日本財団補助金による
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③．中国人研究者・医療技術者招聘助成事業

(1) 虚血性神経傷害のメカニズム解明と治療法の開発に関する研究

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研究発表中または研究中の本人のスナップ写真、及び発表論文等のコピーを添付

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研究テーマ 虚血性神経傷害のメカニズム解明と治療の開発に関する研究

2．日本滞在日程

2000年8月3日 関西空港到着（Mu515便）
同日より国立循環器病センター・レジデント宿舎に寄宿し、研究に従事。
2001年4月より 当センターで脳科学研究員として研究を継続する予定。

3．研究報告

別紙書式により、報告本文4000字以上(英文は2600語以上)で報告して下さい（枚数自由・ワープロ使用）

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研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

論文発表に当っては、*日中医学協会助成事業－日本財団助成金による*旨を明記して下さい。

虚血性神経傷害のメカニズム解明と治療の開発に関する研究

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要 旨

「緒言」マトリックス・メタロプロテアーゼ(matrix metalloproteinase: MMP)が脳傷害に関与するという報告がある。我々は、局所脳虚血による脳梗塞形成に MMP が関与するかを調べた。
「方法」
雄 ICR(18—22ｇ)マウスを用いた。ハロセン麻酔下に左頸動脈を露出し、8—0ナイロンフィラメントを外頸動脈より挿入、先端を 起始部まで進め中大脳動脈を閉塞し、局所脳虚血を誘導した。虚血後の局所脳血流量、動脈圧、動脈血ガス分圧、pH、直腸温をモニターした。 (0.5%カルボメチルセルロースに溶解)を皮下注射により投与した。MMP の活性をゲラチンを基質とする zymography により調べた。中大脳動脈閉塞 24 時間後に脳を取り出し冠状断を作成、2%TTC 溶液により染色し、脳梗塞領域を測定した。各脳断面の脳梗塞領域を加算することにより、脳梗塞体積を算出した。
「結果」zymography を用いて、中大脳動脈閉塞 6 時間後より MMP-9 の活性が虚血脳半球に検出された。MMP 阻害剤 (100mg/kg)の中大脳動脈閉塞 30 分前投与は、中大脳動脈閉塞 24 時間後の MMP-9 活性を有意に減弱させた。また、KB-R7785(0、30、100mg/kg)は、濃度依存性に脳梗塞体積を減少し、100mg/kg 投与では、溶解液投与に比べて有意に脳梗塞体積を縮小させた。 さらに、KB-R7785(100mg/kg)の 3.5 時間間隔による 2 回繰り返し投与では、中大脳動脈閉塞開始 1 時間後の投与でも有意な脳梗塞体積縮小効果が得られた。
「結論」以上の結果は、 MMP-9 の活性化が局所脳虚血による脳梗塞形成に重要な役割を果たしていることを示す。MMP-9 阻害による脳虚血急性期治療の可能性を示唆するものと考えられる。

KEY WORDS

Cerebral ischemia, focal – matrix metalloproteinase – infarct – activity – MCAO

A novel (MMP) inhibitor KB-R7785 attenuates brain damage resulting from focal cerebral ischemia in mice

Abstract

Background and purpose- Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that can degrade all of the extracellular matrix when they are activated. In normal conditions, they are involved in tissue development, bone remodeling, wound healing, implantation, and ovulation and are strictly regulated of their activities by multiple factors. Over-expressions and abnormal activities of MMPs would lead to tissue injury. In brains, the main components of cerebral vascular basement membrane are type IV and V collagen and laminin, fibronectin that are the substrates of gelatinase A (MMP-2) and gelatinase B (MMP-9). It has suggested that MMP-9 and MMP-2 were involved in brain damage after focal cerebral ischemia by disrupting the blood brain barrier, contributing to brain edema, facilitating invasion of inflammatory cell and promoting neuronal cell death. The present study intended to investigate the effect of a new inhibitor of MMPs, KB-R7785, on ischemic stroke.

Methods- Adult male ICR mice (18 to 22 g, n=110) were randomly subdivided into 9 groups treated with KB-R7785 and corresponding vehicle in single pretreatment with different doses (30mg/kg, 100mg/kg,) and in repeated administrations (100mg/kg) at different time points (**A**: 30minutes before and 3 hours after MCAO; **B**: 1 hour and 4.5 hours after MCAO; **C**: 3 hours and 6.5 hours after MCAO). Ischemia was induced by intraluminal permanent MCAO under halothane anesthesia. rCBF and body temperature were monitored during surgery. The infarction volumes at 24 hours after MCAO were determined with 5 equidistant coronal sections stained with 2% TTC. MMPs zymography was conducted with brain tissues from other separate mice subjected to ischemia.

