Vespakinin-M, a natural peptide from Vespa magnifica, promotes functional recovery in stroke mice

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Acute ischemic stroke triggers complex systemic pathological responses for which the exploration of drug resources remains a challenge. Wasp venom extracted from Vespa magnifica (Smith, 1852) is most commonly used to treat rheumatoid arthritis as well as neurological disorders. Vespakinin-M (VK), a natural peptide from wasp venom, has remained largely unexplored for stroke. Herein, we first confirmed the structure, stability, toxicity and distribution of VK as well as its penetration into the blood-brain barrier. VK (150 and 300 µg/kg, i.p.) was administered to improve stroke constructed by middle cerebral artery occlusion in mice. Our results indicate that VK promote functional recovery in mice after ischemia stroke, including an improvement of neurological impairment, reduction of infarct volume, maintenance of blood-brain barrier integrity, and an obstruction of the inflammatory response and oxidative stress. In addition, VK treatment led to reduced neuroinflammation and apoptosis associated with the activation of PI3K-AKT and inhibition of IkBα-NF-κB signaling pathways. Simultaneously, we confirmed that VK can combine with bradykinin receptor 2 (B2R) as detected by molecular docking, the B2R antagonist HOE140 could counteract the neuro-protective effects of VK on stroke in mice. Overall, targeting the VK-B2R interaction can be considered as a practical strategy for stroke therapy.
Acute ischemic stroke (AIS) remains a leading cause of disability and mortality, with an increasing incidence and causing serious harm to human health. Within 8–10 min of ischemic attack, neurons in the ischemic core area immediately undergo irreversible necrosis and a salvageable penumbra, dominated by neuronal apoptosis, is formed around the ischemic core area. Currently, the main therapeutic strategies for AIS focus on restoring cerebral blood flow (CBF) and saving the ischemic penumbra by the administration of thrombolytic drugs such as intravenous recombinant tissue-type plasminogen activator (Supplementary Table 2). However, when intravenous thrombolysis has a time window up to 4.5 h, <3% of stroke patients are able to benefit from these interventions and two-thirds of patients have different degrees of disability. Subsequently, reperfusion by thrombolytic therapy after AIS can also accelerate cerebral injury, resulting in brain edema, brain hemorrhage, and neuronal death. This phenomenon is termed cerebral ischemia/reperfusion (I/R) injury and is implicated in various types of cellular stress, including energy failure, oxidative stress, elevation of the intracellular calcium levels, release of excitatory neurotransmitters, neuroinflammatory response, and apoptosis. The cerebral tissue around the ischemic core area exacerbates progressively within a few days after stroke. Therefore, a working neuroprotective therapy in stroke is still being explored.

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Proteolytic degradation of peptide-based drugs is generally considered as the main defect to limit systemic therapeutic applications. Therefore, high-performance liquid chromatography (HPLC) was used to assess the stability of VK in PBS or plasma at physiological pH (7.4) and temperature (37°C). The results indicated that VK in PBS or plasma was gradually degraded, with the degradation rate of VK in PBS being 62.4% and in plasma was completely degraded at 120 min (Fig. 1b–d). For neuropharmacology, crossing the BBB is a remarkable challenge, and VK was labeled with FITC in the N-terminus. FITC-labeled VK was then administered to stroke mice. After 30 min, these mice were imaged by an in vivo imaging system. Because the BBB is partially compromised by stroke insults, VK was found to slightly accumulate in the ischemic penumbra (Fig. 1e). Brains were collected at 0, 30, 60, 90, and 120 min and the distribution of VK was observed. FITC-labeled VK was seen in the cortex, hippocampus, and striatum (Fig. 1e), while these areas of the brain suffered from massive neuronal cell death after focal ischemia. To further evaluate whether VK was a cell-penetrating peptide, the oxygen-glucose deprivation/reoxygenation (OGD/R) model simulated I/R in vivo was established in HT22 hippocampal neuron cells and FITC-labeled VK was incubated for 120 min. Live-cell imaging suggested that FITC-labeled VK partially crossed the cell membrane for OGD/R-treated (Fig. 1f, Supplementary Fig. 1e, and Supplementary Movie 1), Supplementary Movie 2 as a negative group with FITC. These results indicate that VK can cross the BBB and cell membrane under conditions such as cerebral I/R.
VK alleviates neurological impairment and cerebral infarction during the period of acute cerebral ischemia in mice. According to the Stroke Therapy Academic Industry Roundtable (STAIR) recommendations, we investigated a dose-response experiment of VK treatment on stroke outcomes by middle cerebral artery occlusion/reperfusion (MCAO/R) in mice. In the MCAO/R model, drug treatments and behavior tests were performed according to the methods shown in Fig. 2a. In order to ensure MCAO-induced cerebral ischemia had occurred, laser speckle contrast imaging (LSCI) was used to monitor changes in CBF. CBF was shown to drop by 20–30% of the initial blood flow. During the operation of MCAO, CBF, blood gas, body temperature, and blood pressure were within the normal range, with no difference between the groups. To study the effect of VK on infarct volume, the infarct areas of mice sacrificed at 48 h after MCAO/R were assessed. There was no infarction in the sham group, while the MCAO/R group showed infarction in the striatum, hippocampus, cortex, and caudate nucleus (Fig. 2b, d). VK decreased infarct volume as described in TTC staining (Fig. 2c). Magnetic resonance imaging (MRI) also revealed that VK observably reduced the area of cerebral infarction in MCAO/R mice (Fig. 2d).

