Original Article

Modulation of the Oxidative Stress and Nuclear Factor κB Activation by Theaflavin 3,3'-gallate in the Rats Exposed to Cerebral Ischemia-Reperfusion

(ischemia-reperfusion / middle cerebral artery occlusion / nuclear factor kappa B / reactive oxygen species / theaflavin 3,3'-gallate)

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Abstract. The major pathobiological mechanisms of IR injury include excitotoxicity, oxidative stress, and inflammation. TF$_3$, a major constituent of black tea, possesses biological functions such as anti-oxidative and anti-inflammatory activities. The purpose of this study was to verify the neuronal protective potential of TF$_3$ and its mechanisms against cerebral IR injury in rats. TF$_3$ administration (10 and 20 mg.kg^{-1}) ameliorated the infarct volume. TF$_3$ also decreased the content of MDA and NO. TF$_3$ significantly increased the activity of SOD and GSH-Px, which were reduced by IR injury. Administration of TF$_3$ decreased mRNA and protein expression of COX-2 and iNOS. DNA binding and Western blotting revealed an increase in NF-κB activation and IκB depletion in IR brain tissue. Pretreatment with TF$_3$ markedly inhibited IR-induced increase in nuclear localization of NF-κB, and preserved IκB in the cytoplasm. The results show that TF$_3$ exerts protective effects against cerebral IR injury by reducing oxidative stress and modulating the NF-κB activation.

Introduction

Acute ischemic stroke is the main leading cause of death and long-term disability worldwide. It is obvious

Abbreviations: COX-2 – cyclooxygenase-2, EMSA – electrophoretic mobility shift assay, GSH-Px – glutathione peroxidase, IκB – NF-κB inhibitor, iNOS – inducible nitric oxide synthase, IR – ischemia-reperfusion, MCAO – middle cerebral artery occlusion, MDA – malondialdehyde, NF-κB – nuclear factor κB, NO – nitric oxide, ROS – reactive oxygen species, SOD – superoxide dismutase, TF$_3$ – theaflavin 3,3'-gallate.

that oxidative stress, nitric oxide (NO) neurotoxicity and excessive inflammatory response are implicated in the pathogenesis of cerebral ischemia-reperfusion (IR) injury (Higuchi et al., 1998; Schaller and Graf, 2004). Cerebral IR enhances formation of reactive oxygen species (ROS) in brain tissues (Siesjo, 1992). Moreover, ROS can activate diverse downstream signalling pathways, including the transcription factor nuclear factor kappa B (NF-κB), thus regulating expression of genes encoding a variety of proinflammatory proteins such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Clemens, 2000; Jozsef and Filep, 2003). Overexpression of COX-2 and that of iNOS have recently emerged as important determinants of post-ischemic inflammation, which contributes to the progression of brain damage (Lerouet et al., 2002; Candelario-Jalil et al., 2003).

Despite aggressive research into developing neuroprotective treatments of brain injury, no drugs have been proved successful in advanced clinical trials. Recent studies demonstrate that a number of antioxidants can reduce ROS-mediated reactions and rescue neurons from cerebral ischemic damage in animal models (Clemens, 1991). The protective effects of antioxidants against IR-induced injury may be mediated by down-regulation of NF-κB activity (Shen et al., 2003). It has also been reported that the use of anti-inflammatory compounds has great potential as a therapeutic strategy for neuroprotection following ischemic brain injury (Barone and Feuerstein, 1999).

Theaflavins are natural polyphenols found in black tea, including theaflavin (TF$_1$), theaflavin 3-gallate (TF$_2$A), theaflavin 3'-gallate (TF$_2$B), and theaflavin 3,3'-gallate (TF$_3$) (Gupta et al., 2002). These tea polyphenols possess a broad spectrum of biological functions such as anti-oxidative, anti-bacterial, anti-tu-

mour, anti-viral, anti-inflammatory and cardiovascular protection activities (Mukhtar and Ahmad, 2000; Higdon and Frei, 2003). Studies on RAW 264.7 mouse macrophages have shown that theaflavins, in particular TF$_3$, effectively inhibited activation of transcription factor NF-κB, preventing expression of iNOS (Lin et al., 1999). Therefore, the present study was undertaken to evaluate the neuronal protective potential of TF$_3$ and its mechanisms on middle cerebral artery occlusion (MCAO)-induced focal cerebral IR model in rats.

