

## Original Article

# Ferulic acid inhibits nitric oxide-induced apoptosis by enhancing GABA<sub>B1</sub> receptor expression in transient focal cerebral ischemia in rats

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**Aim:** Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) provides neuroprotection against apoptosis in a transient middle cerebral artery occlusion (MCAo) model. This study was to further investigate the anti-apoptotic effect of FA during reperfusion after cerebral ischemia.

**Methods:** Rats were subjected to 90 min of cerebral ischemia followed by 3 or 24 h of reperfusion after which they were sacrificed.

**Results:** Intravenous FA (100 mg/kg) administered immediately after middle cerebral artery occlusion (MCAo) or 2 h after reperfusion effectively abrogated the elevation of postsynaptic density-95 (PSD-95), neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), nitrotyrosine, and cleaved caspase-3 levels as well as apoptosis in the ischemic cortex at 24 h of reperfusion. FA further inhibited Bax translocation, cytochrome c release, and p38 mitogen-activated protein (MAP) kinase phosphorylation. Moreover, FA enhanced the expression of gamma-aminobutyric acid type B receptor subunit 1 (GABA<sub>B1</sub>) in the ischemic cortex at 3 and 24 h of reperfusion. In addition, nitrotyrosine-positive cells colocalized with cleaved caspase-3-positive cells, and phospho-p38 MAP kinase-positive cells colocalized with nitrotyrosine- and Bax-positive cells, indicating a positive relationship among the expression of nitrotyrosine, phospho-p38 MAP kinase, Bax, and cleaved caspase-3. The mutually exclusive expression of GABA<sub>B1</sub> and nitrotyrosine revealed that there is a negative correlation between GABA<sub>B1</sub> and nitrotyrosine expression profiles. Additionally, pretreatment with saclofen, a GABA<sub>B</sub> receptor antagonist, abolished the neuroprotection of FA against nitric oxide (NO)-induced apoptosis.

**Conclusion:** FA significantly enhances GABA<sub>B1</sub> receptor expression at early reperfusion and thereby provides neuroprotection against p38 MAP kinase-mediated NO-induced apoptosis at 24 h of reperfusion.

**Keywords:** ferulic acid; neuronal nitric oxide synthase; inducible nitric oxide synthase; Bax; p38 mitogen-activated protein kinase; nitric oxide-induced apoptosis

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## Introduction

In regional cerebral ischemic injury, severe ischemic insults will predominantly result in necrosis, while mild ischemic insults will predominantly induce apoptosis<sup>[1]</sup>. Cerebral ischemia/reperfusion (I/R)-induced apoptosis does not merely exacerbate acute cerebral infarction, but is also closely correlated with chronic neurodegenerative diseases<sup>[2,3]</sup>. Excitotoxicity induced by activation of glutamate receptors is the predominant mechanism underlying neuronal apoptosis in stroke<sup>[4]</sup>. Cerebral ischemic damage is associated with excessive release of excitatory glutamate, which subsequently

upregulates not only the expression of neuronal nitric oxide synthase (nNOS), but also inducible nitric oxide synthase (iNOS)<sup>[5,6]</sup>. Numerous studies<sup>[7–10]</sup> have shown that the glutamate receptor-postsynaptic density-95 (PSD-95)-nNOS complex and iNOS produce an excessive amount of nitric oxide (NO) during cerebral I/R injury; NO immediately reacts with superoxide radicals to form peroxynitrite, which subsequently nitrates protein tyrosine residues, generating nitrotyrosine and leading to oxidative/nitrative stress. Toxic NO-mediated oxidative/nitrative stress during cerebral I/R injury may contribute to the activation of the apoptotic cascade<sup>[11]</sup>. *In vitro* studies<sup>[5,12]</sup> have demonstrated that the NO-induced apoptotic signaling cascade involves mitogen-activated protein (MAP) kinase-mediated Bax translocation from the cytosol to the mitochondria and subsequent caspase-3 activation in cultured

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cells.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) is a component of *Anglica sinensis* (olivi) Didl (AS) and *Ligusticum chuaoanxiong* Hort (LC). Both AS and LC have been used to treat stroke in Traditional Chinese Medicine for centuries. In previous studies, FA reduces cerebral ischemic injury by weakening the expression of PSD-95 in the ischemic area and provides neuroprotection against apoptosis partly via inhibiting intercellular adhesion molecule-1 (ICAM-1) mRNA expression in a transient middle cerebral artery occlusion (MCAo) model<sup>[13, 14]</sup>. In addition, FA inhibits glutamate-induced apoptosis through modulation of the MAP kinase signaling pathway in cultured cortical neurons<sup>[15]</sup>. However, the precise mechanism by which FA suppresses apoptosis in transient MCAo remains unclear. Therefore, the purpose of this study was to further characterize the anti-apoptotic effect of FA after 90 min of MCAo.

## Materials and methods

### *In vivo* occlusion model

Adult male Sprague-Dawley rats weighing 300–350 g were employed in this study. Rats were fasted overnight and received water *ad libitum*. All experimental procedures were performed under the guidelines approved by the Care and Use of Laboratory Animals Committee of the China Medical University.

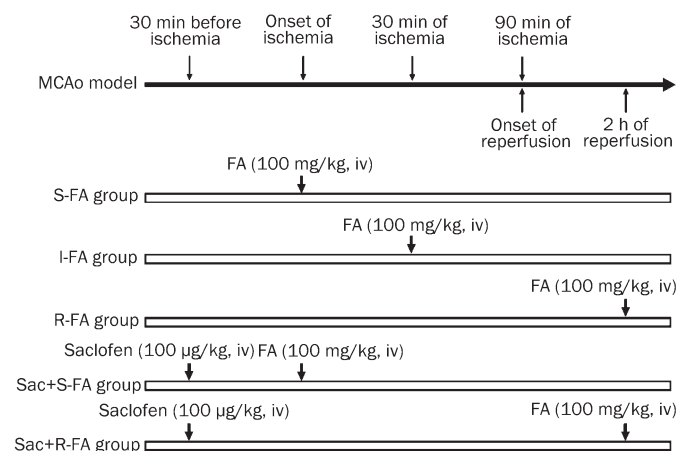
Ischemia was induced by intraluminal suture occlusion of the right middle cerebral artery (MCA) as previously described<sup>[16]</sup>. Briefly, rats were anesthetized with chloral hydrate (400 mg/kg, ip). A PE-50 catheter was then introduced into the right femoral artery to monitor arterial blood pressure and sample blood for analysis of blood gas. The right common carotid artery (CCA) and internal carotid artery (ICA) were exposed through a neck midline incision, and the pterygopalatine artery was ligated near its origin. A 3–0 nylon filament suture, blunted at the tip by a flame and coated with poly-L-lysine (Sigma-Aldrich, USA), was advanced from the right external carotid artery (ECA) through the CCA and up to the ICA for a distance of 20–25 mm, thereby occluding the MCA origin. After 90 min ischemia, the nylon suture was removed, inducing reperfusion. The right distal MCA was exposed via a cranial burr hole (2.5 mm lateral and 2.0 mm posterior to the bregma). The MCA blood flow was monitored by Laser-Doppler flowmetry (DRT4, Moor Instruments Inc, Wilmington, USA) at the pre-ischemia (>500 units), ischemia (<100 units), and reperfusion (>300 units) periods, and the Laser-Doppler flow measurements were used to verify success of the cerebral I/R procedure.

### Physiological parameters

Rectal temperature was monitored using a rectal thermometer and maintained at  $37.0 \pm 0.5$  °C using an electrical heating pad throughout the study. Blood gas parameters, including pH, pO<sub>2</sub>, and pCO<sub>2</sub>, were measured 10 min prior to and 90 min after cerebral ischemia.

