**Baifuzi reduces transient ischemic brain damage through an interaction with the STREX domain of BKCa channels**

S Chi, W Cai, P Liu, Z Zhang, X Chen, L Gao, J Qi, L Bi, L Chen and Z Qi

Stroke is a long-term disability and one of the leading causes of death. However, no successful therapeutic intervention is available for the majority of stroke patients. In this study, we explored a traditional Chinese medicine Baifuzi (Typhonium giganteum Engl.). We show, at first, that the ethanol extract of Baifuzi exerts neuroprotective effects against brain damage induced by transient global or focal cerebral ischemia in rats and mice. Second, the extract activated large-conductance Ca\(^{2+}\) - activated K\(^{+}\) channel (BKCa) channels, and BKCa channel blockade suppressed the neuroprotection of the extract, suggesting that the BKCa is the molecular target of Baifuzi. Third, Baifuzi cerebroside (Baifuzi-CB), purified from its ethanol extract, activated BKCa channels in a manner similar to that of the extract. Fourth, the stress axis hormone-regulated exon (STREX) domain of the BKCa channel directly interacted with Baifuzi-CB, and its deletion suppressed channel activation by Baifuzi-CB. These results indicate that Baifuzi-CB activated the BKCa channel through its direct interaction with the STREX domain of the channel and suggests that Baifuzi-CB merits exploration as a potential therapeutic agent for treating brain ischemia.

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Stroke is a devastating condition that annually affects 15 million people worldwide, and is the leading cause of adult disability in industrialized countries. Although there has been progress in our understanding of the pathophysiology of stroke during the past few decades, stroke continues to pose major therapeutic challenges, both to neuroscientists and to clinicians. For example, a large number of neuroprotective agents have been shown to successfully reduce infarct size in animal models of stroke, but they have failed to demonstrate efficacy in phase III clinical trials in humans. To date, no successful therapeutic interventions are available for the majority of stroke patients. The intravenous recombinant tissue plasminogen activator (rt-PA) is the only drug therapy available for thrombolytic treatment of stroke in the United States, Canada and Europe. However, the requirement to exclude hemorrhagic stroke and the restriction to use within 3 h of the onset of symptoms limit rt-PA therapy to only ~2% of all stroke patients. Therefore, there is an urgent need for new therapeutic options for the treatment of stroke.

One of the options that might offer fertile ground for modern drug development is traditional medicines, because traditional medicines, such as traditional Chinese medicine (TCM), have long time since documented the effects of many natural products on humans, and extensive human experience can provide some degree of comfort with regard to their safety. Clinical application of artemisinin, triptolide, celastrol, capsaicin and curcumin has demonstrated the power and promise of turning traditional medicines into modern drugs. One of the most recent examples is veregen, an extract of green tea leaves, which was approved in 2006 by the US Food and Drug Administration as a new drug to treat perianal and genital condyloma. As a matter of fact, more than half of the pharmaceutical drugs are either natural products or their derivatives.

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**Abbreviations:** 4VO, 4-vessel occlusion; BKCa channel, large-conductance Ca\(^{2+}\) - activated K\(^{+}\) channel; Baifuzi-CB, Baifuzi cerebroside; [Ca\(^{2+}\)]\(_{i}\), intracellular free Ca\(^{2+}\) concentration; e-Baifuzi, ethanol extract of Baifuzi; CTX, charybdotoxin; IPI, interpulse interval; MCAO, middle cerebral artery occlusion; \(P_o\), single channel open probability; PLO, protein lipid overlay; PPF, paired-pulse facilitation; PPR, paired-pulse ratio; SIGD, synaptically induced glial depolarization; STREX, stress axis hormone-regulated exon; TCM, traditional Chinese medicine; TLC, thin layer chromatography

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Dried tubers of the herb *Typhonium giganteum* Engl. (Chinese name: *Baifuzi*), as a TCM, has been recorded in Chinese pharmacopoeia and used for the treatment of brain stroke for a long time. However, unlike industrially manufactured pharmacological drugs used in Western medicine, its active components and molecular targets have not been specified. Obviously, identification of its effects on well-defined molecular targets and the subsequent identification of its active component(s) underlying these effects are important for the evaluation of its therapeutic efficacy. Brain ischemia begins with energy depletion, followed by the disruption of ion homeostasis, membrane depolarization, excessive neurotransmitter (glutamate) release and elevation of intracellular calcium, all of which initiate a neurotoxic cascade that results in neurodegeneration and death of ischemic neurons. The large-conductance Ca$^{2+}$-activated K$^+$ (*BKCa*) channels are widely expressed in the brain and are preferentially located at glutamatergic synaptic terminals. They react to either increases in intracellular Ca$^{2+}$ or membrane depolarization by increasing K$^+$ efflux. Thus, they function as an ‘emergency break’ to limit Ca$^{2+}$ influx for regulating synaptic transmission under conditions of enhanced neuronal excitability. It has been shown that activation of BKCa channels in ischemic cells by BKCa channel openers could minimize neuronal depolarization, limit accumulation of potentially pathological levels of Ca$^{2+}$ from a number of potential sources, reduce neurotransmitter release and significantly attenuate infarct growth during ischemic stroke in animal models. In addition, blockade of BKCa channels enhanced cell damage in organotypic slice cultures after oxygen and glucose deprivation. In this study, we show that the ethanol extract of *Baifuzi* (e-Baifuzi) shows significant neuronal protective activity in the rat and mice model of ischemic stroke. Second, we show that the BKCa channel is the molecular target of the ethanol extract. Third, we identified that *Baifuzi* cerebroside (Baifuzi-CB) is the active component of *Baifuzi* in activating the BKCa channel. Finally, we show that the stress axis hormone-regulated exon (STREX) domain of the BKCa channel, a 59-amino-acid splice insertion located in the cytoplasmic side of the channel, is required for the activation of the BKCa channel by Baifuzi-CB, probably due to direct interaction between Baifuzi-CB and the STREX domain.