Material and Methods

Middle cerebral artery occlusion-induced focal cerebral ischemia

The experiments were performed in accordance with the institutional guidelines on the care and use of experimental animals set by the Chinese Academy of Sciences. TF$_3$ was purchased from Wako Cat (#208-15171, Osaka, Japan) and other chemicals were purchased from Sigma-Aldrich China Inc (Beijing, China). TF$_3$ was injected intraperitoneally (10 and 20 mg.kg^{-1}) 30 min before ischemic occlusion and 30 min before reperfusion. Focal cerebral ischemia was produced by occluding the left middle cerebral artery according to the methods by Longa et al. (1989). Briefly, the rats were anaesthetized with chloral hydrate (400 mg.kg^{-1}, ip). Through a midline neck incision, the left common and external carotid artery were isolated from muscles and coagulated. A 3-0 nylon suture with a blunted tip was inserted into the internal carotid through the external carotid artery stump and advanced up to 21 mm or until resistance was felt. After 2 h of MCAO, the suture was removed to restore blood flow. In the vehicle-treated group, the same surgical procedure was performed except that the suture was introduced into the external carotid artery but not advanced. After surgery, the incision was sutured and the rats were returned to their cage with free access to water and food. After 24-h reperfusion, rats were sacrificed by rapid decapitation under deep anesthesia and the brains were taken out for biochemical estimations.

Neurological score analysis

Neurological scores in the vehicle-treated group and drug-treated group were determined after 24-h reperfusion by an observer blinded to the identity of the groups according to the method of Longa et al. (1989).

Infarct volume analysis

After 24-h reperfusion, whole brains were rapidly removed. Immediately after being weighed, the brains were sliced into 2-mm-thick coronal sections and stained with 2% 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min in the dark, followed by fixation with 10% formalin at room temperature overnight. The sections were photographed with a digital camera (Kodak DC240, Emeryville, CA) connected to a computer. The unstained areas, defined as infarct tissue, were calculated by using an image analysis program. The infarct volume was calculated by measuring the unstained area in each slice.

Biochemical analysis

The estimate of lipid peroxidation of the cerebral cortex was determined by measuring the formed malondialdehyde (MDA). The content of MDA was commonly determined by the modified thiobarbituric acid (TBA) method (Sun et al., 1988). Superoxide dismutase (SOD) activity in the brain homogenate was estimated after 2 h of ischemia and 24 h of reperfusion using the NADH oxidation method reported by Paoletti and Mocali (1990). Glutathione peroxidase (GSH-Px) activity was estimated in brain homogenates in vehicle and TF$_3$-treated rats after 24-h reperfusion as reported by Keller et al. (1998). NO production was measured as nitrite (a stable metabolite of NO) concentrations using the Griess reagent system following Jiancheng Institute of Biotechnology protocols (Jiancheng Institute of Biotechnology, Nanjing, China).

RT-PCR

Total RNA was extracted from cortex using the TRIzol reagent (Sigma Co). cDNA was synthesized according to the manufacturer's instruction for the reverse transcription kit (GIBCO-BRL, Bethesda, MD), and then amplified with a multiplex PCR kit (GIBCO-BRL). Conditions for amplification were as follows: initial denaturation for 2 min at 94°C, 35 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 60 s, and a final extension stop at 72°C for 7 min. The rat specific primers (sense and antisense primers) for iNOS, COX-2, and β-actin were 5'-CGGTGCTGTATTTCCTTACGAGGCGAA-GAAGG-3' and 5'-GGTGCTGTCTGTTAGGAGGT-CAAGTAAAGGGC-3'(iNOS, 259 bp); 5'-CCATGT-CAAAA-CCGTGGTGAATG-3' and 5'-A-TGGGAGTTGGGCAGTCATCAG-3' (COX-2, 374 bp); 5-ATGGATGACGATATCGCTG-3 and 5-ATGAG-GTAGTCTGTCAGGT-3 (β-actin, 568 bp), respectively. Reaction products were then separated on 1.5% agarose gel, stained with ethidium bromide, and visualized by UV transillumination. The HPIAS-1000 software analysis system was used to determine the relative absorbance of mRNA expression.

Preparation of nuclear and cytoplasmic extracts

Nuclear proteins were extracted with some modifications of a previously described procedure (Ogita and Yoneda, 1994). Fresh brain samples were homogenized in 400 μl of hypotonic lysis buffer A (10 mmol/l HEPES, pH 7.9, 10 mmol/l KCl, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1 mmol/l DTT, and 1 mmol/l PMSF). Homogenized tissues were incubated on ice for 5 min, NP-40 was added to a final concentration of 5 g/l, and samples

were vigorously mixed and centrifuged. The cytoplasmic proteins were removed and the pellet nuclei were resuspended in 50 μl buffer C (20 mmol/l HEPES, pH 7.9, 0.4 mol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, and 1 mmol/l PMSF). After 30-min agitation at 4°C, the samples were centrifuged and supernatants, containing nuclear proteins, were transferred to a fresh vial. The protein concentrations of nuclear extracts were determined by Bradford protein assay.

