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LF 16-0687 Ms, a bradykinin B_2 receptor antagonist, reduces ischemic brain injury in a murine model of transient focal cerebral ischemia

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1 Bradykinin promotes neuronal damage and brain edema through the activation of the B_2 receptor. The neuroprotective effect of LF 16-0687 Ms, a B_2 receptor antagonist, has been described when given prior to induction of transient focal cerebral ischemia in rat, but there are no data regarding the consequence of a treatment when given after injury. Therefore, in a murine model of transient middle cerebral artery occlusion (MCAO), we evaluated the effect of LF 16-0687 Ms given prior to and/or after the onset of ischemia on neurological deficit, infarct volume and inflammatory responses including cerebral edema, blood–brain barrier (BBB) disruption and neutrophil accumulation.

2 LF 16-0687 Ms (1, 2 and 4 mg kg^{-1}) administered 0.5 h before and, 1.25 and 6 h after MCAO, decreased the infarct volume by a maximum of 33% and significantly improved the neurological recovery.

3 When given at 0.25 and 6.25 h after MCAO, LF 16-0687 Ms (1.5, 3 and 6 mg kg^{-1}) decreased the infarct volume by a maximum of 25% and improved the neurological score.

4 Post-treatment with LF 16-0687 Ms (1.5 mg kg^{-1}) significantly decreased brain edema (−28%), BBB disruption (−60%) and neutrophil accumulation (−65%) induced by ischemia. Physiological parameters were not modified by LF 16-0687 Ms.

5 These data emphasize the role of bradykinin B_2 receptor in the development of infarct lesion, neurological deficit and inflammatory responses resulting from transient focal cerebral ischemia. Therefore, B_2 receptor antagonist might represent a new therapeutic approach in the pharmacological treatment of stroke.

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Keywords: Bradykinin B_2 receptor antagonist; focal cerebral ischemia; mice; neuroprotection; brain edema; inflammation

Abbreviations: BBB, blood–brain barrier; BWC, brain water content; LF 16-0687 MS, 1-[[3-[(2,4-dimethylquinolin-8-yl)oxymethyl]−2,4-dichlorophenyl] sulfonyl]-*N*-[3-[[4-(aminoimino-methyl)phenyl]carbonyl-amino]propyl]-2(*S*)-pyrrolidine carboxyamide dimesylate salt; MABP, mean arterial blood pressure; MCAO, middle cerebral artery occlusion; MPO, myeloperoxidase; PaO_2, arterial pressure in O_2; $PaCO_2$, arterial pressure in CO_2

Introduction

Bradykinin is generated in plasma and tissue from kininogen by the action of kallikrein (Bhoola *et al*., 1992). All the components of the kallikrein–kinin system have been identified in brain tissue from various species including human (Kizuki *et al*., 1994; Walker *et al*., 1995; Raidoo *et al*., 1996). The actions of bradykinin are mediated through the G-protein coupled B_2 (constitutive) and B_1 (inducible) receptor subtypes; however the B_2 receptor accounts for the majority of acute physiological effects of bradykinin (Marceau, 1995; Regoli *et al*., 1998). The B_2 receptor has been located on cerebral endothelial cells (Wahl *et al*., 1996), neurons (Raidoo & Bhoola, 1997; Chen *et al*., 2000), astrocytes and microglia (Hosli & Hosli, 1993). In neuronal and neuroglial tissues, activation of the B_2 receptor induces the production and release of proinflammatory mediators such as reactive oxygen species (Rosenblum, 1987; Ellis, 1990; Sobey *et al*., 1997), nitric oxide (Katusic *et al*., 1993; Gorlach & Wahl, 1996), prostanoids (Gecse *et al*., 1989), excitatory amino-acid neurotransmitters (Parpura *et al*., 1994; Jeftinija *et al*., 1996) and cytokines (Cunha *et al*., 1992). The release of such mediators triggers inflammatory reactions resulting in cerebral arteriolar dilatation, loss of cerebrovascular autoregulation, endothelial cell lesion and increased cerebral vascular permeability (Whalley & Wahl, 1983; Unterberg *et al*., 1984; Wahl *et al*., 1996). These events lead to edema formation, blood–brain barrier (BBB) disruption and ultimately neuronal injury and cell death (Unterberg *et al*., 1986; Francel, 1992; Walker *et al*., 1995). Bradykinin is considered as an important mediator of inflammatory responses associated to cerebral injury (Unterberg & Baethmann, 1984; Francel, 1992). An increase in plasma bradykinin levels was observed during global cerebral ischemia in rat (Kamiya *et al*., 1993) and

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activation of the kallikrein–kinin pathway was reported in patients with cerebral infarct (Wagner *et al*., 2002). Although a number of experimental studies showed that the nonpeptide B_2 receptor antagonist, LF 16-0687 Ms (Pruneau *et al*., 1999b), reduced cerebral edema and improved neurological function in rodent models of traumatic brain injury including closed head trauma (Pruneau *et al*., 1999a; Verrecchia *et al*., 2000; Rachinsky *et al*., 2001; Kaplanski *et al*., 2002), controlled cortical impact injury (Stover *et al*., 2000) and cold lesion trauma (Schulz *et al*., 2000; Gorlach *et al*., 2001; Plesnila *et al*., 2001), only few studies have investigated the contribution of the B_2 receptor in cerebral ischemia. Relton *et al*. (1997) demonstrated that CP-0597, a peptide B_2 receptor antagonist, significantly reduced brain swelling, infarct size and improved behavioral outcomes and body weight loss in a rat model of transient focal cerebral ischemia. Using the same model, Zausinger *et al*. (2002) reported that LF 16-0687 Ms was neuroprotective, further supporting a detrimental role of B_2 receptor in the development of ischemic brain damage. However, in this study, LF 16-0687 Ms was given before the induction of ischemia, and to date, there are no data regarding the effect of LF 16-0687 Ms administered after the onset of ischemia. In addition, there is no experimental study examining the consequence of a treatment with a B_2 receptor antagonist in ischemic mice brain.