**Results**

**e-Baifuzi protects ischemia-induced brain injury**

e-Baifuzi against ischemia-induced neuronal cell death. Transient global ischemia leads to a dramatic loss of neurons in the CA1 region of the hippocampus. Therefore, we examined whether e-Baifuzi has neuroprotective roles in the CA1 region in the rat model of transient global ischemia, which was made by 20 min middle cerebral artery occlusion (4VO). In the CA1 region of the ischemic hemisphere, the number of pyramidal neurons on the eighth day post-4VO was <50% of the control (2VO rats, n = 8, P < 0.01; Figures 1a and b). Surprisingly, a single injection of e-Baifuzi (5 or 50 mg/kg) at 1 h post-4VO significantly attenuated neuronal loss in a dose-dependent manner (versus vehicle-treated 4VO rats, n = 8, P < 0.01; Figure 1c), although no neuroprotective effect was observed at a dosage of 1 mg/kg (P > 0.05). In contrast, the administration of e-Baifuzi at a dosage of 5 mg/kg did not affect the number of pyramidal neurons in 2VO rats (n = 8, P > 0.05). Furthermore, the administration of e-Baifuzi (5 mg/kg) even at 4–8 h post-4VO also significantly reduced ischemia-induced neuronal loss (versus vehicle-treated 4VO rats, n = 8, P < 0.01; Figure 1c), whereas it could not protect neuronal cells when administered at 24 h post-4VO (n = 8, P > 0.05). These results suggested that e-Baifuzi could protect the brain from transient global cerebral ischemia and that its therapeutic time window may last up to 8 h after the onset of ischemia.

e-Baifuzi improves ischemia-induced deficits in spatial memory. Neuronal damage induced by transient global ischemia is accompanied by severe impairments in performance on hippocampally dependent spatial learning tasks. To investigate whether e-Baifuzi could improve the behavioral disorder induced by transient global cerebral ischemia, we evaluated its effects on the performance of rats in a Morris water maze test. Results showed that rats subjected to 20 min global cerebral ischemia (4VO) significantly extended the escape latency to the hidden platform compared with 2VO rats (n = 8, P < 0.01; Figure 1d), whereas the swimming speed was not detectably different between them (data not shown). In agreement with its neuroprotection, e-Baifuzi (5 mg/kg) reduced ischemia-induced prolongation of the escape latency when administered between 1 and 8 h post-4VO (versus vehicle-treated 4VO rats, n = 8, P < 0.01). In contrast, it did not affect the escape latency of 2VO rats at a dosage of 5 mg/kg (n = 8, P > 0.05). Thus, e-Baifuzi could improve spatial memory impairment induced by global cerebral ischemia.

e-Baifuzi reduces MCAO-produced cerebral infarction. To determine the effect of e-Baifuzi against focal cerebral ischemia-induced injury, we used a mouse model of middle cerebral artery occlusion (MCAO) by the intraluminal filament insertion technique. Infarct size was measured at 24 h of reperfusion as described previously. As shown in Figure 2a and b, a single injection of e-Baifuzi (5 mg/kg) at 1 h post-MCAO remarkably reduced the hemispheric infarct, from 42 ± 2% in vehicle (saline)-injected MCAO mice (n = 10) to 19 ± 5% in e-Baifuzi-injected MCAO mice (n = 11, P < 0.01), indicating that e-Baifuzi could reduce cerebral infarct induced by focal ischemia-induced damage.

**e-Baifuzi protects the brain from ischemic injury through activation of BKCa channels**

e-Baifuzi activates BKCa channels. The above study confirmed the protective role of e-Baifuzi in ischemic injury. As BKCa channels have been shown to have an important role in ischemic stroke, we explored whether the BKCa channel is a potential target for the effect of e-Baifuzi. Whole-cell configuration was used to investigate the effect of e-Baifuzi on the whole-cell currents of BKCa channels expressed in CHO cells. All test experiments (n = 28) showed an increase in current amplitude with the application of e-Baifuzi. Figure 3a clearly shows increases in whole-cell BKCa channel currents from the same cell in
response to e-Baifuzi at a concentration of 0.45 µg/µl. At a voltage of +100 mV, 0.45 µg/µl e-Baifuzi increased the current two and threefold at an intracellular Ca$^{2+}$ concentration of 0.1 and 1 µM, respectively (Figure 3b). At the single channel level, e-Baifuzi increased the single channel open probability ($P_o$) from 2.1 ± 0.9% of control ($n = 11$) to 18.4 ± 6.3% in the presence of 0.45 µg/µl e-Baifuzi ($n = 8$) without affecting its amplitude (Figure 3c and d). These results indicated that e-Baifuzi could activate the BK$_{Ca}$ channel and implied that the BK$_{Ca}$ channel is the molecular target of Baifuzi.