The neurological function of mice following MCAO/R was assessed by a series of behavioral tests to observe the effects of VK on sensorimotor and cognitive recovery. The vehicle-treated mice exacerbated deficits in mobility of the left limbs (as shown in the Longa test) as compared to the sham group (Fig. 2e). However, the administration of VK improved neurological deficits compared to vehicle-treated mice (Fig. 2e). In sensorimotor asymmetry assessments, vehicle-treated stroke mice showed a poor grip strength and impaired motor coordination as compared to the sham group (Fig. 2f, g). However, VK treatment improved functional performance and ameliorated the sensorimotor asymmetry of mice as assessed by the rotarod test and grip test (Fig. 2f, g). Furthermore, to confirm the effects of VK on cognitive function, the classic Morris water maze test was constructed (Fig. 2h–k). Compared with the sham group, an observably increased escape latency to find the platform was found in stroke mice (Fig. 2h); however, VK treatment accelerated the spatial learning ability as observed by a decreased escape latency on day 11–14 (Fig. 2h, j). In the probe trial, VK-treated mice exhibited more crossovers and spent more time in the platform quadrant (spatial memory ability) as compared to the vehicle group (Fig. 2i, k). Collectively, these findings indicate that VK therapy reduces infarct volume and alleviates neurological impairment after MCAO/R in mice.

VK blocks oxidative stress and restores energy metabolism in the brain after MCAO/R in mice. Recent data support the view that oxidative stress, including lipid peroxidation and free-radical damage, is a mediator of cerebral I/R injury. In addition, BK (but not VK) shows antioxidant action in cardiovascular disease.

To evaluate the effects of VK on anti-oxidative damage in stroke mice, we measured markers of lipid peroxidation levels and free-radical damage levels in the brain cortex after a stroke at 48 h (Fig. 3a). Compared with the sham group, the activity of superoxide dismutase (SOD) was reduced in vehicle-treated stroke mice whereas lipid peroxide (LPO) and malondialdehyde (MDA) levels were increased; VK enhanced SOD activity and decreased content of MDA and LPO (Fig. 3b–d). Interestingly, for glutathione, no obvious difference was found between groups (Fig. 3e). To further explain whether VK inhibited oxidative stress...
VK maintains BBB integrity after MCAO/R in mice. BBB destruction during cerebral I/R is an important pathological change that exacerbates cerebral edema and cerebral infarction. With regards to its structure, a destructive BBB was characterized by a dissolved or fractured basement membrane, exfoliated tight joints (TJs) and endothelialcytes (ECs), vacuolar or swelled mitochondria, and extremely swollen astrocyte end feet. Functionally, increased BBB permeability can cause brain edema, which aggravates stroke. Therefore, we assessed BBB permeability by the Evans blue (EB) method after stroke (Fig. 4a). The stained area of the right hemisphere obviously increased in the vehicle group (Fig. 4b). The vehicle-treated group had a remarkably increased area of EB extravasation, while VK reversed the effect of MCAO-induced on BBB permeability (Fig. 4c). BBB function depends on the integrity of its components, mainly the microvascular endothelial cells and astrocytes involved in its formation (Fig. 4d). We confirmed that VK could maintain BBB integrity (Fig. 4e). These results collectively demonstrated that VK protects against cerebral ischemia injury by maintaining the BBB permeability after stroke in mice.

VK reduces the secretion of proinflammatory cytokines from activated microglia. Cerebral ischemia mediates a rapid microglial response and an abnormal activation of microglia is considered a mark of neuroinflammatory responses. OGD/R treatment reduced the viability of BV2 microglia cells, which was reversed by VK treatment in a dose-dependent manner (Supplementary Fig. 4a). Additionally, OGD/R increased the expression of proinflammatory cytokines (Supplementary Table 4) in neurons, we used a reactive oxygen (ROS) kit (DCFH-DA). ROS levels were increased in neurons exposed to the OGD/R model as compared with untreated cells or controls (Fig. 3g, h). Moreover, 24-h pretreatment with VK could rescue the OGD-induced ROS levels, which suggests that VK directly restrained oxidative stress in HT22 cells.

Compounds capable of maintaining energy dynamics in the ischemic penumbra may be attractive therapeutics for stroke therapy. Therefore, kits tested adenosine triphosphate synthase (ATPase) and lactic acid (LD). An increased ATP activity was observed in the VK-treated groups compared with the vehicle-treated group (Fig. 3f). These findings suggest that VK could increase energy production in neural cells by promoting mitochondrial ATP production. Given the link between neuronal metabolism and cell function, OGD/R model was established to reveal the role of VK on neuron bioenergetics. The dose-dependent stimulation of VK decreased glycolysis (extracellular acidification rate) and accelerated the mitochondrial oxygen consumption rate in neurons (Fig. 3i–l). These results indicate that VK blocks oxidative stress and restores energy metabolism in the brain after MCAO/R in mice.

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Fig. 3 Effects of VK on oxidative stress and energy metabolism in stroke mice. 

**a** Experimental design: in addition to the sham group, mice (8–10 weeks, male) were subjected to MCAO for 60 min. Mice were randomized into four groups: sham group, vehicle group, and VK (150 and 300 mg/kg) groups. Mice were administrated VK at 0, 4, 22.5, and 46.5 h after MCAO/R as assessed by Kits. After reperfusion for 48 h, ischemic tissue was collected. 

