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ORIGINAL ARTICLE

The effect of procyanidin on expression of STAT1 in type 2 diabetes mellitus SD rats with focal cerebral ischemia

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Abstract

OBJECTIVES: Diabetes with cerebral infarction is a common disease that severely impacts health. This study investigated the effect of procyanidin (PC) on the expression of signal transducers and activators of transcription (STAT1) in type 2 diabetes mellitus SD rats with focal cerebral ischemia. We then explored the protective mechanisms of PC in type 2 diabetes mellitus SD rats with focal cerebral ischemia, to provide theory evidence for its clinical application.
METHODS: We set up a type 2 diabetes mellitus-MCAO model, evaluated neurological function, and used immunohistochemistry methods to measure the activity of STAT1.
RESULTS: The brain expression of STAT1 in rats of the sham-operation group was low, but more STAT1 positive cells were found in normal rats with ischemia and in rats with both type 2 diabetes and ischemia when groups were compared with the sham-operation group ($p<0.01$). Compared with rats that had type 2 diabetes and ischemia, the numbers of STAT1 positive cells after low, medium and high-doses of PC were all decreased ($p<0.01$), whereby the mid and high-dose groups showed a more substantial decrease ($p<0.01$) and with no variance between the two groups ($p>0.05$).
CONCLUSIONS: These results indicate that PC has a neuroprotective effect on type 2 diabetes mellitus-MCAO; this may be through decreasing the expression of STAT1, which influences the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway that may inhibit apoptosis to relieve neurological impairment.

INTRODUCTION

Diabetes is an independent risk factor for ischemic cerebrovascular disease with the risk of cerebral infarction being 2–3 times higher than that for non-diabetics (Capes *et al.* 2001). Type 2 diabetes with cerebral infarction is a common disease that seriously harms health and has a poor prognosis with high mortality, which brings a heavy burden to society and families. So far, many drugs have been trialled but outcomes remain poor.

The JAK/STAT pathway is an important pathway for many cytokines and remains an active research focus. The cytokines activate JAK after combination with a receptor and then activate STAT1, finally inducing target gene expression. Recent studies have shown that the JAK/STAT pathway is one of the common pathways of physiological and pathological processes in the human body, and it is closely related with the onset, prevention and cure of many diseases (Klampfer 2006; Shouda *et al.* 2006; Folch Puy *et al.* 2006).

Cerebral ischemia causes the release of a large number of cytokines and growth factors that can activate the JAK/STAT pathway. It has been confirmed that cerebral ischemia can increasingly induce STAT1 protein expression (Cao, 2006) and then activate the JAK/STAT pathway. STAT1 can restrain cell growth, mediate the signal transduction of apoptosis, and also down-regulate the expression of the c-Myc promoter, which participates in apoptosis induction (Ramana *et al.* 2000).

This study aimed to investigate the effect of PC on expression of STAT1 in type 2 diabetes mellitus SD rats with focal cerebral ischemia. We then studied the protective mechanisms of PC in type 2 diabetes mellitus SD rats with focal cerebral ischemia, in order to provide theory evidence for its clinical application.

MATERIALS AND METHODS

Animal, drug, reagent

SD rats were provided by the Experimental Animal Center of Liaoning Medical College, housed at a constant temperature and with a 12-hour light-dark cycle. This study was carried out in strict accordance with the recommendations of the Guide for the National Science Council of the Republic of China. PC was provided by Tianjin Jianfeng Natural Product R&D Co., Ltd (batch number: 6050804), China; STZ was provided by Sanland Chemical Co., Ltd, USA.

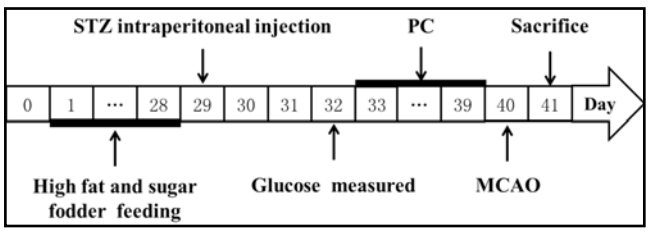

Fig. 1. Experimental design. The arrows indicate the timing of high fat and sugar fodder feeding, STZ intraperitoneal, glucose measured, PC administration, MCAO, and sacrifice.

Experimental groups

Male adult SD rats (body weight: 180–240 g) were randomly divided into the sham-operation group, the normal rats with ischemia group, the type 2 diabetes rats with ischemia group, or the PC low, mid and high-dose groups with 15 rats per group. The total number of rats used in this study was 90. Rats of the PC groups were treated with intragastric administration of PC after creation of the diabetes model. Once per day for 1 week, and at 1 hour before the middle cerebral artery occlusion (MCAO) surgery, rats were treated with intragastric administration once again. The doses of PC for low, mid and high-dose groups were: 50 mg/kg.time, 100 mg/kg.time, 200 mg/kg.time, and the PC powder was prepared as a suspension with distilled water.