Therefore, the main purpose of the present study was to evaluate the potential therapeutic value of LF 16-0687 Ms administered after the onset of ischemia on neurological deficit, histological damage and inflammatory responses including cerebral edema, BBB disruption and neutrophil accumulation following transient middle cerebral artery occlusion (MCAO) in mice.

Methods

Drugs

LF 16-0687 Ms (1-[[3-[(2,4-dimethylquinolin-8-yl)oxymethyl]-2,4-dichlorophenyl]sulfonyl]-*N*-[3-[[4-(aminoiminomethyl)-phenyl]carbonyl-amino]propyl]-2(S)-pyrrolidinecarboxyamide dimesylate salt) was synthesized as a mesylate salt at Laboratoires Fournier (Daix, France). This compound was dissolved in saline and injected subcutaneously ($10\,ml\,kg^{-1}$) in all the experiments. Doses of LF 16-0687 Ms used in the present study refer to the base form.

Transient focal cerebral ischemia

All experiments were performed on male Swiss mice weighing 22–25 g (Charles River, France) housed under a controlled temperature, 12 h light/dark cycle and with access to food and water *ad libitum*. Animal care was in compliance with French regulations on protection of animals used for experimental and other scientific purposes (D2001-486), as well as with the EEC regulations (Official Journal of European Community L358 12/18/1986).

Mice were anesthetized with chloral hydrate (Sigma, France) at the dose of $400\,mg\,kg^{-1}$ (i.p. $10\,ml\,kg^{-1}$). Body temperature was maintained at $37\pm0.5^{\circ}C$ by means of a heating blanket and a heating lamp throughout the entire experimental procedure. Focal cerebral ischemia was induced by left MCAO with the use of an intraluminal filament technique, as previously described (Ding-Zhou *et al*., 2002). Briefly, the left common and external carotid arteries were isolated and ligated with a 4-0 silk suture through a midline neck incision. An arteriotomy was fashioned in the common carotid artery just proximal to the carotid bifurcation. A 6-0 nylon monofilament (Ethicon), blunted at the tip with an open flame, was introduced through this incision into the internal carotid artery and advanced approximately 13 mm distal to the carotid bifurcation for occlusion of the origin of the middle cerebral artery. The thread was carefully withdrawn 15 min after MCAO. After recovery from anesthesia, animals were returned to their cages and placed in a heating incubator at 29°C with free access to food and water during the entire postoperative period (24 or 48 h). In sham-operated mice, the carotid arteries were prepared surgically, but the filament was not inserted (Kamii *et al*., 1994).

Experimental protocols

Study I: effect of pre- and postischemic treatment with LF 16-0687 Ms Treatment with LF 16-0687 Ms or its vehicle (saline) was started 0.5 h before ischemia, and repeated 1.25 and 6 h after the onset of ischemia. Mice were randomly assigned to five groups: group 1, non-operated mice; group 2, ischemic mice treated with saline; groups 3–5, ischemic animals treated with LF 16-0687 Ms at the dose of 1, 2 and $4\,mg\,kg^{-1}$. The mice thus received a cumulative dose of 3, 6 and $12\,mg\,kg^{-1}$ respectively. This dosing schedule has been selected according to the studies of Zausinger *et al*. (2002). These authors have shown that LF 16-0687 Ms at low dose, given 0.5 h before ischemia, at onset of reperfusion and 6 h after reperfusion, reduced ischemic brain damage in rats subjected to 1.5 h MCAO. At 48 h after ischemia, neurological deficit and brain infarction were determined.

Study II: effect of postischemic treatment with LF 16-0687 Ms Treatment with LF 16-0687 Ms or its vehicle (saline) was given 0.25 and 6.25 h after the onset of ischemia. Mice submitted to MCAO were randomly assigned to five groups: group 1, non-operated mice; group 2, ischemic mice treated with saline; groups 3–5, ischemic mice treated with LF 16-0687 Ms at the dose of 1.5, 3 and $6\,mg\,kg^{-1}$. Thus, here again, the mice received a cumulative dose of 3, 6 and $12\,mg\,kg^{-1}$ respectively. Neurological deficit and infarct volume were determined 48 h after ischemia.