## Experiment A

Rats were randomly divided into 7 groups: immediately after ischemia plus FA (S-FA), 30 min after ischemia plus FA (I-FA), 2 h after reperfusion plus FA (R-FA), vehicle, sham, pretreatment with saclofen in the S-FA (Sac+S-FA), and pretreatment with saclofen in the R-FA (Sac+R-FA) groups. Rats in the S-FA group were subjected to MCAo and simultaneously intravenously administered FA (Sigma-Aldrich) at a dose of 100 mg/kg. After 90 min of ischemia, rats were subjected to reperfusion and sacrificed at 24 h of reperfusion. Rats in the I-FA group were subjected to the same procedure as rats in the S-FA group; however, FA was administered 30 min after cerebral ischemia. Rats in the R-FA groups were subjected to the same procedure as rats in the S-FA group; however, FA was administered 2 h after reperfusion. Rats in the vehicle group were subjected to the same procedure as rats in the S-FA group, but did not receive FA. Rats in the sham group underwent the same operation as rats in the vehicle group, but the MCA origin was not occluded. Rats in the Sac+S-FA and Sac+R-FA groups were administered 100 µg/kg intravenous saclofen (Sigma-Aldrich) prior to 30 min of cerebral ischemia after which they were subjected to the same procedure as rats in the S-FA and R-FA groups, respectively. The methods of drug administration for the FA-treated groups were shown in Figure 1.



**Figure 1.** The scheme of ferulic acid (FA) administration.

### Immunohistochemistry (IHC) analysis

After 90 min of cerebral ischemia, rats were sacrificed under deep anesthesia at 24 h of reperfusion. Rats were transcardially perfused with 200 mL of 0.9% saline and 200 mL of 4% paraformaldehyde (PFA, pH 7.4). Rat brains were removed quickly and postfixed in 4% PFA followed by 30% sucrose (wt/vol) for 3 d after which they were cut into 15 µm sections using a cryostat. Brain sections were rinsed with Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich) containing 0.01% Tween-20 and immersed in 3% H<sub>2</sub>O<sub>2</sub>/methanol for 15 min to inhibit endogenous peroxidase activity. Thereafter, sections were incubated with 10% normal animal serum

(Zymed, CA, USA) for 20 min at room temperature (RT). The sections were incubated in moist chambers with rabbit anti-PSD-95 (1:100 dilution, #2507 Cell Signaling Technology), rabbit anti-nNOS (1:500 dilution, 61-7000 Zymed), mouse anti-iNOS (1:500 dilution, N32020 Transduction Laboratories), mouse anti-nitrotyrosine (1:100 dilution, MAB5404 Chemicon), rabbit anti-cleaved caspase-3 (17kD, 1:100 dilution, #9661S Chemicon), or guinea pig anti-Gamma-aminobutyric acid type B subunit 1 (GABA<sub>B1</sub>) (1:1000 dilution, AB1531 Chemicon) antibodies for 1 h at RT. Following incubation with the appropriate secondary antibody and avidin-biotin peroxidase complexes (ABC kit, Zymed, CA, USA), sections were colored with a 3,3'-diaminobenzidine (DAB) kit (Zymed, CA, USA), and counterstained with hematoxylin. The stained sections were mounted with mounting media (Assistant-Histokitt, Germany), and the immunopositive cells were detected by microscopic analysis (Axioskop 40, Zeiss). Negative controls for PSD-95, nNOS, iNOS, nitrotyrosine, and cleaved caspase-3 staining on adjacent serial sections in the vehicle group were incubated without primary antibodies. In addition, the negative control for GABA<sub>B1</sub> staining was performed using the adjacent section in the S-FA group and omitting the primary antibody.

#### Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay

TUNEL analysis was used to identify cells with nuclear DNA fragmentation in the ischemic cortex. TUNEL staining was performed according to the manufacturer's instructions (QIA33 Calbiochem, USA). Briefly, the brain sections adjacent to those used in IHC analysis were incubated with 20 µg/mL proteinase K for 20 min at RT, rinsed with TBS and incubated with 1×TdT equilibration buffer for 30 min at RT. They were then incubated with a TdT labeling reaction mixture for 1.5 h at 37 °C. After addition of the stop solution and blocking buffer, sections were incubated with 1×conjugate solution for 30 min at RT, and TUNEL-positive cells were visualized using a DAB kit (Calbiochem). Finally, sections were counterstained with methyl green (Calbiochem).

#### Western blot analysis

Twenty four hours after reperfusion, the rats were anesthetized with choral hydrate. The rat brains were removed and coronally sectioned from -4.3 to +1.7 mm bregma. The brain was separated into right cortex, right striatum, left cortex and left striatum. The right cortex was weighed and homogenized in the cytosolic extraction buffer (#K256-100 BioVision, USA). Lysates were centrifuged at 700×g for 10 min at 4 °C. The supernatant was transferred to a new tube and centrifuged at 10000×g for 30 min at 4 °C. The supernatant was collected and saved as the cytosolic fraction; the pellet was resuspended in 100 µL of the mitochondrial extraction buffer (#K256-100 BioVision, USA) and saved as the mitochondrial fraction. Protein concentrations of the cytosolic and mitochondrial fractions were determined by using a Bio-Rad assay. The samples were boiled at 100 °C in sodium dodecyl sulfate (SDS) gel loading

buffer for 10 min and loaded onto a 10% SDS polyacrylamide gel. After electrophoresis, separated proteins were electrotransferred to a nitrocellulose membrane (Hybond-c Extra, Amersham Biosciences, UK) in transfer buffer. The membrane was incubated in 5% skim milk containing 0.1% Tween 20 for 60 min at RT to block nonspecific binding after which they were incubated with rabbit anti-cleaved caspase-8 (1:1000 dilution, 3259-100 BioVision), rabbit anti-Bax (1:1000 dilution, #2772 Cell Signaling Technology), rabbit anti-cytochrome c (1:1000 dilution, #4272 Cell Signaling Technology), rabbit anti-cleaved caspase-3 (1:1000 dilution, #9661S Cell Signaling Technology), rabbit anti-p44/42 MAPK (extracellular signal-regulated kinase; ERK) (1:1000 dilution, #9102 Cell Signaling Technology), rabbit anti-phospho-p44/42 MAPK (ERK) (1:1000 dilution, #9101 Cell Signaling Technology), rabbit anti-SAPK/c-Jun N-terminal protein kinase (JNK) (1:1000 dilution, #9252 Cell Signaling Technology), rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) (1:1000 dilution, #9251S Cell Signaling Technology), rabbit anti-p38 MAP kinase (1:1000 dilution, #9211 Cell Signaling Technology), or rabbit anti-phospho-p38 MAP kinase (Thr180/Tyr182) (1:1000 dilution, #9212 Cell Signaling Technology) polyclonal antibodies overnight at 4 °C, respectively. In addition, the transferred membranes were probed with monoclonal antibodies specific for actin (1:5000 dilution, MAB1501 Chemicon) as an internal control for the cytosolic fraction and COX IV (1:5000 dilution, AB14744-100 Abcam) as an internal control for the mitochondrial fraction overnight at 4 °C. After washing, membranes were incubated with anti-rabbit HRP-linked IgG (1:5000 dilution, Jackson ImmunoResearch) or anti-mouse HRP-linked IgG (1:10000 dilution, Santa Cruz Biotechnology), or HRP-conjugated anti-biotin antibody (1:5000 dilution, Cell Signaling Technology) in phosphate buffered saline for 1 h at RT. Proteins were detected using an enhanced chemiluminescence reagent (Amersham Biosciences, UK) according to the manufacturer's instructions. Densitometric analysis was carried out using Gel-Pro Analyzer software. The optical density was calculated, and the levels of proteins were expressed as the densitometric ratio of proteins to actin or COX IV.