Model building

The rats were first fed with a high fat and high sugar feed [10% lard, 2.5% cholesterol, 20% sucrose, 2% sodium cholate, 65.5% normal feed (Guo *et al.* 2002)] for 4 weeks to induced insulin resistance, and low doses of streptozotocin (STZ, 25 mg/kg) by a one-off intraperitoneal injection. Three days later, the rats' tail vein blood glucose was measured, with the rats with a fasting plasma glucose >16.7 mmol/L being diagnosed with type 2 diabetes.

Rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (0.3 ml/100 mg), and the left common carotid artery (CCA) was exposed and ligatured at the bifurcation of the ipsilateral CCA (medial end) and the external carotid artery (ECA), at about 5 mm from the CCA end shear opening. A nylon wire (diameter 0.235 mm, 6 cm long) with a rounded tip made by heating was inserted from the shear opening; the insert depth was 18±0.5 mm calculated from the crotch of the CCA and advanced until the origin of the left MCA was occluded. Occlusion success was denoted by the immediate appearance of a left-sided Horner's sign. Rats with subarachnoid hemorrhage were excluded. The preparation of rats for the sham group was the same as above except the nylon wire was not inserted. During surgery, rectal temperature was held at 37.0–37.5 °C with a heating pad and light bulbs. Brain tissue samples were collected twenty-four hours after the onset of occlusion (Figure 1).

Neurologic impairment score

Twenty-four hours after MCAO, we used the Bederson (Bederson *et al.* 1986) criterion to evaluate neuroethology: grade 5 scored 0: normal (no dysfunction); grade 4 scored 1: the contralateral upper limb cannot completely stretch; grade 3 scored 2: the resistance decreased when the rat is pushed to the opposite side; grade 2 scored 3:

circling towards the contralateral side when the tail is grasped; grade 1 scored 4: circling automatically; grade 0 scored 5: decreased consciousness and no spontaneous activity.

Immunohistochemisty

Brain tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and embedded in paraffin. The paraffin sections were washed with PBS, blocked with 1% normal goat serum in PBS for 20 mins, and then reacted with anti-STAT1 antibody (diluted 1:100; Boster, China) incubated at 4 °C for 15 hour. After rinsing with PBS, sections were reacted with biotin-conjugated goat anti-rabbit IgG (1:200 in PBS) at 37 °C for 30 mins, washed with PBS, followed by incubation with an avidin-biotin-peroxidase complex at 37 °C for 30 mins from a SABC Elite kit (Boster, China). Sections were rinsed with PBS and stained with diaminobenzidine tetrahydrochloride (Boster, China) solution with 0.03% hydrogen peroxidase for 5 mins. Sections were counterstained with hematoxylin and dehydrated in graded alcohol. Slides were observed under a microscope and then photographed.

Tab. 1. Neurologic impairment score ($\bar{x} \pm s$).

Group	Rats	Score
Sham	15	0
Normal rats-ischemia	15	3.07±0.59†
Type 2 diabetes rats-ischemia	15	3.73±0.70†‡
PC low-dose	15	2.40±0.51^{l}
PC mid -dose	15	1.80±0.56^{m}
PC high-dose	15	1.47±0.52^{h}

Compared with sham-operation group, $^{\dagger}p<0.01$; compared with normal rats with ischemia group,$^{\ddagger}p<0.01$; compared with type 2 diabetes rats with ischemia group, $^{l}p<0.01$, compared with PC low-dose group, ^{m}p, $^{h}p<0.01$.

Tab. 2. STAT1 positive cells ($\bar{x} \pm s$, N=8).

Group	Dose (mg/kg)	STAT1 positive cells
Sham		4.10±1.26
Normal rats-ischemia		18.50±2.11†
Type 2 diabetes rats-ischemia		20.13±1.36†‡
PC low-dose	50	17.25±0.99^{l}
PC mid-dose	100	13.67±1.88^{m}
PC high-dose	200	12.92±1.74^{h}

Compared with sham-operation group, $^{\dagger}p<0.01$; compared with normal rats with ischemia group, $^{\ddagger}p<0.01$; compared with type 2 diabetes rats with ischemia group, $^{l}p<0.01$, compared with PC low-dose group, ^{m}p, $^{h}p<0.01$.

Statistical analysis

A quantitative estimate of the number of STAT1-immunopositive cells was obtained by counting cells on every 6th section in the pyramidal layer of the cerebral cortex on the ischemic side. Positive cells were counted under a 40× objective lens using ImageJ software and were averaged to provide a mean positive cell number.