EMSA analysis

Nuclear protein extracts of brain tissues were analysed by EMSA for NF-κB nuclear translocation as previously described (Clemens et al., 1997). The EMSA binding reaction mixture contained 8 μg protein of nuclear extracts, 2 μg of poly(deoxyinosinic-deoxycytidylic acid) (Sigma Co), and [^{32}P]-labelled double-stranded oligonucleotide containing the binding motif of the NF-κB probe (4,000 cpm) in binding buffer (10 mmol/l HEPES, pH 7.9, 50 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l DTT, 100 ml/l glycerol, and 0.2 g/l albumin). The sequence of the double-stranded oligomer used for EMSA was 5'-AGTTGAGGGGACTTTC-CCAGGC-3'. The reaction was incubated for 30 min at room temperature before separation on 5% polyacrylamide gel, followed by autoradiography. For supershift assay, the nuclear extracts were incubated with antibodies – the p50 and p65 subunits of NF-κB complex (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 25 °C and analysed by EMSA. As controls, the nuclear extracts were also treated with normal rabbit IgG.

Western blot analysis

The cortices of brains were removed and used for Western analysis. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) and all samples were adjusted to an equal protein content before analysis. Samples (30 μg of total protein) were separated on 8% denaturing polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (80 V, 90 min; transfer buffer 25 mM Tris, 190 mM glycine, 20% methanol, 0.5% sodium dodecyl sulphate) by an electroblotter (Bio-Rad). After being blocked for 2 h at room temperature in blocking buffer (5% nonfat milk in 20 mM Tris/HCl, pH 7.6, 140 mM NaCl, 0.5% Tween 20), membranes were incubated overnight at 4°C with primary antibodies against anti-phospho-STAT-1Tyr-701 (Zymed, South San Francisco, CA), or anti-STAT-1 (Santa Cruz Biotechnology). Membranes were then washed (in 20 mM Tris/HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20) and incubated with a peroxidase-conjugated secondary antibody at room temperature for 50 min. The immunoblots were visualized using Western blotting luminal reagent (Cell Signal Corp, Danvers, MA). The density of the protein band was scanned and analysed with an image analyser.

Statistical analysis

Unless otherwise stated, all the results were finally presented as means ± S.E.M. Statistical differences between different groups were assessed by a one-way analysis of variance and Student-Newman-Keuls test. The P value less than 0.05 was considered statistically significant.

Results

Physiological parameters

The physiological variables were measured and presented in Table 1. The rectal temperature was monitored and maintained at about 36.5 to 37.2°C. Blood gases and blood pH were measured by pH/blood gas analyser iSTAT. Mean blood pressure (MBP) was measured using a XBP1000 NIBP system.

Effect of TF$_3$ on neurological scores

After 2 h of MCAO and 24 h of reperfusion, the neurological scores in control rats were measured to 2.68, which were significantly decreased to 1.32 and 1.05, respectively, by the treatment with TF$_3$ (10 and 20 mg.kg^{-1}) (P < 0.01, Fig. 1). These results demonstrated that administration of TF$_3$ exhibited a substantial improvement in rat neurobehavioral impairment induced by IR.

Effect of TF$_3$ on cerebral infarction

The infarct volume was evaluated in the coronal brain sections which were stained with TTC. Two hours of MCAO and 24 h of reperfusion showed an infarct vol-

Table 1. Physiological parameters

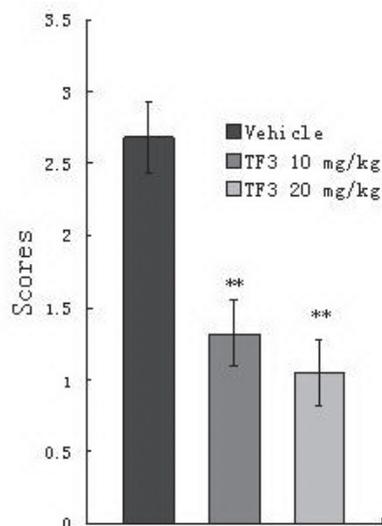| Group	Vehicle (N = 8)		Sham (N = 8)		TF$_3$ (10 mg.kg^{-1} N = 8)		TF$_3$ (20 mg.kg^{-1} N = 8)	
	Basal	30-min Rep	Basal	30-min Rep	Basal	30-min Rep	Basal	30-min Rep
Rectal Temp (°C)	37.2 ± 0.3	37.4 ± 0.3	36.8 ± 0.2	36.6 ± 0.2	36.5 ± 0.3	36.6 ± 0.3	36.7 ± 0.2	36.3 ± 0.3
PCO$_2$ (mm Hg)	56.9 ± 7.6	56.4 ± 7.2	55.7 ± 8.0	55.78± 8.2	53.4 ± 9.2	54.2 ± 7.8	54.7 ± 6.9	52.4 ± 8.7
PO$_2$ (mm Hg)	41.5 ± 7.8	40.7 ± 7.9	44.0 ± 5.2	44.4 ± 5.0	42.5 ± 8.9	40.1 ± 8.8	43.5 ± 9.7	44.2 ± 8.9
pH	7.3 ± 0.1	7.2 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.2	7.3 ± 0.1
MBP (mm Hg)	106 ± 14	109 ± 15	101 ± 10	103 ± 11	102 ± 17	104 ± 15	104 ± 13	103 ± 18

