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NMR analysis of the rat neurochemical changes induced by middle cerebral artery occlusion

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ABSTRACT

Stroke is a leading cause of death and disability, affecting millions of people worldwide with almost 80% of them as ischemic stroke and understanding the multiple mechanisms underlying cerebral ischemia is essential for development of effective treatments. To understand metabolic changes induced by focal brain ischemia, we conducted a comparative analysis of metabolic composition of cerebral tissue from rats with sham-operation and middle cerebral artery occlusion (MCAO) using high-resolution nuclear magnetic resonance (NMR) spectroscopy. More than 40 metabolites were assigned including organic acids, amino acids, carbohydrates, choline, pyrimidine and purine metabolites. Our results showed that MCAO led to significant level decreases for glutamate, glutamine, aspartate, γ-aminobutyrate (GABA), taurine, malate, fumarate, acetate, phosphocreatine, and purine and pyrimidine metabolites such as inosine, hypoxanthine, xanthine, uracil and UDP/UTP, together with significant level increases for glucose in focal brain tissue extracts. This demonstrated that experimental ischemic stroke in rats caused extensive perturbation in tricarboxylic acid cycle, GABA shunt, and metabolisms of choline and nucleic acids. These findings provided essential information for our understandings of MCAO-caused biochemical alterations and demonstrated the metabolite composition analysis as a useful tool for understanding the neurochemistry of stroke.

1. Introduction

Stroke is a serious cause of both morbidity and mortality affecting the health of over 16 million people worldwide every year [1] and has become a heavy burden on both the concerned families and community. To be more specific, more than 5 million man and women died and another 5 million were disabled by stroke annually [2]. In United States alone, the direct and indirect costs caused by stroke were estimated to be more than 40 billion U.S. dollars in 2007 [3]. The total losses caused by ischemic stroke from 2005 to 2050 is projected to reach 2.2 trillion dollars [4]. With the rise of aging population, the burden will also be increased greatly in developing countries including China. Almost 80% of all strokes are ischemic cerebral events in both developed and developing countries [5]. Despite the prevalence and severe consequences, however, there are few effective treatments available for ischemic stroke. The only FDA-approved clot-busting drug, tissue plasminogen activator, is required to be administered within 3 h from the onset of stroke. However, such requirement is not easy to meet. In United State, for instance, only less than 5% ischemic stroke patients can reach hospitals in time and be considered for this treatment. So far, the pathogenesis is not clearly understood and thus comprehension of the stroke related pathophysiology is essential for disease prediction and effective therapeutic screening to minimize the severity of ischemic damage.

Focal brain ischemia results from an abrupt blood flow reduction below a critical threshold because of obstruction of a major brain artery. The restricted delivery of oxygen and glucose impairs the ATP synthesis and this energy failure can cause the ischemic cascades such as acidosis [6], inflammation [7], production of reactive oxygen species [8], necrosis, apoptosis and eventually neuronal death [9]. Metabolic disturbance plays an important role in brain ischemic injury, including an increase in anaerobic glycolysis, excessively release of glutamate to extracellular space [10], perturbation of glutamate–glutamine cycle [11] and turnover of

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protein synthesis [12]. Therefore, metabolic analysis ought to be a useful approach for understanding the biochemical aspects of stroke.

Magnetic resonance spectroscopy (MRS) has been proven as a powerful tool to investigate the metabolic alterations in ischemic stroke *in vivo*. Previous *in vivo* studies showed that cerebral ischemia led to the level changes in lactate, creatine, N-acetylaspartate (NAA) and choline [13–16]. However, with low sensitivity and limited resolution, the detectable and assignable metabolites from MRS were limited. High-resolution nuclear magnetic resonance (NMR) spectroscopy offers a better alternative to acquire more metabolic information since these techniques are capable of simultaneously detecting a wide range of metabolites and their changes associated with physiological and genetic alterations. Multivariate data analysis approaches [17–19], such as principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), are normally employed to uncover and visualize the metabolic alterations relative to the biological disturbance of interests. Such combination has become the technical basis for so-called metabonomics [20,21]. In practice, metabonomics typically makes use of high-resolution NMR or mass spectrometry (MS) analyses to provide metabolic profiles of biological samples such as urine, blood and seminal fluid. Consequently, metabonomics has been successfully used as an important tool for understanding pathophysiology [22–25], identifying biomarkers [26,27], disease diagnosis [23,28] and mechanistic aspects of toxicity [29–31].

MCAO has been employed as one of classic models to study the pathogenesis of focal brain ischemia in term of changes in gene expression, transcriptions and proteins. More recently, NMR and high performance liquid chromatography (HPLC) were used to observed metabolic changes under ischemic condition. These works showed that cerebral ischemia caused level increases for adenosine at 4 h [32] and for inosine and hypoxanthine 6 h after occlusion in extracellular fluid of striatum [33].