**Figure 1** Effects of e-Baifuzi on global cerebral ischemia. (a) Representative sections in 2VO and 4VO rats treated with vehicle or e-Baifuzi (0, 1, 5 and 50 mg/kg) at 1 h post-4VO. (b) Bar graphs represent the number of surviving pyramidal neurons of hippocampal CA1 in 2VO (open bars) and 4VO rats (hatched bars). Histological changes in ischemic hippocampal CA1 were assessed on the eighth day post-4VO. (c) Time window of e-Baifuzi neuroprotection post-4VO. A single injection (i.p.) of e-Baifuzi (5 mg/kg) is administered at 1, 4, 8 or 24 h post-4VO. *P < 0.05, **P < 0.01. (d) Effects of e-Baifuzi on 4VO-impaired special memory. The Morris water maze test was carried out at 3–7 days post-4VO. Bar graphs represent the results of escape latency to reach the hidden platform on the fifth day of Morris water maze training. It must be noted that a single administration of e-Baifuzi within 1–8 h post-4VO decreases the ischemia-induced death of neuronal cells and prolongation of latency. so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; BF, e-Baifuzi.

e-Baifuzi reduces presynaptic glutamate release. Excessive glutamate release and subsequent excitotoxicity are major pathological factors leading to neurodegeneration and death of ischemic neurons. Previous studies have reported that BK$_{Ca}$ channels located at glutamatergic presynaptic terminals regulate presynaptic Ca$^{2+}$ entry, which in turn regulates presynaptic glutamate release. Therefore, we investigated the effect of e-Baifuzi on presynaptic glutamate release. First, the excitatory postsynaptic potential (EPSP) slopes in the presence of e-Baifuzi (0.45 µg/µl) were consistently less than those in its absence.
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Figure 2 Effects of e-Baifuzi on brain infarct after MCAO. (a) Representative section of vehicle-treated sham, vehicle-treated MCAO and e-Baifuzi (5 mg/kg)-treated MCAO rats. (b) Bar graphs represent the mean infarct volume percentage in sham-op (open bars) and MCAO-mice (hatched bars) treated with vehicle or e-Baifuzi (5 mg/kg) at 1 h post-MCAO. **P < 0.01

Figure 3 e-Baifuzi activates BKCa channels. (a) Representative current trace of whole-cell BKCa channel current before (control) and after 10 min of extracellular application of 0.45 μg/μl e-Baifuzi. The current was activated by rectangular voltage steps from −80 to +100 mV for 200 ms with 10 mV increment. (b) Statistics on ratio values (Io/I) of the whole-cell BKCa current before (I0) and after (I) application of 0.45 μg/μl e-Baifuzi at +100 mV in the presence of 0.1 and 1 μM intracellular Ca2+, respectively. (c) Representative single channel recordings of the BKCa channels in excised inside-out configuration at +50 mV. Channel openings are shown as an upward deflection. Arrows indicate the level that all the channels are at their closed state. (d) Statistical data for open probability (Po) of the single BKCa channels in the presence of 0.45 μg/μl e-Baifuzi (Figure 4a, P < 0.05; n = 10 slices per 5 rats). In addition, the reduction of the EPSP slope completely recovered within 10 min after e-Baifuzi washout (n = 12 slices per 6 rats; Figure 4b), indicating that e-Baifuzi reversibly suppresses synaptic transmission. Second, paired-pulse facilitation (PPF), a sensitive indicator of presynaptic glutamate release, was measured by delivering two successive stimuli with 25–150 interpulse intervals (IPIs). Compared with that before the addition of e-Baifuzi, the paired-pulse ratio (PPR) at 50–75 IPI was markedly increased after addition of e-Baifuzi (0.45 μg/μl) (P < 0.05, n = 15 slices per 7 rats; Figure 4c). Furthermore, we measured the synaptically induced glial depolarization (SIGD), an indicator of the amount of presynaptic glutamate release,22 in the presence of CNQX (10 μM)/AP5 (50 μM) (see the Materials and Methods’ section). Consistent with the result of PPF, the area of SIGD was significantly reduced by perfusion of e-Baifuzi (0.45 μg/μl) (P < 0.05, n = 12 slices per 6 mice), and could be recovered after the washout of e-Baifuzi (Figure 4d). Thus, e-Baifuzi could reversibly reduce presynaptic glutamate release, which in turn might protect the brain from the excitotoxicity of excessive glutamate release.

Blockade of BKCa channels abolishes the neuroprotection and glutamate release induced by e-Baifuzi. If the BKCa channel is the molecular target, then its blocker should suppress the neuroprotective effect of e-Baifuzi. To determine whether the neuroprotection of e-Baifuzi is associated with BKCa channel activation, charybdotoxin (CTX, 400 nM) was administered (i.v.c) for 30 min before the injection of e-Baifuzi (5 mg/kg) at 1 h post-4VO. Blockade of the BKCa channel by CTX pretreatment significantly prevented the neuroprotection of e-Baifuzi (Figure 5a, P < 0.01, n = 8), whereas CTX alone had no significant effect on either 2VO or 4VO rats in comparison with the corresponding vehicle-treated control groups. As CTX is a specific blocker of the BKCa channel, the result strongly suggests that the BKCa channel is the molecular target of e-Baifuzi for its neuroprotective effect. Furthermore, we found that administration of 100 nM CTX could prevent e-Baifuzi from reducing the area of SIGD (P < 0.05, n = 12 slices per 6 rats; Figure 5b). These results strongly suggest that e-Baifuzi protects ischemia-induced brain injury by opening BKCa channels to decrease presynaptic glutamate release, and gives further evidence that the BKCa channel is the molecular target of Baifuzi.