**b–d** The superoxide dismutase (SOD) activity and malondialdehyde (MDA), lipid peroxide (LPO), and glutathione (GSH-PX) levels in the brain were determined by the use of biochemical kits. Adenosine triphosphate (ATP) synthase (β) and lactic acid (LD) (f) were detected to examine the effect of VK treatment on energy metabolism after reperfusion injury. **g** HT22 cells stained with DCFH-DA for flow cytometry, using without DCFH-DA as a negative control for the FAC5 gating strategy. **h** Quantification of DCFH-DA-positive cells as the mean ± SEM from three independent experiments. DCFH-DA (10 μM). **i,j** Real-time changes in the O2 consumption rate of neurons in response to treatment with the indicated concentrations of VK for 24 h. Cells were treated with 2 μM of oligomycin, 5 μM of carbonyl cyanide-precipitatedfluorophosphorohydroxylidrazine (FCCP), and 1 μM of rotenone and antimycin, as indicated by the three red arrows. **k,l** To assess the extracellular acidification rate, cells were treated with 1 μM of rotenone and antimycin and 50 mM of 2-deoxy-o-glucose (2-DG) as indicated by the two red arrows. Statistical analyses were performed using the Kruskal-Wallis test with the Dunn post hoc test or two-way repeated-measures ANOVA followed by Bonferroni’s post hoc test. Data (animal experiment) represent the mean ± SD, n = 5–7.

BV2 cells and VK remarkably blocked these effects (Supplementary Fig. 4b). The surgical operation, administration time, and detection methods were displayed in detail (Fig. 5a). Consistent with the above results, in vivo, the MCAO-induced model increased the release of the proinflammatory cytokines in the peripheral (Fig. 5b–e) and cortex of the ischemic hemisphere (Fig. 5f–i), including IL-1β, TNF-α, IL-6, and IL-8. However, in comparison with the vehicle-treated group, VK treatment decreased IL-1β, TNF-α, IL-6, and IL-8 levels in the peripheral and cortex of the ischemic hemisphere (Fig. 5b–i). Additionally, an increase in IL-10 levels was observed in VK-treated and sham mice compared to vehicle-treated mice (Fig. 5j, k). Immunohis-tochemical staining (IHC) was performed to observe the morphology of microglia labeled by an anti-IBA1 antibody. Cerebral I/R insult conferred a marked elevation of IBA1 expression in the cerebral cortex of mice, which was greatly blunted by the administration of VK (Fig. 5l and Supplementary Fig. 4c). From a morphological perspective, the microglia in the sham group were in a resting state characterized by small cell size and elongated branches; in the vehicle group, microglia became larger with shorter and thicker axons, and thus were in the activated state. The morphology of microglia in the VK group was between that of the previous groups. To further assess the effects of VK on activated microglia in stroke, TNF-α co-localization with IBA-1 was performed by immunofluorescence (Supplementary Fig. 4d).

We also isolated these microglia for phenotype study by flow cytometry to analyse the expression of CD45 + F4/80 + CD11b + MHCII+ (Fig. 5o and Supplementary Fig. 5). In brief, VK suppressed the excessive activation of microglia (Fig. 5m–o, Supplementary Fig. 4d, and Supplementary Fig. 6). These results suggest that VK inhibits the neuroinflammatory response and provides a new approach for the treatment of ischemic stroke.

**VK protects neurons against cell death and axonal injury in stroke mice.** In the pathophysiological process of AIS development, the destruction of neurovascular unit (NVU) homeostasis is complicated by the regulation of temporal and spatial networks during in AIS. We explored the effects of VK on BBB damage and activated microglia. Herein, we built an OGD/R model of HT22 hippocampal neuron cells, which can remarkably decrease cell activity and cause apoptosis in HT22 cells; VK treatment enhanced cell activity (Supplementary Fig. 7a) and restrained apoptosis (Supplementary Fig. 7b, c). To further evaluate the pathological changes of neurons in stroke brains, we conducted IHC with anti-NeuN antibody and Nissl staining (Fig. 6a). The positive cells of NeuN were decreased in vehicle-treated mice when compared with the sham group. The number of NeuN-positive cells in the VK-treated group was greater than that in the
vehicle-treated group (Supplementary Fig. 7d). For Nissl staining, at high magnification, typical pyknotic neurons or dark neurons were observed in selected cortex and hippocampus areas in vehicle-treated mice following stroke. Interestingly, morphologically intact neurons were broadly seen in similar brain areas of mice in the sham and VK treatment groups (Fig. 6b). The results observably demonstrated that VK therapy inhibited cell death following ischemic damage. To confirm the functional effects of VK on neuronal activity in the peri-infarct zone, the intrinsic excitability of pyramidal neurons was recorded by electrophysiology after MCAO/R (Fig. 6c, d). Decreases in neuronal excitability could lead to the poor activation of pyramidal