Furthermore, the effect of LF 16-0687 Ms given postischemia was investigated on cerebral edema, BBB disruption and neutrophil accumulation. In each experiment, four groups were studied: a group of non-operated mice and a group of sham-operated mice served as controls, and two ischemic groups were treated with either saline or LF 16-0687 Ms at the dose of $1.5\,mg\,kg^{-1}$ given 15 min and 6.25 h after MCAO (total dose of LF 16-0687 Ms $3\,mg\,kg^{-1}$). Cerebral edema and the BBB disruption were examined 24 h after ischemia, and neutrophil accumulation was evaluated 48 h after MCAO. These time points were chosen on the basis of our preliminary studies concerning the time course of these ischemic outcomes in our model (Ding-Zhou *et al*., 2002; unpublished observations).

Evaluation of neurological deficit

Sensorimotor neurological deficit was assessed by a grip test (Hall, 1985). Each mouse was picked up by the tail and placed on a taut string 60 cm long suspended 40 cm above a table. Grip score was measured as the length of time (in seconds) that the mouse remained on the string in some manner (using one or more paws, tail, tail plus paws), for a maximum of 30 s. Each experiment was conducted randomly and blindly. Neurological examinations were assessed only once in non-operated, vehicle- and LF 16-0687 Ms-treated ischemic mice, so the neurogical evaluation was not performed before the treatment. This choice is made on the basis of studies of Hall (1985) who has reported that if mice were placed repeatedly on the string, some of them could be made to perform better. This phenomena is probably related to the effect of training and memory. So it is useful to allow each mouse an equal opportunity when performing this neurological test.

Measurement of infarct volume

After neurological score evaluation, mice were killed with an overdose of sodium pentobarbitone (200 mg kg^{-1}, i.p. , Sanofi, France), their brains were removed and sectioned into six 1 mm-thick coronal slices using a tissue Chopper (McIlwain, The Mickle laboratory Engineering, England). Coronal brain slices were immediately immersed into 2% 2,3,5,-triphenyltetrazolium chloride (Sigma, France) for 20 min at room temperature in the dark followed by fixation in a 4% paraformaldehyde solution (Prolabo, France) overnight prior to analysis (Bederson *et al*., 1986). The infarction area, outlined in white, was measured on the posterior surface of each section using a computer image analysis system (Imstar, Paris, France) and corrected for brain edema according to Golanov & Reis (1995). Infarct volume, expressed in mm^3, was calculated by a linear integration of the corrected lesion areas.

Evaluation of cerebral edema

Cerebral edema was determined by measuring the brain water content (BWC) according to the wet–dry method (Hatashita *et al*., 1988). The mice were killed and brains were immediately removed and placed on a frozen plate. Tissue samples were dissected out from infarct areas in ischemic mice and from corresponding areas in sham-operated and non-operated animals. Samples were immediately weighed to obtain wet weight. Then, samples were dried in a desiccating oven at 110°C for 24 h and weighed again to obtain the dry weight. BWC was calculated as follows: BWC (%) = (wet weight − dry weight) × 100/wet weight.

Evaluation of BBB permeability

The integrity of the BBB was investigated using Evans blue extravasation (Chan *et al*., 1991; Ikeda *et al*., 1994). Evans blue at 1% in saline (100 μl) was injected into the tail vein. After 60 min, the chest wall was opened under sodium pentobarbitone anesthesia (55 mg kg^{-1}, i.p.). Animals were perfused transcardially with saline at 100 mmHg pressure until blue color was absent from the effluent. Brains were then removed, tissue samples were dissected out as described above, weighed, placed in 400 μl of pure formamide (Sigma, France) and incubated for 72 h in the dark at 50°C. The optical density of the formamide solution was measured at 620 nm. Data are expressed as μg Evans blue g^{-1} tissue.

Evaluation of neutrophil accumulation

Myeloperoxidase (MPO) activity was used as an indicator of neutrophil accumulation in brain parenchyma (Barone *et al*., 1991). Mice were anesthetized with sodium pentobarbitone (55 mg kg^{-1}, i.p.) and transcardially perfused with 200 ml of saline at 100 mmHg. Brains were removed, samples were dissected out as previously described and immediately frozen at −40°C until the assay. Frozen tissue samples were weighted and homogenized with an ultra thurax (Ika Labortehnik, Germany) in 20 w v^{-1} of 5 mmol l^{-1} potassium phosphate buffer (4°C, pH 6) followed by centrifugation for 30 min at 25,000 × *g* at 4°C. Supernatants were discarded, and the pellets were washed again as described above. MPO was extracted by suspension in 0.5% hexadecyltrymethylammonium bromide in 10 w v^{-1} 50 mmol l^{-1} potassium phosphate buffer (pH 6, 25°C). The samples were immediately frozen in methyl-2 butane at −40°C, and three freeze–thaw cycles were performed with 10 s sonication (Vibra Cell, Fischer Bioblock Scientific, France) between each cycle at 25°C. Samples were then incubated at 4°C for 20 min and centrifuged at 12,500 × *g* for 15 min at 4°C. An aliquot of 7 μl of the supernatant was then allowed to react at 25°C with 200 μl of mixed solution containing 0.167 mg ml^{-1} *o*-dianisidine dihydrochloride (Sigma, France) and 0.0005% hydrogen peroxide (Sigma, France) in 50 mmol l^{-1} potassium phosphate buffer (pH 6).

The kinetic change in absorbance at 460 nm during 2 min was assayed using a colorimetric microplate reader (Dynex, MRX France). One unit of MPO activity was defined as the degradation of 1 μmol of hydrogen peroxide per minute at 25°C and was expressed in units per gram weight of wet tissue (U MPO g^{-1}).

