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Plasma free fatty acid profile is dramatically and acutely changed under ischemic stroke in the mouse model

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Abstract

Although plasma biomarkers would facilitate rapid and accurate diagnosis of ischemic stroke for immediate treatment, no such biomarkers have been developed to date. In the present study, we tested our hypothesis that plasma free fatty acids (FFA) are altered at early stages of acute ischemic stroke. Plasma was collected from mice 2 h after permanent middle cerebral artery occlusion (pMCAo) onset, as well as from sham-operated and control animals. After 2 hours, pMCAo significantly changed the plasma FFA profile with the most dramatic 2-to 3-fold relative increase in very long n-3 and n-6 FFA including 20:4n-6, 22:4n-6, 22:5n-6, and 22:6n-3. Changes in plasma FFA profile are consistent with FFA liberation from brain phospholipid hydrolyzed under ischemic insult. These results identify, for the first time, the plasma FFA profile as a potential biomarker for an early ischemic stroke within the therapeutic window for thrombolytic treatment. Further studies are required to confirm its specificity and sensitivity in clinical settings.

Keywords

ischemic stroke; lipids; plasma; free fatty acids; biomarkers

Introduction

Immediate treatment can play a major role in the outcome of ischemic stroke and helps to improve prognosis in many cases (Davis et al., 2006). One of the factors affecting immediate treatment is the rapid and accurate diagnosis of ischemic stroke. In addition to clinical and imaging studies, plasma biomarkers may significantly aid in the early management of ischemic stroke. Importantly, the only FDA-approved therapeutic treatment for ischemic stroke is tissue plasminogen activator administration within 3–4.5 hours of stroke onset. Thus, the ideal biomarker for ischemic stroke would be readily detectible within this window for intravenous thrombolysis (del Zoppo et al., 2009).

To date, the majority of potential stroke biomarkers studied are of a peptide/protein or nucleotide nature and do not meet this requirement (Ahmad et al., 2012). One of the reasons

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Conflict of Interest:
None

is that these biomarkers are hydrophilic and do not readily cross the blood-brain barrier (BBB). Thus, brain-specific peptides/proteins such as S100b and neuron-specific enolase do not increase in plasma until 10–18 hours post-ischemia and do not correlate with infarct volume prior to 24 hours after injury (Ahmad et al., 2012; Fassbender et al., 1997). Many other brain-specific proteins studied have similar limitations (Ahmad et al., 2012; Fassbender et al., 1997; Kövesdi et al., 2010; Wunderlich et al., 2006), while proteins such as copeptin found elevated under acute stroke conditions are highly non-specific for the brain and may serve as a more general marker of physiologic stress (Katan et al., 2009; Nickel et al., 2012). Another promising approach is utilization of blood gene expression analysis to identify the etiology of acute ischemic stroke (Jauch et al., 2017). However, the timing of RNA expression that requires blood collection 24 hours after stroke onset (Jauch et al., 2017) may add limitations to this approach to meet “the ideal ischemic stroke biomarker” requirements.

However, lipid biomarkers may overcome these limitations. Lipids are hydrophobic and readily cross the BBB, and they account for about 10% of brain wet weight (O’Brien and Sampson, 1965). They have a brain-specific profile with one of the highest arachidonic (20:4n-6), docosahexaenoic (22:6n-3), and adrenic acid (22:5n-6) concentrations esterified in the *sn*-2 position of glycerophospholipids (Carrié et al., 2000a; Carrié et al., 2000b; Marcheselli et al., 1988). Additionally, alterations in brain lipids develop immediately upon ischemic injury through fatty acid release by a phospholipase A_2 family (PLA_2) (Bazan, 1970, 1971; Brose et al., 2016; Brose et al., 2011; Golovko and Murphy, 2008; Hamilton et al., 2007). Recently, using a targeted quantification, a brain-specific sphingolipid was identified as a promising biomarker for brain damage 24 h following ischemic stroke onset, demonstrating the feasibility of using lipid biomarkers to diagnose stroke (Sheth et al., 2015).

