Day 30</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Plasma EETs (nmol/l) ( \bar{n} = 13 )</td>
<td>80.71 ± 10.67</td>
<td>51.29 ± 9.98</td>
<td>0.02</td>
</tr>
<tr>
<td>Plasma 20-HETE (pmol/l) ( \bar{n} = 13 )</td>
<td>1391 ± 226</td>
<td>699 ± 152</td>
<td>0.004</td>
</tr>
<tr>
<td>Plasma F₂-isoprostanes (pmol/l) ( \bar{n} = 28 )</td>
<td>4747 ± 1153</td>
<td>2737 ± 327</td>
<td>0.06</td>
</tr>
</tbody>
</table>
damage and elevated cortical blood flow during vascular occlusion [22,23]. EETs are also produced and released by perivascular nerves and may play a role in neurogenic relaxation of the cerebral vasculature [16]. We observed elevated levels of both EETs and DiHETEs in the stroke patients compared with controls. The increased circulating DiHETEs could suggest that stroke patients had increased sEH activity. An alternative explanation is that all CYP450 metabolites are increased with the release of fatty acids that accompanies ischaemic stroke [2]. Interestingly, at 30 days post-stroke, the plasma levels of EETs had been attenuated. It is unclear what change there was in plasma DiHETEs as the number available for analysis was too small. Again these changes may be due to an acute activation of the enzyme following ischaemia, a reduction in the availability of substrate or homeostatic response.

Ischaemic brain injury is thought to result from a cascade of events that includes inflammation, free radical formation and cell death [1]. The metabolism of arachidonic acid by the CYP450 enzyme system may also be a significant source of oxidative damage [24]. The present study supports this hypothesis with stroke patients having elevated plasma F₂-isoprostane concentrations compared with controls. At 30 days post-stroke, this marker of oxidative damage was reduced, suggesting the free radical formation that accompanies ischaemic brain injury is an acute response. This is in agreement with two previous studies that observed elevated plasma F₂-isoprostanes in the hours following acute ischaemic stroke, which were not observed at later time points [20,25]. Although we saw no significant association between plasma F₂-isoprostanes and smoking, this may be due to the small number of smokers (n = 9) in the stroke population.

The present study is the first to examine individual circulating plasma CYP450AAM in a human stroke population and compare this with age- and gender-matched controls. Although the study is limited by the small number of participants, potential for selection bias, interval to collection of samples and possibility that clinical treatment may have influenced CYP450AAM, our findings suggest a role for both CYP450AAM and oxidative damage in the pathogenesis of stroke, certainly in the days immediately following ischaemia. Although the follow-up analysis was limited by the small number of samples available and the possibility that interval treatment may have further influenced CYP450AAM, there is a suggestion that CYP450AAM levels were attenuated at 30 days post-stroke. Research investigating the effect of CYP450AAM on long-term outcome and whether this can be improved by treatment is warranted. Further elucidation of the role of CYP450AAM should include exploration of the role of function polymorphisms in the CYP4F2 gene that have been shown to be associated with increased BP and incidence of ischaemic stroke in Swedish men [11].

In conclusion, the present study has shown elevated plasma 20-HETE, EETs, DiHETEs and oxidative stress in acute ischaemic stroke, compared with healthy age- and gender-matched controls.

AUTHOR CONTRIBUTION

Natalie Ward was responsible for the study design, sample analysis, interpretation of data, and drafting and preparation of the paper. Kevin Croft, David Blacker, Graeme Hankey, Anne Barden, Trevor Mori, Ian Puddey and Christopher Beer were responsible for study design, interpretation of data and critical review of paper prior to submission.

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