

# LF 16-0687 Ms, a New Bradykinin B<sub>2</sub> Receptor Antagonist, Improves Neurologic Outcome but Not Brain Tissue Prostaglandin E<sub>2</sub> Release in a Rat Model of Closed Head Trauma Combined with Ethanol Intoxication

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**Background:** LF 16-0687 Ms previously was reported to improve Neurologic Severity Score (NSS) and decrease cerebral edema and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) release after closed head trauma (CHT) in rats. Here, we examined whether these beneficial effects of LF 16-0687 Ms are altered when CHT is accompanied by acute ethanol administration.

**Methods:** Six groups of rats (n = 8 per group) were examined during combination of the following experimental con-

ditions: CHT versus sham operation, LF 16-0687 Ms 3 mg/kg subcutaneously versus saline, and ethanol 2 g/kg versus saline.

**Results:** After CHT, brain water content decreased and NSS improved with ethanol + LF 16-0687 Ms as compared with values after saline or ethanol. PGE<sub>2</sub> release decreased with ethanol (147 ± 59 pg/mg tissue) but not with ethanol + LF 16-0687 Ms (286 ± 194 pg/mg tissue).

**Conclusion:** Ethanol does not affect

the improvement of NSS and the decrease of cerebral edema seen with LF 16-0687 Ms after CHT, but does reverse the ability of LF 16-0687 Ms to minimize the increase of PGE<sub>2</sub> release. In intoxicated patients, bradykinin antagonist therapy may improve post-CHT outcome without altering PGE<sub>2</sub> release.

**Key Words:** Bradykinin B<sub>2</sub> antagonist, Cerebral edema, Ethanol, Head injury, Neurologic outcome, Prostaglandin, Rat.

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Head injury from motor vehicle crashes secondary to alcohol intoxication is a serious health care problem. It is estimated that more than half of the fatal motor vehicle crashes in the United States involve individuals who have been drinking, and that head injury is the major cause of death in these cases.<sup>1</sup> Numerous studies have examined treatments designed to improve neurologic outcome after head injury, but none has examined the interaction between acute ethanol intoxication and putative “neuroprotective” treatment. On the basis of previous studies of brain injury in which ethanol was given without other “neuroprotective” treatment, it is difficult to predict whether ethanol should augment or impair the effect of such treatment because eth-

anol was reported to improve outcome measures in some models of brain injury but to worsen outcome in others.<sup>2–6</sup>

One treatment designed to improve neurologic outcome after head injury is the antagonism of bradykinin B<sub>2</sub> receptors. Potential beneficial effects of B<sub>2</sub> receptor blockade include decreased release of glutamate, reactive oxygen species, and nitric oxide; suppression of *N*-methyl-D-aspartate receptor-mediated increase in neuronal calcium; decreased blood-brain barrier (BBB) permeability; and reduced formation of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and cerebral edema.<sup>7–13</sup> Recently, we reported that LF 16-0687 Ms, a new nonpeptide B<sub>2</sub> receptor antagonist, improved neurologic outcome and decreased brain edema and PGE<sub>2</sub> formation after closed head trauma (CHT) in rats.<sup>9,14</sup> It is not known whether ethanol interferes with the beneficial effects of B<sub>2</sub> receptor blockade. The present study was designed to assess whether the beneficial effects of LF 16-0687 Ms after CHT (i.e., improved neurologic outcome and decreased cerebral edema and PGE<sub>2</sub> formation) persist when acute ethanol intoxication is combined with CHT.