Results- The zymogram showed that MMP-9 is activated initially 3 hours after MCAO and maximized at 24 hours after ischemia. Single injection of KB-R7785 attenuated infarction size in a dose dependant manner; by 20% and 39% with 30 mg/kg and 100 mg/kg, respectively. In repeated administration groups, KB-R7785 showed decreasing lesion ranges in a certain time window. The lesion volumes were significantly decreased in group A and B ($p<0.01$, Scheffe and t test) but not in group C. There were no differences in body temperature and %rCBF reductions between each group.

Conclusions- We demonstrated that the new inhibitor of MMPs KB-R7785 has a beneficial effect against ischemic brain injury, and our data provides further evidence that MMP-9 is activated after cerebral ischemia, contributing to brain infarct formation.

Introduction

Matrix metalloproteinases (MMPs) are a group of zinc-containing protease that can proteolytically degrade and remodel extracellular matrix components (EMC). They have wide substrate specificity and provide tissue with the collective ability to degrade collagens, laminin, gelatin, elastin fibronectin, and proteoglycans. According to their substrate specificity, MMPs are classified as collagenase (Type I collagenase/MMP-1); gelatinase A (72-kDa Type IV collagenase/MMP-2); stromelysins (MMP-3, -10 and -11); uterine metalloproteinase (PUMP-1/MMP-7); neutrophil collagenase (MMP-8); and gelatinase B (92-kDa Type IV collagenase/MMP-9) (11). MMPS physiologically benefits tissue development, wound healing, bone growth, ovulation and angiogenesis; meanwhile they would pathologically promote a variety of disease processes such as tumor invasion and metastasis, rheumatoid arthritis and atherosclerosis as well as multiple sclerosis, Alzheimer's disease and malignant gliomas in central nervous system (13). On account of this feature, MMPs are tightly controlled by multiple steps. Initially they are all released as latent forms that may be proteolytically activated by cleavage of amino-terminal propeptides

at a conserved sequence. This cleavage can be mediated by serine protease or plasmin or undergo autocatalytic activation. The activities of MMPs are regulated by gene expression, proenzyme activation, and inhibition of active enzymes by their specific tissue inhibitors of metalloproteinases (TIMPs).

Recently, MMPs have been implicated in pathogenesis of brain injury after cerebral ischemia (1,5,7,9). Among MMPs, MMP-2 and MMP-9 attack type IV collagen, laminin, and fibronectin, which are the major components of the basal lamina around the cerebral blood vessels and define blood brain barrier (13). It has been reported that the expression and activities of MMP-2 and MMP-9 was elevated after onset of stroke in human patients (2). Animal model study also showed that level of MMP-9 in ischemic brain was increased quite early and was related to the destruction of blood brain barrier, contributing to the increases of permeability of BBB and promoting brain edema (9). MMPs may also promote tissue invasion of neutrophils and macrophages, and contribute to hemorrhages that result after reperfusion of ischemic tissue (7). It suggested that MMP-9 may be a potential target of therapeutical treatment (12,10). Related studies have shown that some inhibitors of MMPs can reduce infarct size after ischemia and MMP-9 gene knock-out mouse showed reduced brain damage compared with that in wild-type mouse (4, 10). But so far there's no report showing a direct evidence in vivo that synthetic inhibitor of MMPs exert its neuroprotection by downregulating the activity of MMP. In this study, we investigated the effect of a new synthetic inhibitor of MMPs, KB-R7785 (6,8), on focal cerebral ischemia in mice in different dose and determined the therapeutic time window and further we compared the activities of MMP-9 between drug-treated group and vehicle group using gelatinase zymography and activity assay.