**Fig. 4** Protective effect of VK on the blood–brain barrier (BBB) in stroke mice. a Experimental design: in addition to the sham group, mice (8–10 weeks, male) were subjected to MCAO for 60 min. Mice were randomized into four groups: sham group, vehicle group, and VK (150 and 300 µg/kg) groups. Mice were administered VK at 0, 4, 22.5, and 46.5 h after MCAO/R and BBB integrity was assessed by Evans blue (EB) and transmission electron microscope (TEM). b Brain samples were stained with EB. c EB content in damaged (right) hemisphere was quantized. d Schematic of BBB. The BBB mainly consists of endothelial cells (EC), pericytes (PC), astrocytes (Ast), neurons, tight junctions (TJs), and the basement membranes (BM). The main components of the BBB are cerebral microvascular ECs joined by TJs, thus restricting exogenous molecules into the brain. e The structure of the BBB was observed by TEM. In the sham group (e1–3), the capillary morphology was regular, the EC and TJ were complete, the thickness of the BM was uniform and continuous around the outside of EC, and the structure of mitochondria (Mt) was clear. When the BBB was destroyed (e4–6), the BM and TJ were largely dissolved and shed, and the Ast showed extreme edema; there were large electronic blank areas and the Mt was loose or vacuolar, which were relieved by VK treatment (e7–12). The marks with different symbols indicate the constituent cells or matrix of the BBB. Scale bars: 2 µm. Data are expressed as the mean ± SD, n = 5. Values were analyzed using one-way analysis of variance (ANOVA) with the Tukey multiple comparisons test.
neurons and a subsequent decline in the excitatory synaptic drive, thus further aggravating the ischemic pathology. Here, MCAO induction reduced the frequency of miniature excitatory post synaptic currents (mEPSCs), which is suggestive of stroke-induced excitotoxicity (Fig. 6e, f), yet showed no obvious effects on the amplitude of mEPSCs (Fig. 6g), in agreement with previous studies. However, pyramidal neurons in VK-treated mice showed a higher action potential threshold at 48 h after MCAO compared with the vehicle group (Fig. 6e). Moreover, VK also showed no obvious effects on the amplitude of mEPSCs after stroke in mice (Fig. 6f). These data indicate that VK treatment preserves the excitability of pyramidal neurons after MCAO/R in mice. Activated microglia are now generally accepted to release both protective and cytotoxic factors, through which they can...
Fig. 6 VK protects neurons against cell death and axonal injury in stroke mice. a Experimental design: in addition to the sham group, mice (8-10 weeks, male) were subjected to MCAO for 60 min. Mice were randomized into four groups: sham group, vehicle group, VK (150 and 300 µg/kg) groups. Mice were administered VK at 0, 4, 24, and 48 h after MCAO/R as assessed cell death and axonal injury by IHC staining for NeuN41, Nissl staining, and patch-clamp whole-cell recording. b Nissl staining for the mice hippocampus (CA1 region, interaural: 2.10–2.58 mm, bregma −1.46 to −1.22 mm) and cortex in each group was displayed. Position of stimulating and recording electrodes for conduction velocity measurements in (c, d). e Representative firing patterns of pyramidal neurons from mouse neocortex elicited by depolarizing current steps after MCAO/R. All miniature excitatory post synaptic currents (mEPSCs) were recorded at a holding potential of −80 mV. f Cumulative frequency plots of the amplitude (left) and quantitative analysis of the frequency of AMPA receptor-mediated mEPSCs (right). g Cumulative frequency plots of the amplitude (left) and quantitative analysis of the amplitude of AMPA receptor-mediated mEPSCs (right). Results are expressed as the mean ± SEM, n = 5–7. Statistical significance was determined by one-way ANOVA and Bonferroni test as post-hoc comparisons. h Schematic showing that primary cortical neurons obtained from fetal C57BL/6 mice of embryonic day 16–17.5 and primary microglia isolated from C57BL/6 mice at postnatal day 1–2 were co-cultured with or without VK. For the co-culture system, (I) cell supernatant was collected to measure proinflammatory factors. Data are mean ± SD (n = 4).

impact on neuronal functions and viability42,43. In this study, cortical neurons and microglial isolated and purified from C57bl/6 mice were co-cultured, and OGD/R models were established to investigate the protective effect of VK in vitro (Fig. 6h). The result of proinflammatory cytokines showed that VK protects neurons, and avoids persecution of proinflammatory secreted by microglia (Fig. 6i).

Reduced neuroinflammation and apoptosis by VK treatment are associated with active PI3K–AKT and inhibitory IκBα–NF-κB signaling pathways. To investigate the mechanisms by which VK improved functional recovery after stroke, ischemic tissue from stroke mice was collected and subjected to a phospho-antibody array. These results suggested that PI3K–AKT and inhibitory IκBα–NF-κB signaling pathway (Fig. 7d–h), eventually promoting functional recovery. Briefly, VK treatment stimulated MCAO-mediated PI3K (p85α) and AKT phosphorylation (Fig. 7d–f). In addition, increased phosphorylation levels of IκBα and NF-κB were seen in the vehicle-treated group compared to the sham group, while VK (300 µg/kg) reduced IκBα and NF-κB levels (Fig. 7d, g, h). Additionally, VK treatment also partially down-regulated the phosphorylation levels of p38 and ERK1/2 compared to vehicle treatment (Supplementary Fig. 8). The data from Western blot analysis were consistent with a phospho-antibody array. These results suggest that PI3K–AKT signaling is activated to suppress IκBα and NF-κB levels in stroke mice by VK treatment and is related to reductions in inflammation and apoptosis.