Effect of LF 16-0687 Ms on physiological variables

The carotid artery was cannulated to study the effect of LF 16-0687 Ms on mean arterial blood pressure (MABP), arterial blood gases and pH. The arterial catheter was connected to a pressure transducer (EMKA Technologies). Once MABP was stabilized, animals were treated with either LF 16-0687 Ms at the dose of 1.5, 3 and 6 mg kg^{-1} or its vehicle. MABP was recorded during 2 h. Arterial blood samples were collected 15, 30, 60, 90 and 120 min after treatment to analyze pH, partial pressure in arterial oxygen and carbon dioxide (PaO_2 and $PaCO_2$), using a blood gas and pH analyzer (ABL 330, Radiometer, France). Body temperature was maintained at 37 ± 0.5°C by means of a heating blanket throughout the experiment.

Data expression and statistical analysis

Data are expressed as means ± s.e.m. (standard error of mean) of *n* observations, where *n* represents the number of animals or samples. Comparisons between multiple groups were evaluated by one-way analysis of variance (ANOVA) followed by protected least-significant difference (PLSD) Fisher's test. The effect of surgical procedure was first evaluated by comparing sham-operated group *versus* the non-operated

group using one-way ANOVA followed by Dunnett's test. Comparisons between sham-operated and ischemic groups were then evaluated by one-way ANOVA followed by PLSD Fisher's test. When measures were repeated (physiological variables), data were analyzed by a two-factorial ANOVA for repeated measures. A P-value less than 0.05 was considered to be statistically significant.

Results

Study I: effect of pre- and postischemic treatment with LF 16-0687 Ms

Effect on neurological deficit At 48 h after MCAO, the grip score reflected a severe impairment of sensory motor function in vehicle-treated ischemic mice as compared with non-operated mice (9.8 ± 4.0 *versus* 29.7 ± 0.3 s, $P<0.001$) (Figure 1a). LF 16–0687 Ms improved the grip score in a dose-related manner with a significant effect at 2 and 4 mg kg^{-1} (22.3 ± 3.4 and 25.2 ± 3.1 s, $P<0.01$ *versus* vehicle-treated ischemic mice).

Effect on infarct volume At 48 h after transient MCAO, an infarct developed in the cerebral cortex and the striatum (80 ± 4 mm^3) (Figure 1b). Administration of LF 16-0687 Ms at 1, 2 and 4 mg kg^{-1} significantly reduced the total infarct volume by 33% (54 ± 10 mm^3, $P<0.01$), 23% (62 ± 7 mm^3, $P<0.05$) and 33% (54 ± 7 mm^3, $P<0.01$) respectively.

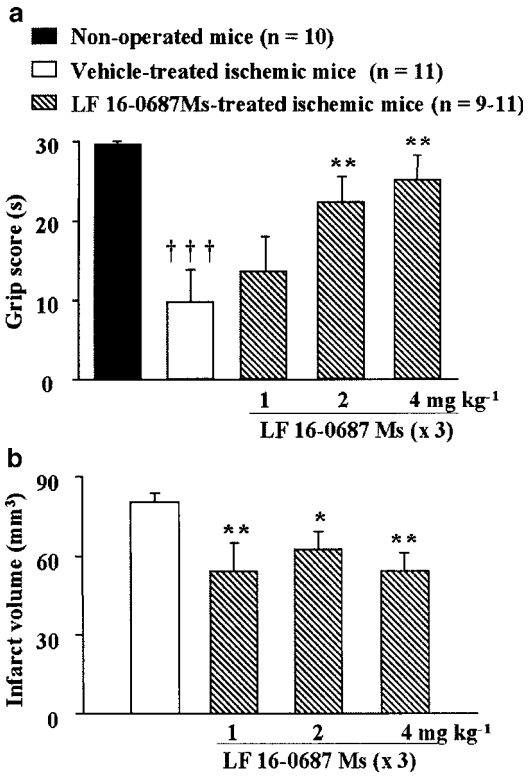

Figure 1 Effect of pre- and postischemic treatment with LF 16-0687 Ms on (a) grip test and (b) infarct volume 48 h after MCAO. Vehicle and LF 16-0687 Ms at 1, 2 and 4 mg kg^{-1} were given (s.c.) 0.5 h before, 1.25 and 6 h after MCAO. Experimental groups consisted of 9–11 mice. Values are means ± s.e.m. †††$P<0.001$ *versus* non-operated mice, *$P<0.05$ and **$P<0.01$ *versus* vehicle-treated mice.

Study II: effect of postischemic treatment with LF 16-0687 Ms

Effect on neurological deficit At 48 h after transient MCAO, LF 16-0687 Ms given 0.25. and 6.25 h postischemia at doses of 1.5, 3 and 6 mg kg^{-1} improved the grip score (23.1 ± 2.4 s, $P<0.001$; 21.9 ± 2.1 s, $P<0.001$ and 20.1 ± 2.9 s, $P<0.01$ *versus* 8.8 ± 3.3 s in vehicle-treated ischemic mice, respectively) (Figure 2a).