Materials and methods

Focal cerebral ischemic model in mice

One hundred forty-seven adult male ICR mice (18 to 22 g) were used in the study. The procedures performed following the National Cardiovascular Center's guidelines for animal care and experiments. The focal ischemia was induced by permanent intraluminal middle cerebral artery occlusion method. Mice were anesthetized with 1.0% halothane in 70% N_2O and 30% O_2 using a vaporizer (Halowick, Muraco Medical, Tokyo, Japan). The right femoral artery was cannulated for measuring blood pressure and collecting arterial blood sample. Rectal and temporal muscular temperatures were maintained at 37℃ with a thermostat and a heating pad (NS-TC, Neuroscience, Tokyo, Japan; and BAT-12, Physitemp, NJ). Following a midline skin incision and exposing of left carotid artery, an 8-0 nylon monofilament coated with a silicon resin/hardener mixture was introduced into the left internal carotid artery through left external carotid artery, up to the origin of the middle cerebral artery and left for 24 hours. Regional cerebral blood flow (rCBF) was monitored with laser Doppler flowmetry during operations (to confirm the induction of focal ischemia and to control the reductions of CBF between groups within a comparable range). Sham control animals were just subjected to exposing of left carotid artery without occluding middle cerebral artery. All the animals were kept in a 32℃ incubator (Thermocare, Incline Village, NV) for 3 hours after surgery.

Administration of matrix metalloproteinase inhibitor KB-R7785

KB-R7785 (New Drug Discovery Research Lab. Of Kanebo, Ltd, Osaka, Japan) was suspended in 0.5% carboxymethyl cellulose (CMC) and administered subcutaneuously. To

determine its efficacy on infarct volumes, single as well as twice dose were administered in separate group. Single dose was given 30 minutes before ischemia onset with different dosage (30mg/kg n=15, 100mg/kg n=15) in each subgroup; Twice administration (100mg/kg per dose) was also subdivided into 3 subgroups by different time point at which drug were given. To prepare for tissue extracts used in testing the effect of KB-R7785 on inhibition of MMP activity, another separate single-dose group (100mg/kg, n=8) was treated. Every corresponding vehicle control received 0.5% carboxymethyl cellulose (CMC).

Infarct measurement

Mice were killed 24 hours after ischemia onset. The brain was cut into 5 coronal 2-mm-thick sections, and stained with 2% 2.3.5-triphenyltetrazolium chloride (TTC) for 30 minutes and then fixed in 10%formalin solution for more than 3 hours. Infarct volumes were measured with standard computer-assisted image analysis technique.

Preparations of brain tissue extracts

To determine the gelatinase activity at different time point in ischemic brain, mice were killed at 1,3,6,12,24 hours after MCAO (n=4 per time point). While in single treated mice (n=8) and its corresponding control (n=7), this procedure was performed only at 24 hours. Sham-operated control (n=2) was also performed at 24 hours. The brains were removed immediately and divided into ispilateral ischemic and contralateral noischemic hemispheres.

Hemispheric tissue was quickly frozen in liquid nitrogen and then stored at −80℃until use. Tissue samples were homogenized on ice with a Potter glass homogenizer in lysis buffer (50mM Tris-HCl pH 7.6, 150mM NaCl, 0.5% IGAPAL, 0.1% BRIJ, 0.1% deoxycholic acid) including protease inhibitors (10μg/ml leupeptin, 2μg/ml aprotinin, and 1mM PMSF). The homogenates were then centrifuged at 14,000×g for 15 minutes at 4℃.

Supernatants were collected, and the total protein concentrations of them were determined by Bradford assay using a Beckman spectrophotometer (Bio-Rad Laboratories, Hercules, CA, U. S. A.).

MMP zymography

Ten microliters (10ul) of total protein extract from per sample was prepared and standardized the protein concentration to 3ug/uL. Ten microliters of 2×sample buffer (125mM Tris-HCl, 20% glycerol, 2% sodium dodecyl sulfate, 0.005% bromophenol blue) was added to each protein extract. The prepared samples (30ug/20ul) were loaded on lanes of 7.5% polyacrylamide gel containing 1mg/ml gelatin as substrate. Electrophoresis was performed with a Tris-glycine running buffer at 4mA constant current for 1.5 to 2 hours. The gel was removed and incubated in renaturing buffer (2.5% Triton-X 100 in distilled water) at room temperature with gentle agitation for 1 hour. Replaced renaturing buffer with developing buffer (50mM Tris-HCl pH 7.5, 200mM NaCl, 5mM $CaCl_2$, 0.2% BRIJ 35), the gel was incubated on a rotary shaker at 37℃ for 20 minutes then for 24 hours with refreshed developing buffer. After developing, the gel was stained with 0.5% Coomasie Blue R-250 for 30 minutes and then destained in 30% methanol and10% acetic acid properly.