## MATERIALS AND METHODS

### Experimental Design

The experiments were approved by the Animal Care Committee of Ben-Gurion University of the Negev (Beer-Sheva, Israel). Sixty-one spontaneously breathing Sprague-Dawley rats weighing 200 to 300 g were anesthetized with a mixture of halothane and room air (10 L/min). The rectal

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temperature was maintained at 37°C using a heating pad, and anesthesia was considered as sufficient for surgery when corneal reflexes were abolished. After the scalp was infiltrated with bupivacaine 0.5%, it was incised and reflected laterally. In 25 rats (groups 1–3; random, balanced design), CHT was not delivered and the scalp incision was sutured (sham-operated groups). In another 36 rats (groups 4–6), a cranial impact of 0.5 J was delivered by a silicone-coated rod that protruded from the center of a free-falling plate as previously described.<sup>15</sup> It was previously determined that the energy imparted to the skull through the stereotaxically guided plate was linearly related to the distance of the fall, so that the nonpenetrating impact caused a reproducible brain injury.<sup>15,16</sup> The impact point was 1 to 2 mm left of the midline on the skull's convexity. After CHT, the incision was sutured.

After sham or CHT, all rats were laid on their left side to check righting reflex and recovery time as part of the assessment of Neurological Severity Score (NSS), which was evaluated 1 hour after CHT. The NSS was determined as previously described by a blinded observer.<sup>17</sup> Points were assigned for alterations of motor functions and behavior so that the maximal score of 25 represents severe neurologic dysfunction and a score of 0 indicates an intact neurologic condition. Specifically, the following were assessed: ability to exit from a circle (three-point scale), gait on a wide surface (three-point scale), gait on a narrow surface (four-point scale), effort to remain on a narrow surface (two-point scale), reflexes (five-point scale), seeking behavior (two-point scale), beam walking (three-point scale), and beam balance (three-point scale). After CHT, six rats became apneic and died, and five animals had an NSS < 10 or > 20. These animals were excluded from the study. Animals were excluded on the basis of previous results with this model indicating that with NSS < 10 the neurologic impairment is so minor that "protective" treatment cannot significantly decrease NSS, whereas with NSS > 20 the injury is so severe that no treatment can prevent worsening impairment.<sup>18</sup> The remaining 25 animals were assigned to groups 4 through 6 using a random, balanced design.

The 1-hour assessment of NSS was followed immediately by subcutaneous injection of 3 mg/kg of either LF 16-0687 Ms (groups 3 and 6) or its vehicle, 0.9% saline (groups 1, 2, 4, and 5), and intraperitoneal injection of 2 g/kg of ethanol (groups 2, 3, 5, and 6) or 0.9% saline (groups 1 and

4). The experimental conditions for the six groups are given in Table 1. In this model, assessment of NSS at 1 hour is not used as an outcome measure but instead is used to ensure that the initial severity of injury resulting from CHT does not differ between groups. Pilot studies indicated that when ethanol 2 g/kg was given before a CHT of 0.5 J, the NSS at 1 hour after CHT increased to near maximal values in all rats. With all NSS values at or near maximal, it is no longer possible to use NSS to ensure equivalency of initial severity of injury between groups. Accordingly, in the present study administration of ethanol was delayed until after the determination of NSS at 1 hour after CHT. Although not establishing intoxicating plasma concentrations of ethanol before CHT (as would occur in patients with acute alcohol intoxication before head injury), our methodology did provide intoxicating concentrations of ethanol during the treatment period (see below).

After treatment with ethanol, LF 16-0687 Ms, and/or vehicle, animals were returned to their cages and given free access to food and water. Rats were treated through a gastric tube with 200 mg/kg paracetamol every 8 hours after CHT. At 24 hours, the assessment of NSS was repeated. After the 24-hour assessment of NSS, all rats were killed using a guillotine. Brains were then immediately removed and placed on a frozen plate.

### Brain Edema

Brain tissue samples up to 50 mg were dissected out from areas adjacent to the zone of macroscopic damage in the left hemisphere and from the corresponding area in the right hemisphere. In sham-operated animals, brain tissue samples were dissected from corresponding areas of both hemispheres. These small tissue samples were used for determination of water content and PGE<sub>2</sub> release. Water content was determined from the difference between wet weight (WW) and dry weight (DW). Specifically, after WW of fresh brain tissue samples was obtained, samples were dried in a desiccating oven at 105°C for 24 hours and weighed again to obtain DW. Tissue water content (%) was calculated as [(WW – DW) × 100]/WW.