Fig. 1. Neurological deficit analysis after 2 h of MCAO and 24 h of reperfusion in vehicle- and TF$_3$ (10 and 20 mg.kg^{-1})-treated groups.
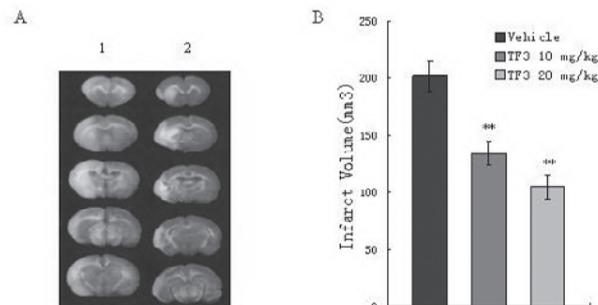**P < 0.01 as compared to the vehicle-treated group

Fig. 2. (A) Representative coronal brain sections stained with TTC after 2 h of MCAO and 24 h of reperfusion showing infarction. Lanes 1–2: vehicle, TF$_3$ (20 mg.kg^{-1}). (B) Volume of infarction after 2 h of MCAO and 24 h of reperfusion in vehicle and TF$_3$ (10 and 20 mg.kg^{-1})-treated groups.
** P < 0.01 as compared to the vehicle-treated group

ume of 201.24 ± 22.68 mm^3. The infarct volume was decreased to 133.96 ± 13.45 mm^3 and 104.66 ± 10.09 mm^3 in TF$_3$-treated groups (10 and 20 mg.kg^{-1}), respectively (P < 0.01, Fig. 2).

Effect of TF$_3$ on lipid peroxidation

The estimate of lipid peroxidation was obtained by measuring the formed MDA. MDA levels in the vehicle-treated group were elevated after 2 h ischemia and 24 h reperfusion. As compared to the vehicle-treated group, the levels of MDA significantly decreased in the TF$_3$-treated groups (10 and 20 mg.kg^{-1}) (P < 0.01, Table 2).

Effect of TF$_3$ on NO production

Nitrite was used as an indicator of NO production, due to the short half-life of NO.

The nitrite concentration in cortical tissue homogenates was significantly increased in the vehicle-treated group. As shown in Table 2, TF$_3$ (10 and 20 mg.kg^{-1}) treatment significantly suppressed the increase of NO formation (P < 0.01, Table 2).

Effect of TF$_3$ on SOD activity

SOD activity was observed in the cerebral cortex after 2-h ischemia and 24-h reperfusion. In the vehicle-treated group, SOD activity was decreased as compared to the sham-operated group. IR-induced decrease of the SOD activity was reverted by TF$_3$ (10 and 20 mg.kg^{-1}) treatment (P < 0.05, P < 0.01, Table 2).

Effect of TF$_3$ on GSH-Px activity

GSH-Px activity was estimated in the cerebral cortex after 2-h ischemia and 24-h reperfusion. In the vehicle-treated group, GSH-Px activity was decreased as compared to the sham-operated group. TF$_3$ (10 and 20 mg.kg^{-1}) treatment inhibited the IR-induced decrease of GSH-Px activity (P < 0.05, P < 0.01, Table 2).

Effect of TF$_3$ on protein expression of iNOS and COX-2

To confirm whether the inhibition of NO production is due to lower enzymatic activity or decreased protein expression of iNOS, we further studied the effect of TF$_3$ on iNOS protein expression by Western blotting. In addition to iNOS, we have also studied the effect of TF$_3$ on the expression of the COX-2 pro-

Table 2. Effect of TF$_3$ on levels of MDA and NO, activities of SOD and GSH-Px

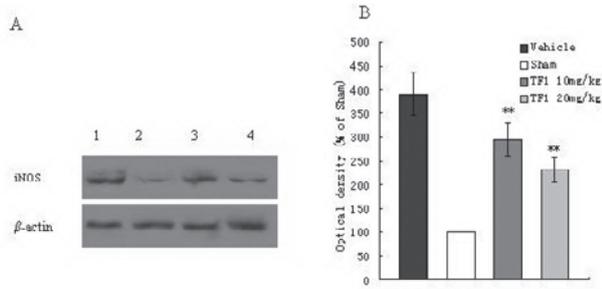| Parameters	Dose (mg.kg^{-1})	MDA (nmol/mg protein)	NO (µmol/g protein)	SOD (U/mg protein)	GSH-Px (U/mg protein)
Vehicle (N = 8)	-	4.96 ± 1.77	9.67 ± 1.26	0.325 ± 0.052	22.5 ± 3.4
Sham (N = 8)	-	1.53 ± 0.14**	5.64 ± 0.85**	0.683 ± 0.071**	46.3 ± 6.7**
TF$_3$ (N = 8)	10	3.29 ± 1.55 **	7.41 ± 1.19**	0.469 ± 0.057*	34.6 ± 6.2*
TF$_3$ (N = 8)	20	2.48 ± 1.23 **	6.54 ± 1.12**	0.568 ± 0.063**	37.1 ± 6.9**