Statistical analysis

Data were expressed as mean±SEM. For statistical analysis of infarct volumes in each group, an ANOVA was followed by Scheme and student's t test. For physiological parameters, t test was used. All these statistical analysis were performed with Statview J-4.5 software. Statistical significance was accepted when $p<0.05$.

Results

KB-R7785 inhibits MMP-9 activity after permanent cerebral ischemia

First we examined the MMP activities ischemic brain .We observed a light band of 98 kD molecular weight which should reflect the activity of gelatinase B (MMP-9) on blue background zymographic gel was initially detected at 6 hours after MCAO and increased its density with time course, reached to its peak at 24 hours after ischemia whereas in contralateral hemispheres this band was defective (Figure 1).

The effect of KB-R7785 on inhibiting the activity of MMP-9 was then investigated using this technique. Compared with vehicle control (n=9), pretreatment with KB-R7785 (100mk/kg, n=10) 30 minutes before MCAO showed a reduced band 98 kD representing the inhibition of MMp-9 activity (Figure 2A). Densitometric analysis showed that KB-R7785 significantly inhibited activity of MMP-9 ($p<0.05$; figure 2B).

KB-R7785 reduces brain infarction

Next we examined whether KB-R7785 protects brain against cerebral ischemia. Pretreatment with KB-R7785 30 minutes before MCAO reduced brain infarction in a dose dependent manner (Figure 3). Compared with vehicle-control group (n=15), high dose-treated (100mg/kg) group significantly reduced the infarct size by 39% ($p<0.01$, n=16). There were no differences in body temperature and %rCBF reductions as well as mean arterial blood pressure and blood gas analysis between each group (Table 1).

Delayed treatment of KB-R7785 at 1 hour is protective

Finally, to evaluate the therapeutic time window of KB-R7785, repeated administrations

(100mg/kg per dose) with an interval of 3.5 hours were performed at 3 different time points in 3 groups. Group A was injected 30 minutes before and 3 hours after MCAO; group B was administrated 1 hour and 3.5 hours after MCAO while group C was given 3 hours and 6.5 hours after ischemia. Each group was paired with corresponding vehicle control in which mice only received 0.5% CMC solution with same volume and administration as their KB-R7785 partners. The comparisons of infarct volumes in each group were as follows: $160.6\pm7.8mm^3$ (n=9) vs $97.2\pm5.7\ mm^3$ (n=10, $p<0.001$); $144.5\pm7.5\ mm^3$ (n=12) vs $107.6\pm5.6\ mm^3$ (n=10, $p<0.05$); $157.4\pm9.0\ 6\ mm^3$ (n=9) vs $147.3\pm5.2\ mm^3$ (n=10, $p>0.05$) (Figure 4). The data suggested that KB-R7785 exerted its effect of reducing infarct size when it was administered between the time points of 30 minutes before and 1 hour after induction of cerebral ischemia. Beyond 3 hours after cerebral ischemia onset, it showed no protective effect on ischemic brain injury.

Discussion

The present study demonstrated that (I) KB-R7785 has a protective effect on ischemic stroke by reducing the infarct size; (II) KB-R7785 can inhibit the level of MMP-9 activity after onset of cerebral ischemia. Apart from these, KB-R7785 didn't apparently affect the physiological parameters and showed no detectable side effect.. It has been well defined by others that MMP-9 was involved in the pathogenesis of brain damage (10) but the mechanism underlying the effect of KB-R7785 remains unclear. Our data just provided a possibility that KB-R7785 may exert its brain protection by inhibiting the activity of MMP-9 after ischemia. As we have known, the activation of MMPs after ischemia involves multifactorial events. Some immediate early gene and cytokines such as c-fos, c-jun and tumor necrosis factor-α are increased in ischemia These factors can bind to the AP-1binding site of gelatinase B (MMP-9) and further promote activation

of MMP-9 (12). It has been demonstrated that KB-R7785 can effectively inhibit the expression of TNF-α (6, 8). This indirectly explained that KB-R7785 may exert its brain protection by inhibiting the activation of MMP-9.

