Effect of β-Hydroxybutyrate, a Cerebral Function Improving Agent, on Cerebral Hypoxia, Anoxia and Ischemia in Mice and Rats

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ABSTRACT—Although improving energy metabolism in ischemic brain has been accepted for the treatment of cerebrovascular diseases, administration of glucose, as an energy substrate, would aggravate ischemic brain damage via activating anaerobic glycolysis, which leads to lactate accumulation. β-hydroxybutyrate (BHB) is one of the ketone bodies that can be utilized as an energy source during starvation. The purpose of our study was to define the protective effects of BHB on brain damage induced by hypoxia, anoxia and ischemia. The isotonic solution of BHB administered 30 min before the induction of ischemia at doses over 50 mg·kg⁻¹·h⁻¹ showed remarkable protective effects against hypoxia and anoxia. BHB administered immediately after a bilateral carotid artery ligation at a dose of 30 mg·kg⁻¹·h⁻¹ significantly suppressed the elevation of cerebral water and sodium contents as well as maintaining high ATP and low lactate levels. In contrast, glycerin, a hypertonic agent, substantially reduced the water content but did not show any significant effect on other parameters. We demonstrated that BHB, unlike glycerin, when used as an energy substrate in ischemic brain, has protective effects on cerebral hypoxia, anoxia and ischemia-induced metabolic change.

Keywords: β-Hydroxybutyrate, Glycerin, Anti-anoxic effect, Cerebral ischemia, Cerebral energy metabolism

The brain is a surprisingly active tissue in terms of glucose metabolism; almost all the energy supplied to maintain its vital functions are derived from glucose oxidation. Restriction of the glucose supply and metabolism under such conditions as hypoxia and ischemia would result in brain damages (cerebral edema and infarct). However, epidemiological data have shown that hypoglycemia was associated with higher risks of strokes and that ischemic cerebral injury was, at least partly, attributed to hyperglycemia as well (1 – 3). Furthermore, it has been verified in several animal models that glucose administration accelerated brain damage caused by cerebral ischemia (4 – 6). The elevated glucose anaerobic metabolism under hypoxia and ischemia conditions (6 – 9) causes tissue lactate accumulation which subsequently leads to an increased H⁺ intracellular concentration level. It is thus conclusive to state that glucose containing solutions have or might have harmful effects in an ischemic brain.

Ketone bodies are known for their energy source uses in states of starvation (10). β-Hydroxybutyrate (BHB, Fig. 1), a representative type of ketone body, is produced by degradation of fatty acids in the liver. Recent studies have reported the utilization of BHB as an energy substrate in head trauma patients (11) and hemorrhage shock rats (12). Go et al. reported that fasting, characterized by features like hypoglycemia and ketosis, protected rats from developing brain infarction following hypoxia-ischemia (13). The rats were found to have an increased level of BHB in their blood compared with rats that had a normal

Fig. 1. Chemical structure of (R)-(−)-sodium β-hydroxybutyrate (BHB).
dietary regimen. It was therefore supposed that exogenously administered BHB had beneficial effects on the ischemic brain as it decreased lactate accumulation by means of restraining glucose utilization.

This study describes the cerebroprotective effect of BHB on hypoxia, anoxia and ischemia in mice and rats. Additionally, we also tried to clarify the effect of BHB on cerebral energy metabolism and edema formation after global cerebral ischemia in rats subjected to bilateral common carotid artery ligation (BLCL). The effect of glycerin, a hypertonic agent, on cerebral hypoxia and ischemia was also studied.

MATERIALS AND METHODS

Reagents

BHB was synthesized in the research laboratories of Shimizu Pharmaceutical Co., Ltd. (Shizuoka). The ATP assay kit was obtained from Roche Molecular Biochemicals (ATP Bioluminescence Assay Kit HS II; Mannheim, Germany). The lactate assay kit was obtained from Kyowa Co., Ltd. (Determiner LA, Tokyo). All other chemicals and enzymes used in this study were obtained from Wako Pure Chemicals (Osaka) and of reagent grade.

Administration of solutions

Isotonic solutions of sodium BHB were prepared at concentrations of 0.06%, 0.2%, 0.5%, 0.6%, 1.0% and 2.0% and intravenously administered to animals at a rate of 5 mL·kg⁻¹·h⁻¹. These concentrations correspond to 3, 10, 25, 30, 50 and 100 mg·kg⁻¹·h⁻¹ of BHB, respectively (Table 1). Saline was administered as a vehicle control at the same administration rate. Glycerin solution (Glycerin) as a hypertonic fluid, containing 10% glycerin, 5% fructose and saline, was administered at a rate of 5 or 10 mL·kg⁻¹·h⁻¹.