#### IHC co-staining

Brain sections subjected to single IHC analysis of cleaved caspase 3, GABA<sub>B1</sub> and phospho-p38 MAP kinase (1:200 dilution, Cell Signaling Technology) expression were further incubated with diluted normal blocking serum (Vector Laboratories, CA, USA). After 25 min at RT, sections were incubated with mouse anti-nitrotyrosine (1:200 dilution, MAB5404 Chemicon) antibody for 1.5 h at 37 °C. In addition, the sections subjected to phospho-p38 MAP kinase single staining were incubated with mouse anti-Bax (1:100 dilution, 3331-100 BioVision) antibody for 1.5 h at 37 °C and washed with DPBS. After incubation with diluted biotinylated secondary antibody and ABC-AP reagent (AK-5002, Vectastain), sections were stained with alkaline phosphatase substrate solution (SK-5300, Vector Blue), dried, and mounted with mounting media (Assistant-Histokitt, Germany). Finally, the immunopositive cells were

detected by microscopic analysis (Axioskop 40, Zeiss).

## Experiment B

Rats were randomly divided into 5 groups: S-FA, I-FA, R-FA, vehicle, and sham groups. Rats were subjected to the same experimental procedure as described in Experiment A; however, they were sacrificed at 3 h of reperfusion.

## IHC analysis

After 90 min cerebral ischemia, rats were sacrificed under deep anesthesia at 3 h of reperfusion. Rats were transcardially perfused with 200 mL of 0.9% saline and 200 mL of 4% PFA (pH 7.4). Rat brains were removed quickly and postfixed in 4% PFA followed by 30% sucrose (wt/vol) for 3 d after which they were cut into 15  $\mu$ m sections using a cryostat. Brain sections were subjected to the IHC procedure described in Experiment A and incubated with anti-nNOS, anti-iNOS, or anti-GABA<sub>B1</sub> antibodies.

## Statistical analysis

Data are expressed as mean  $\pm$  standard deviation (SD). All variables are approximately normal distribution and the parametric test such as analysis of variance (ANOVA) is appropriated. Data from all experimental groups were compared using one-way ANOVA followed by *post-hoc* analysis using the Scheffe's test. A probability value less than 0.05 was considered as statistically significant.

## Results

### Physiological parameters

Blood gas parameters, including pH, pO<sub>2</sub>, and pCO<sub>2</sub> were measured 10 min before and 90 min after cerebral ischemia. There were no significant differences in blood gas parameters among the experimental groups ( $P > 0.05$ ; Table 1), indicating that ischemia as well as FA alone or combined with saclofen did not affect the physiological parameters.

### Effects of FA on PSD-95, nNOS, iNOS and nitrotyrosine levels at 24 h of reperfusion

All immunopositive cells were evaluated within the square (1 mm<sup>2</sup>) located in the penumbra area of the brain coronal section (0.92 mm posterior to bregma). PSD-95-, nNOS-, iNOS-, and nitrotyrosine-positive cells were present in extremely large

**Table 1.** Summary of selected physiological parameters 10 min before and 90 min after cerebral ischemia.  $n=4$ . Mean  $\pm$  SD.

	pH	pO <sub>2</sub> (mmHg)	pCO <sub>2</sub> (mmHg)
10 min before cerebral ischemia			
Sham	7.32 $\pm$ 0.04	90.4 $\pm$ 5.3	30.0 $\pm$ 3.6
Vehicle	7.27 $\pm$ 0.03	99.6 $\pm$ 7.4	29.2 $\pm$ 6.3
S-FA	7.30 $\pm$ 0.03	94.4 $\pm$ 7.9	29.3 $\pm$ 6.9
I-FA	7.28 $\pm$ 0.03	100.2 $\pm$ 7.0	23.7 $\pm$ 3.6
R-FA	7.27 $\pm$ 0.01	101.2 $\pm$ 9.3	24.1 $\pm$ 4.8
Sac+S-FA	7.27 $\pm$ 0.04	102.2 $\pm$ 7.3	27.0 $\pm$ 6.1
Sac+R-FA	7.30 $\pm$ 0.03	94.5 $\pm$ 11.0	27.8 $\pm$ 3.1
90 min after cerebral ischemia			
Sham	7.32 $\pm$ 0.04	95.8 $\pm$ 3.3	27.7 $\pm$ 3.7
Vehicle	7.31 $\pm$ 0.04	100.0 $\pm$ 10.6	21.8 $\pm$ 2.4
S-FA	7.31 $\pm$ 0.03	93.6 $\pm$ 6.4	22.0 $\pm$ 4.4
I-FA	7.32 $\pm$ 0.04	102.2 $\pm$ 10.3	20.9 $\pm$ 4.5
R-FA	7.29 $\pm$ 0.03	99.2 $\pm$ 12.0	20.2 $\pm$ 4.6
Sac+S-FA	7.32 $\pm$ 0.03	92.0 $\pm$ 8.9	24.5 $\pm$ 3.0
Sac+R-FA	7.34 $\pm$ 0.02	89.3 $\pm$ 10.2	23.4 $\pm$ 2.3

numbers in the ischemic cortex of the vehicle groups ( $P < 0.01$  vs sham group) at 24 h of reperfusion; a reduction in PSD-95-, nNOS-, iNOS-, and nitrotyrosine-positive cells was observed in the S-FA group as compared to the vehicle group ( $P < 0.01$ ). In addition, fewer PSD-95-, nNOS-, iNOS-, and nitrotyrosine-positive cells were detected in the R-FA groups as compared to the vehicle group ( $P < 0.01$ ; Figure 2; Table 2); there were no significant differences in the number of immunopositive cells between the I-FA and vehicle groups ( $P > 0.05$ ; Figure 2; Table 2).

The Sac+S-FA and Sac+R-FA groups were undertaken to examine the effect of FA under the saclofen pretreatment condition. No differences in the expression of PSD-95, nNOS, iNOS, and nitrotyrosine in the ischemic cortex among the vehicle, Sac+S-FA, and Sac+R-FA groups were observed ( $P > 0.05$ ; Figure 2; Table 2).