In the present study, we investigated the use of plasma free fatty acid (FFA) profile as a potential biomarker for early ischemic stroke. Two hours of permanent middle cerebral artery occlusion (pMCAo) significantly changed the plasma FFA profile with the most dramatic 2-to 3-fold relative increase in very long n-3 and n-6 FFA. These results are the first to identify plasma FFA profiles as a potential biomarker for early ischemic stroke within the therapeutic window for thrombolytic treatment. Further studies are required to confirm its specificity and sensitivity in clinical settings.

Methods

Animals

All animal use was approved by the University of North Dakota IACUC (protocol #1503–8). C57BL/6 male mice (22–25g, 8–10 weeks old) were provided standard laboratory chow and water ad libitum.

pMCAo Model and Blood Collection

Animals were fasted overnight before the experiment, and a 2-hour pMCAo was induced using an intraluminal filament as previously described (Jackman et al., 2011). Animals were

not given food after the surgery. Briefly, mice were anesthetized with 1.75–2.25% isoflurane, and a standardized microfilament (702345PK5Re, Doccol Corporation, Sharon, MA) was inserted into the internal carotid occlude the origin of middle cerebral artery (MCA). Body temperature was maintained between 36.5^0C to 37.5^0C using a heating pad, and was measured with a probe placed rectally. Cerebral blood flow was measured using a Moor Instruments (Axminster, Devon, UK) DRT4 laser Doppler monitor with the sensor placed in the ischemic center (2 mm posterior, 5 mm lateral to the bregma). If blood flow was reduced by less than 80% (20% of the pre-ischemia values), animals were excluded from analysis on the basis of incomplete ischemia. Importantly, the blood flow was recorded from the probe placed on the surface of the scull. Therefore, the meninges blood flow interfered with the cortex flow recording, and this method of monitoring does not allow to detect a 100% blood flow reduction in the ischemic core. A similar surgical procedure was performed in the sham-operated group, except the filament was inserted and immediately withdrawn from the vessel without advancing it to the MCA origin. Two hours after surgery, the animals were anesthetized with ketamine/xylazine (i.p., 100mg and 10 mg per kg, respectively) and blood was collected by a cardiac puncture into heparinized microfuge tubes placed on ice. Control animals were not subjected to surgery prior to blood collection. Plasma was separated by centrifugation and immediately extracted for FFA analysis.

Plasma FFA Extraction and Analysis

Ten μL of plasma was extracted with 90 μL of methanol containing 0.02% BHT (Brose et al., 2013; Brose et al., 2014) and a mixture of internal standards (Cambridge Isotope Laboratories, Tewksbury, MA). For each sample, we used 1 μg of palmitic acid 16:0-$^{13}C_{16}$ for saturated fatty acids (FA) quantification, 100 ng of 18:1-$^{13}C_{18}$ for monounsaturated FA, and 100 ng of 20:4n-6-$^{2}H_{8}$ for polyunsaturated FA (PUFA). After centrifugation, 10 μL of supernatant was injected into the ultra-high-pressure liquid chromatography - mass spectrometry (UPLC-MS) system for analysis.

UPLC-MS analysis was performed as previously described (Brose et al., 2014; Wang et al., 2014). The UPLC system consisted of a Waters ACQUITY UPLC pump with a well-plate autosampler (Waters, Milford, MA) equipped with an ACUITY UPLC HSS T3 column (1.8 μM, 100 A pore diameter, 2.1×150 mm, Waters) and an ACUITY UPLC HSS T3 Vanguard precolumn (1.8 μM, 100 A pore diameter 2.1 × 5 mm, Waters). One microliter of a sample was injected onto the column. The column temperature was 55°C and the autosampler temperature was 8°C.

Solvent A consisted of acetonitrile : water (40 : 60) with 10 μM ammonium acetate and 0.025% acetic acid. Solvent B was acetonitrile : 2-propanol (10 : 90) containing 10 μM ammonium acetate and 0.02% acetic acid. The flow rate was 0.3 mL/min, and the initial %B was 30%. At 0.1 min %B was increased to 54% over 10 min, to 99% over another 10 min, held at 99% for 8 min, and then returned to initial conditions over 0.5 min. The column was equilibrated for 2.5 min between injections.