Animals

Male ddY mice weighing 25 to 35 g (5-week-old) and Wistar rats weighing 160 to 180 g (6-week-old) were purchased from Japan SLC, Inc. (Hamamatsu). These animals were housed at 23 ± 3°C with a humidity of 55 ± 15% and light-dark cycle on 12 h/day (07:00 to 19:00). The rats and mice, housed in a group of 3 and 5 animals per cage, respectively, were given water ad libitum and were fed a specific diet (MF; Oriental Yeast, Tokyo). They were subjected to the same conditions for a duration of at least 7 days prior to executing the experiments. They underwent an overnight fast before the experiments but had free access to water. All animals received humane care in compliance with the “Guiding Principles for the Care and Use of Laboratory Animals” formulated by The Japanese Pharmacological Society.

Table 1. Composition of BHB solutions

| BHB conc. (%) | Dose (mg·kg⁻¹·h⁻¹) | BHB⁻ | Na⁺ | Cl⁻ |
|--------------|-------------------|------|-----|-----|
| 0.06         | 3                 | 4.62 | 154.0 | 149.38 |
| 0.2          | 10                | 15.4 | 154.0 | 138.6 |
| 0.5          | 25                | 38.5 | 154.0 | 115.5 |
| 0.6          | 30                | 46.2 | 154.0 | 107.8 |
| 1.0          | 50                | 77.0 | 154.0 | 77.0 |
| 2.0          | 100               | 154.0 | 154.0 | 154.0 |

N₂ gas-induced hypoxia in mice

Five mice were put into a plastic desiccator chamber (2.5 L). The hypoxia was induced by having the mice exposed to a gaseous mix of 96% N₂ and 4% O₂ at a flow rate of 5 L/min. The O₂ concentration in the chamber was monitored with an Acid-Base Analyzer (ABL-30; Radiometer, Copenhagen, Denmark). The survival time was defined as latency to the arrest of respiration from the onset of hypoxia. BHB, Glycerin or saline was continuously infused for 30 min through the tail vein using an animal holder (KN-324B; Natsume, Tokyo) before the onset of hypoxia.

KCN-induced anoxia in rats

The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a polyethylene tube 50 (PE-50) was inserted into the left femoral vein for continuous drug administration. One day after the surgery, anoxia was induced by an injection of 3 mg/kg KCN (3 mg/mL) at a rate of 0.5 mL/min for 2 min into the tail vein 30 min after the onset of infusion. The survival time was defined as latency to the arrest of respiration from the administration of KCN.

Global cerebral ischemia model in rats

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a PE-50 tube was inserted into the left femoral vein for continuous drug administration. One day after the surgery, the animals were anesthetized using a face mask in a mixture of 30% O₂ and 70% N₂ at an initial concentration of 3.5%, which was subsequently reduced down to 1.5 – 2.0% during the experimental procedure. The body temperature was monitored by a rectal probe and maintained at 37.5 ± 0.5°C during the operation with a heating blanket regulated by an animal blanket system (MK-900; Muromachi, Tokyo). A midline incision was made in the neck and bilateral common carotid arteries were carefully exposed and separated from the surrounding connective tissue and nerve fibers. Global cerebral ischemia was induced by BLCL. In sham-operated rats, bilateral common carotid arteries were exposed according to the
same procedure indicated above and the skin incision was closed without BLCL. A PE-50 tube was inserted into the tail artery to monitor blood pressure and to collect blood samples. Blood pressure and arterial blood gases (pH, $P_{O_2}$, $P_{CO_2}$) were measured just before and 3 h after BLCL.

**Measurement of cerebral water and Na$^+$ content**

Immediately after BLCL, BHB was intravenously infused for a period of 6 h. Once the 6 h-period had elapsed, the rats were sacrificed, had their brains excised and their cerebellum discarded. The cerebral water content was determined by measuring the percentage difference of the brain weights before and after drying the tissue samples for 24 h at 105°C. The dried samples were digested in 5 mL of 2 N HNO$_3$ for 1 week. Cerebral sodium content as mEq/kg dry weight was measured using an atomic absorption spectroscopy (AA-6700F; Shimadzu, Kyoto) at 589.0 nm.

In some rats, the intravenous infusion of BHB was initiated 3 h after BLCL and continued on until they were sacrificed.

**Measurement of tissue energy metabolites**

The intravenous administration of BHB was started immediately after BLCL and continued for 3 h before sacrificing the rats by microwave irradiation at 5 kW for 2 s (TMW-6402C; Toshiba, Tokyo). The brains were excised and homogenized in 5 mL of ice-cold 6% trichloroacetic acid (TCA) with a homogenizer (Nichionn Ika Kikai, Physcotron, Tokyo). After 15-min centrifugation (3000 rpm, 4°C), the supernatant was collected as the sample solution. ATP levels were measured by the luciferin-luciferase bioluminescence method using an ATP photometer Lumat (LB9507; EG&G Berthold, Berlin, Germany). Lactate levels were measured by the lactate dehydrogenase enzymatic method using Cobas Fara (Roche Diagnostics, Mannheim, Germany). ATP and lactate levels were expressed as $\mu$mol/g brain.