### Incubation of Brain Slices and PGE<sub>2</sub> Assay

At the same time brain tissue samples were obtained for determination of brain tissue water content, brain tissue sam-

**Table 1** Experimental Groups

Group No.	No. of Rats Meeting Inclusion Criteria	Closed Head Trauma Delivered	Ethanol 2 g/kg Given	LF16-0687 Ms 3 mg/kg Given	No. of Rats Surviving at 24 H
1	8	No	No	No	8
2	8	No	Yes	No	8
3	9	No	Yes	Yes	8
4	9	Yes	No	No	8
5	8	Yes	Yes	No	8
6	8	Yes	Yes	Yes	8

ples (approximately 30 mg each) from the contused area and corresponding area in the right hemisphere were excised. Each sample was incubated for 2 hours in Krebs-Henseleit buffer containing 0.2% glucose and equilibrated with 95% oxygen and 5% carbon dioxide (pH, 7.35–7.4) at 37°C.<sup>19</sup> At the conclusion of the incubation period, the buffer solution was frozen at –20°C for later determination of PGE<sub>2</sub> by radioimmunoassay as previously described.<sup>20</sup> Anti-PGE<sub>2</sub> bovine serum albumin (Bio-Makor, Israel),<sup>3</sup> H-PGE<sub>2</sub> (Amersham, United Kingdom), PGE<sub>2</sub> (for standard curve) (Sigma, United States), and charcoal (Merck, United States) were used. Protein content was determined using the Lowry method (enzyme-linked immunosorbent assay).

### Plasma Ethanol Concentrations

In a separate experiment, 15 rats were anesthetized and CHT was delivered as outlined above. At 1 hour after CHT, rats were randomly assigned to receive intraperitoneal injection of ethanol 1.5 g/kg (n = 5), 2 g/kg (n = 5), or 3 g/kg (n = 5). These doses were selected because they include the range of doses previously reported to cause acute intoxication in rats.<sup>21–24</sup> Blood samples were obtained before injection of ethanol; 5, 15, and 30 minutes after injection; and 1, 2, 4, 8, and 12 hours after injection of ethanol for determination of plasma ethanol concentration.

### Drugs

LF 16-0687 Ms (1-[[[3-[(2,4-dimethylquinolin-8-yl)oxymethyl]-2,4-dichlorophenyl] sulfonyl]-N-3-[[4-(aminomethyl)phenyl] carbonylamino] prophy]-2(S)-pyrrolidinecarboxamide dimesylate) was synthesized at Laboratoires Fournier. Doses of LF 16-0687 Ms used in the present study refer to the base form of the drug.

### Statistical Analysis

Parametric data are given as means ± SD. A multiway analysis of variance followed by a Student-Newman-Keuls

test was performed for water content and PGE<sub>2</sub> release. NSS data are given as median and range and were compared between groups using the Kruskal-Wallis test followed by a Mann-Whitney *U* test. Differences were considered as significant at *p* < 0.05.

### RESULTS

The release of PGE<sub>2</sub> in rats receiving CHT + saline (no ethanol or LF 16-0687 Ms) was increased by about five- to sixfold compared with that in sham-operated rats (Table 2). In the group receiving CHT + ethanol, PGE<sub>2</sub> release (147 ± 59 pg/mg tissue) was decreased compared with that in the group receiving CHT + saline. However, in the group receiving CHT + ethanol + LF 16-0687 Ms, PGE<sub>2</sub> release (286 ± 194 pg/mg tissue) was not significantly different from that in the group receiving CHT + saline.