Baifuzi-CB is the major active ingredient

The above studies indicated that e-Baifuzi had a significant neuroprotective effect on the ischemic brain through activation of BKCa channels. This result prompted us to seek the active ingredient(s) of Baifuzi. We isolated Baifuzi-CB from e-Baifuzi,23 which was verified by 1H and 13C NMR spectra (Supplementary Figures 1b and c). They appeared as one point on a normal silica gel thin layer chromatography (TLC) plate (Supplementary Figure 1a). Although phytochemical studies have shown that Baifuzi contains several other chemicals, including amino acids, fatty acids, choline, uracil, daucosterol and dipalmin,23 we began our studies by focusing on Baifuzi-CB, because our previous study showed that sulfatide, a kind of cerebroside, can potentely activate BKCa channels.24 In whole-cell patch-clamp recordings, Baifuzi-CB dose-dependently increased BKCa channel currents. Intracellular application of 10 μM Baifuzi-CB increased the relative BKCa channel current (IIo) from 0.80 ± 0.06 of control to...
3.32 ± 0.88 (Figure 6a). On the other hand, extracellular application of 10 μM Baifuzi-CB increased the relative BKCa channel current from 0.70 ± 0.07 of control to 1.31 ± 0.15 (Figure 6b), suggesting that the action site of Baifuzi-CB is easier to access from the intracellular side. To examine whether the activation of BKCa channels by Baifuzi-CB was due to some diffusible intracellular signal molecules, the effect of Baifuzi-CB on BKCa channels was further examined at a single channel level in excised outside-out patches. As shown in Figure 6c, application of 10 μM Baifuzi-CB to the outside-out patch enhanced $P_o$ from $3.1 \pm 1.7\%$ to $12.0 \pm 3.2\%$. Activation of BKCa channels by Baifuzi-CB in patches excised from the cell membrane strongly suggests that their actions are not mediated by diffusible intracellular signal molecules. On the basis of the amount of Baifuzi-CB purified from e-Baifuzi, it is calculated that e-Baifuzi contains 0.87% of Baifuzi-CB. Thus, 0.45 μg/ml of e-Baifuzi reduces presynaptic glutamate release. (a) Stimulus–response curve, in which the EPSP slope were evoked by stimulating the Schaffer collateral-CA1 path with a current from 0.1 to 1.0 mA in the absence and presence of e-Baifuzi (0.45 μg/ml), was constructed by plotting the EPSP slope against stimulus intensity (0.1–1.0 mA). (b) Reversible inhibition of the EPSP slope by a transient application of e-Baifuzi (0.45 μg/ml). Horizontal hatched bar indicates the duration of e-Baifuzi application. (c) Effect of e-Baifuzi on presynaptic glutamate release. Paired-pulse facilitation (PPF) is plotted against various IPIs. (d) Sample traces of SIGD were obtained just before, during and after the application of e-Baifuzi (0.45 μg/ml). Hippocampal slices were stained with RH155, a voltage-sensitive dye. SIGD: synaptically induced glial depolarization. Horizontal hatched bar indicates the duration of e-Baifuzi application. **$P<0.01$
Baifuzi corresponds to 5.1 μM of Baifuzi-CB (MW ~ 720 Dalton). Comparing Figure 3a and 6b, the channel activated by 10 μM Baifuzi-CB is at the same level as that of 0.45 μg/ml e-Baifuzi, indicating that Baifuzi-CB is the major active component responsible for the activation of the BKCa channel.

Molecular mechanism of BKCa channel activation by Baifuzi-CB

STREX is required for BKCa channel activation by Baifuzi-CB. It has been shown in our previous studies that STREX is important for activation of BKCa channels by lipid-soluble chemicals.24,25 Besides, it has been demonstrated that inclusion of the STREX exon facilitates voltage- and Ca2+-dependent activation of BKCa channels.26 To test whether STREX contributes to the activation of the BKCa channel by Baifuzi-CB, we studied its effect on the STREX-deleted mutant of the channel. Both intracellularly (1 μM, Figure 7) and extracellularly (10 μM, Figure 6b) applied Baifuzi-CB significantly increased whole-cell current for wild-type BKCa channels. The relative whole-cell current was increased from 0.80 ± 0.06 of control to 1.57 ± 0.28 in the presence of 1 μM Baifuzi-CB applied intracellularly (Figure 7b), whereas extracellularly applied Baifuzi-CB (10 μM) increased the current from 0.70 ± 0.06 of control to 1.31 ± 0.15 (Figure 6b). In contrast, the STREX-deleted mutant BKCa channel could not be activated by Baifuzi-CB, whether it was applied intracellularly (Figure 7b) or extracellularly (Supplementary Figure 2), indicating that STREX is required for BKCa channel activation by Baifuzi-CB.