*P < 0.05
**P < 0.01 as compared to vehicle-treated group

Fig. 3. (A) Protein expression of iNOS was assessed by using Western blot. Lanes 1–4: vehicle, sham, TF_3 (10 mg. kg^{-1}), TF_3 (20 mg.kg^{-1}). Compared to the vehicle-treated group, the protein level of iNOS was significantly decreased following treatment with TF_3 (B). Immunoblots were quantified by densitometric scanning, normalized with β-actin and expressed as percentage of sham. Protein samples were obtained from eight rats in each group. Results are representative of duplicate samples of three separate experiments and the densitometric evaluations are means of three independent experiments.
**P < 0.01 as compared to the vehicle-treated group

Fig. 5. (A) mRNA expression of iNOS was performed by using RT-PCR. Lanes 1–4: vehicle, sham, TF_3 (10 mg. kg^{-1}), TF_3 (20 mg.kg^{-1}). Compared to the vehicle-treated group, mRNA expression of iNOS was significantly decreased following treatment with TF_3 (B). Immunoblots were quantified by densitometric scanning, normalized with β-actin and expressed as percentage of sham. RNA samples were obtained from eight rats in each group. Results are representative of duplicate samples of three separate experiments and the densitometric evaluations are means of three independent experiments.
**P < 0.01 as compared to the vehicle-treated group

tein. The brain tissue obtained from the sham-operated animals showed low protein expression of iNOS and COX-2. After 2 h of MCAO and 24-h reperfusion, the protein expression of iNOS and COX-2 remarkably increased in the ischemic hemisphere in the vehicle-treated group as compared with the sham-operated group. TF_3 treatment could reduce protein expression of COX-2 and iNOS in a dose-dependent manner (Figs. 3, 4).
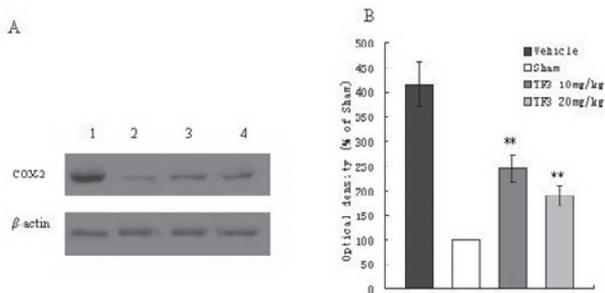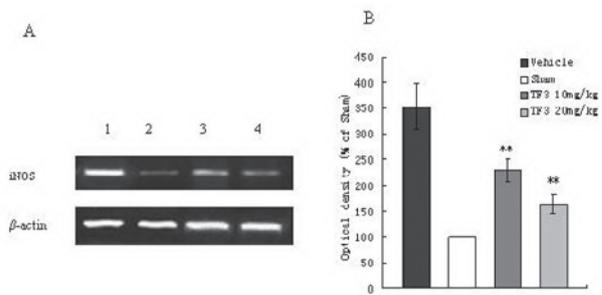
Effect of TF_3 on mRNA expression of iNOS and COX-2

Since iNOS and COX-2 expression was decreased at the protein level, we investigated whether the decrease in NO production was due to down-regulation of *iNOS* gene expression at the mRNA level using RT-PCR. The brain tissue obtained from the sham-operated group showed low mRNA expression of iNOS and COX-2.
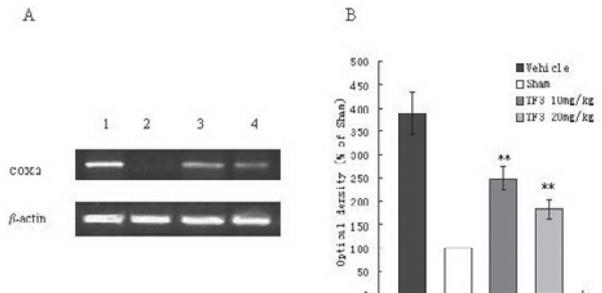