FA were quantified using a quadrupole time-of-flight mass spectrometer (Q-TOF, Synapt G2-S, Waters) with electrospray ionization in negative ion mode as described previously (Brose et al., 2014; Wang et al., 2014). MassLynx V4.1 software (Waters) was sused for

instrument control, acquisition, and sample analysis. FA were quantified against corresponding internal standards using generated standard curves.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad, San Diego; CA). Statistical comparisons were determined using ANOVA with Tukey's *post-hoc* test with statistical significance defined as <0.05. All values are expressed as mean ± SD, n=4 animals per group.

Results and Discussion

Although plasma biomarkers would facilitate rapid and accurate diagnosis of early ischemic stroke for immediate treatment, no such biomarkers have been developed to date. Compared to other molecules, FA have a number of advantages as potential stroke biomarkers. They are hydrophobic and readily cross the BBB, have a brain-specific profile, and are released from esterified from within minutes upon ischemia onset.

Previously, using animal models, plasma lipids were analyzed 24 h after transient MCAo (tMCAo) under reperfusion conditions when brain FA may be washed out from the necrotic regions through alterations to the BBB. Under these conditions, 20:4n-6 was increased 2-fold (Rodriguez de Turco et al., 2002), while 22:6n-3 was unaffected. Conflicting with this study, free 20:4n-6 was decreased by 25%, while most n-3 PUFA were increased in plasma in the other report (Paik et al., 2009), although no ANOVA analysis was performed for statistical comparison between the groups in this report. In addition, plasma ceramides were also elevated 24 h after tMCAo (Sheth et al., 2015). In humans, plasma FFA level were positively correlated with the lesion volume when measured 2 days after ischemic stroke event (Chung et al., 2017). However, neither FFA composition nor early time-points were addressed in this study. Importantly, the ideal biomarker for ischemic stroke would be detectible within few hours upon stroke onset to allow intravenous thrombolysis treatment. However, plasma FFA composition at this early time point, as well as under pMCAo when there is a limited reperfusion, has not been previously addressed in animal studies or in clinical settings.

To evaluate plasma FFA profile as a potential biomarker for early ischemic stroke within the therapeutic window for thrombolytic treatment, we analyzed, for the first time, plasma FFA composition 2 h after pMCAo onset (Fig. 1 and 2). The relative concentration (in mole%) of saturated FFA (SFA) was significantly decreased ~35%, while brain-abundant monounsaturated FA (MUFA) and PUFA were significantly increased ~1.6- and ~1.4-fold, respectively, in plasma compared to the control and sham-operated groups. Importantly, there were no differences in plasma FFA between the control and sham-operated animals, indicating that the surgery itself did not have an effect on the observed FFA alterations in plasma. Because the absolute concentration (in nmole/mL) of SFA was unaffected by pMCAo (Fig. 2), the relative decrease in SFA was the result of an increase in plasma-free MUFA and PUFA rather than increased SFA consumption under stroke conditions. The most significantly altered MUFA was 18:1n9 (1.6-fold increase), and among PUFA - 20:4n-6, 22:4n-6, 22:5n-6, and 22:6n-3 (an increase of 1.8- to 2.3-fold compared to the sham and

control groups, Fig. 1). This is consistent with immediate phospholipase activation in the ischemic brain (Bazan, 1971; Carrié et al., 2000a) that release brain-abundant FA esterified in phospholipids. Although possible, it is unlikely that plasma FFA are elevated through the liberation from other tissues including adipose tissue. The only significant site of FFA liberation into plasma under physiological and fasting conditions is adipose tissue (Eaton et al., 1969; Mortiaux and Dawson, 1961), however released adipose free fatty acids have a different fatty acid profile with very low mole % of long chain PUFA (Halliwell et al., 1996; Hodson et al., 2008; Malcom et al., 1989), making free fatty acids released from brain distinguishable from this pool in plasma.