B2R antagonist HOE140 counteracts the neuro-protective effects of VK on stroke in mice. B2R is neuroprotective in brain ischemic insults and increases the migration of glial cells via activated B2R44,45. To reveal VK-B1R or B2R interaction, the HDOCK server for integrated protein–protein docking was performed as described46. Known three-dimensional structure of VK and the three-dimensional structure of B1R or B2R, we predicted VK-B1R or B2R interaction sites, and the peptide docking model of VK and B2R with the highest score (Fig. 8a–d). The docking summary of the top 10 models were also displayed (Supplementary Figs. 9 and 10). Notably, VK, composed of BK and its analogs, was the first neurotoxin component isolated from wasp venom. To determine whether VK binds to B1R or B2R,
Fig. 7 Reduced neuroinflammation and apoptosis by VK treatment were associated with PI3K-AKT-mediated NF-κB inhibition. a Layout of antibody array. b Scanned image of antibody microarray and the protein expression levels were tested with antibody microarray analysis (c). d–h Western blot analysis revealed that there were changes in the phosphorylation levels of PI3K (p85α), AKT, IkBa, and NFkB in the VK groups compared to the vehicle group.
Lys-(des-Arg9-Leu8)-BK and HOE140, which are respectively inhibitors of B1R or B2R, were used to treat stroke mice (Fig. 8e). Longa test and TTC staining were conducted to assess the effects of B1R or B2R on functional recovery after MCAO/R. Compared with vehicle treatment, VK treatment attenuated neurological deficits and infarct size (Fig. 8f–i). Similarly, a combination treatment of VK and Lys-(des-Arg9-Leu8)-BK improved functional injury. However, the major pharmacological effects of VK were blocked by HOE140 preconditioning in stroke (Fig. 8f–i). The EB extravasation assay demonstrated that the combined administration of VK and HOE140 damaged the BBB integrity, as evidenced by less EB extravasation in ischemic hemispheres (Fig. 8j, k). Mechanically, HOE140 could inhibit VK-mediated AKT activation (Supplementary Fig. 11).
antagonist HOE140, which could suppress the upregulation of AKT signaling triggered by VK under OGD/R condition, could offset the protective effects of VK. These data suggest that targeting the VK-B2R interaction may be a practical strategy for stroke therapy.

Discussion

Herein, VK from Vespa magnifica was identified by Edman sequencing, MS/MS, and amino acid sequence determination and shown to be completely degraded in plasma at 120 min, suggesting an interval time of VK administration. In this study, 150 and 300 μg/kg body weight were effective dose, these dose supports evidence from previous observations. We also confirmed that safe doses of VK were <96 mg/kg in acute toxicity tests of mice, suggesting 640 times the effective dose (150 μg/kg) is still safe. Additionally, VK was shown to penetrate the BBB and live-cell imaging systems showed that VK is a cell-permeable peptide (CPP). For neuropharmacology, crossing the BBB is a remarkable event of AIS involve a dynamic developmental process and the pathological process involves complex temporal and spatial cascades of NVU. The induction of MCAO aggravated the severity of the CBF deficit in the first few pivotal hours during MCAO in the vehicle group, which led to a different evolution of brain injury. Hence, a successful stroke model was confirmed by LSCI. Herein, we reported that VK effectively improved the sensorimotor and cognitive recovery and reduced the infarct size in the mouse model of MCAO/R. The AIS is caused by complex pathological physiological mechanisms and has different clinical manifestations in human than in mice. The presence of standardized scores for sensory nerves and motor behavior in rats provides an advantage in functional behavioral assessment since mice and human have similar cerebral blood circulation. We demonstrated that VK improved the sensorimotor and cognitive recovery in stroke by the Longa test, grip test, rotarod test, and the Morris water maze. The potential mechanism for VK to improve functional recovery was explored. Shortly after onset of focal cerebral ischemia, neurovascular dysfunction is manifested by the disruption of BBB integrity and function. At the cellular level, endothelial cells rapidly convert into a pro-inflammatory/pro-thrombotic state via the upregulation of protease-activated receptor 1 (PAR1) and tissue factor (TF) as well as matrix metalloproteinase (MMP) gene expression in the ischemic core and boundary, which facilitates inflammation and BBB disruption. A reduction infarct size and the maintenance BBB integrity were mainly provided by VK treatment.

Furthermore, lipid peroxidation damage caused by stroke was restrained by VK treatment. Previous studies have shown that BK protects against oxidative stress-induced endothelial cell senescence and BK can also be beneficial after ischemic stroke. Interestingly, VK was the first neurotoxin component isolated from wasp venom, with this small peptide playing a considerable role in regulating blood pressure, inflammation, and renal and cardiac function. We demonstrated that VK exhibited an antioxidant activity in stroke mice. The generation of reactive oxygen species is increased in activated microglia and the ensuing oxidative damage can induce an uncontrolled inflammatory condition. Therefore, compounds with antioxidant activity may be beneficial for stroke and neuroinflammatory conditions. Although several studies report that BK likely causes a specific cascade of inflammatory responses in the CNS, it has also been shown to possess anti-inflammatory and neuroprotective effects, suppressing the release of inflammatory cytokines from in vitro microglia assays. Similarly, our data validated that VK restrained the pro-inflammatory response and promoted the expression of anti-inflammatory mediators in OGD-induced BV2 cells and mouse primary microglia cells.