Twenty-four hours after CHT, edema developed in the injured left hemisphere as indicated by a significant increase in water content. In sham-operated rats, ethanol or ethanol + LF 16-0687 Ms had no effect on brain water content (Fig. 1). In the CHT groups, brain water content in the CHT + ethanol group was not significantly different from that in the CHT group. In contrast, adding LF 16-0687 Ms to CHT + ethanol markedly and significantly reduced the edema in the left hemisphere of rats receiving CHT (80.0 ± 1.9%) as compared with the untreated CHT group (82.9 ± 1.5%).

In rats receiving CHT + saline, NSS was 15 (range, 12–18) and 13 (range, 11–15) at 1 and 24 hours after injury, respectively. NSS values in rats receiving CHT + ethanol were not significantly different from those in rats receiving CHT + saline. By comparison, when LF 16-0687 Ms was added to CHT + ethanol, NSS was 16 (range, 13–19) and 7 (range, 3–11) at 1 and 24 hours, respectively. The improvement in NSS at 24 hours in the CHT + ethanol + LF 16-0687 Ms group was significantly greater than that in the CHT + saline and CHT + ethanol groups, indicating that LF 16-0687

**Table 2** Prostaglandin E<sub>2</sub> Release, Cerebral Edema, and Neurological Severity Score

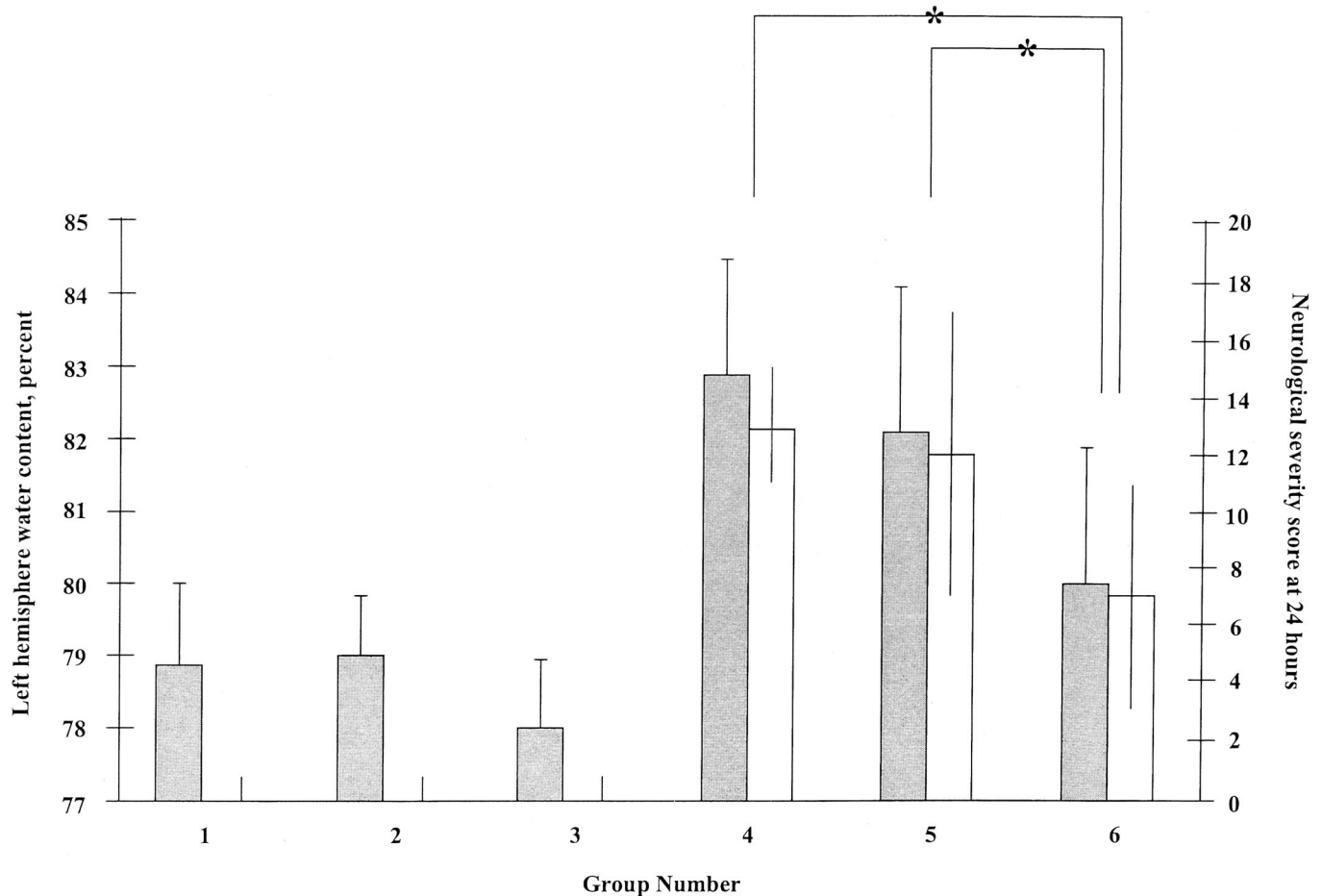
Group No.	Experimental Conditions	PGE <sub>2</sub> Release, Mean ± SD (pg/mg)	Brain Water Content, Mean ± SD (%)		Median Neurologic Severity Score (Range)	
			Left Hemisphere	Right Hemisphere	1 H	24 H
1	Sham + saline	66 ± 26	78.9 ± 0.9	79.0 ± 0.7	0 (0–3)	0 (0–1)
2	Sham + ethanol	43 ± 28	79.0 ± 0.8	79.2 ± 1.2	0 (0–3)	0 (0–1)
3	Sham + ethanol + LF	35 ± 16	78.0 ± 0.9	78.1 ± 0.5	0 (0–3)	0 (0–1)