Additional factors that may potentially limit FFA diagnostic value are human plasma FFA variability/fluctuations and time required for FFA analysis. Although absolute concentration of total FFA is quite variable with ~60% relative standard deviation (Choi et al., 2014; Chung et al., 2017), individual FFA relative concentrations (mole %) are within 10–20% for most plasma FFA (Ågren et al., 1995; Hodson et al., 2008; Jacobsen et al., 1983; Melchert et al., 1987; Umhau et al., 2009; Yli-Jama et al., 2002), indicating that variability is not a limiting factor for using plasma FFA as biomarkers. In addition, long chain free PUFA are not significantly affected by fed/fated state because dietary FA are transported in esterified form, and FFA liberated from adipose tissue do not significantly affect human plasma PUFA (Halliwell et al., 1996). The estimated time for FFA analysis including plasma collection, extraction, and LC-MS analysis, is ~1.5 hour that is within the time required for magnetic resonance imaging, however this time may be decreased in the future with the development of express methods for FFA profiling. However, further validation of plasma FFA profiling specificity for stroke compared to other clinical conditions is needed.

Our results unveil the plasma FFA profile as a potential biomarker for early ischemic stroke within the therapeutic window for thrombolytic treatment. Further studies are required to confirm its specificity and sensitivity in clinical settings.

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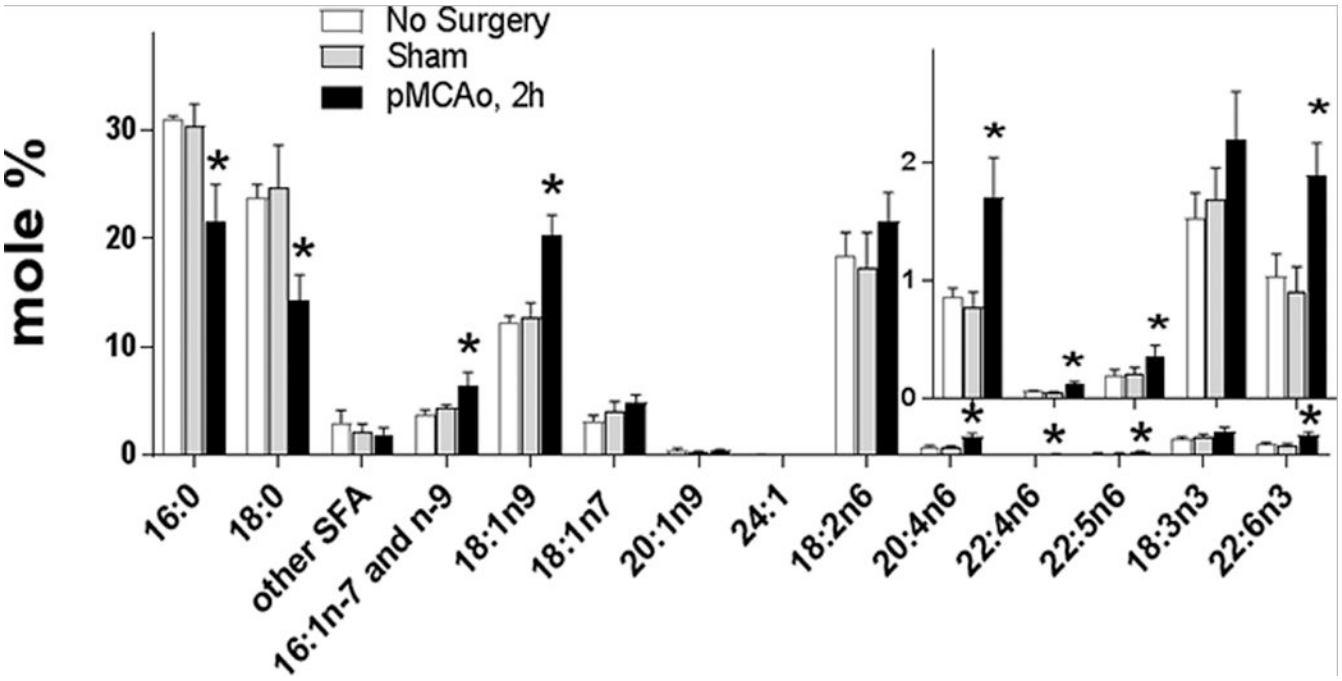

Figure 1.

