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Rapamycin ameliorates brain metabolites alterations after transient focal ischemia in rats

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ABSTRACT

Rapamycin has been shown to protect against middle cerebral artery occlusion (MCAo) induced ischemic injury. In this study, the neuroprotective effect of rapamycin on the metabolic changes induced by MCAo was evaluated using nuclear magnetic resonance (NMR) spectroscopy of brain tissues. MCAo in rats was induced by insertion of nylon filament. One hour after ischemia, rapamycin (250 μg/kg, i.p.) in dimethyl sulfoxide was administered. Reperfusion was done 2 h after ischemia. Twenty-four hours after ischemia phospholipase A_2 (PLA_2) levels and metabolic changes were assessed. Perchloric acid extraction was performed on the brain of all animals ($n=7$; sham, vehicle; DMSO and rapamycin 250 μg/kg) and the various brain metabolites were assessed by NMR spectroscopy. In all 44 metabolites were assigned in the proton NMR spectrum of rat brain tissues. In the vehicle group, we observed increased lactate levels and decreased levels of glutamate/glutamine, choline containing compounds, creatine/phosphocreatine (Cr/PCr), taurine, myo-inositol, γ-amino butryic acid (GABA), N-aspartyl aspartate (NAA), purine and pyrimidine metabolites. In rapamycin treated rats, there was increase in the levels of choline containing compounds, NAA, myo-inositol, glutamate/glutamine, GABA, Cr/PCr and taurine as compared to those of vehicle control ($P<0.05$). Rapamycin treatment reduced PLA_2 levels as compared to vehicle group ($P<0.05$). Our findings indicated that rapamycin reduced the increased PLA_2 levels and altered brain metabolites after MCAo. These protective effects might be attributed to its effect on cell membrane metabolism; glutamate induced toxicity and calcium homeostasis in stroke.

1. Introduction

Stroke is a serious cause of mortality and morbidity affecting about 16 million people worldwide every year (Strong et al., 2007). After the onset of ischemia, multiple mechanisms contribute to brain injury including excitotoxicity, activation of phospholipases, generation of free radicals and activation of inflammatory cascade (Phillis and O'Regan, 2003; Mattson et al., 2000). The pathophysiology and biochemistry implicated in the stroke injury have not been fully elucidated. To understand the biochemical basis in stroke, metabolic analysis may serve as a useful proposition.

Intravenous recombinant tissue plasminogen activator is the only approved therapy for treatment of ischemic stroke (Adams et al., 2005). However, patients who receive this drug within the initial therapeutic window have a high risk of intracranial hemorrhage (Külkens and Hacke, 2007), disruption of blood brain barrier, seizures and progression of neuronal damage (Tsirka et al., 1997; Wang et al., 1998; Zhuo et al., 2000). Rapamycin is an immunosuppressive and anti-proliferative agent, shown to act through mammalian target of rapamycin (mTOR) receptors and inhibit cell survival, proliferation, differentiation, apoptosis and autophagy (Manning and Cantley, 2007). It has been shown to afford improvement in behavioral and learning in the animal models of traumatic brain injury, Alzheimer disease and stroke (Erlich et al., 2007; Caccamo et al., 2010; Chauhan et al., 2011).

Proton (1H) *in-vivo* magnetic resonance spectroscopy (MRS) has been used to study the *in-vivo* alterations in the brain biochemistry in several neurological conditions including brain tumors (Brandão and Castillo, 2013), multiple sclerosis (Arnold et al.,1990) and stroke (Lin et al., 2014; Cvoro et al., 2010). By employing MRS spectroscopy, cerebral ischemia induced changes in levels of brain metabolites such as lactate, N-acetyl aspartate (NAA), choline (Cho) and creatine (Cr) have been detected in

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stroke patients (Lin et al., 2014; Cvoro et al., 2010) and also in the experimental models of stroke (van der Toorn et al., 1996; Yang et al., 2012).

One of the experimental models, which depicts transient stroke similar to, that observed in humans is the middle cerebral artery occlusion (MCAo) model. This model is easy to perform in rodents and is widely used to study the potential of drugs in the treatment of stroke (Chauhan et al., 2011, 2012; Rogers et al., 1997). We have previously demonstrated the protective effect of rapamycin in the MCA occlusion induced focal ischemia in rats (Chauhan et al., 2011). This neuroprotection by rapamycin was evident in the biochemical, neuro-imaging and functional outcomes. In the present study, our objective has been to explore the mechanism of neuroprotection by rapamycin on the brain biochemistry in the focal cerebral ischemia by estimating the concentration of various metabolites using *in -vitro* 1H NMR spectroscopy of brain tissues.

2. Materials and methods

2.1. Experimental groups

Male Wistar rats weighing 230–250 g ($n=7$) were obtained from the Central Animal Facility of All India Institute of Medical Sciences, New Delhi, India. The animals were maintained under standard laboratory conditions with natural dark–light cycle and were allowed free access to standard dry rat diet and tap water. All experimental procedures performed in rats were reviewed and approved by the Institutional Animal Ethics Committee.