After the onset of cerebral ischemia stroke, ATP levels expeditiously decline during the first 5 min and fall to ~15–30% of the
ATP concentration measured in the non-ischemic hemisphere over the first 2 h after ischemia onset. In the present study, VK therapy partially recovered energy metabolism in the ischemic hemisphere as determined by the detection of ATP and LDH content. Furthermore, to illustrate the effect of VK on energy metabolism in neurons, the glycolysis and oxidative phosphorylation pathways in neurons were also evaluated by Seahorse after stroke. Increasing evidence indicates that the neurological impairment in stroke is mediated through inflammatory reactions and pro-inflammatory cytokines. Moreover, the administration with BK after transient forebrain ischemia in rats could provide 97% neuroprotection as well as a decrease in inducible nitric oxide synthase-negative cells and caspase 3 expression and an inhibition of the release of cytokotic cytochrome. These data were in agreement with the results presented herein. In this study, we also found that treatment with VK ameliorated neuronal damage as evidenced by IHC and Nissl staining. I/R in rat brain generated a 10-fold increase in glutamate concentration during ischemia, which progressively returned to baseline after 30 min of reperfusion. This increase in extracellular glutamate levels is one of the major factors inducing cell death after brain ischemia. Importantly, the neuroprotective role of VK has also been reinforced by evidence of preserving the excitability of pyramidal neurons. This finding suggests that VK is able to inhibit neuronal apoptosis and synapse injury after stroke.

The PI3K–Akt pathway has been reported to play a marked role in the neuroprotective effects against cerebral ischemia. Previous studies have confirmed that PI3K–Akt signaling has anti-oxidative, anti-neuroinflammatory, and anti-apoptotic properties in neurons. In contrast, the intracerebroventricular injection of LY294002, an inhibitor of AKT, reduces the phosphorylation levels of Akt and deteriorates neuronal damage after I/R. Therefore, drugs that boost Akt activity may symbolize a new class of therapeutics against AIS, which is in agreement with our experimental data demonstrating that VK could dramatically stimulate the PI3K–Akt pathway to exert neuroprotective activity. NF-kB is a central regulator of neuroinflammatory response and promotes the pro-inflammatory M1 activation of microglia. Modulating the activity of NF-kB could potentially suppress neuroinflammatory processes in ischemic stroke. In this study, we found that VK inhibited MCAO-induced phosphorylated IkBα and NF-kB p65 activation. Additionally, the protein microarray indicated that the MCAO-induced stroke model in mice could up-regulate the phosphorylation levels of IkBα (S32), NF-kB (S536), P38 (T180/Y182), Casp7 (D198), and JNK (T183), which were obviously downregulated by VK treatment. Go rich-collection analysis reveals that these processes are involved in a variety of protein functions and cell localization. However, whether the neuroprotective effect of VK is caused by active PI3K–Akt-mediated NF-kB–IkBα inhibition remains unknown.

B1R and B2R both belong to the G protein-coupled receptor (GPCR) family, with B2R mediating most of the physiological functions of kinin. It has been increasingly appreciated that BK and Lys-BK could act as a ‘double-edged sword’. By inhibiting oxidative stress and apoptosis, the systemic or local delivery of human TK protects against mouse myocardial I/R injury via B2R. Similarly, we described that the neuroprotective effects of VK on stroke in mice was inhibited by HOE140, the specific antagonist of B2R. We also conducted the autodock experiment to evaluate the combination of VK and B2R. Therefore, it is presumed that the activation of VK-B2R could be protective in CNS ischemic insults. The single substitution of serine for threonine in this compound results in enhanced action when compared to BK. In terms of pharmacodynamic activity, a previous study has shown that VK exhibits remarkable anti-nociceptive effects when injected directly into the rat CNS; it is approximately three times more potent and remains active longer than BK. These results can be explained by a more stable conformation in its secondary structure and/or the fact that the modification may protect against hydrolysis through neuronal kininases, preserving the effect of the peptide on B2R. As a candidate compound acting in the CNS, the stability of VK in plasma needs to be improved by structural modification and pharmacy studies.

Finally, we only evaluated the neuroprotective effect of VK (150 and 300 μg/kg) during the period of acute cerebral ischemia in male mice. However, efficacy studies were not performed in female mice, and the protective effect of longer period time (such as 14d, 21d, 28 d) of VK on stroke severity was not demonstrated in this study. Human stroke occurs in the context of sex, aging, diabetes, hypertension, heart disease, and the use of concomitant medications, sex as a biological variable should be also considered in our future research. As far as the NVU responses are concerned, stroke is the most well-examined CNS disease because stroke pathophysiology shows relatively biphasic phenomena.

Under the acute phase after stroke onset, ischemic injury results in an abrupt deprivation of nutrient supplies that quickly leads to irreversible damage in the core of the affected area. On the other side, remodeling signaling such as angiogenesis and synaptic remodeling may occur in the partly preserved peri-infarct area (so-called penumbra) during the chronic phase. In future, the protective effects of VK on chronic phase of ischemic stroke will be studied and proven.

In conclusion, our data reveal that VK promotes functional recovery in mice after ischemia stroke, including an improvement of neurological impairment, a reduction of infarct volume, protection of the BBB, and the inhibition of inflammatory responses and oxidative stress. In addition, we found that reduced neuroinflammation and apoptosis by treatment with VK were associated with PI3K–AKT-mediated NF-kB inhibition. Simultaneously, the B2R antagonist HOE140 could counteract the neuro-protective effects of VK on stroke in mice. Taken together, these findings reveal that targeting the VK–B2R interaction can be considered as a practical strategy for stroke therapy (Fig. 9).