Effect on infarct volume At 48 h after transient MCAO, administration of LF 16-0687 Ms at 1.5, 3 and 6 mg kg^{-1} significantly reduced the total infarct volume by 25% (65 ± 5 mm^3, $P<0.01$), 25% (65 ± 5 mm^3, $P<0.01$) and 17% (71 ± 6 mm^3, $P<0.05$), respectively, compared with vehicle-treated ischemic mice (86 ± 3 mm^3) (Figure 2b). This histological protection was more prominent in the cortical infarct area (25–35%) than in the striatal lesion (10–14%).

Effect on brain water content At 24 h after transient MCAO, the BWC in the ischemic area was significantly increased in vehicle-treated mice compared with that of sham-operated mice (85.8 ± 0.5 *versus* 80.1 ± 0.1%, $P<0.001$) (Figure 3). LF 16-0687 Ms at a dose of 1.5 mg kg^{-1}, given 0.25 and 6.25 h after MCAO reduced the BWC increase (84.2 ± 0.7%, $P<0.05$ *versus* vehicle-treated mice). The BWC

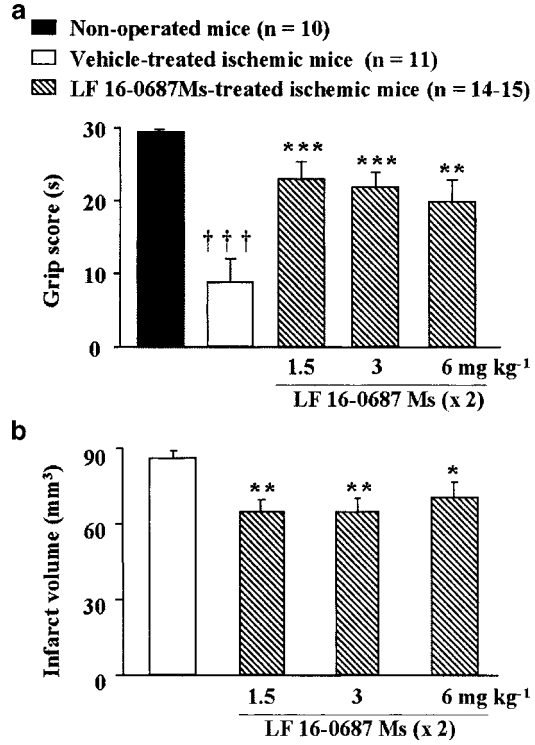

Figure 2 Effect of postischemic treatment with LF 16-0687 Ms on (a) grip test and (b) infarct volume 48 h after MCAO. Vehicle and LF 16-0687 Ms at 1.5, 3 and 6 mg kg^{-1} were given (s.c.) 0.25 and 6.25 h after MCAO. Experimental groups consisted of 10–15 mice. Values are means ± s.e.m. †††$P<0.001$ *versus* non-operated mice, *$P<0.05$, **$P<0.01$ and ***$P<0.001$ *versus* vehicle-treated mice.

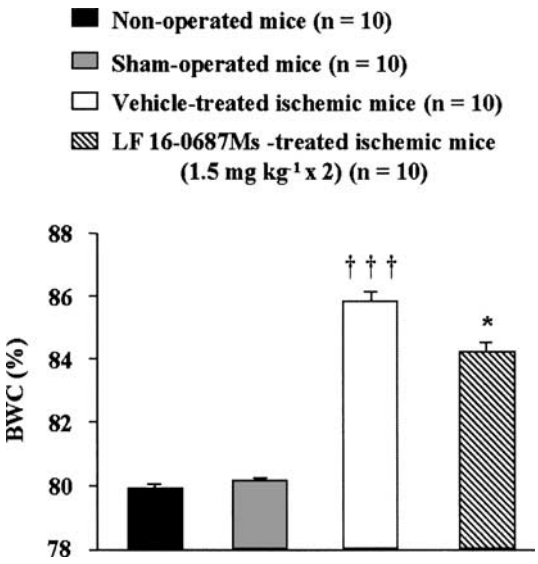

Figure 3 Effect of LF 16-0687 Ms on the brain water content (BWC) measured 24 h after MCAO. LF 16-0687 Ms (1.5 mg kg^{-1}) was given (s.c.) 0.25 and 6.25 h after MCAO. Experimental groups consisted of 10 mice. Values are means ± s.e.m. †††$P<0.001$ *versus* sham-operated mice, *$P<0.05$ *versus* vehicle-treated mice.

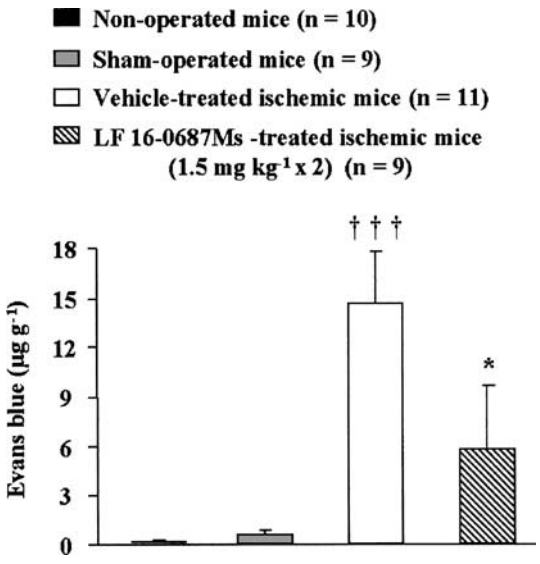

Figure 4 Effect of LF 16-0687 Ms on Evans blue extravasation measured 24 h after MCAO. LF 16-0687 Ms (1.5 mg kg^{-1}) was given (s.c.) 0.25 and 6.25 h after MCAO. Experimental groups consisted of 9–11 mice. Values are means ± s.e.m. †††$P<0.001$ *versus* sham-operated mice, *$P<0.05$ *versus* vehicle-treated mice.

in sham-operated mice was not significantly different from that of non-operated mice.