4	CHT + saline	350 ± 133 <i>p</i> < 0.05 <sup>a</sup>	82.9 ± 1.5 <i>p</i> < 0.05 <sup>a</sup>	80.6 ± 2.4	15 (12–18)	13 (11–15)
5	CHT + ethanol	147 ± 59 <i>p</i> < 0.05 <sup>a,b</sup>	82.1 ± 2.0 <i>p</i> < 0.05 <sup>a</sup>	80.0 ± 0.9	15 (13–17)	12 (7–17)
6	CHT + ethanol + LF	286 ± 194 <i>p</i> < 0.05 <sup>a</sup>	80.0 ± 1.9 <i>p</i> < 0.05 <sup>a,c</sup>	78.7 ± 1.4	16 (13–19)	7 (3–11) <i>p</i> < 0.05 <sup>c</sup>

PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; LF, LF 16-0687 Ms; CHT, closed head trauma to the left hemisphere.

<sup>a</sup> Significantly increased as compared with corresponding sham group.

<sup>b</sup> Significantly decreased compared with CHT + saline group.

<sup>c</sup> Significantly decreased compared with CHT + saline and CHT + ethanol groups.



**Fig. 1.** On the two vertical scales, left hemisphere water content (gray bars) is given as mean  $\pm$  SD, and NSS at 24 hours (open bars) is given as median and range. Group number is given on the horizontal scale. LF, LF 16-0687 Ms; CHT, closed head trauma to the left hemisphere. The experimental conditions for the groups were as follows: 1, sham + saline; 2, sham + ethanol; 3, sham + ethanol + LF; 4, CHT + saline; 5, CHT + ethanol; and 6, CHT + ethanol + LF. Left hemisphere water content and NSSs were increased in the CHT groups as compared with corresponding sham groups. Among the CHT groups, the decrease of left hemisphere water content and NSS with LF (group 6) as compared with saline (group 4) or ethanol (group 5) was statistically significant (\*).

Ms promoted a partial recovery of neurologic function at 24 hours.