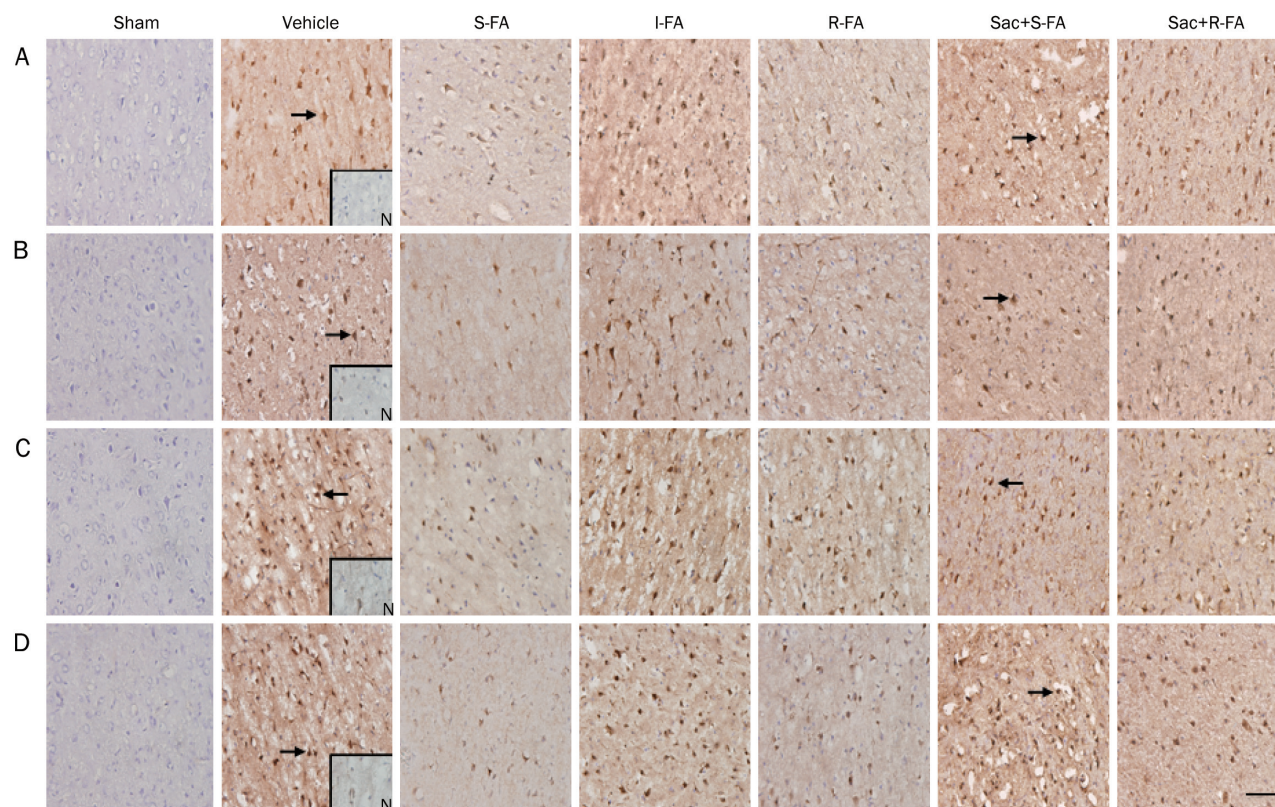
### Effects of FA on the expression of cleaved caspase-3 and TUNEL-positive cells at 24 h of reperfusion

Cleaved caspase-3 expression (17/19 kDa) in the ischemic cortex was increased by 2.9-fold in the vehicle group *versus* the

**Table 2.** The expression of PSD-95-, nNOS-, iNOS-, nitrotyrosine-, cleaved caspase-3-, and TUNEL-positive cells (counts/mm<sup>2</sup>).  $n=4-5$  per group. Mean  $\pm$  SD. <sup>c</sup> $P < 0.01$  vs sham group; <sup>f</sup> $P < 0.01$  vs vehicle group.

	Sham	Vehicle	S-FA	I-FA	R-FA	Sac+S-FA	Sac+R-FA
PSD-95	0 $\pm$ 0	233 $\pm$ 22 <sup>c</sup>	119 $\pm$ 17 <sup>f</sup>	272 $\pm$ 41	133 $\pm$ 25 <sup>f</sup>	233 $\pm$ 37	254 $\pm$ 29
nNOS	0 $\pm$ 0	212 $\pm$ 24 <sup>c</sup>	98 $\pm$ 10 <sup>f</sup>	230 $\pm$ 30	109 $\pm$ 18 <sup>f</sup>	223 $\pm$ 39	258 $\pm$ 35
iNOS	0 $\pm$ 0	247 $\pm$ 24 <sup>c</sup>	102 $\pm$ 20 <sup>f</sup>	245 $\pm$ 29	129 $\pm$ 15 <sup>f</sup>	218 $\pm$ 40	240 $\pm$ 20
Nitrotyrosine	0 $\pm$ 0	239 $\pm$ 17 <sup>c</sup>	111 $\pm$ 19 <sup>f</sup>	224 $\pm$ 25	107 $\pm$ 17 <sup>f</sup>	224 $\pm$ 24	247 $\pm$ 28
Cleaved caspase-3	0 $\pm$ 0	233 $\pm$ 26 <sup>c</sup>	117 $\pm$ 20 <sup>f</sup>	269 $\pm$ 31	149 $\pm$ 16 <sup>f</sup>	229 $\pm$ 39	266 $\pm$ 48
TUNEL	0 $\pm$ 0	305 $\pm$ 50 <sup>c</sup>	134 $\pm$ 20 <sup>f</sup>	361 $\pm$ 34	169 $\pm$ 36 <sup>f</sup>	292 $\pm$ 56	295 $\pm$ 69





**Figure 2.** Effects of FA on PSD-95 (A), nNOS (B), iNOS (C), and nitrotyrosine (D) levels at 24 h of reperfusion. N, negative control of stain. Scale bar=100  $\mu$ m.

sham group ( $P<0.05$ ) at 24 h of reperfusion, and the expression of cleaved caspase-3 in the S-FA group was 0.5-fold lower than that observed in the vehicle group ( $P<0.05$ ). Additionally, analysis of cleaved caspase-3 expression in the R-FA group revealed a 0.5-fold reduction as compared to the vehicle group ( $P<0.05$ ). No statistical difference was observed in the expression of cleaved caspase-3 between the I-FA and vehicle groups ( $P>0.05$ ; Figure 3). Increased cleaved caspase-3- and TUNEL-positive cells in the ischemic cortex were observed in the vehicle group ( $P<0.01$  vs sham group) at 24 h of reperfusion; however, significant reductions were observed in the S-FA group as compared with the vehicle group ( $P<0.01$ ). Fewer cleaved caspase-3 and TUNEL-positive cells were also detected in the R-FA group as compared to the vehicle group ( $P<0.01$ ; Figure 3; Table 2); however, no significant differences were observed among the vehicle, Sac+S-FA, and Sac+R-FA group ( $P>0.05$ ; Figure 3; Table 2). Finally, nitrotyrosine-positive cells colocalized with cleaved caspase-3-positive cells, and the distribution patterns of the double labeled cells were similar to those of nitrotyrosine- or cleaved caspase-3-positive cells (Figure 2, 3, and 4).

#### Effects of FA on the expression of cleaved caspase-8, Bax and cytochrome c at 24 h of reperfusion

Using Western blot analysis and subcellular fractionation, no marked difference was observed among the sham, vehicle,