Effect on Evans blue extravasation Evans blue content in brain tissue was similar in sham-operated and non-operated mice ($0.6\pm0.3\,\mu g\,g^{-1}$ tissue *versus* $0.1\pm0.0\,\mu g\,g^{-1}$ tissue) (Figure 4). At 24 h after MCAO, Evans blue content in the area of infarction was markedly increased in the vehicle-treated group ($14.6\pm3.1\,\mu g\,g^{-1}$ tissue, $P<0.001$ *versus* sham-operated mice). In mice treated with 1.5 mg kg^{-1} of LF 16-0687 Ms, 0.25 and 6.25 h after MCAO, Evans blue content was reduced by 60% ($5.8\pm3.9\,\mu g\,g^{-1}$ tissue, $P<0.05$ *versus* vehicle-treated group).

Effect on brain myeloperoxidase activity At 48 h after ischemia, vehicle-treated mice exhibited a massive increase in MPO activity compared with sham-operated mice (0.189 ± 0.004 *versus* $0.010\pm0.003\,U\,g^{-1}$, $P<0.001$) (Figure 5). When LF 16-0687 Ms was given at a dose of 1.5 mg kg^{-1}, 0.25 and 6.25 h after MCAO, the increased MPO activity was reduced by 65% ($0.066\pm0.025\,U\,g^{-1}$, $P<0.001$ *versus* vehicle-treated mice). There was no significant difference between MPO activity in sham-operated and non-operated mice.

Effect on physiological variables Administration of LF 16-0687 Ms at 1.5, 3 and 6 mg kg^{-1} did not result in any effect on MABP, PaO_2, $PaCO_2$ and pH (Table 1). Physiological variables were within the normal physiological ranges throughout the experiment without significant difference between vehicle-treated and LF 16-0687 Ms-treated animals.

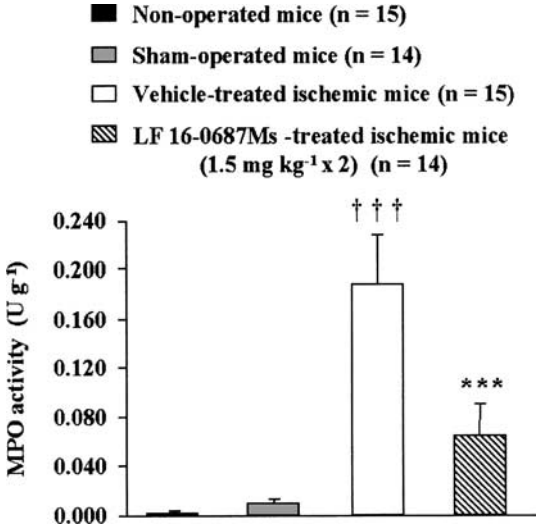

Figure 5 Effect of LF 16-0687 Ms on myeloperoxidase activity (MPO) measured 48 h after MCAO. LF 16-0687 Ms (1.5 mg kg^{-1}) was given (s.c.) 0.25 and 6.25 h after MCAO. Experimental groups consisted of 14–15 mice. Values are means ± s.e.m. †††$P<0.001$ *versus* sham-operated mice, ***$P<0.001$ *versus* vehicle-treated mice.

Discussion

In the present study, we evaluated the neuroprotective effect of LF 16-0687 Ms, a selective bradykinin B_2 receptor antagonist, in mice submitted to transient focal cerebral ischemia. We demonstrated that LF 16-0687 Ms reduced the infarct volume and the neurological deficit even when the treatment was initiated after the onset of MCAO. Furthermore, we showed that LF 16-0687 Ms decreased cerebral edema, BBB disruption and neutrophil accumulation following ischemia. These results suggest that bradykinin, through the activation of B_2 receptor, plays a detrimental role in the development of ischemic damage and support the development of LF 16-0687 Ms as a powerful therapeutic approach of stroke.

LF 16-0687 Ms is a selective nonpeptide antagonist of the bradykinin B_2 receptor (Pruneau *et al*., 1999a) which is about 1000-fold more potent than CP-0127, another B_2 receptor antagonist (Cheronis *et al*., 1992; Pruneau *et al*., 2000) and