Fig. 4. (A) Protein expression of COX-2 was assessed by using Western blot. Lanes 1–4: vehicle, sham, TF_3 (10 mg. kg^{-1}), TF_3 (20 mg.kg^{-1}). Compared to the vehicle-treated group, the protein level of COX-2 was significantly decreased following treatment with TF_3 (B). Immunoblots were quantified by densitometric scanning, normalized with β-actin and expressed as percentage of sham. Protein samples were obtained from eight rats in each group. Results are representative of duplicate samples of three separate experiments and the densitometric evaluations are means of three independent experiments.
**P < 0.01 as compared to the vehicle-treated group

Fig. 6. (A) mRNA expression of COX-2 was performed by using RT-PCR. Lanes 1–4: vehicle, sham, TF_3 (10 mg. kg^{-1}), TF_3 (20 mg.kg^{-1}). Compared to the vehicle-treated group, mRNA expression of COX-2 was significantly decreased following treatment with TF_3 (B). Immunoblots were quantified by densitometric scanning, normalized with β-actin and expressed as percentage of sham. RNA samples were obtained from eight rats in each group. Results are representative of duplicate samples of three separate experiments and the densitometric evaluations are means of three independent experiments.
**P < 0.01 as compared to the vehicle-treated group

After 2 h of MCAO and 24-h reperfusion, the mRNA expression of iNOS and COX-2 remarkably increased in the ischemic hemisphere in the vehicle-treated group as compared to the sham-operated group. TF₃ treatment could reduce mRNA expression of iNOS and COX-2 in a dose-dependent manner. This inhibition of mRNA correlated with the inhibition of protein expression by TF₃ (Figs. 5, 6).

Effect of TF₃ on NF-κB activation

Western blot and EMSA were used to determine the status of the NF-κB complex in cerebral cortex. Western blot analysis of homogenates prepared from nuclear extracts of cerebral cortex was performed with anti-p65 and -p50 antibodies. NF-κB subunits p65 and p50 were increased after 2-h ischemia and 24 h of reperfusion. TF₃ significantly suppressed translocation of both subunits p65 and p50. As shown in Fig 3, NF-κB binding activity by EMSA increased in vehicle-treated cerebral cortex of nuclear extracts. TF₃ (10 and 20 mg.kg⁻¹) significantly inhibited the NF-κB binding activity in a dose-dependent manner (Figs. 7, 8, 9).

Effect of TF₃ on protein expression of IκB

The activation of NF-κB is thought to occur secondary to the proteolytic degradation of cytosolic IκBa, allowing free NF-κB to translocate to the nucleus where it binds to specific promoter sequences and initiates gene transcription. As pretreatment with TF3 inhibited NF-κB activation in cerebral tissues, we determined whether the protective effect against IR-induced cerebral injury might be related to its effect in preserving cytosolic IκB. Western blot analysis of homogenates prepared from cytoplasm extracts of cerebral cortex
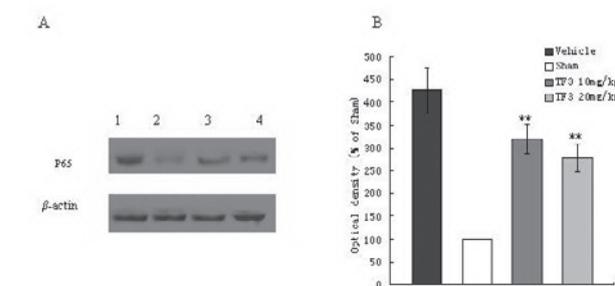

Fig. 8. (A) Protein expression of p65 was assessed by using Western blot. Lanes 1–4: vehicle, sham, TF₃ (10 mg. kg⁻¹), TF₃ (20 mg.kg⁻¹). Compared to the vehicle-treated group, the protein level of p65 was significantly decreased following treatment with TF₃ (B). Immunoblots were quantified by densitometric scanning, normalized with β-actin and expressed as percentage of sham. Protein samples were obtained from eight rats in each group. Results are representative of duplicate samples of three separate experiments and the densitometric evaluations are means of three independent experiments.
**P < 0.01 as compared to the vehicle-treated group

showed that protein expression of IκB was weaker in samples from vehicle-treated rats, while it was stronger in the cytoplasm with TF₃ treatment. TF₃ (10 and 20 mg.kg⁻¹) increased protein expression of IκB in a dose-dependent manner (Fig. 10).