During the 24-hour period that followed CHT, two rats (one in group 3 and one in group 4) died. In the 15 rats that received ethanol 1.5, 2, or 3 g/kg solely to permit measurement of plasma ethanol concentrations, concentrations rose quickly after administration of ethanol and then declined progressively over the ensuing 12 hours. Peak ethanol concentrations occurred at 5 minutes and were  $208 \pm 104$ ,  $280 \pm 41$ , and  $450 \pm 46$  mg/dL, respectively, exceeding the blood concentration previously reported to be intoxicating in rats.<sup>24</sup> Plasma ethanol concentrations at 12 hours were  $56 \pm 22$ ,  $59 \pm 23$ , and  $71 \pm 27$  mg/dL, respectively, significantly different from baseline only in the 3-g/kg group.

## DISCUSSION

LF 16-0687 Ms is a novel, nonpeptide, potent, selective B<sub>2</sub> receptor antagonist.<sup>25,26</sup> In contrast with peptide deriva-

tives such as Hoe 140, LF 16-0687 Ms is devoid of partial agonist activity.<sup>27</sup> The principal findings of this study were that acute ethanol intoxication does not change LF16-0687 Ms-induced improvement of NSS or decrease of cerebral edema but reverses the effect of the drug on reduction of PGE<sub>2</sub> release. Our findings that LF 16-0687 Ms improved NSS and decreased edema is consistent with previous reports that antagonism of B<sub>2</sub> receptors improved NSS and decreased edema in rats receiving CHT + saline and extends this effect to a situation mimicking alcohol intoxication.<sup>9</sup> However, the present finding that adding LF 16-0687 Ms to CHT + ethanol did not decrease PGE<sub>2</sub> release differs from a previous report that adding LF 16-0687 Ms to CHT + saline significantly decreased post-CHT release of PGE<sub>2</sub>.<sup>14</sup>

It is not clear why LF 16-0687 Ms blocked the increase of PGE<sub>2</sub> release after CHT + saline in a previous study but not after CHT + ethanol in this study.<sup>14</sup> In the previous study, PGE<sub>2</sub> release after CHT + saline was  $368 \pm 186$

pg/mg tissue, and after CHT + LF 16-0687 Ms it was  $77 \pm 65$  pg/mg tissue.<sup>14</sup> In the present study, PGE<sub>2</sub> release after CHT + saline was  $350 \pm 133$  pg/mg tissue and was decreased to  $147 \pm 59$  pg/mg tissue after CHT + ethanol. In other words, both LF 16-0687 Ms alone and ethanol alone decreased PGE<sub>2</sub> release after CHT. By comparison, PGE<sub>2</sub> release with CHT + ethanol + LF 16-0687 Ms was  $286 \pm 194$  pg/mg tissue, greater than CHT + ethanol (present study) or CHT + LF 16-0687 Ms (previous study).<sup>14</sup>

Previous reports on interactions between ethanol and bradykinin suggest some mechanisms that might play a role in the effects of combining ethanol and LF 16-0687 Ms. In neural tissue, positive interactions include reports that ethanol reduced the magnitude of bradykinin-mediated intracellular calcium mobilization, and [3H] inositol phosphate formation and guanine nucleotide-binding protein associated with phospholipase C in neuroblastoma cells, and formation of inositol 1,4,5-trisphosphate, and hydrolysis of phosphoinositides in neuroblastoma-glioma cells.<sup>28-31</sup> Negative interactions include reports that ethanol had no effect on binding to bradykinin receptors or on bradykinin-stimulated activation of phospholipase C in neuroblastoma-glioma cells or inositol lipid metabolism in astroglial cells.<sup>31,32</sup> In nonneural tissue, ethanol did not attenuate bradykinin-induced activation of mitogen-activated protein kinase or intracellular calcium mobilization in pheochromocytoma cells; decreased bradykinin-stimulated [3H] phosphatidic acid in myoblasts and arachidonic acid release in canine kidney cells; and enhanced bradykinin-induced calcium mobilization in rat liver epithelial cells and pheochromocytoma cells, nitric oxide synthase activity in bovine pulmonary artery endothelial cells, and nitric oxide release from and vasodilation of rings of bovine pulmonary artery and vein.<sup>33-39</sup>