**Methods**

**The purification and identification of VK.** VK was purified and identified as described previously. In brief, crude venom from *Vespa magnifica* (Smith) was collected from Yunnan Province, China. The lyophilized crude venom was dissolved in deionized water (1 mg/ml) and filtered, then loaded onto a Sepax Bio C18 column (21.2 × 250 mm, 10 μm). To purify VK, HPLC was put on a Waters 2535 system equipped with a manual injector and two-solvent system: (A) acetonitrile with 0.1% trifluoroacetic acid (TFA) and (B) water with 0.1% TFA. The effluent fractions corresponding to the chromatographic peak was manually collected in a tube and lyophilized for subsequent detection. Mass measurements of the separations were executed using a matrix-assisted laser desorption/ionization time of flight mass spectrometry (140,000 at 200 M/Z at a scan rate of 1.5 Hz, Q Exactive™, Thermofisher Scientific, USA). The molecular mass of the sample was determined by the reflection method with positive ion mode. The amino acids were confirmed by tandem mass spectrometry (MS/MS) and automatic amino acid analyzer. The purified component was sequenced with Edman degradation.

**VK stability in plasma.** VK was mixed with PBS or plasma to a final concentration of 0.735 mM and incubated (37 °C, 750 rpm; Thermomixer, Eppendorf AG, Hamburg, Germany) for 0, 30, 60, 90, or 120 min. The samples were filtered with a 0.45 μm membrane and analyzed by HPLC (Agilent 1260, column: Sepax Bio C18 (4.6 x 250 mm, 5 μm); detection wavelength: 215 nm, flow rate: 1.0 ml/min, loading volume: 20 μL). VK in PBS or plasma was detected by quantifying the peak areas relative to the initial peak areas (0 min). All stability tests were performed at least in triplicate.

**Study approval.** All experimental procedures and animal housing in this study were designed and conducted in accordance with the approval of the Institutional Animal Care and Use Committee of Xiamen University, China (Animal Ethics nos.: XMULAC20200122). Adult male C57BL/6J mice weighing 22–25 g (8–10 weeks) were obtained from Xiamen University Laboratory Animal Center. All mice were housed in a specific pathogen-free facility under a 12-h light/dark cycle in a temperature-controlled environment (22–25 °C) with a humidity of 40–70% and
had free access to food and water. We used 357 mice for the study (Supplementary Table 1).

Focal cerebral ischemia model. Experimental ischemic stroke was performed by MCAO using published protocols\(^6,7,8\) with some modifications. In detail, mice (8–10 weeks)\(^\text{9,10}\) were deeply anesthetized with 1.5% isoflurane in a mixture of 30% O\(_2\) and 69% N\(_2\)O. Conditions of the blood gas were maintained at constant levels throughout the operation (P\(_{O2}\), 120 ± 10 mmHg; P\(_{CO2}\), 35 ± 3 mmHg) and temperatures in the temporal muscle and rectum were also maintained at 37.5 ± 0.2 °C and 37.0 ± 0.1 °C, respectively. Body temperature was kept in the normal range (36.5–37.5 °C) with a heating lamp and a heating pad during surgery. All surgical supplies and instruments were sterilized. The internal common carotid artery (CCA) and the external carotid artery were then gently separated. The blood flow of CCA was blocked with a sterile nylon suture (No.: MSMC21B120PK50, Ruiwode Bio-technology Co., Ltd., China), which was inserted into middle cerebral artery from CCA. The mice in the sham group underwent the same surgery without a nylon suture. After 60 min, the suture was removed and the blood circulation was recovered, MCAO/R was induced. Regional CBF was monitored in all stroke animals by LSCI (PeriScan PSI System; PERIMED, Stockholm, Sweden) to confirm a successful occlusion.

Exclusion criteria. Exclusion criteria in mice were performed as described above\(^,9,10\) (1) mice with a CBF reduction of <70% were excluded from further assessments; (2) no neurological deficits at 3 h after MCAO/R; (3) postmortem examination shows subarachnoid hemorrhage.

Drug treatment. Mice were randomly divided into four groups: (1) the sham group, (2) MCAO group (vehicle treated), and test drug groups (3) MCAO + VK (150 µg/kg, i.p.), and (4) MCAO + VK (300 µg/kg, i.p.). After MCAO, mice were treated with normal saline or VK 0, 4, 22.5, and 46.5 h.
Transmission electron microscopy. Frontal cortex samples were isolated from mice with stroke and fixed in 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4), treated with 10% gelatin solution in sodium cacodylate buffer, and incubated with 2% osmium tetroxide. Sections were cut using a Leica EM TP ultramicrotome (Leica Microsystems) at 40 nm and placed in 25-mm-thick sections at the level of the frontal cortex (bregma: 1.0 to −1.94 mm) using a cryostat microtome (Leica CM1950). The sections were subsequently stained with Nissl staining.

MRI. After MCAO/R, mice were treated with VK (300 μg/mL, i.p.) at 0, 4, 22.5, and 46.5 h. Following sequential dehydration by 10%, 20%, and 30% ETOH, brains were put in frozen section embedding medium (SAKURA Tissue-Tek O.C.T. Compound, Japan) and cut into 25-mm-thick sections at the level of the frontal cortex (bregma: 1.0 to −1.94 mm) using a cryostat microtome (Leica CM1950). The sections were subsequently stained with Nissl staining.

