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Protective Effect of Pharmacological Preconditioning of Total Flavones of Abelmoschl Manihot on Cerebral Ischemic Reperfusion Injury in Rats

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Abstract: The present study was to investigate the effect of pharmacological preconditioning of total flavones of *Abelmoschl Manihot* (TFA) on cerebral ischemic reperfusion injury in rats. Rat cerebral ischemia/reperfusion injury was induced by occluding the right middle cerebral artery (MCA). The infarct size was determined by staining with 2,3,5-triphenyl tetrazalium chloride (TTC). The serum malonaldehyde (MDA), nitric oxide (NO) and lactate dehydrogenase (LDH) levels were measured by using spectrophotometry; Inducible NO synthase (iNOS) mRNA expression was detected by RT-PCR method. The percentage of cerebral infarction volume was 28.1 ± 0.8 in the model group, while TFA or nimodipine (Nim) pretreatment 36 hours prior to the ischemic insult significantly decreased the infarction volume. Increases of serum LDH activity and MDA level were observed after ischemia/reperfusion, but these changes were inhibited in rats pretreated with either TFA (20, 40, 80, 160 mg/kg) or Nim, indicating a delayed protective effect of TFA preconditioning on cerebral ischemic reperfusion injury. In addition, the serum NO level and the cerebral iNOS mRNA were up-regulated, suggesting a possible mechanism for the protective effect of TFA pretreatment on cerebral ischemic reperfusion injury.

Keywords: Total Flavones of Abelmoschl Manihot (TFA); Pharmacological Preconditioning; Cerebral; Ischemia/Reperfusion Injury; RT-PCR.

Introduction

The term "brain tolerance" is described as a phenomenon of transient resistance to a lethal insult evoked by preconditioning with a mild insult of short duration. Preconditioning stimulus, while inadequate to damage neurons, somehow increases their ability, and sometimes the ability of the whole organism as well, to survive subsequent lethal challenge

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(Barone *et al*., 1998). The first description of brain tolerance was provided by Kitagawa *et al*. (1990). A series of recent studies have described another relevant phenomenon termed "chemical preconditioning." Based on the accumulated experimental and clinical data, the brain tolerance subsequently reinforced by pharmacological intervention might become a successful prophylactic strategy against serious brain insults in patients (Grasso *et al*., 2006). Abelmoschl Manihot is one of the plants that is rich in flavones, such as quercetin, hyperin and rutin. Recent studies have shown that total flavones of Abelmoschl Manihot (TFA) have protective effects against cerebral ischemic injury in rabbit and rat (Guo and Chen, 2002; Gao *et al*., 2003). Also, pharmacological preconditioning of TFA (PPA) has been shown to possess a protective effect against rabbit myocardial ischemia-reperfusion injury (Fan *et al*., 2005). However, there is no information about the effect of PPA on cerebral ischemic injury. By using a rat model of cerebral ischemia, we set out to investigate the effect of PPA on cerebral ischemic indexes (cerebral infarction size, serum LDH, MDA level) and its mechanism.

Materials and Methods

Drugs and Reagents

TFA (content of flavones over 85%) was provided by the Institute of Medicine of Anhui Province. Nimodipine (Nim) was provided by the Fangming factory of Shandong (Shandong, China). 2, 3, 5-triphenyl tetrazalium chloride (TTC), MDA, LDH and NO test kits were purchased from Nanjing Jiancheng Bilolgical Company (Nanjing, China). The primers of iNOS and β-actin were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China).

Animals

This investigation conforms to the regulations stipulated by Anhui Medical University Animal Care Committee which follows the protocol outlined in *The Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH publication No. 85-23, revised 1996).

Total of 72 healthy rats (male to female: 1:1) weighing 220–250 g were obtained from the Animal Center of Anhui Medical University. The animals were allowed free access to food and drinking water. The rats were randomly divided into the following 9 groups: sham, model, control, ischemic preconditioning (IP), TFA 20 mg/kg, TFA 40 mg/kg, TFA 80 mg/kg, TFA 160 mg/kg and Nim 2 mg/kg. Rat cerebral ischemia/reperfusion (I/R) injury was induced at 36 hours after above treatment.