Interaction between Baifuzi-CB and STREX. We investigated the manner in which Baifuzi-CB is sensed by STREX, which is located at the cytoplasmic side, to activate the BKCa channel. A simple explanation may be that STREX interacts directly with Baifuzi-CB and consequently conveys the signal to gate the channel. To test whether the STREX domain is sufficient for Baifuzi-CB binding, we performed a protein lipid overlay (PLO) assay. No interaction was detected between STREX and POPE (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol (POPG) or DL-a-Phosphatidylcholine Dipalmitoyl (DPPC). In contrast, the STREX domain of the BKCa channel could interact with both
**Discussion**

In this study, we show that e-Baifu on ischemic brain damage through BK channel

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**Figure 8** Direct interaction between Baifu on ischemic brain damage through BK channel and the STREX domain of the BKCa channel. (a) The ability of STREX to bind various lipid-soluble materials. The concentration of the lipid-soluble material was increased from right to left and spotted onto nitrocellulose membranes. (b) Effect of POPE, POPG and DPPC on BKCa channels at +20 mV. (c) Intracellularly applied Baifu on ischemic brain damage through BK channel (1 μM) on the single wild-type BKCa channels (left) and on the channels with an overexpression of STREX fragments (BKCa/STREX = 1:2, q/q) (right) at +10 mV. (d) Statistical summary for the experiment in panels b and c.
Many BK<sub>Ca</sub> channel modulators have been reported to prevent neuronal degeneration after ischemia in preclinical studies. However, there is no approval of a single BK<sub>Ca</sub> channel modulator for clinical use so far. For example, BMS-204352, one of the potent BK<sub>Ca</sub> channel activators described to prevent neuronal degeneration after ischemia, has failed to improve the clinical outcome in patients affected by stroke.  

In this study, we demonstrated that *Baifuzi* could effectively reduce brain damage in both focal and global ischemia. We are aware that there is no guarantee that *Baifuzi* will be effective in the clinical treatment of stroke. However, the following reasons may suggest its potential of being regarded as a therapeutic agent: (1) *Baifuzi* has been effectively used for the treatment of brain stroke for a long time in China. (2) Cerebrosides are rich in the brain, implying that *Baifuzi-CB* may have a target-specific effect on neurons. (3) As an analog of membrane cerebroside, *Baifuzi-CB* may be transported by the endogenous transport machinery to target cells to fulfill its action.

Lipid–protein interactions are of fundamental importance, as they partly determine the structures of integral membrane proteins and their activities. Lipid regulation of channels has been demonstrated for NMDA receptor channels, transient receptor potential channels, inward rectifier K<sup>+</sup> channels, voltage-dependent K<sup>+</sup> channels, BK<sub>Ca</sub> channels and Twik-related K<sup>+</sup> channels. The KcsA potassium channel only opens if the membrane contains a small amount of an anionic lipid, and lipid association has actually been resolved in the crystal structure of KcsA K<sup>+</sup> channels. Therefore, it is not surprising that *Baifuzi-CB* can activate BK<sub>Ca</sub> channels. In agreement with this result, we have previously found that sulfatide, a kind of cerebroside, can potently activate BK<sub>Ca</sub> channels. In this study, we found that the BK<sub>Ca</sub> channel can be activated by *Baifuzi-CB* even at the excised membrane, implying that channel activation was not due to some intracellular diffusible signals. Besides, *Baifuzi-CB* could not increase at the intracellular Ca<sup>2+</sup> level in hippocampal neurons (data not shown), suggesting that BK<sub>Ca</sub> channel activation is not due to an increase in intracellular Ca<sup>2+</sup> concentration. Then, how does *Baifuzi-CB* activate the channel? There are at least three possibilities, which are not exclusive with each other: (1) The STREX domain of the BK<sub>Ca</sub> channel is attracted toward the plasma membrane because of the direct interaction with *Baifuzi-CB*, leading to a stabilization of the open state. This is similar to what has been proposed for the PI(4,5)P<sub>2</sub>-dependent gating of inward-rectifier K<sup>+</sup> channels. (2) *Baifuzi-CB* alters the thickness of the membrane, and thus makes the BK<sub>Ca</sub> channel easy to open. This mechanism has been shown for BK<sub>Ca</sub> channel activation by phosphatidylcholines (PCs) of varying chain lengths. (3) *Baifuzi-CB* may create stress in the membrane because of its large head group, as we reported previously for the BK<sub>Ca</sub> channel activated by membrane stress created with amphiphilic lipids. Obviously, further studies are required to judge which one or their combinations is correct.

In both global and focal ischemia, restriction of blood flow to the brain limits the delivery of oxygen and glucose to neurons, causing energy depletion. This is followed by a cascade of neurochemical events, including disruption of ion homeostasis, excessive release of glutamate, overload of intracellular Ca<sup>2+</sup>, release of free radicals, inflammatory changes and finally cell death of neurons, glia and endothelial cells. These injury cascades are interconnected in complex ways, as one event in the cascade can cause or can be caused by multiple other events, and cells suffering from different severity levels of ischemia may undergo different chemical processes. On the other hand, AKT has been shown to have a prominent part in signaling networks that result in the modulation of cellular proliferation, apoptosis and survival. Recently, activation of AKT has been demonstrated to be important in neuronal survival after cerebral ischemia. The neuroprotective effects of nerve growth factor, osteopontin, erythropoietin, as well as preconditioning and post-conditioning to reduce ischemia-induced brain injury, are mediated by AKT. In our study, we demonstrated that *Baifuzi-CB* could activate the BK<sub>Ca</sub> channel and reduce the release of glutamate, which is responsible for the neuroprotective effect of *Baifuzi*. Therefore, it would be very interesting to study whether the AKT pathway is involved in the effect of *Baifuzi* on brain ischemia.