Table 1 Physiological variables for saline and LF 16-0687 Ms-treated mice

	MABP (mmHg)	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	*pH*
Vehicle				
Before	89±8	122±6	37.5±2.2	7.38±0.03
15 min after	89±9	118±6	40.3±2.8	7.38±0.02
30 min after	87±9	122±3	42.5±1.6	7.34±0.02
60 min after	90±9	121±3	37.1±2.0	7.33±0.03
90 min after	90±7	121±5	41.6±2.0	7.35±0.03
120 min after	90±5	116±6	39.4±2.2	7.39±0.02
LF 16-0687 Ms (1.5 mg kg^{-1})				
Before	87±5	122±6	37.5±2.2	7.38±0.03
15 min after	90±5	113±2	40.5±2.1	7.39±0.03
30 min after	93±3	118±5	37.8±1.2	7.38±0.03
60 min after	90±5	114±6	38.6±2.6	7.33±0.03
90 min after	86±7	125±3	40.1±2.2	7.34±0.03
120 min after	87±8	120±4	38.7±1.8	7.38±0.01
LF 16-0687 Ms (3 mg kg^{-1})				
Before	87±7	122±6	37.5±2.2	7.38±0.03
15 min after	91±7	117±3	36.7±1.0	7.36±0.03
30 min after	90±5	120±6	40.5±1.8	7.34±0.03
60 min after	89±5	119±7	39.1±2.0	7.34±0.03
90 min after	84±7	113±7	41.9±1.9	7.34±0.03
120 min after	89±7	111±4	40.0±2.5	7.38±0.03
LF 16-0687 Ms (6 mg kg^{-1})				
Before	90±6	122±6	37.5±2.2	7.38±0.03
15 min after	91±6	112±6	42.3±2.6	7.36±0.02
30 min after	94±6	120±7	42.0±3.1	7.34±0.01
60 min after	92±7	119±7	41.2±2.3	7.33±0.03
90 min after	88±7	120±7	42.3±3.0	7.34±0.03
120 min after	90±7	114±5	40.3±2.4	7.40±0.03

Values of MABP, $PaCO_2$, PaO_2 and pH were measured before and after treatment of LF 16-0687 Ms (1.5, 3 and 6 mg kg^{-1}) or its vehicle. There were no significant differences between groups. Values are means±s.e.m. ($n=6$ per group).

which is devoid of partial agonist activity (Pruneau *et al*., 1999b) in contrast with Hoe 140, another B_2 receptor antagonist (Hock *et al*., 1991; Wirth *et al*., 1991; Feletou *et al*., 1994). Pharmacokinetic studies in mice indicated that LF 16-0687 Ms given s.c. had a rapid onset of action with a maximal plasma value achieved at 1 h. In agreement with previous observations (Pruneau *et al*., 1999a; Stover *et al*., 2000; Plesnila *et al*., 2001), we showed that LF 16-0687 Ms had no adverse effects on MABP, blood gases and pH.

The mechanism by which bradykinin contibutes to the development of ischemic brain injury remains unclear. Previous studies that investigated the differential role of B_1 and B_2 receptors after focal cerebral ischemia (Relton *et al*., 1997) and cold cortical injury (Gorlach *et al*., 2001) demonstrated that blockade of the B_2 receptor was protective against neuronal deficit and survival in agreement with the present study, while the B_1 receptor was apparently not involved. In the brain of mammals, B_2 receptor is present on endothelial cells, glial cells and neurons (Walker *et al*., 1995; Raidoo *et al*., 1996; Chen *et al*., 2000). Activation of this receptor results in stimulation of the membrane phosphatidyl–inositol cascade, activation of protein kinase C leading to generation of inositol-1,4,5,-triphosphate and diacylglycerol (Raidoo & Bhoola, 1998). Activation of these intracellular second-messenger systems ultimately leads to the production of prostaglandins (Gecse *et al*., 1989), nitric oxide (Katusic *et al*., 1993; Gorlach & Wahl, 1996) and free radicals (Rosenblum, 1987; Ellis, 1990; Sobey *et al*., 1997), which activate Ca^{2+}-dependent K^+ channels resulting in an increase of neuronal intracellular calcium levels (Parpura *et al*., 1994; Sobey *et al*., 1997). These endogenous substances are well-known mediators of cell damage. Bradykinin also triggers the production of other inflammatory mediators such as cytokines (Cunha *et al*., 1992; Schwaninger *et al*., 1999), P-selectin (Rozsa *et al*., 1998) and platelet-activating factor (McIntyre *et al*., 1985; Satoh *et al*., 1995). In addition, bradykinin has been shown to induce glutamate and aspartate release from cultured astrocytes (Parpura *et al*., 1994; Jeftinija *et al*., 1996) and neurons (Rydh-Rinder *et al*., 2001), these two excitatory amino acids playing a detrimental role in ischemic situations. All of these actions are believed to contribute to pathophysiological disturbance following cerebral ischemia including the development of cerebral edema, an increase of BBB permeability, neutrophil accumulation and ultimately cell destruction (Francel, 1992). These deleterious actions of bradykinin together with the identification of bradykinin receptor throughout the brain (Hosli & Hosli, 1993; Wahl *et al*., 1996; Raidoo *et al*., 1996; Raidoo & Bhoola, 1997; Chen *et al*., 2000) as well as the activation of the kallikrein–kinin system following cerebral injury (Maier-Hauff *et al*., 1984; Kamiya *et al*., 1993; Makevnina *et al*., 1994), provide a strong rationale for the anti-ischemic action of LF 16-0687 Ms. LF 16-0687 Ms was shown to attenuate brain damage and edema formation in rodent models of traumatic brain injury including closed head injury (Pruneau *et al*., 1999a; Verrecchia *et al*., 2000; Kaplanski *et al*., 2002), controlled cortical impact injury (Stover *et al*., 2000) and cryogenic brain lesion (Schulz *et al*., 2000; Plesnila *et al*., 2001). In a rat model of transient focal