Relative plasma free fatty acid alterations 2 h after pMCAo onset.

Plasma free fatty acids were analyzed two hours after permanent middle cerebral artery occlusion (pMCAo), in sham operated, and control animals using a UPLC-MS method against stable isotope labeled internal standards. Data are mean ± SD (n=4). * - Statistically different ($p<0.05$) from other groups using ANOVA with Tukey *post-hoc* test.

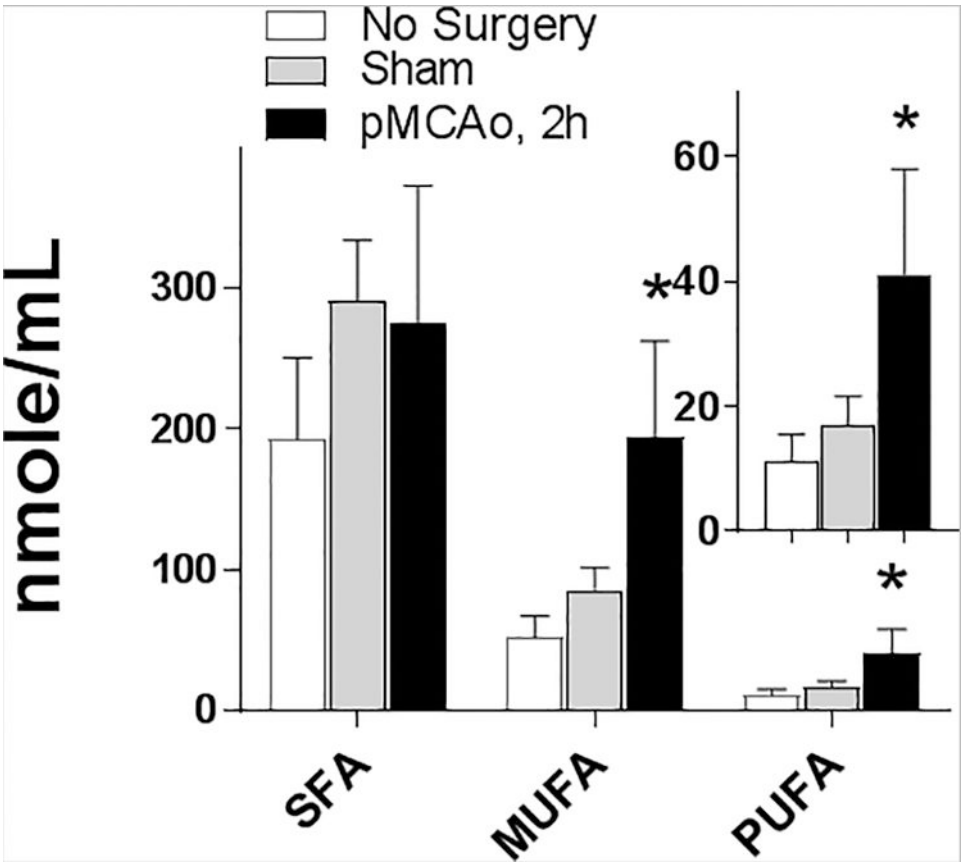

Figure 2.

Absolute plasma free fatty acid concentration 2 h after pMCAo onset.

Plasma free fatty acids were quantified two hours after permanent middle cerebral artery occlusion (pMCAo), in sham operated, and control animals using a UPLC-MS method against stable isotope labeled internal standards. SFA: the sum of 14:0, 16:0, 18:0, 20:0, 22:0, and 24:0; MUFA: the sum of 16:1n-7, 16:1n-9, 18:1n-7, 18:1n-9, 20:1n-9, and 24:1; PUFA: the sum of 18:2n-6, 18:3n-3, 20:4n-6, 22:4n-6, 22:5n-6, and 22:6n-3. Data are mean ± SD (n=4). * - Statistically different ($p<0.05$) from other groups using ANOVA with Tukey *post-hoc* test.