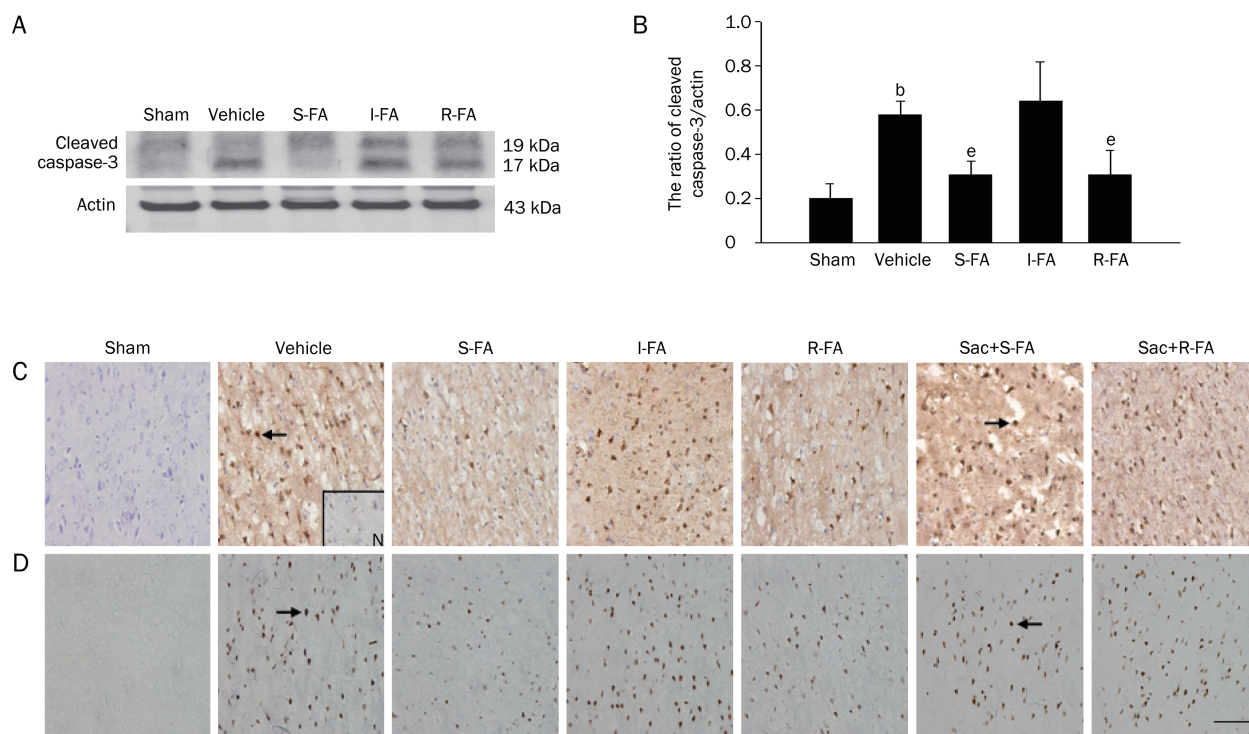
S-FA, I-FA, and R-FA groups with regard to the expression of cleaved caspase-8 in the ischemic cortex at 24 h of reperfusion ( $P>0.05$ ). Analysis of whether Bax translocated from the cytosol to the mitochondria of cells in the ischemic cortex 24 h after reperfusion revealed increased mitochondrial Bax in the vehicle group as compared to the sham group (1.5-fold,  $P<0.05$ ). However, mitochondrial Bax expression in the S-FA and R-FA groups was reduced as compared to the vehicle group (both 0.7-fold,  $P<0.05$ ; Figure 5). No difference in mitochondrial Bax expression was observed between the I-FA and vehicle groups ( $P>0.05$ ; Figure 5).

The release of cytochrome c from the mitochondria to the cytosol in response to Bax translocation in the ischemic cortex was also analyzed. Significant enhancement of cytochrome c in the cytosolic fraction of the vehicle group as compared with the sham group was detected (1.6-fold,  $P<0.05$ ). Moreover, the expression of cytosolic cytochrome c was diminished in the S-FA and R-FA groups as compared with the vehicle group (both 0.7-fold,  $P<0.05$ ; Figure 5). There was no difference in the level of cytosolic cytochrome c between the I-FA and vehicle groups ( $P>0.05$ ; Figure 5).

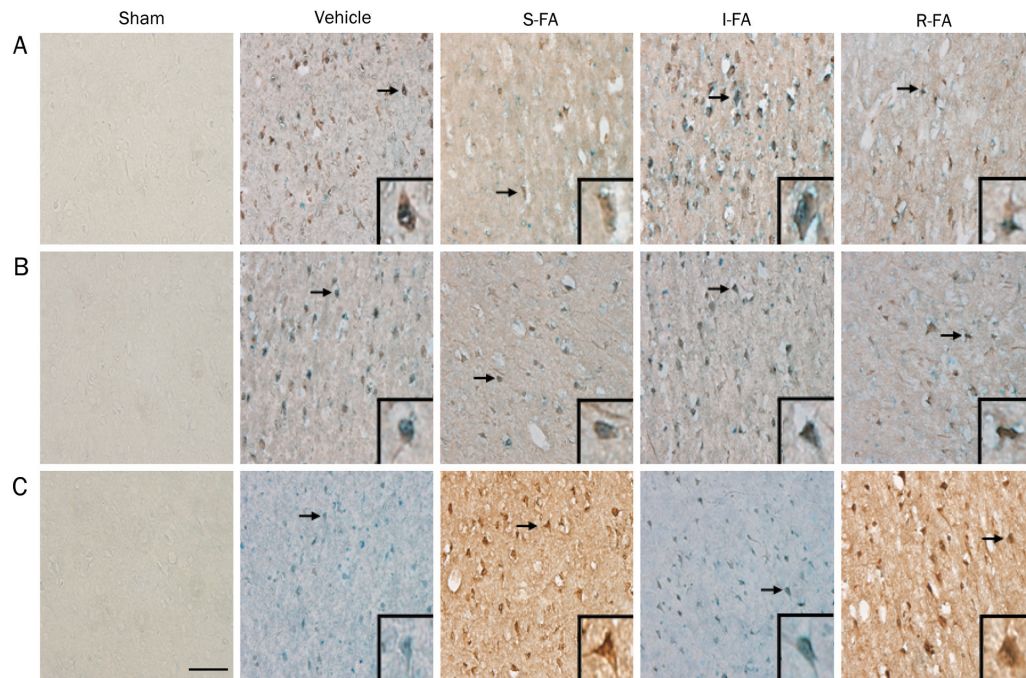
#### Effects of FA on the phosphorylation of ERK, JNK and p38 MAP kinase at 24 h of reperfusion

Phosphorylation of ERK, JNK, and p38 MAP kinase in the ischemic cortex was detected by Western blot analysis.





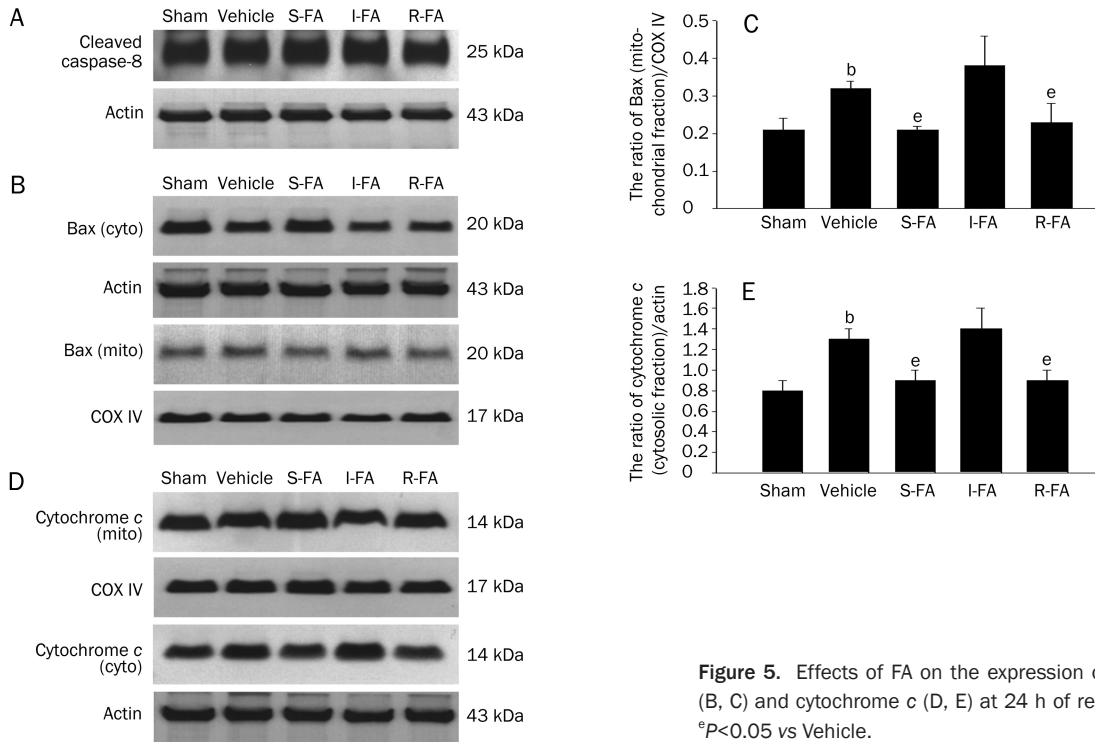
**Figure 3.** Effects of FA on the expression of cleaved caspase-3 (A, B, and C) and TUNEL-positive cells (D) at 24 h of reperfusion. <sup>b</sup> $P < 0.05$  vs Sham, <sup>e</sup> $P < 0.05$  vs Vehicle.