All values are expressed as mean ± standard deviation. Differences between means were analyzed using one-way ANOVA. The values from different groups of animals were compared using a two-tailed, unpaired Student's t-test. A $p<0.05$ was considered statistically significant.

RESULTS

Neurologic impairment score

The neurological impairment score in the normal rats with ischemia group and the type 2 diabetes rats with ischemia group was increased more than in the sham-operation group ($p<0.01$), and the score increased more notably in the type 2 diabetes rats with ischemia group ($p<0.01$); The PC groups all had improved neurological impairment scores, with the mid and high-dose groups having a more obvious effect, but there was no statistically significant difference between the two groups (Table 1).

The different doses of PC influenced the expression of STAT1 positive cells in type 2 diabetes mellitus SD rats with focal cerebral ischemia

The expression of STAT1 in the normal rats with ischemia group (18.50±2.11) and type 2 diabetes rats with ischemia group (20.13±1.36) was increased more than the sham-operation group (4.10±1.26) ($p<0.01$), and in the type 2 diabetes rats with ischemia group the expression increased more obviously ($p<0.01$); The low, medium and high-doses of PC groups all had reduced expression of STAT1 [(17.25±0.99), (13.67±1.88) and (12.92±1.74)(all $p<0.01$)], with the mid and high-dose groups having a more obvious effect ($p<0.01$), but there was no statistically significant difference between the two groups ($p>0.05$) (Table 2, Figure 2).

DISCUSSION

We now pay more attention to the the central nervous system damage caused by diabetes, as diabetes can cause peripheral neuropathy, enhance the incidence of stroke, and also have other complications such as hyperlipidemia, accelerated artherosclerosis, hypertension, autonomic neuropathy, proliferating small vessel disease in brain, depressed erythrocyte deformability, raised platelet adhesion reactions and other hematological disorders. These factors can all aggravate ischemic brain injury.

The JAK/STAT pathway is an important signal transduction pathway. It transmits ecto-polypeptide

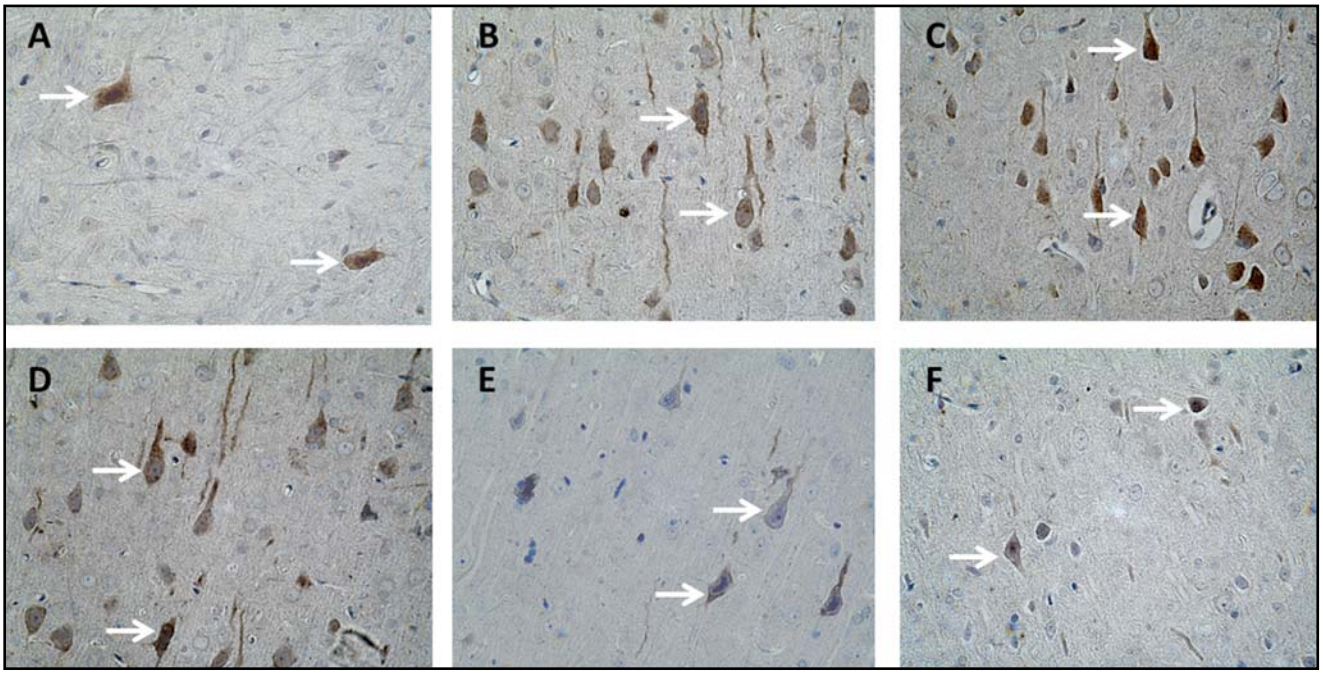

Fig. 2. Immunohistochemistry method to measure the activity of STAT1 a: sham-operation group; b: normal rats with ischemia group; c: type 2 diabetes rats with ischemia group; d: PC low-dose group; e: PC mid-dose group; f: PC high-dose group.