In summary, we demonstrated that e-*Baifuzi* provided significant neuroprotection, not only in protecting neurons against cell death but also in promoting behavioral recovery in the animal model of stroke. We identified the BK<sub>Ca</sub> channel as a molecular target for the therapeutic action of *Baifuzi*, and that *Baifuzi-CB* is the active component that effectively activates BK<sub>Ca</sub> channels. Through BK<sub>Ca</sub> channel activation, e-*Baifuzi* limited glutamate release, which is involved in excitotoxicity during ischemic damage. These data provide evidence that e-*Baifuzi* attenuates ischemia-induced neuronal damage by enhancing the activation of BK<sub>Ca</sub> channels. Its neuronal protective effect provides pharmacological rationale for the traditional use of *Baifuzi* in stroke. As the natural source of *Baifuzi*, the medicinal tubers of *T. giganteum* Engl., has a long history in Chinese traditional medicine, it is hoped that *Baifuzi-CB* will be a well tolerated, promising therapeutic candidate for the treatment of ischemic brain stroke and other forms of neuronal damage.

**Materials and Methods**

**Extraction and isolation.** Tubers of *Baifuzi* were collected from Yuxian County (Henan Province, China). The dried material was powdered and then extracted with 95% ethanol for several days with shaking. The extract was concentrated to yield e-*Baifuzi*. e-*Baifuzi* was chromatographed on silica gel, then on ODS to afford e-*Baifuzi-CB*, which was confirmed to be a mixture of cerebrosides on the basis of H<sup>1</sup> and C<sup>13</sup> NMR spectra (Supplementary Figure 1b and c). TLC experiment indicated that e-*Baifuzi-CB* is one of the major spots of e-*Baifuzi* (Supplementary Figure 1a). The detailed process was described in our previous paper.

**Experimental animals.** Male Sprague-Dawley rats (weighing 200–250 g, Oriental Bio Service Inc., Nanjing, China) and male mice (weighing 25–30 g, Oriental Bio Service Inc.) were used throughout the study. Animals were housed in a light-controlled room under a 12 h light–dark cycle starting at 0700 hours and kept at 25°C. They were given unrestricted access to food and water. All procedures were in accordance with the guidelines of the Institute for Laboratory Animal Research of the Nanjing Medical University.

**Preparation of the global brain ischemia model.** We used a 4VO method for transient global ischemia as described elsewhere. Briefly, using sodium pentobarbital anesthesia (60 mg/kg, intraperitoneally (i.p.)), both common
cardiovascular (CCAs) of rats were dissected free, and the incision was closed.
Immediately after this procedure, both vertebral arteries (VAs) between the first and second cervical vertebrae were exposed and electro-cauterized completely using a bipolar cautery under an operating microscope (SZH-ILLB, Olympus, Tokyo, Japan). After 24 h, the common arteries were occluded with aneurysm clips for 20 min. The clips were then removed, and blood flow through the arteries was confirmed before the wound was sutured. Rectal temperature was continually monitored and maintained at ~ 37°C using a heating pad until the animal had fully recovered from anesthesia. Sham operation (control) groups had a permanent bilateral occlusion of VAs (2VO rats) that was carried out in the same manner as in 4VO rats, except that the CCAs were not occluded. All rats were allowed to survive for 8 days after the onset of cerebral ischemia.

**Preparation of the focal cerebral ischemia model.** Focal cerebral ischemia was induced by MCAO for 60 min using the intraluminal filament insertion technique, as described previously.56 All mice were anesthetized with 2% halothane, and were then maintained with 1% halothane in 70% N2O and 30% O2 using a vaporizer. Briefly, a polyl-lyxine (0.1% per weight volume)-coated nylon monofilament thread (3/0 G with the tip heat blunted to a diameter of 0.104 mm) was inserted through the external carotid artery and advanced into the internal carotid artery to occlude the origin of the MCA (~ 12 mm). Adequate vascular occlusion and reperfusion was monitored in the front parietal cortex of the occluded side by Perimed PF5050 (Perimed, Jarfalla, Sweden) multichannel laser Doppler flowmetry. Body and head temperatures were maintained at 37 ± 0.5°C using a homeothermic blanket and water pads. Arterial blood pressure and gases were monitored through a femoral catheter. After 60 min of occlusion, the filament was withdrawn to allow for reperfusion, and animals recovered from anesthesia and survived for 24 h. Sham-operated animals were treated identically, except that MCAs were not occluded after the neck incision.

**Drug administration.** Animals subjected to cerebral ischemia treatment or sham operation were blinded to drug-treated groups. e-Baifuizi was diluted in 0.9% saline to a final concentration of 1.0% ethanol that was used as vehicle control. CTX, sham operation were blinded to drug-treated groups. e-

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**Behavioral analysis.** Animals were trained in the standard Morris water maze task.77 For the Morris water maze task, a pool (90 cm diameter, 45 cm height) made of black-colored plastic was prepared, and was filled with water (20 ± 1°C) that was rendered cloudy with nonionic detergent. Swimming paths were analyzed by using a computer system using a video camera (AXIS-90 Target2; Neuroscience, Inc., Tokyo, Japan). In the hidden-platform test, the platform (7 cm in diameter) was submerged 1 cm below the water surface. The first day of training began with a free swim, in which the swim speed was assessed in the absence of the platform, and then immediately after the free swim, mice were given four training trials per day over 5 days, in which they were given 90 s to swim to the hidden platform. If a mouse did not find the platform in the allotted time, it was placed on the platform for 15 s. The average swim speed (cm/s) and latency (s) to reach the platform were scored on all trials and analyzed. Data were obtained from groups of 10 mice throughout the experiments.