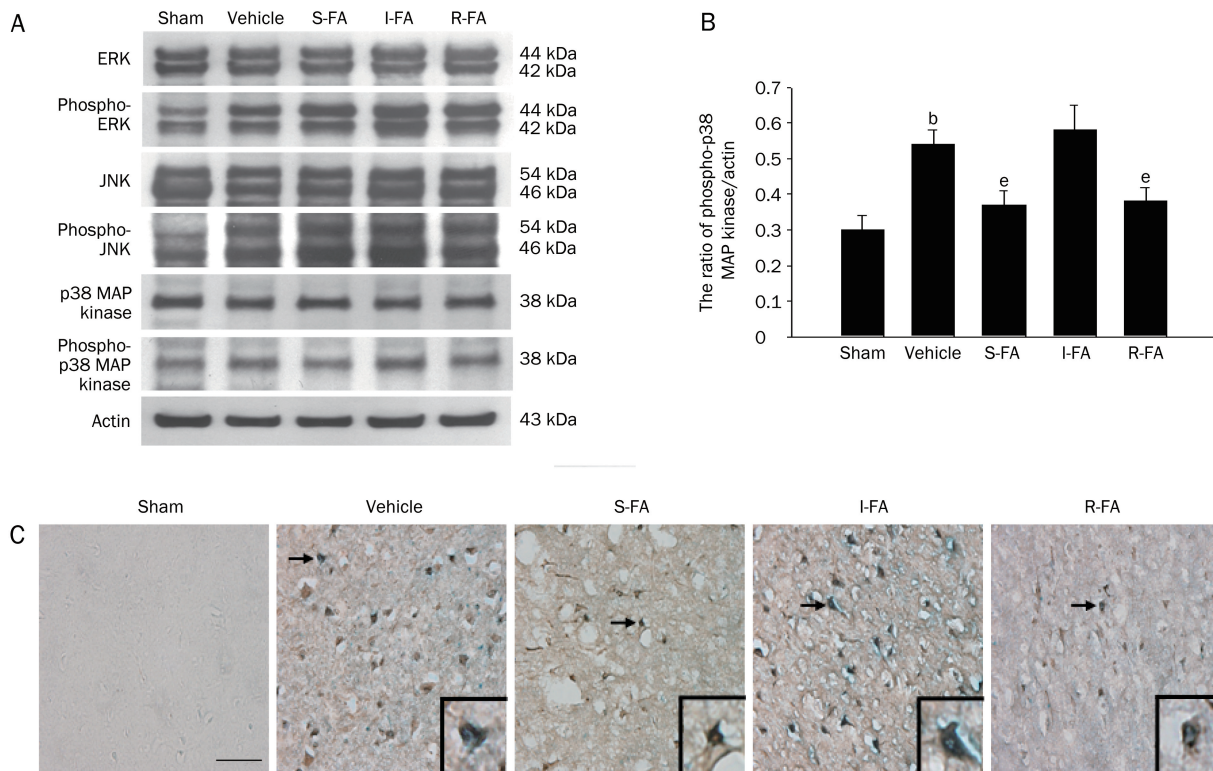
**Figure 4.** Effect of FA on nitrotyrosine-cleaved caspase-3 (A), nitrotyrosine-phospho-p38 MAP kinase (B), and nitrotyrosine-GABA<sub>B1</sub> (C) double labeled cells at 24 h of reperfusion.

Among the vehicle, S-FA, I-FA, and R-FA groups, no significant differences in the levels of ERK or phospho-ERK were observed at 24 h of reperfusion ( $P > 0.05$ ). Similarly, no marked differences in JNK or phospho-JNK expression were detected between the vehicle and FA-treated groups ( $P > 0.05$ ; Figure 6). Although there was no difference in p38 MAP kinase expres-

sion among the sham, vehicle, S-FA, I-FA, and R-FA groups at 24 h of reperfusion, the level of phospho-p38 MAP kinase in the ischemic cortex of the vehicle group was 1.8-fold higher than that observed in the sham group ( $P < 0.05$ ). In addition, a 0.7-fold reduction in phospho-p38 MAP kinase in both the S-FA and R-FA groups was detected as compared to the



**Figure 5.** Effects of FA on the expression of cleaved caspase-8 (A), Bax (B, C) and cytochrome c (D, E) at 24 h of reperfusion. <sup>b</sup> $P < 0.05$  vs Sham. <sup>e</sup> $P < 0.05$  vs Vehicle.



**Figure 6.** Effects of FA on the phosphorylation of ERK, JNK, and p38 MAP kinase (A, B), and phospho-p38 MAP kinase-bax double labeled cells (C) at 24 h of reperfusion. <sup>b</sup> $P < 0.05$  vs Sham, <sup>e</sup> $P < 0.05$  vs Vehicle.

vehicle group (both  $P < 0.05$ ); the expression of phospho-p38 MAP kinase was similar between the I-FA and vehicle groups

( $P > 0.05$ ; Figure 6).

To test the relationship between oxidative/nitrative stress



and the Bax-mediated mitochondrial apoptotic pathway, nitrotyrosine-phospho-p38 MAP kinase colocalization analysis and phospho-p38 MAP kinase-Bax colocalization analysis were performed at 24 h of reperfusion. Phospho-p38 MAP kinase-positive cells colocalized with nitrotyrosine- and Bax-positive cells, and the expression patterns of nitrotyrosine-phospho-p38 MAP kinase and phospho-p38 MAP kinase-Bax double labeled cells were similar to those of phospho-p38 MAP kinase detected by Western blot analysis (Figure 4 and 6), revealing a correlation among nitrotyrosine, phospho-p38 MAP kinase, and Bax expression.

#### Effects of FA on the expression of GABA<sub>B1</sub> receptors at 3 and 24 h of reperfusion

In the ischemic cortex, very few GABA<sub>B1</sub> receptor-positive cells were found in the vehicle groups at 3 h of reperfusion ( $P>0.05$  vs sham group). However, the number of GABA<sub>B1</sub> receptor-positive cells in both the S-FA and R-FA groups was larger than that observed in the vehicle group ( $39\pm6$  vs  $13\pm6$  and  $49\pm11$  vs  $13\pm6$ , respectively; both  $P<0.01$ ; Figure 7). Additionally, no significant difference was found in GABA<sub>B1</sub> receptor-positive cells between the I-FA and vehicle groups ( $P>0.05$ ; Figure 7). At 24 h of reperfusion, GABA<sub>B1</sub> receptor-positive cells were present in the ischemic cortex of the vehicle group as compared to the sham group ( $128\pm16$  vs  $0\pm0$ ;  $P<0.01$ ). However, there was a significant enhancement of GABA<sub>B1</sub> receptor-positive cells in the ischemic cortex of the S-FA and R-FA groups as compared with the vehicle group ( $257\pm22$  vs

$128\pm16$  and  $258\pm33$  vs  $128\pm16$ , respectively; both  $P<0.01$ ; Figure 7). Moreover, no differences in GABA<sub>B1</sub> receptor-positive cells were detected between the I-FA and vehicle groups ( $P>0.05$ ; Figure 7).