Rat Cerebral Ischemic Injury Model

The rat I/R injury was induced by a middle cerebral artery occlusion (MCAO) (Longa *et al*., 1989) under 10% chloral hydrate anesthesia (350 mg/kg, ip). The basic surgical

procedure of blocking blood flow into the middle cerebral artery with an intraluminal fishing thread introduced through the internal carotid artery. After 90 min of ischemia, the fishing thread was withdrawn and the MCA ischemic territory was reperfused for 120 min. The body temperature was maintained at 37ºC during the experiment with a heat lamp. Except for surgical operation, sham group rats were not exposed to ischemic insult. Model Group rats were subjected to 90 min MCAO followed by 120 min reperfusion. IP group rats received the treatment of occluding middle cerebral artery twice for 5 min with 5 min interval 36 hours prior to the cerebral I/R injury. TFA or Nim groups rats were subjected to the cerebral I/R injury at 36 hours after administration of TFA or Nim by perfusing intravenously twice for 5 min with 5 min interval. The control group rats were given identical pretreatment with normal saline 36 hours prior to the ischemic insult. The animals were sacrificed by decapitation after reperfusion for 120 min. The blood samples were collected and the brains were harvested. The cerebral infarction size was determined by staining of TTC.

Measurement of NO and MDA Levels and LDH Activity

Blood was collected into test tubes and centrifuged for 10 min at 4000 g. The serum was transferred into three other clean tubes. NO and MDA levels were measured at 550 nm by Griess method (Kitamura *et al.*, 1995) and at 532 nm by TBA method (Stacey *et al*., 1980), respectively. LDH activity was detected at 440 nm by spectrophotometry (Wang *et al*., 1999).

Determination of Infarction Size (Yang *et al*., 1998)

The rat brain was quickly removed and briefly cooled in refrigerator with a temperature of about −20ºC for 10 min. The brain was sliced coronally in a 2 mm interval with a brain matrix. The sliced sections were then subsequently stained with 2% TTC at 37ºC for 30 min and then fixed in 10% buffer formalin for 10 min. Stained coronal sections were scanned with a camera, and the pictures were traced and digitized using a computerized planimetry technique (Image J, version 1.33u) to analysis percentage of infarction volume.

Total infarct volumes were calculated by summation of the infracted area in 6 brain slices (2 to 14 mm from frontal pole) and integrated by the thickness (2 mm).

Reverse Transcriptase Polymerase Chain Reaction (*RT-PCR*) (Kwak *et al*., 2005)

Total RNA was isolated from frozen specimens using the chloroform-avantin-alcohol method with TRIzol. The cDNA synthesis was carried out with TaqMan reverse transcription reagent in a 10 µl reaction volume containing 1 µL total RNA, $MgCl_2$, 10 × RT buffer, RNase free dH_2O, dNTP mixture, RNase inhibitor, AMV reverse transcriptase, random 9 mers of or oligo dT-adaptor primer. After preincubation for 2 min at 94ºC, 35 cycles of

amplification (94ºC for 30 sec, 59ºC for 30 sec and 72ºC for 45 sec) were performed and β-actin was used for an internal control. The sense and antisense oligonucleotides for iNOS were 5′-agagaagggcgcgttgtggtagtt-3′ and 5′-tgtcagatggcaagggttcaggtg-3′, respectively. The sense and antisense oligonucl-eotides for β-actin were 5′-cacgatggaggggccggactcatc-3′ and 5′-taaagacctctatgccaacacagt-3′.

Statistical Analysis

The data were expressed as mean ± SD, and analyzed by one-way repeated measure ANOVA and student's t-test for comparisons between groups. $p < 0.05$ was considered statistically significant.

Results

Effect of PPA on the Infarction Size

The MCAO induced severe cerebral ischemic injury as indicated by the measurement of the cerebral infarction size in untreated rats of the model group (28.1 ± 0.9%) and control group (28.4 ± 1.2%). IP markedly decreased the cerebral infarction size (16.6 ± 1.3%, $p < 0.01$). TFA pretreatment also significantly decreased the cerebral infarction size in 20 mg/kg group (24.1 ± 1.0%, $p < 0.01$), in 40 mg/kg group (22.7 ± 2.8%, $p < 0.05$), in 80 mg/kg group (19.8 ± 0.9%, $p < 0.01$) and in 160 mg/kg group (18.3 ± 1.6%, $p < 0.01$) compared to the control group. Pretreatment with 2 mg/kg Nim had a similar effect in reducing infarction size (17.6 ± 1.4%, $p < 0.01$, Table 1).