cerebral ischemia, Zausinger *et al*. (2002) have reported that 4 mg kg^{-1} of LF 16-0687 Ms given 0.5 h before ischemia, at onset of reperfusion and 6 h after reperfusion (i.e. 12 mg kg^{-1} day^{-1}) significantly attenuated brain damage and cerebral edema, while higher doses failed to affect infarct volume. This significant dose-dependent effect of LF 16-0687 Ms, together with other consistent findings in traumatic brain injury (Verrecchia *et al*., 2000; Plesnila *et al*., 2001), led us to the dosing regime used in the present experiments. The neuroprotective effects of LF 16-0687 Ms were first confirmed by using a similar pre- and post-treatment strategy. Then, we examined the effect of a post-treatment alone. One of the principal findings of this study is that LF 16-0687 Ms at low doses, given even after the onset of MCAO, can still reduce the infarct volume and improve neurological function with a similar magnitude as to the treatment begun before MCAO. In accordance with our finding, it was shown in a rat model of brain trauma that initiation of LF 16-0687 Ms treatment either 1 h or prior to after the insult was similarly efficient in improving neurological function recovery and in reducing cerebral edema (Rachinsky *et al*., 2001). Verrecchia *et al*. (2000) reported that protection could even be accomplished when the onset of treatment was delayed up to 2 h after closed head injury in mice.

The time course of brain edema formation and BBB damage has been previously studied in our murine model of transient MCAO. We showed that the BWC significantly increased 4 h after MCAO, an effect that remained significant for up to 48 h (unpublished observation), and that Evans blue extravasation occurred 6 h after MCAO with a maximum at 24 h (Ding-Zhou *et al*., 2002). The reduction of cerebral edema and of BBB disruption after a postischemic treatment with LF 16-0687 Ms suggests that BBB damage, leading predominantly to vasogenic brain edema, is a target of LF 16-0687 Ms. Previous data showed that the progression of edema closely correlated with the rise of plasma and tissue bradykinin levels after global ischemia in rat (Kamiya *et al*., 1993). Accordingly, a significant enhancement of brain kininogen content, which was accompanied by protein leakage and increase of cerebral water content, was detected in rat after fluid percussion injury (Ellis *et al*., 1989). These results are consistent with other reports, showing that bradykinin synthesis inhibitors and B_2 receptor antagonists decreased cerebral edema in both global and transient focal cerebral ischemia in rat (Kamiya *et al*., 1993; Relton *et al*., 1997; Zausinger *et al*., 2002). Cytotoxic edema is also known to participate in brain swelling and neuronal death. However, the present study did not allow to assess if the reduction of brain edema by LF 16-0687 Ms is also due to reduction of the cytotoxic edema.

The inflammatory reaction is a major contibutor to progression of ischemic infarction (Sornas *et al*., 1972; Kochanek & Hallenbeck, 1992; Matsuo *et al*., 1995; Liao *et al*., 2001) and some reports support the view that bradykinin is a chemotactic agent for neutrophils (Bhoola *et al*., 1992; Bhoola, 1996; Carl *et al*., 1996). As we previously observed a maximum neutrophil accumulation 48 h after MCAO (unpublished data), this led us to investigate the effect of the blockade of B_2 receptor on neutrophil accumulation. We found that the MPO activity increase induced by MCAO was significantly inhibited by the post-treatment with LF 16-0687 Ms, suggesting that the inhibition of neutrophil accumulation participated in the neuroprotective effect of LF 16-0687 Ms. Interestingly, B_2 receptor has been localized on the external surface of neutrophils (Haasemann *et al*., 1994). Reduction of MPO activity in a model of traumatic shock in intestinal tissue (Christopher *et al*., 1994) and attenuation of neutrophil rolling and sticking following global cerebral ischemia (Lehmberg *et al*., 1998, 2000) have also been reported with CP-0127, another B_2 receptor antagonist. Similarly, the neutrophil accumulation induced by either bradykinin or the venom of *Phoneutria nigriventer* spider in rat pleural exudate (Costa *et al*., 2002) and the increased leukocyte adhesion induced by high concentration of bradykinin in rat mesentery (Shigematsu *et al*., 1999) were markedly attenuated by Hoe-140, another B_2 receptor antagonist. In addition to its local production in ischemic inflamed tissue and transudation from plasma due to BBB leakage, bradykinin can also reach inflammatory sites through infiltrating neutrophils which represent another effective way of delivering high concentrations of bradykinin at the site of inflammation (Bockmann & Paegelow, 2000; Bhoola *et al*., 2001). In this respect, there are data suggesting that neutrophils contain the complete system for the generation of bradykinin (Gustafson *et al*., 1989; Bockmann & Paegelow, 2000).

In conclusion, our results show that a post-treatment with LF 16-0687 Ms significantly reduced infarct volume, neurological deficit and inflammatory responses including cerebral edema, BBB disruption and neutrophil accumulation. The present findings provide further support for the therapeutic development of LF 16-0687 Ms in stroke and emphasize the key role of bradykinin B_2 receptor as a mediator of secondary brain damage.

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