Electrophysiological recordings and assessment of excitatory inputs. Mice were treated with VK (150 and 300 μg/mL, i.p.) at 0, 4, 22.5, and 46.5 h after MCAO/R. Pyramidal neurons in layers 2–3 of the mouse neocortex located ~1 mm² were prepared as previously described. Following incubation, slices were transferred to a recording chamber in which oxygenated artificial cerebrospinal fluid was warmed to 32°C and superfused over the submerged slices at 2 mL/min. Recordings were collected from the pyramidal neurons, with mEPSCs recorded at 3 Hz. mEPSCs were recorded at 300–350 μV amplitude. For mEPSC recording, the composition of artificial cerebrospinal fluid (in mM) was as follows: 120 K-glutamate, 20 KCl, 2 MgCl₂, 0.1 EGTA, 10 sodium phosphocreatine, 0.2 leupeptin, 4 Mg-ATP, 0.3 Na₃GTP, and pH 7.3 (290 mosmol). mEPSCs were recorded at a holding potential of −60 mV to inhibit action potential-mediated excitatory postynaptic currents. For mEPSC recording, the composition of artificial cerebrospinal fluid (in mM) was as follows: 120 K-glutamate, 20 KCl, 2 MgCl₂, 0.1 EGTA, 10 sodium phosphocreatine, 0.2 leupeptin, 4 Mg-ATP, 0.3 Na₃GTP, and pH 7.3 (290 mosmol). mEPSCs were recorded at a holding potential of −60 mV to inhibit action potential-mediated excitatory postynaptic currents. For mEPSC recording, the composition of artificial cerebrospinal fluid (in mM) was as follows: 120 K-glutamate, 20 KCl, 2 MgCl₂, 0.1 EGTA, 10 sodium phosphocreatine, 0.2 leupeptin, 4 Mg-ATP, 0.3 Na₃GTP, and pH 7.3 (290 mosmol). mEPSCs were recorded at a holding potential of −60 mV to inhibit action potential-mediated excitatory postynaptic currents. For mEPSC counting, the primary antibody was omitted during immunostaining. Images were acquired by confocal microscopy (Zeiss LSM 880 or FV1000 MPE-B, Olympus) and processed with ImageJ (NIH, Bethesda, MD, USA).

Cytokine enzyme-linked immune sorbent assays. Mice were decapitated under anesthesia at 48 h after MCAO/R. The IL-1β (Cat# 88-5019-88), TNF-α (Cat# 88- 7798-99), IL-6 (Cat# 88-7964-88) and IL-8 (Cat# 88-8880-22) were purchased from Thermo Fisher Scientific. The levels of these proteins were measured by enzyme-linked immune sorbent assays (ELISA) kits, according to the manufacturer’s recommendations.

FITC-labeled VK and live-cell imaging. VK (Gly–Arg–Pro–Hyp–Gly–Phe–Ser–Pro–Phe–Arg–Ile–Arg–ASP–NH2) was labeled with FITC in the N-terminus. VK (100 nm) was added to each well and, after 15 min of incubation at 37°C, live-cell images were acquired and processed by confocal microscopy (Zeiss LSM 880). Live-cell images were recorded for 90 min. The negative group was FITC treated without labeled VK.

Fluorescence imaging of VK. A successful MCAO model was established and these mice were used for subsequent experiments. Immediately after surgery, FITC or FITC-labeled VK were administered intravenously and mice were killed after 30 min to isolate the brain and imaged by an imaging system (IVIS Lumina II). For fluorescent imaging, the brain was immediately collected and kept in the dark for 0, 30, 60, and 120 min after the intravenous injection of VK. Frozen sections were prepared according to the above method (see Morphological Assessment). Images were analyzed by confocal microscopy (FV1000 MPE-B, Olympus).

The HDOC server for integrated protein–protein docking. Protein–protein and protein–DNA/RNA docking based on a hybrid algorithm of template-based modeling and ab initio free docking as consulted http://hdoc.phys.hust.edu.cn/.

Statistical analysis. Statistical analysis was conducted using Graph Pad Prism 8 software. In order to determine the normal distribution of the sample, the Kolmogorov–Smirnov test was conducted. If the sample was normal distribution, then statistical analysis was conducted among multiple groups using one-way analysis of variance (ANOVA), followed by the Dunnett test or Student’s t test. If the sample was not normal distribution, then a Kruskal–Wallis test was performed. A statistical difference was established at P < 0.05.
**Research Reporting Summary** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All data is included in the article and supplementary information. Source data of figures are provided in Supplementary Data 1. The protein microarray and uncropped western blots are provided in Supplementary Figs. 12-19. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
Z.H.R. designed the project and wrote the manuscript. Z.H.R., W.M., G.Y., H.F.R., L.J.M., X.D., W.Q. and L.W.D. constructed stroke mice. W.M., G.Y. and Z.H.R. performed behavioral tests, Immunofluorescence, Immunostaining, Western blot, Transmission electron microscope, and data analysis. L.J.G. performed four coagulation tests. Z.H.R. performed Flow cytometry. W.X.M. and X.H. performed platelet function tests. Z.H.R. and W.X.M. performed data analysis of antibody arrays. Z.H.R. and W.M. performed primary neuron and microgria culture. C.J.D., Z.C.G. and Z.Y. helped with data analysis, interpretation, and supervised the project. Z.C.G., C.J.D. and Z.Y. were responsible for review and editing. Z.H.Y. provided the source and identification of VK.

Competing interests
The authors declare no competing interests.

Additional information
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