Effect of PPA on Serum LDH Activity and MDA Level

The significant elevations of serum LDH activity and MDA level were detected in the model group and the control group. IP markedly inhibited the elevations of serum LDH

Table 1. Effect of PPA on the Cerebral Infarction Size in Rats

Group	Dose (mg/kg)	Percentage of Infarction Volume (%)	Inhibitory Rate (%)
Sham	–	0.0 ± 0.0	–
Model	–	$28.1 \pm 0.9^{\Delta}$	–
IP	–	$16.6 \pm 1.3^{**}$	40.9
Control	–	$28.4 \pm 1.2^{\Delta}$	–
NIM	2	$17.6 \pm 1.4^{*}$	37.4
TFA	20	$24.1 \pm 1.0^{**}$	14.2
	40	$22.7 \pm 2.8^{*}$	19.2
	80	$19.8 \pm 0.9^{**}$	29.5
	160	$18.3 \pm 1.6^{**}$	34.9

Mean ± SD, n = 8. $^{\Delta}p < 0.01$ compared to sham, $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control.

Table 2. Effects of PPA on Serum Activity of LDH and Contents of MDA and NO in Rats

Group	Dose (mg/kg)	LDH (U/L)	MDA (nmol/ml)	NO (μM)
Sham	–	3881.2 ± 595.1	5.9 ± 1.3	46.5 ± 6.1
Model	–	6695.7 ± 203.9$^{\Delta}$	9.7 ± 1.6$^{\Delta}$	48.5 ± 6.1
IP	–	4060.9 ± 512.8**	5.9 ± 1.5**	86.5 ± 11.1**
Control	–	6060.8 ± 706.1$^{\Delta}$	8.4 ± 1.2$^{\Delta}$	57.8 ± 11.8
Nim	2	3914.1 ± 784.3**	5.6 ± 1.2**	103.2 ± 19.6**
TFA	20	6124.7 ± 361.7*	8.7 ± 1.3*	67.6 ± 5.9*
	40	5443.1 ± 543.4*	8.3 ± 0.9*	92.3 ± 17.6*
	80	4894.1 ± 459.8**	4.9 ± 0.6**	118.9 ± 11.8**
	160	4354.4 ± 467.6**	4.2 ± 0.6**	167.5 ± 16.3**

Mean ± SD, n = 8. $^{\Delta}p < 0.01$ compared to sham, $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control.

Table 3. The Effect of PPA on the Expression of iNOS mRNA

Group	Dose (mg/kg)	iNOS/β-actin (OD)
Sham	–	0.00 ± 0.00
Model	–	0.35 ± 0.04*
TFA	160	0.75 ± 0.06**

Mean ± SD, n = 6. $^{*}p < 0.05$ compared to normal, $^{**}p < 0.01$ compared to model.

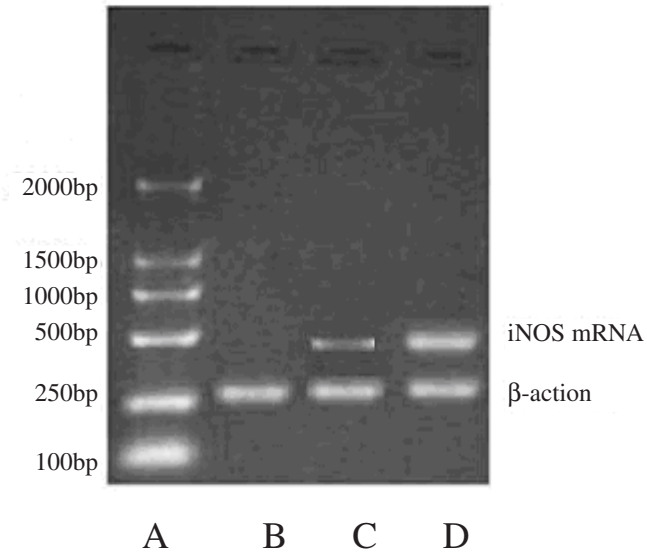

Figure 1. Expression of iNOS mRNA. A: DNA marker, B: Sham, C: Model, D: TFA 160 mg/kg.

activity and MDA level compared to the model group ($p < 0.01$). The pretreatment with TFA (20, 40, 80, 160 mg/kg) or Nim (2 mg/kg) had a similar effects ($p < 0.05$ or $p < 0.01$) as that seen in the IP group (Table 2).

Effect of PPA on Serum NO Content

In the range of 20~160 mg/kg, the pretreatment with TFA significantly increased serum NO content ($p < 0.05$ or $p < 0.01$), IP and the pretreatment of Nim (2 mg/kg) had similar effects (Table 2).

Effect of PPA on the Expression of iNOS mRNA

The pretreatment with 160 mg/kg TFA markedly potentiated the cerebral ischemia/reperfusion-induced increase of expression of iNOS mRNA in rat cerebrum compared to the model group ($p < 0.01$); there was also a significant increase of expression of iNOS mRNA in rat cerebrum compared to the sham group ($p < 0.05$, Table 3, Fig. 1).