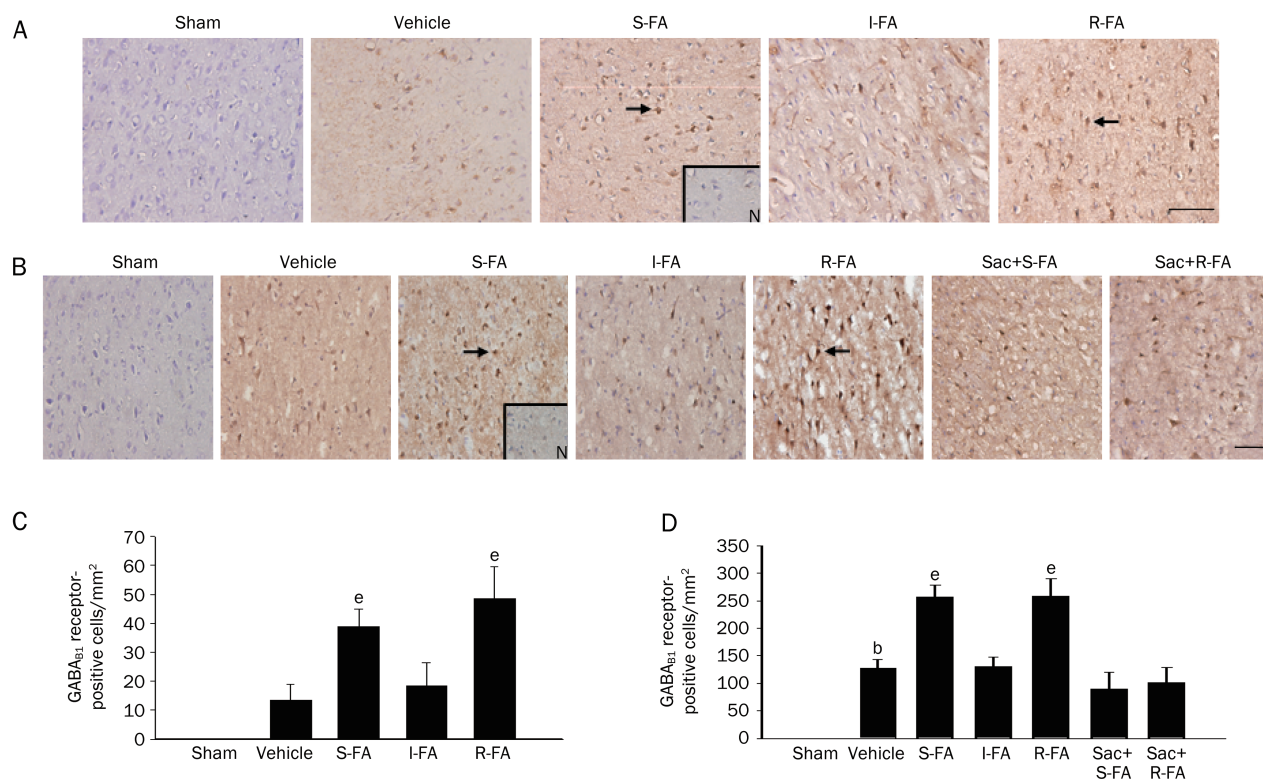
The effect of saclofen, a GABA<sub>B</sub> receptor antagonist, on the number of GABA<sub>B1</sub> receptor-positive cells in the Sac+S-FA and Sac+R-FA groups was also determined, and no significant differences among the Sac+S-FA, Sac+R-FA, and vehicle groups in response to GABA<sub>B1</sub> receptor expression were observed ( $P>0.05$ ; Figure 7), indicating that saclofen pretreatment decreased the number of GABA<sub>B1</sub> receptors in the Sac+S-FA and Sac+R-FA groups at 24 h of reperfusion.

Upon examining the number of GABA<sub>B1</sub> receptor- and nitrotyrosine-positive cells by double staining in the ischemic cortex at 24 h of reperfusion, we found that GABA<sub>B1</sub> receptor-positive cells were abundant in the S-FA and R-FA groups. However, nitrotyrosine-positive cells were obviously present in the vehicle and I-FA groups (Figure 4). The mutually exclusive expression of these two molecules indicated that there is a negative relationship between GABA<sub>B1</sub> receptor and nitrotyrosine.

Finally, there was no significant difference in nNOS or iNOS-positive cells in the ischemic cortex among the experiment groups at 3 h of reperfusion (data not shown).

#### Discussion

Convincing evidence has implicated that glutamate-induced intracellular calcium overload resulting in massive NO pro-



**Figure 7.** Effects of FA on the expression of GABA<sub>B1</sub> receptors at 3 (A, C) and 24 h (B, D) of reperfusion. <sup>b</sup> $P<0.05$  vs Sham, <sup>e</sup> $P<0.05$  vs Vehicle.



duction plays a key role in neuronal apoptosis in hypoxic/ischemic brain injury<sup>[4, 17]</sup>. PSD-95, a scaffolding protein, is involved in glutamate receptor interaction with nNOS at post-synaptic sites, and the glutamate receptor-PSD-95-nNOS complex facilitates nNOS activation and subsequent NO production<sup>[8, 18]</sup>. In addition, iNOS makes a prominent contribution to glutamate-induced peroxynitrite formation after cerebral ischemic insult<sup>[19]</sup>. Peroxynitrite can induce single- and double-strand breaks in DNA, resulting in apoptosis. Thus, nitrotyrosine as a marker for peroxynitrite-mediated oxidative/nitrative stress is also an early marker of apoptosis caused by cerebral I/R injury<sup>[7]</sup>. In the present study, PSD-95, nNOS, iNOS, nitrotyrosine, and cleaved caspase-3 levels as well as apoptosis were significantly increased in the ischemic cortex at 24 h of reperfusion, whereas FA administered immediately after cerebral ischemia (S-FA group) or 2 h after reperfusion (R-FA group) abrogated the elevation of PSD-95, nNOS, iNOS, nitrotyrosine, cleaved caspase-3, and apoptosis. However, administration of FA 30 min after cerebral ischemia (I-FA group) failed to decrease the expression of these molecules. In addition, colocalization of nitrotyrosine- and cleaved caspase-3-positive cells markedly increased in the ischemic cortex at 24 h of reperfusion; however, it was suppressed by FA treatment in the S-FA and R-FA groups. These results are consistent with previous reports which demonstrated that nNOS and iNOS expression are markedly increased in the ischemic area 24 h after cerebral ischemia, and pharmacologically-selective inhibitors of the glutamate receptor-PSD-95-nNOS complex and iNOS reduce nitrotyrosine formation, attenuate caspase-3 activation, and ameliorate ischemic brain injury<sup>[6, 20-22]</sup>. On the basis of these findings, we posit that FA exerts its protective effect against PSD-95, nNOS, and iNOS-mediated oxidative/nitrative stress (nitrotyrosine) in the ischemic cortex of the S-FA and R-FA groups at 24 h of reperfusion, and the anti-oxidative/nitrative effect of FA further contributes to the inhibition of caspase-3-dependent apoptosis.

There are two major pathways of caspase-dependent apoptosis: the intrinsic and extrinsic pathways. The intrinsic apoptotic pathway involves the cytochrome *c*-mediated caspase cascade, and the extrinsic apoptotic pathway is mediated by death receptors and caspase-8 activation<sup>[23]</sup>. Earlier studies have documented that NO-induced apoptosis could be mediated by mitochondrial-dependent caspase activation in cultured cells<sup>[22, 24]</sup>. The mitochondrial apoptotic pathway could be initiated by the action of Bax, a member of the Bcl-2 family of proteins. Bax normally resides in the cytosol and translocates to the mitochondria in response to the apoptotic stimulation. Bax translocation induces the release of cytochrome *c* through opening mitochondrial permeability transition pores, and subsequently cleaving and activating caspase-3, the major apoptotic executioner, which in turn initiates apoptosis<sup>[25, 26]</sup>. In examining the expression of key apoptosis-related molecules, we found that Bax translocation from the cytosol to the mitochondria obviously occurred in the ischemic cortex at 24 h of reperfusion, triggering cytochrome *c* release from the mitochondria to the cytosol, caspase-3 activation, and apoptosis.