Elevation of acetate and decreases of glutamine and aspartate were also observed in the ischemic tissue 6 h after occlusion [34–36]. NMR measurements of cerebral ischemic tissue extracts indicated that focal ischemia caused decrease in aspartate, glutamate, NAA and total creatine at 24 h after occlusion [34]. However, these studies were fragmented in terms of the metabolisms and the holistic metabolic responses of cerebral tissues to focal brain ischemia remained to be fully understood.

In this work, we applied the NMR-based metabonomic strategy to search for the metabolic variations in ischemic brain tissue following experimental stroke induced by an intraluminal filament occlusion of the middle cerebral artery. The aim of this study is to further define the metabolic features associated with the occlusion of middle cerebral artery.

2. Materials and methods

2.1. Chemicals

2,3,5-Triphenyltetrazolium chloride (TTC) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Sodium chloride, $K_2HPO_4{\cdot}3H_2O$ and $NaH_2PO_4{\cdot}2H_2O$ (all in analytical grade) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); D_2O (99.9% in D) and sodium 3-trimethlysilyl [2,2,3,3-D_4] propionate (TSP) were purchased from Cambridge Isotope Laboratories (Miami, FL). K_2HPO_4/NaH_2PO_4 buffer (0.1 M, pH 7.4) was employed for NMR studies due to their good solubility and storage stability [37], which contained D_2O (10%, v/v) as a field lock and TSP (0.03 mM) as chemical shift reference.

2.2. Animal handling

Animal procedures were conducted in accordance with the National Guidelines for Experimental Animal Welfare (Ministry of Science and Technology of People's Republic of China, 2006). Male Sprague-Dawley rats were purchased from Vital River Laboratories (Beijing, China) and housed in groups of five and allowed to acclimate for at least five days in a temperature-controlled environment (24 °C) with lighting between 07:00 and 19:00. All animals had free access to food and water throughout the study and were randomly assigned to sham-operated and MCAO groups with body weigh of 248–260 g after acclimation.

2.3. Focal brain ischemia models

Permanent focal cerebral ischemia was induced using the filament model as described previously [38]. Briefly, rats were anesthetized with 10% chloral hydrate. Through a midline neck incision, the right external and internal carotid arteries were dissected from the surrounding connective tissue. A nylon monofilament coated with paraffin wax was inserted into the right common carotid artery and advanced into the right internal carotid artery until mild resistance was felt, indicating that the filament was positioned properly and occluded blood flow to the middle cerebral artery. Sham-operated rats were subjected to the same surgical procedure except that filaments inserted into the right common carotid artery were 0.5 cm. Nylon monofilaments were soaked in gentamicin injection and saline for 30 min, respectively, and washed with saline before used. All surgical procedures were kept in a sterile environment. The operated animal body temperature was kept about 37 °C until neurological evaluation. Neurological evaluation was performed 2 h and 26 h after occlusion of middle cerebral artery according to a 5-points scale described in a previous report [39]. Briefly, a score of 0 means no neurological deficit; a score of 1 indicates failure to extend left forepaw fully; a score of 2 means circling to the left on the ground; a score of 3 means falling to the left when walking; rats with a score of 4 could not walk spontaneously and with disturbance of consciousness. Rats scored 1–3 at 2 h post occlusion were considered as successful MCAO models.

In order to estimate the infarct volume for tissue extraction, three rats were subjected to MCAO with infarct volume visualized by TTC staining as previously described [39]. Briefly, 2 mm-thick coronal sections of brain tissue were stained in 2% TTC for 30 min at 37 °C following 12 h immersion in 10% formalin. Normal cerebral tissue was stained red whereas the infarct tissue unstained.

2.4. Tissue extraction procedure

Twenty-six hours after insertion of filaments, rats were sacrificed with decapitation and brain tissues were quickly removed, snap-frozen in liquid nitrogen and stored at −80 °C until further analysis. During extraction, the ischemic part of right brain supplied blood flow by the middle cerebral artery was removed, weighed and then ground in liquid nitrogen. 600 μl CH_3OH/H_2O (1:1) was added to the ground brain tissue and allowed to thaw for 10 min followed with 30 s intermittent sonication (10 s sonication and 10 s break, repeated for three times) in an ice bath. After centrifugation at 11180 × *g* at 4 °C for 10 min, the supernatant was transferred into a new EP tube and the pellet was re-extracted twice with the same procedure. The supernatants from three extractions were combined. Methanol was removed from the pooled supernatant under vacuum in a Savant SpeedVac concentrator (Thermo SAVANT, SC110A-230) for 18 h and then lyophilized in a freeze-drier for at least 24 h. The dried powder samples were added with 600 μl Na–K phosphate buffer (0.1 M, pH 7.4) containing 10% D_2O and 0.03 mM TSP. After centrifugation (11180 × *g*) at 4 °C for 5 min,