Discussion

It is well-known that ischemia/reperfusion induces neuronal injuries through several pathophysiological mechanisms (Gwag *et al*., 1995; Chen *et al*., 2000; Chong and Feng, 1999), including intracellular calcium overload, lipid peroxidation and free radical production, which finally triggers cerebral ischemic injury. As an endogenous form of neuroprotection against ischemia/reperfusion injury, IP has been described to be a biphasic event: the acute phase is limited to 1–3 hous after a brief ischemic stimulus, and the delayed phase emerges 24 hours later and may last up to 72 hours (Rejdak *et al*., 2001). We have previously reported that TFA produced acute neuroprotection against rat cerebral ischemia/reperfusion injury (Wen *et al*., 2006). In the present study, we have found that: 1) The pretreatment of TFA has a delayed neuroprotection against cerebral I/R injury in rats; 2) The delayed neuroprotection of TFA is involved in inhibiting lipid peroxidation and stimulating NO release.

LDH is a very important metabolic enzyme in neuron and is released into blood stream by injured neuron. Thus, increasing activity of LDH released from cerebral cells into blood serum may reflect the damage of neurons (Shang *et al*., 2006). MDA, the product of lipid peroxidation of cell membrane, is another index to evaluate cerebral ischemic injury. In this study, rat cerebral I/R injury is detected after 90 min MCAO followed by 60 min reperfusion, as indicated by the formation of cerebral infarction, the increases in serum LDH activity and MDA level. The pretreatment with TFA or Nim 36 hours prior to the ischemic insult significantly inhibited the increases of serum LDH activity and MDA level and reduced cerebral infarction size indicating a delayed protective effect of PPA against rat MCAO and reperfusion-induced cerebral I/R injury.

NO, a short-lived diffusible molecule produced from arginine by NO synthase (NOS), has a wide range of biological functions including modulation of vascular tone, regulation of local cell growth, and protection of the vessel from injurious consequences of platelets and cells circulating in blood (Gonon *et al*., 2004). In the central nervous system NO acts as a neuromediator with many physiological functions, including the formation of memory, neurotransmitter release and reuptake, neurodevelopment, synaptic plasticity, cerebral blood flow and regulation of gene expression. NO is synthesized from three NOS isoforms: neuronal NOS (nNOS), endothelial NOS, and inducible NOS (iNOS). Under normal physiological conditions, only constitutively expressed eNOS is believed to be active. In pathological conditions, iNOS is activated and induced to sustained amount of NO (Kwak *et al*., 2005). Growing lists of conditions, many of them risk factors are assiociated with diminished release of NO. Diminished NO bioactivity may facilitate vascular inflammation that could lead to oxidation of lipoproteins and foam cell formation, the precursor of the atherosclerotic plaque. Recently, convincing evidences that NO plays an important role in the pathogenesis of neuronal injury during cerebral ischemia have been reported (Gonzalez-Barrios *et al*., 2002; Jiang *et al*., 2002). However, each NOS isoform plays a unique role in pathogenesis of cerebral ischemia. NO derived from iNOS may be an endogenous neuroprotectant after traumatic brain injury in rats and mice. In a model of controlled cortical impact with secondary hypoxemia, the treatment of rats with the iNOS inhibitors significantly exacerbated deficits in cognitive performance and increased neuron loss in hippocampus, and iNOS(-/-) mice showed markedly worse performance than iNOS(+/+) mice (Sinz *et al*., 1999). The generation of new neurons in the adult mammalian hippocampus is thought to play a role in repairing the brain after injury. There is a study indicated that expression of iNOS is necessary for ischemia-stimulated cell birth in the dentate gyrus and that activation of iNOS may provide a possible strategy for functional recovery from cerebral ischemic insult (Zhu *et al*., 2002). In the present study, a significant increase of expression of iNOS mRNA was observed by using RT-PCR method, the result is in agreement with above reports.

Flavones have been shown to increase endothelial NOS activity and NO production (Delaflotte *et al*., 1984; Harris *et al*., 1997). In this study, the increases in serum NO content and cerebral iNOS mRNA after PPA were observed, indicating the stimulation of NO release may be, at least partially, involved in the delayed protection of PPA against cerebral ischemia/reperfusion.

In summary, the present study was the first to show that PPA has a delayed protective effect against cerebral I/R injury in rats, and its mechanism may be involved in inhibiting lipid peroxidation and stimulating NO release.

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