On the contrary, FA significantly reduced cerebral I/R injury-induced Bax translocation, cytochrome *c* release, caspase-3 activation, and apoptosis in the ischemic cortex of the S-FA and R-FA groups at 24 h of reperfusion. Several reports have shown that elevated Bax levels in association with increased cytochrome *c* release is found in the ischemic area at 24 h after reperfusion, and agents with an ability to reverse the increased level of Bax could effectively inhibit cerebral I/R-induced apoptosis<sup>[27, 28]</sup>. Thus, FA may protect against NO-induced apoptosis at least partially by way of regulation of Bax translocation, but not through the modulation of cleaved caspase-8; the expression of cleaved caspase-8 remained unchanged in the ischemic cortex at 24 h of reperfusion.

MAP kinase family members include ERK, JNK, and p38 MAP kinase. Sustained phosphorylation of JNK and p38 MAP kinase is associated with the Bax-mediated mitochondrial apoptotic pathway in the *in vitro* studies using cultured neurons and *in vivo* studies of focal cerebral ischemia<sup>[29-31]</sup>. In the present study, the expression of phospho-p38 MAP kinase was markedly greater in the ischemic cortex at 24 h of reperfusion, whereas FA treatment in the S-FA and R-FA groups selectively downregulated the level of phospho-p38 MAP kinase but failed to curtail the expression of phospho-ERK and phospho-JNK. Additionally, upon examination of phospho-p38 MAP kinase-nitrotyrosine colocalization and phospho-p38 MAP kinase-Bax colocalization, we observed that phospho-p38 MAP kinase-positive cells colocalized with nitrotyrosine- and Bax-positive cells, which were markedly increased in the ischemic cortex; however, the number of these double labeled cells was obviously reduced upon FA treatment in the S-FA and R-FA groups. Previous studies have elucidated that p38 MAP kinase signaling transduction pathway initiates Bax translocation, thereby inducing NO-induced apoptosis in cultured neurons, and treatment with the p38 MAP kinase inhibitor effectively interrupts NO-induced cell death<sup>[29, 32]</sup>. Thus, our present results strongly suggest that Bax translocation from the cytosol to the mitochondria is subject to regulation by p38 kinase in response to NO-induced apoptosis, whereas FA clearly suppresses Bax-mediated apoptosis by decreasing the level of phospho-p38 MAP kinase in the ischemic cortex at 24 h of reperfusion.

Gamma-aminobutyric acid (GABA) is the chief inhibitory neurotransmitter in the mammalian central nervous system and acts on GABA type A (GABA<sub>A</sub>) and type B (GABA<sub>B</sub>) receptors<sup>[33]</sup>. GABA<sub>B</sub> receptors, heterodimeric G-protein-coupled receptors composed of GABA<sub>B1</sub> and GABA<sub>B2</sub>, act presynaptically by inhibiting Ca<sup>2+</sup> channels and postsynaptically by regulating inwardly-rectifying K<sup>+</sup> channels. Thus, GABA<sub>B</sub> receptors play a pivotal role in protecting against glutamate-induced apoptosis in a rat model of transient focal cerebral ischemia<sup>[34]</sup>. A number of studies have demonstrated a decrease in GABA<sub>B</sub> receptor expression 24 h after cerebral ischemia and that GABA<sub>B</sub> agonists could exert neuroprotection against glutamate-induced excitotoxicity via enhancing GABA<sub>B</sub> receptors<sup>[10, 33, 35, 36]</sup>. In the present study, we found that FA firstly increased the expression of GABA<sub>B1</sub> receptors in the

S-FA and R-FA groups at 3 h of reperfusion in spite of reduced nNOS and iNOS expression in the ischemic cortex. At 24 h of reperfusion, the expression of GABA<sub>B1</sub> receptors was robustly increased in these two FA-treated groups. Intriguingly, the distribution patterns of GABA<sub>B1</sub> receptors were contrary to those of PSD-95, nNOS, iNOS, and nitrotyrosine in the ischemic cortex of the vehicle and FA-treated groups at 24 h of reperfusion. Further validation of this difference was acquired from GABA<sub>B1</sub>-nitrotyrosine double staining, which revealed the mutually exclusive expression of GABA<sub>B1</sub> and nitrotyrosine. Our work confirms those previous studies<sup>[10, 33, 35, 36]</sup> and further elucidates that the effect of FA against p38 MAP kinase-mediated apoptosis in the S-FA and R-FA groups is due to the enhancement of GABA<sub>B1</sub> receptor expression at 24 h following reperfusion.

To gain further insight into the possible role of GABA<sub>B1</sub> receptors in attenuating NO-induced apoptosis in the FA-treated groups, we examined the action of saclofen, which is utilized to block GABA<sub>B</sub> receptor during cerebral ischemia, in the S-FA and R-FA groups and found that the administration of saclofen at 30 min before MCAo completely abolished the neuroprotection of FA against NO-induced apoptosis at 24 h of reperfusion. Thus, the enhancement of GABA<sub>B1</sub> receptor expression at 3 h of reperfusion might be the early beneficial action of FA against p38 MAP kinase-mediated NO-induced apoptosis at 24 h of reperfusion.

The collateral circulation is built up for the preferential protection of the penumbra area after initial cerebral ischemia; however, adenosine triphosphate (ATP) depletion and energy failure, which lead to suppression of GABA<sub>B</sub>-mediated hyperpolarization, rapidly occur within 30 min after ischemia, and the brain energy state returns to nearly normal throughout the infarct territory upon reperfusion<sup>[37–39]</sup>. Experimental studies have elucidated that the GABA<sub>B</sub> agonist effectively reduces glutamate release in response to GABA<sub>B</sub> receptor expression in the ischemic area 20 to 40 min after cerebral ischemia<sup>[36]</sup>; FA and its metabolites are sharply decreased in plasma and rapidly excreted in urine 0.5 to 1.5 h after administration of FA in rats<sup>[40]</sup>. Thus, we conclude that in the S-FA group, FA rapidly enhances GABA<sub>B1</sub> receptors within 30 min after ischemia; in the R-FA group, FA might increase GABA<sub>B1</sub> expression at 2 h of reperfusion under the condition of the energy state recovery, and the S-FA and R-FA groups subsequently increase GABA<sub>B1</sub> receptors at 3 and 24 h of reperfusion. In the I-FA group, one of the possible explanations for the opposite effect on GABA<sub>B1</sub> receptor expression is that FA fails to enhance GABA<sub>B1</sub> expression under the condition of ATP depletion at 30 min of ischemia, and FA might be excreted in urine at the initial reperfusion, resulting in decreased GABA<sub>B1</sub> expression at 3 and 24 h of reperfusion. However, further research is needed to clarify this finding.

In summary, FA significantly enhances the expression of GABA<sub>B1</sub> receptors at 3 h of reperfusion and thereby provides neuroprotection against p38 MAP kinase-mediated NO-induced apoptosis at 24 h of reperfusion. Previous studies indicated that the administration of FA at the beginning of

cerebral ischemia time point possesses anti-oxidative and anti-apoptotic effects in MCAo model<sup>[14, 41]</sup>. Our data further suggest that FA provides a potential therapeutic modality to extend the time window in ischemic stroke-induced apoptosis and is a promising drug worthy of further investigation.

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### Author contribution

Chin-yi CHENG and Shan-yu SU performed research, analyzed data and wrote the paper. Nou-ying TANG, Tin-yun HO and Wan-yu LO contributed new reagents and analytic tools. Ching-liang HSIEH designed research.

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