signal information to the target promoter of intranuclear targets, plays a dual role as signal transducer and transcription activator, and acts as a simple transcriptional control mechanism that does not need secondary messengers (Klampfer 2006; Shouda *et al.* 2006; Folch Puy *et al.* 2006). STAT1 is activated in neurons after cerebral ischemia and contributes to ischemic brain injury (Takagi *et al.* 2002; West *et al.* 2004).

Some researches presume that STAT1 promotes apoptosis (Chin *et al.* 1997; Stephanou *et al.* 2001). Takagi (Takagi *et al.* 2002) and coworkers found the STAT1–/– mouse has protection from ischemia as their results show that the STAT1–/– mouse infarct size was smaller than that of Wild Type (WT) mice, and their neurological impairment was lessened with fewer TUNEL positive cells after ischemia. The activation of caspase-3 seen in the STAT1–/– mouse is much lower than that of the WT mouse. Stephanou (Stephanou *et al.* 2000) reported that STAT1 can cut down the expression of Bcl-2 and Bcl-x, which are important anti-apoptosis genes in ischemic reperfusion injury of brain. This finding indicates that STAT1 may promote apoptosis in cerebral ischemic injury. Thorsten R. Doeppner and his colleague also found that caspase-3-dependent activation of STAT1 might be involved in the aggravation of brain injury in ephrin-B3–/–mice (Doeppner *et al.* 2011). Henrik Ahlenius (Ahlenius *et al.* 2012) and his colleagues found that LNK (an adaptor protein) expression after stroke increased in the subventricular zone (SVZ) through the transcription factors linked to STAT1. The LNK protein attenuated insulin-like growth factor 1 signaling by inhibition of AKT phosphorylation, resulting in reduced neural stem cell and progenitor cell proliferation.

Cerebral ischemia can lead to STAT1 protein phosphorylation and nuclear translocation, participating in the process of cerebral ischemic injury through regulation of the transcription and phosphorylation of caspase-3 (the apoptosis and cell death related protein).

From 2000, more reports consistently describe the protective action of some plant compounds such as polyphenols in brain injury, mainly underlining the importance of their antioxidant effect. As previously described, the JAK/STAT pathway plays a significant role in the transduction of cytokine signals and it is supposed to play a critical role in brain injury. Changes in the activation and/or gene expression of STAT1 have been observed in the brains of rats either after focal cerebral ischemia with or without reperfusion (West *et al.* 2004; Sun *et al.* 2007), indicating their relevant involvement.

Procyanidin is an antioxidant with a bulk activated phenolic hydroxyl group that functions as an anti-oxidant and removes various kinds of activated oxygen free radicals. It depresses capillary permeability and may have many pharmacological actions in the cardiovascular system (Yamaguchi *et al.* 1999; Sinatra & DeMarco 1995). Recent study has identified that it can be applied to treat and prevent myocardial ischemia-reperfusion trauma through removal of the oxyradical from the ischemic vascular endothelial cell (Facino *et al.* 1999), thus PC ought to have good effect on cerebrovascular disease.

In our study, the expression of STAT1 in normal rats with ischemia and in type 2 diabetes rats with ischemia

was increased compared with the sham-operation group ($p<0.01$), and expression increased substantially more in rats with type 2 diabetes and ischemia ($p<0.01$), which demonstrates that STAT1 plays a significant role in diabetes with cerebral ischemia. We presume its mechanism in rats with type 2 diabetes and cerebral ischemia involves massive cytokine and growth factor release, which induces the STAT1 protein activation and nuclear translocation, then activation of the JAK/STAT1 pathway, to regulate the transcription and phosphorylation of caspase-3, to promote apoptosis.

Each PC group showed down-regulated expression of STAT1 in the ischemic lateral cerebral cortex of the rats with type 2 diabetes and cerebral ischemia, and the mid and high-dose groups' effect is more obvious. We presume PC represses the expression of STAT1, then restrains the JAK/STAT1 pathway, reducing its apoptotic effect, leading to a neuroprotective effect.

Down-regulation of excessive activation of STAT1 could be one of the most relevant features of neuroprotection. Further elucidation of the precise molecular mechanisms of anti-STAT1 action is urgent. The identification of PC's inhibitory action on STAT1 activation may open the window to the development of new safe drugs against cerebral ischemia in diabetes and many other diseases with similar etiologies.

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