**Electrophysiological analysis** 

**Slice preparation.** Rats were decapitated under deep anesthesia with ethyl ether. Brains were rapidly removed and coronal brain slices (400 μm) were cut using a vibrating microtome (Micoslicer DTK 1500, Dousaka EM Co., Kyoto, Japan) in ice-cold cutting solution composed of (in mm) 94 sucrose, 30 NaCl, 4.5 KCl, 1.0 MgCl2, 26 NaHCO3, 1.2 NaH2PO4, and 10 d-glucose with pH 7.4. The hippocampal slices were continuously incubated in artificial cerebrospinal fluid (ACSF) at 36 ± 1°C for more than 60 min to allow the slices to recover. ACSF was composed of (in mm) 124 NaCl, 2 CaCl2, 4.5 KCl, 1 MgCl2, 26 NaHCO3, 1.2 NaH2PO4, and 10 d-glucose. pH was adjusted to 7.4. Both the cutting solution and ACSF were oxygenated with a gas mixture of 95% O2 and 5% CO2.

**Field potential recording.** For recordings, the slice was transferred into a recording chamber and perfused continuously with oxygenated ACSF at a flow rate of ~1 ml/min. The experiments were conducted at 30 ± 1°C. Briefly, orthodromic stimuli were delivered using an electrically polished bipolar tungsten electrode that was placed in the outer third of the molecular stratum of hippocampal dentate gyrus to stimulate the lateral perforant path. Constant current pulses (0.05 Hz) were supplied by a stimulator (SEN-3301, Nihon Kohden, Tokyo, Japan). EPSP was recorded from the middle third of the molecular stratum with a 4–5 MΩ resistance glass microelectrode that was filled with 0.9% NaCl and connected to a neutralized, high input-impedance preamplifier with a high-pass filter at 5 kHz. The level of test stimulus was set to evoke ~50% of a minimum stimulus intensity that evoked a saturating EPSP in each slice. Afferent stimulation was delivered with bipolar glass micropipettes. Signals were amplified using a differential AC amplifier (A-M Systems, model 1700, Seattle, WA, USA). EPSPs were digitized and saved using the pCLAMP system (Axon Instrument Inc., Foster City, CA, USA). Baseline stability of baseline recordings was established by deliverying single pulses (1 per min, 0.1 ms pulse width at an intensity yielding half-maximal EPSP amplitude for a given slice) for 15–30 min before collection of input/output functions. Baseline synaptic transmission was assessed by averaging the response to five pulses (from 0.1 to 1.0 mA) delivered at a rate of 0.05 Hz. PPF was examined at a range of latencies (25–150 ms) and a stimulus intensity of half-maximal for elicitation of EPSP.

**Optical recordings.** All slices used in optical recording were stained with a voltage-sensitive dye (RH155 0.2 mg/ml, Nippon Kanko Shikiso Kenkyujo, Okayama, Japan) for 15 min, and were kept in a chamber containing oxygenated ACSF for at least 30 min for recovery as described previously.55 The slices were then transferred into a recording chamber and placed on the stage of an Olympus inverted microscope (IMT-2, Nikon, Tokyo, Japan). Test stimulus was set at 50% maximal stimulus intensity to evoke optical EPSP (op-EPSP) in each slice. Light from a tungsten-halogen lamp (type JC-24/200 W, Kondo Philips, Tokyo, Japan) was collimated, and an interference filter with a transmission maximum at 700 ± 10 nm (Olympus Optical, Tokyo, Japan) was used. Changes in light absorption associated with membrane potential changes were measured using an optical recording system equipped with a high-speed diode camera with metal oxide (MOS) image sensor (128 × 128 pixels, each of which receives light from a 25 μm × 25 μm sample area) and a data processing unit (HR Deltaron-1700 Fujif; Fuji Photo Film, Tokyo, Japan). To minimize bleaching of the dye molecules and
photodynamic damages to slices, a heat-absorption filter was placed on the slices, and the light path to the slice was opened only long enough to measure optical responses (1–2 s). In each trial, a background image recorded for 16 ms before electrical stimulation was stored as a reference image. To analyze the change in neuronal activities over time at a given region, data from each pixel were stored and retrieved, and the amplitude of the optical signal (indicated as a percentage change in optical absorbance) was plotted as a function of time. Signals obtained from six pixels in str. molecular were averaged. RH155 dye is reported to stain glial cells preferentially over neuronal cells. Optical recording with the RH155 dye is used to monitor glial depolarization by the electrogenic glutamate transporter (GLT-1) uptaking the released glutamate. When AMPA and NMDA receptors are blocked, a delayed depolarizing response can be recorded, which is completely abolished by the specific GLT-1 blocker. Thus, the size of SIGD is considered to reflect the probability of presynaptic glialute release.