MCAo was performed according to the procedure described previously (Chauhan et al., 2011; Koizumi et al., 1986). The rats were randomly divided into 3 groups, the first was sham group (no MCA occlusion and no drug treatment, $n=7$); the second was MCA occluded and vehicle treated group (dimethyl sulfoxide, i.p., $n=7$) whereas the third group was treated with rapamycin with a dose of 250 μg/kg, i.p. dissolved in DMSO (dosage was selected from our previous study by Chauhan et al. (2011), one hour after occlusion. The rats were euthanized after 24 h and their brains were then removed for the estimation of phospholipase A2 levels and for *in-vitro* NMR spectroscopy.

2.2. Measurement of phospholipase A_2 concentration

The quantitative measurement of PLA_2 in the rat brain was performed using the commercial rat PLA_2 ELISA kits (Cayman Chemicals, USA). All procedures were performed according to the manufacturer's instructions.

2.3. Perchloric acid extraction

For performing the *in vitro* NMR, the water-soluble metabolites were extracted from the excised rat brain cortex tissues using perchloric acid extraction (PCA) as previously described earlier (Payen et al., 1996; Sharma et al., 2003). 3-Trimethyl silyl propionic acid (TSP) (0.5 mM) was added to the sample that served both as a chemical shift reference and concentration standard for the proton NMR studies (Sharma et al., 2003).

2.4. In vitro NMR spectroscopy

Proton NMR of tissue specimens was carried out on a 700 MHz NMR spectrometer (Agilent) Technologies, U.K. The data was acquired using a standard 5 mm dedicated multinuclear broad-band inverse probe at 25 °C. The residual water was suppressed using a pre-saturation pulse. The chemical shifts of the resonances were referenced to TSP at 0 ppm. The sample in the NMR tube was placed in the center of the receiver coil and shimmed on the deuterium lock signal. The one-dimensional spectra with water suppression were acquired using a single 90° pulse over a spectral width of 7716 Hz using 32K data points, 64 scans and a relaxation delay of 14 s. Two-dimensional correlated spectroscopy (COSY) and the total correlation spectroscopy (TOCSY) were also carried out. The typical parameters used for TOCSY experiments were: data points 2K in F2 dimension, spectral width 7716 Hz and a relaxation delay of 2 s. The number of $t1$ increments was 256 and 64 free induction decays per increment were acquired. The concentrations of the metabolites were determined by comparing the integrated intensity of the isolated resonances of the compounds of interest with that of the TSP signal (Sharma et al., 2003).

2.5. Statistical analysis

Data are represented as mean ± S.D. Analysis of variance (ANOVA) with Bonferroni *post-hoc* analysis was used for comparing the parameters. $P<0.05$ was taken as the level of significance.

3. Results

3.1. Effect of rapamycin on PLA2 levels

Two hours of ischemia demonstrated significant increase in PLA_2 levels in vehicle group as compared to sham (Fig. 1) ($P<0.05$) as assessed after 24 h. Treatment with rapamycin reduced the PLA_2 levels in the rapamycin group as compared to the vehicle treated group (Fig. 1).

3.2. Brain metabolites assignments and concentrations

Figs. 2 and 3 show the representative aliphatic and aromatic regions of the one- dimensional 1H NMR spectrum of the perchloric acid extract of cortex tissue of control, ischemia and rapamycin treated rats.

The resonance assignments were carried out using coupling connectivities observed in two-dimensional TOCSY and compared with the chemical shift values of metabolites of tissue extracts of muscle, brain and plasma (Yang et al., 2012; Sharma et al., 2003; Chen et al., 2012).

A total of 44 metabolites were unambiguously assigned from the PCA extract of the brain tissue of the normal and ischemic rats. The chemical shifts of the metabolites are presented in Table 1.

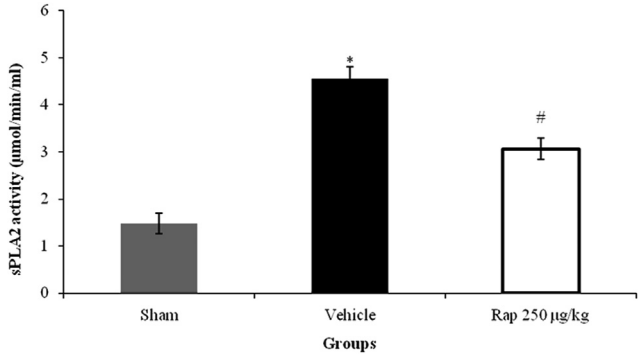

Fig. 1. Shows the effect of rapamycin on the PLA_2 levels. $^{*}P<0.05$ as compared to sham; $^{\#}P<0.05$ as compared to vehicle. Data is presented as mean ± S.D. The levels of brain PLA_2 were investigated 24 h after ischemia. There is an increase in the brain PLA_2 level in the vehicle as compared to sham group at 24 h. With the rapamycin 250 μg/kg treatment, the PLA_2 levels were significantly reduced. A mortality of 14.2% (1/7) was observed in the vehicle treated group and no mortality was observed in the rapamycin group.