Current study also determined the time course of MMPs expression and therapuetical time window of KB-R7785. The data showed that MMP-9 was initially expressed at 6 hours after ischemic insult and peaked at 24 hours. Although this result appeared much delayed than other's data, it still reflected some tendency that MMP-9 was expressed and activated at early period. And this time course was consistent with that of TNF-α elevation. The therapeutic time window indicated that KB-R7785 could protect brain injury when it was injected 30 minutes before and 1 hour after MCAO. Considering the time it would take for the drug to be absorbed, we may postulate that KB-R7785 protected brain injury by inhibiting the early activation of MMP-9. This temporal relationship between time course of MMP–9 activation and therapeutical time window of KB-R7785 provide further indirect evidence that KB-R7785 protect brain injury by inhibiting MMP-9 activity.

In conclusion, our study demonstrated that KB-R7785, a novel inhibitor of MMPs has a dramatic effect of protecting brain tissue from injury, the result suggested that MMPs inhibition may be a potential strategy in stroke treatment and provide another evidence that MMPs are involved in the pathology of cerebral ischemia.

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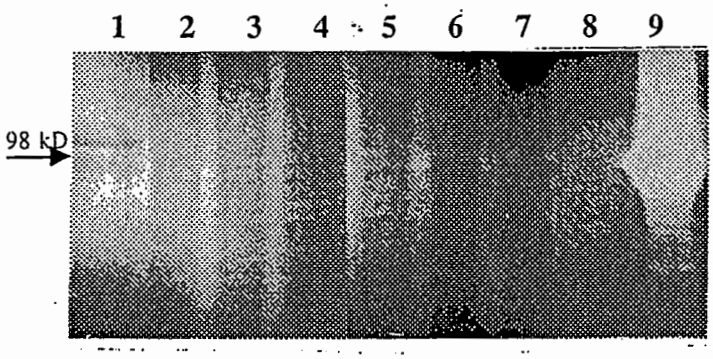

Figure 1 The gelatin zymogram showing time-dependent change in MMP-9 activity after brain ischemia. Lanes are as follows: 1, molecular weight marker; 2, sham; 3, 1hour; 4, 3hour; 5, 6 hour; 6, 12 hour; 7, 24 hour; 8, blank; 9, standard MMP-9. The band at 98 kD level initially appeared at 6 hour and further increased until 24 hour after ischemia.

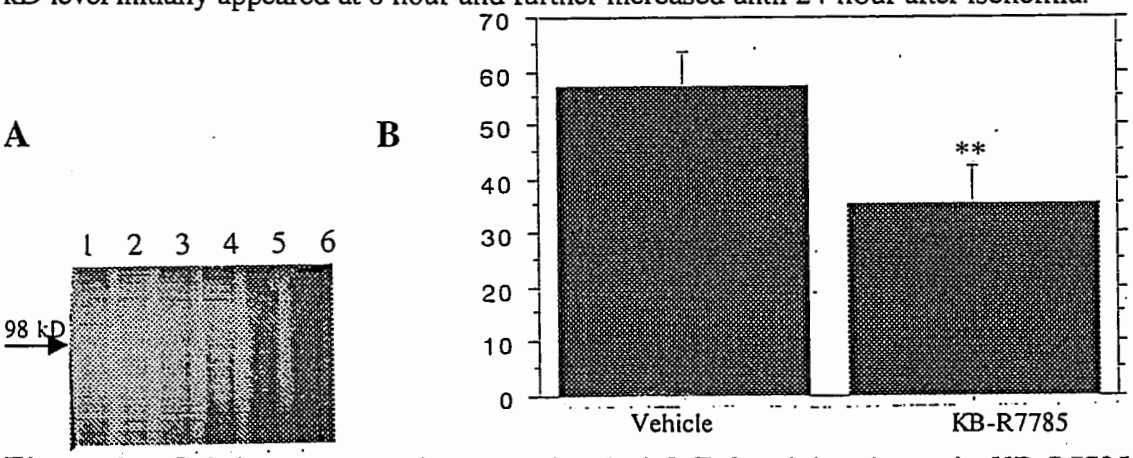

Figure 2 Gelatin zymogram demonstrating the MMP-9 activity change in KB-R7785 treated mice. (A) Lane 1, 2, 3 represent vehicle-control group and Lane 4, 5, 6 represent KB-R7785 treated group. All of these brain samples were taken at 24 hours after MCAO. From the zymogram we can easily identify that 98 kD band in vehicle group appeared more bright than that in KB-R7785 treated group, indicating that KB-R7785 inhibits activity of MMP-9 in ischemic brain. (B) Comparison of the density of 98 kD band reflecting MMP-9 activity between vehicle- and KB-R7785 treated mice. N = 9 and 10 for vehicle and KB-R7785, respectively. Data are mean ± SEM. **, $p < 0.01$ as compared with controls.