Patch-clamp recordings. Whole-cell currents were measured using a conventional tight seal whole-cell recording technique at room temperature. Pipettes had resistances at a range of 2–5 MΩ in recording solution containing (in mM) 154 KCl, 10 EGTA, 10 HEPES (pH 7.3 with KOH) and CaCl₂ adjusted to the desired [Ca²⁺]₀. The intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ) was 0.1 μM if not stated otherwise. The extracellular solution was Hanks’ balanced salt solution (Sigma, St Louis, MO, USA): 1.3 CaCl₂, 0.8 MgSO₄, 5.4 KCl, 0.4 KH₂PO₄, 136.9 NaCl, 0.3 Na₂HPO₄, 10 glucose and 2.4 NaHCO₃. Compensation for cell capacitance and series resistance was made automatically using EPC-9 or EPC-10 amplifiers; only recordings with stable series resistances <25 MΩ were included in the study. Currents were amplified using EPC-9 or EPC-10 patch-clamp amplifiers (HEKA, Lambrecht, Germany), sampled at 2–5 kHz and filtered at 1.5–2.9 kHz using a 4-pole low-pass Bessel filter. Program packages Patchmaster 2.1 and TAC 4.1 (HEKA) were used for data acquisition and analysis. The probability of a single channel being open (Pₒ) was simply calculated from the total time spent in the open state divided by the total time of recording for the patches containing a single channel. Continuous recordings of 2000–4000 ms were used to estimate Pₒ. When multiple channels were present in a patch, Pₒ was calculated from the amplitude height (Hₒ): Pₒ = (1 – e⁻ⁿ), where Pₒ is the fraction of area under the closed state, and N is the number of channels. To avoid underestimating the number of channels in a patch as less as possible, the number of active single channels in the patch was counted at the condition in which the maximum number of channels was observed. Both e-Baifu zi and Baifu zi-CB activate chick BKCa expressed in CHO cells, as well as rat BKCa expressed in HEK293 cells, at both the single channel and whole-cell level. However, for convenience, we present data at the single channel level for chick BKCa and at the whole-cell level for rat BKCa channels. Both e-Baifu zi and Baifu zi-CB have low aqueous solubility and were dissolved in 100% dimethyl sulfoxide (DMSO) as a stock solution and diluted in intracellular or extracellular solutions to obtain different concentrations as required. The final concentration of DMSO was <0.5% per assay, which had no effect on the BKCa channel.

Plasmid construction and STREX–SBP fusion protein purification. The STREX gene of the chick BKCa channel was amplified from pcDNA3.1-BKCa and subcloned into POCI3(+) (Novagen, Madison, WI, USA). The STREX–SBP (streptavidin-binding peptide) fusion protein was constructed by insertion of a peptide linker (Ser-Gly)₅ between the STREX and SBP tag. After culturing in LB medium containing 0.1 mg/ml ampicillin at 37°C and 200 r.p.m./min, cells were induced to express the STREX–SBP fusion protein by adding 0.25 mM IPTG until OD₆₀₀ = 0.4. Cells were harvested by centrifugation at 8000 r.p.m./min for 10 min, ultrasonicated and centrifuged at 12 000 r.p.m./min for 30 min at 4°C to remove the cell fragments. The supernatant was applied to a Ni²⁺-nitrilotriacetic acid (Ni²⁺-NTA) column (Amersham Life Sciences, Buckinghamshire, UK). Gradient imidazole was then used to elute the protein. Its purity was analyzed by SDS-PAGE. The purified protein was stored in buffer containing 50 mM Tris-HCl, pH 8.2, 200 mM NaCl, 1 mM 1,4-dithiothreitol (DTT) and 20% glycerol at –70°C until use.

PLO assay. Five types of lipid-soluble chemicals, including e-Baifu zi, Baifu zi-CB, POPE (Avanti, Alabama, USA), POPG (sodium salt, Avanti) and DPPC (Sigma) were diluted and spotted on a Hybond-C extra nitrocellulose membrane (Amersham Life Sciences) with concentration increased from right to left and air-dried for 1 h at room temperature as described previously. Lipid spots were blocked in buffer 1 (fatty acid-free BSA buffer 40 mg/ml, 25 mM Tris-HCl pH 7.9, 150 mM NaCl) with gentle shaking for 2 h at room temperature and incubated overnight with 9.2 μM STREX–SBP fusion protein in buffer 2 (fatty acid-free BSA 10 mg/ml, 25 mM Tris-HCl pH 7.9, 150 mM NaCl) at 4°C. Taken as a positive control, the purified STREX–SBP fusion protein was also incubated with lipid spots. The membrane was washed in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM NaH₂PO₄, pH 7.4) with 0.05% Tween-20, and then incubated in a 1 : 3000 dilution of streptavidin-alkaline phosphatase (Promega, Co., Madison, WI, USA) for 1 h. The enzyme was bound to the membrane through interaction between streptavidin and the SBP tag. The protein bound to the lipids was detected with a mixture of 4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT, Ameresco, Solon, OH, USA) as a color developer substrate.

Data analysis/statistics. Data were retrieved and processed with the software Micro cal Origin 6.1 (OriginLab, Northampton, MA, USA). Group data were expressed as means ± S.E. Experimental results were compared among groups by ANOVA, followed by Bonferroni’s post hoc test. Statistical analysis was performed using Stata7 software (STATA Corporation, TX, USA). Differences at P < 0.05 were considered to be statistically significant.

Conflict of interest

Professors M Francesca Cordeiro, Frederick W Fitzke and Stephen E Moss are named inventors on a patent application covering the technology disclosed in this report.

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