Discussion

In our studies, we have used a focal cerebral ischemic animal model involving MCAO followed by reperfusion. This model closely reproduces clinical ischemic
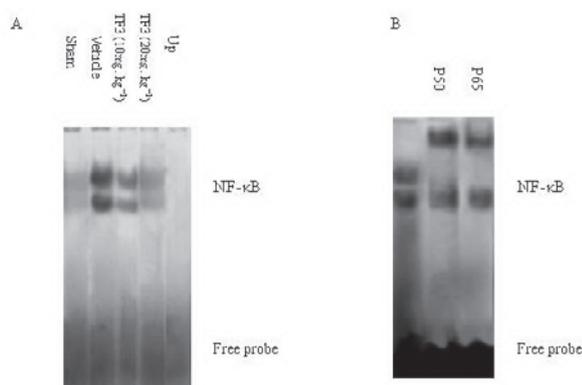

Fig. 7. (A) Nuclear extracts were obtained from cortex and analysed for NF-κB activity by EMSA. Each lane represents nuclear extracts from the vehicle, sham or TF₃ (10 mg. kg⁻¹, 20 mg.kg⁻¹) group. Administration of TF₃ decreased the NF-κB-binding activity. (B) For supershift assay, nuclear extracts from brain cortex were incubated with antibodies against individual components of the NF-κB complex for 30 min prior to EMSA analysis.
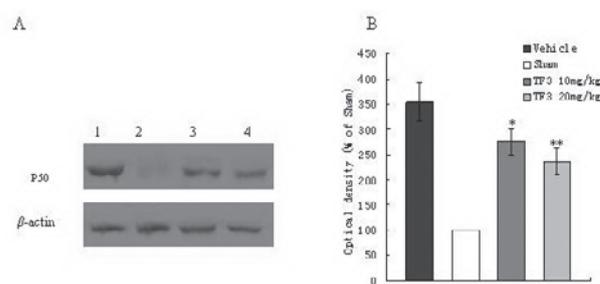

Fig. 9. (A) Protein expression of p50 was assessed by using Western Blot. Lanes 1–4: vehicle, sham, TF₃ (10 mg. kg⁻¹), TF₃ (20 mg.kg⁻¹). Compared to the vehicle-treated group, the protein level of p50 was significantly decreased following treatment with TF₃ (B) Immunoblots were quantified by densitometric scanning, normalized with β-actin and expressed as percentage of sham. Protein samples were obtained from eight rats in each group. Results are representative of duplicate samples of three separate experiments and the densitometric evaluations are means of three independent experiments.
*P < 0.05
**P < 0.01 as compared to vehicle-treated group

Fig. 10. (A) Protein expression of IκBα was assessed by using Western Blot. Lanes 1–4: vehicle, sham, TF$_3$ (10 mg.kg^{-1}), TF$_3$ (20 mg.kg^{-1}). Compared to the vehicle-treated group, the protein level of IκBα was significantly increased following treatment with TF$_3$. (B) Immunoblots were quantified by densitometric scanning, normalized with β-actin and expressed as percentage of sham. Protein samples were obtained from eight rats in each group. Results are representative of duplicate samples of three separate experiments and the densitometric evaluations are means of three independent experiments.
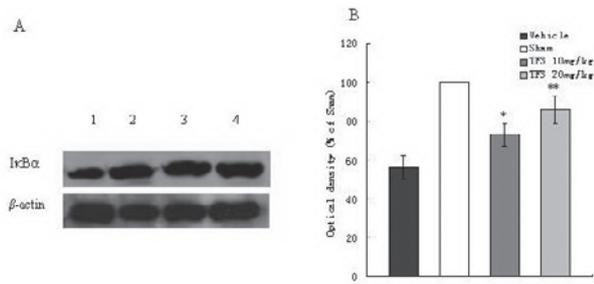*P < 0.05
**P < 0.01 as compared to the vehicle-treated group.

brain damage, showing the oxidative stress and the inflammation observed in human ischemic stroke patients (Ginsberg and Busto, 1989). Catechins and theaflavins are two groups of natural polyphenols found in green tea and black tea, respectively (Leung et al., 2001). Choi et al (2004) reported that epigallocatechin gallate (EGCG), a major constituent of catechins, protected brain damage against transient middle cerebral artery occlusion in rats. In our previous work (Cai et al., 2006), we demonstrated that theaflavin (TF$_1$), a major constituent of theaflavins, significantly protected neurons from cerebral ischemia-reperfusion injury by limiting leukocyte infiltration and expression of ICAM-1, at least in part, through reducing the phosphorylation of STAT-1. In the current study, we also found that TF$_3$, another major constituent of theaflavins, showed protective effects against brain injuries by MCAO-induced focal cerebral IR model in rats. It is well known that IR enhances formation of ROS in brain tissues (Siesjo, 1992). Excessive production of ROS, such as superoxide anion, hydroxyl radical, hydrogen peroxide and nitric oxide, may contribute to brain injury. The brain is very susceptible to the injury caused by oxidative stress, due to the high rate of oxidative metabolic activity, high polyunsaturated fatty acid contents, relatively low antioxidant capacity, and inadequate neuronal cell repair activity (Traystman et al., 1991). ROS have been indicated as one of the earliest and most important components of brain injury after cerebral IR. Overproduction of ROS results in oxidative damage, including lipid peroxidation, protein oxidation and DNA damage, which can lead to cell death (Floyd, 1999). Excessive production of ROS can be partially scavenged by endogenous antioxidant activities, including SOD that catalyzes the dismutation of the superoxide anion, GSH-Px and catalase (CAT) that mediate the breakdown of hydrogen peroxide (Bannister et al., 1987). Therefore, enhancement of the antioxidant activities in brain tissues may be potentially beneficial for the neuronal recovery from IR injury (Kitagawa et al., 2002). In the present study we demonstrated that cerebral IR caused a significant increase in the level of MDA and a decrease of the GSH-Px and SOD activities in the IR brain tissues. Our results also showed that administration of TF$_3$ prior to IR significantly decreased ROS production and the level of MDA. Similarly, TF$_3$ offered significant protection against the depletion of GSH-Px and SOD activity induced by IR. These data suggest that the protective effects of TF$_3$ against IR injury are partially due to their ability to reduce oxidative stress.