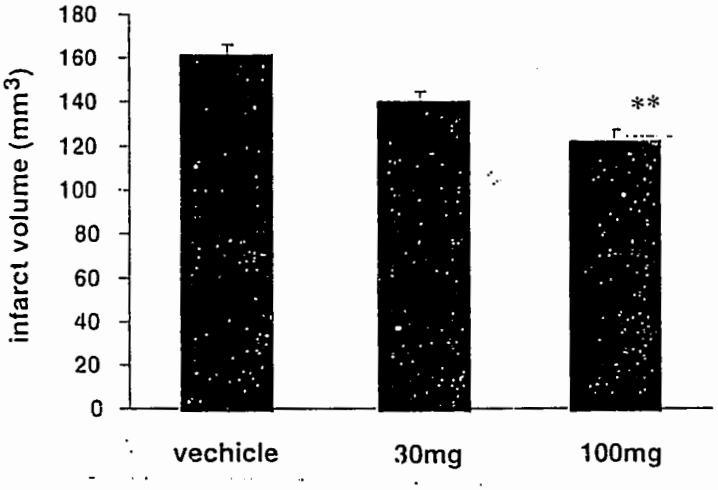

Figure 3 KB-R7785 reduces infarct volume in a dose-dependent manner when administered 30 minutes before MCAO. Infarct volume was determined at 24 hours after MCAO as described in methods. Treatment with KB-R7785 (100mg/kg) significantly decreased infarct volume compared with vehicle. n = 15-17 in each group. Data are mean±SEM. **, $p < 0.01$ as compared with controls.

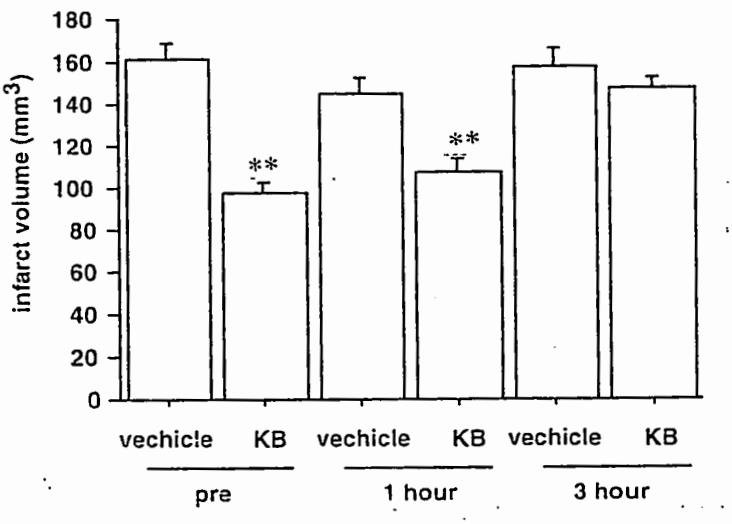

Figure 4 Delayed administration of KB-R7785 at 1 hour after MCAO protect brain against ischemia. Mice received initial injection of vehicle or KB-R7785 (100mg/kg) at 30min before (pre), or 1 or 3 hour after MCAO and then the second injection 3.5 hours thereafter. Infarct volume was determined at 24 hours after MCAO. KB-R7785 injection initiated at 30 min before and 1 hour after MCAO significantly reduced infarct volume compared with their vehicle-injected control. n = 7-9 in each group. Data are mean ± SEM. **, $p < 0.01$ as compared with controls

Table 1 physiological parameters 30 minutes after MCAO

	Vehicle	30mg/kg	100mg/kg
MABP (mmHg)	72±3	74±2	73±2
reduction of CBF (%)	19±4	18±5	20±4
Arterial PH	7.32±0.02	7.35±0.01	7.33±0.01
Arterial Pco_2 (mmHg)	44±2	45±2	45±1
Arterial Po_2 (mmHg)	156±6	152±11	155±10
Rectal temperature (℃)	37.1±0.3	37±0.2	37.2±0.3
Blood glucose (mg/dl)	166±4	160±3	165±5

Data are mean ± SEM, n = 15 in each group.