Under the experimental conditions of this study, LF 16-0687 Ms retained its previously reported beneficial effects of decreasing brain tissue edema formation and improving NSS after CHT.<sup>9,14</sup> A dissociation between edema and NSS as compared with PGE<sub>2</sub> release in models of CHT is not a finding unique to this study. In a model of CHT in rats, indomethacin and the thromboxane A<sub>2</sub> synthetase inhibitor OKY-046 previously were reported to decrease PGE<sub>2</sub> release without significantly altering cerebral edema or NSS.<sup>16,40</sup> Decreased edema and improved NSS, as seen here with LF 16-0687 Ms after CHT + ethanol, also was reported previously when the *N*-methyl-D-aspartate receptor antagonists NPS 846, magnesium, HU-211, and NPS 1506 were given after CHT.<sup>41-45</sup> In contrast, the cyclo-oxygenase inhibitors indomethacin and dexamethasone, methylprednisolone, and Nimesulide failed to decrease edema or improve NSS when given after CHT.<sup>16,46,47</sup>

Regarding potential clinical application of B<sub>2</sub> receptor antagonism for head injury, the present results suggest that acute ethanol intoxication does not impair the early beneficial effects of LF 16-0687 Ms on cerebral edema and neurologic status. Among the various mediators that are thought to be

involved in secondary brain damage after head trauma, bradykinin has been proposed as an essential factor, particularly in the induction of BBB dysfunction and increased vascular permeability. An intracarotid infusion or cortical superfusion of bradykinin induced a leakage of Na<sup>+</sup>-fluorescein, which was blocked by a B<sub>2</sub>, but not a B<sub>1</sub>, receptor antagonist, suggesting that the increase of BBB permeability was mediated by B<sub>2</sub> receptors located on both luminal and abluminal sides of endothelial cells.<sup>48</sup> Bradykinin also caused arterial dilatation, which was mediated by activation of endothelial B<sub>2</sub> receptors and which may contribute to plasma extravasation.<sup>12,49</sup> Activation of endothelial B<sub>2</sub> receptors resulted in the release of prostanoids, nitric oxide, and hydrogen peroxide, which activate Ca<sup>2+</sup>-dependent K<sup>+</sup> channels.<sup>10-12,50</sup> An increase in brain tissue bradykinin content was demonstrated after cortical cold lesion and concussive brain injury.<sup>51,52</sup> Blockade of the kinin-kallikrein system using soybean trypsin inhibitor and aprotinin reduced hemispheric swelling after a cortical cold injury lesion in the rabbit.<sup>53</sup> Finally, in glial cells, bradykinin stimulated the release of excitatory amino acids.<sup>7,8</sup>

In summary, in rats receiving CHT without accompanying intraperitoneal ethanol administration, LF 16-0687 Ms previously was reported to improve NSS and decrease brain water content and release of PGE<sub>2</sub> from injured brain.<sup>14</sup> In the present study in which rats received CHT combined with an intoxicating dose of ethanol, ethanol did not affect the decrease of brain water content or improvement of NSS produced by LF 16-0687 Ms treatment, but did reverse the decrease of PGE<sub>2</sub>. On the basis of the results of previous cell culture studies, we speculate that the negative interaction between ethanol and LF 16-0687 Ms regarding post-CHT PGE<sub>2</sub> release may be related in part to alteration of calcium mobilization, inositol phosphate formation, guanine nucleotide binding, hydrolysis of phosphoinositides, or release of arachidonic acid or nitric oxide.<sup>23-31,36,38,39</sup>

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