It has been clearly shown that ROS regulate the expression of many pro-inflammatory genes, including *COX-2* (Wang et al., 2004; Rodrigo et al., 2005) and *iNOS* (Floyd, 1999; Rodrigo et al., 2005), in brain tissues. The role of inflammation in the cerebral ischemic damage has been reported in humans and various animal models of stroke (Iadecola et al., 2001), and its importance in stroke has been highlighted by observations that anti-inflammatory compounds or deletion of proinflammatory genes are neuroprotective (Barone and Feuerstein, 1999). During IR injury, NO produced by iNOS reacts with ROS and produces peroxynitrites, which have deleterious effects on neuronal survival (Warner et al., 2004). We showed here that, in the rat cortex following IR, NO production was high. Pre-treatment with TF$_3$ markedly attenuated the content of NO. To confirm whether the inhibition of NO production was due to lower enzymatic activity or decreased protein expression of iNOS, we further studied the effect of TF$_3$ on mRNA and protein expression of iNOS by RT-PCR and Western blotting, respectively. We found that the mRNA and protein expression of iNOS were inhibited by TF$_3$. The mRNA and protein expression of COX-2 in rat brain were also affected by IR, and administration of TF$_3$ was correlated with lower levels of mRNA and protein expression of COX-2.

Proinflammatory genes are mainly controlled by NF-κB, which is also upregulated in experimental stroke (Mattson and Camandola, 2001). NF-κB is an important transcription factor that plays a key role in mediating inflammatory response to pro-inflammatory cytokines and ROS in animal models of experimental stroke. It is a family of hetero- or homodimer proteins with DNA-binding and transcription activation. In unstimulated cells, p50:p65 is sequestered in the cytoplasm by inhibitory proteins known as NF-κB inhibitors (IκBs). When cells are exposed to stimuli, IκB is phosphorylated by an upstream IκB kinase (IKK), which leads to its ubiquitination and proteosomal degradation. This process liber-

ates p50:p65, which translocates to the nucleus and induces transcription of several genes, including *iNOS*.

Many factors are known to activate NF-κB, such as ROS (Mattson et al., 1997), calcium overload (Simon et al., 1984), and various cytokines (Liu et al., 1994), which have been implicated as causative agents in IR insults. Previous data have shown that PDTC, a special inhibitor of NF-κB, can strongly protect neurons from IR injury, and suggest that NF-κB is involved in ROS-induced injury (Shen et al., 2003). The ability of PDTC to inhibit NF-κB activation suggests that ROS may be a second messenger system for activation of NF-κB. Moreover, some antioxidants, such as melatonin (Blondeau et al., 2001), have been proposed and used as potential therapeutic agents against oxidative stress-induced neuronal cell death; NF-κB may therefore be a therapeutic target for brain ischemia.

Our data suggest that activation and nuclear translocation of NF-κB are important for IR-induced brain injury in rats. The accumulation of p65 and p50 in the nuclear fraction of the vehicle-treated group were significantly decreased in the TF₃-treated group. Our findings also support a role for IκBα because activation of NF-κB during IR-induced brain injury was accompanied by depletion of IκBα. The protective effects of TF₃ against IR-induced injury may be mediated by down-regulation of NF-κB activation. However, we could not rule out the possibility that suppression of NF-κB activation by TF₃ might be a secondary event through its antioxidative effect. More precise mechanisms need further investigations.

In conclusion, we show here for the first time that TF₃ exerts protective effects against IR-induced brain injury by reducing oxidative stress and modulating the NF-κB activation. Acute stroke is a multi-component disorder, and its treatment will involve agents antagonizing multiple mechanisms. Therefore, TF₃, which acts as an antioxidant and redox modulator in a variety of systems, produces effective neuroprotection and represents a new class of naturally occurring agents with potential use in the therapy of human ischemic stroke.

Acknowledgments

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