**Measurement of regional cerebral blood flow**

The intravenous administration of BHB was started 30 min prior to BLCL and continued on until the animals were sacrificed. Regional cerebral blood flow (rCBF) was measured using a Laser-Doppler Flowmetry (FLO-N1; Neuroscience, Tokyo). Rats were anesthetized with urethane (600 mg/kg, i.m.) and $\alpha$-chloralose (40 mg/kg, i.p.). An incision was made at the midline of the scalp and a Doppler-flow probe was implanted 3-mm posterior and 3-mm lateral to the bregma. The rCBF value was calculated and expressed as a percentage of the baseline value.

**Statistical analyses**

All data were expressed as the mean ± S.E.M. Statistical analyses were made using the unpaired Student’s $t$-test and Dunnett’s multiple test for comparison between two groups and among groups, respectively. Differences were considered to be statistically significant when $P<0.05$.

**RESULTS**

**N$_2$ gas-induced hypoxia**

The protective effect of BHB on 96% N$_2$ / 4% O$_2$-induced hypoxia is shown in Fig. 2. Survival time of saline-treated mice was $220.27 \pm 25.83$ s. BHB significantly ($P<0.01$) prolonged the survival time in a dose-dependent manner and the mice treated with the maximum dose of BHB (100 mg·kg$^{-1}$·h$^{-1}$) were found to show about a 1.5 times longer survival time (380.53 ± 46.09 s) than did saline-treated mice. Mice treated with Glycerin showed a tendency have a prolonged survival time (260.53 ± 38.96 s) as compared to saline-treated animals, but this was not statistically significant.

**KCN-induced anoxia**

Survival time in KCN-induced anoxia is shown in Fig. 3. BHB significantly ($P<0.01$) prolonged survival time at doses of 50 ($58.8 \pm 2.4$ s) and 100 ($57.5 \pm 1.5$ s) mg·kg$^{-1}$·h$^{-1}$ as compared to saline (44.6 ± 1.2 s), whereas Glycerin failed to prolong any survival time (48.2 ± 1.6 s).

**Global cerebral ischemic model**

Physiological parameters are summarized in Table 2. There were no significant differences in each value before

![Fig. 2.](image-url)
BLCL among all groups. After BLCL, moderate hyperventilation was observed in all groups, which was characterized by a 5–10 mmHg decrease in $\text{PaCO}_2$ and a pH increase of 0.1–0.2. Among all groups, however, there were no significant differences in the blood gas values.

Cerebral water and Na$^+$ contents

As shown in Fig. 4, cerebral water content at 6 h after BLCL was 80.28 ± 0.18% in BHB (30 mg·kg$^{-1}$·h$^{-1}$)-treated rats and 79.91 ± 0.11% in the Glycerin (10 mL·kg$^{-1}$·h$^{-1}$)-treated rats, being statistically lower than that of saline-treated rats (81.86 ± 0.18%). Thus, both drugs inhibited the increase in cerebral water content by nearly the same degree. As shown in Fig. 5, cerebral Na$^+$ content was 221.0 ± 10.9 mEq/kg in sham-operated rats. Six hours after BLCL, cerebral Na$^+$ content significantly (P<0.01) rose to 342.6 ± 15.4 mEq/kg in saline-treated rats, while BHB dramatically suppressed the increase at all doses. In Glycerin-treated rats, however, there was no significant effect on cerebral Na$^+$ content.

Cerebral tissue metabolites (ATP, lactate) levels

Cerebral tissue levels of energy metabolites, ATP and lactate, are shown in Figs. 6 and 7, respectively. Tissue ATP and lactate levels in sham-operated rats were 1.85 ± 0.13 and 0.61 ± 0.12 μmol/g, respectively. Three hours after BLCL, tissue ATP and lactate levels in saline-treated rats significantly (P<0.01) changed to 0.52 ± 0.10 and 11.39 ± 0.85 μmol/g, respectively. BHB given at 30 mg·kg$^{-1}$·h$^{-1}$ alleviated these aggravated changes; i.e., tissue ATP levels rose to 1.46 ± 0.11 μmol/g and lactate levels decreased to 1.36 ± 0.17 μmol/g with statistical

**Table 2.** Physiological parameters before and 3 h after BLCL

|            | (n) | MABP (mmHg) | $\text{PaO}_2$ (mmHg) | $\text{PaCO}_2$ (mmHg) | pH        |
|------------|-----|-------------|-----------------------|------------------------|-----------|
| Saline     | 6   | before 111 ± 7.1 | 106.2 ± 8.3 | 34.2 ± 1.8 | 7.36 ± 0.02 |
|            |     | after 116 ± 9.4   | 120.8 ± 6.7 | 27.6 ± 3.0 | 7.42 ± 0.01 |
| BHB        | (30 mg·kg$^{-1}$·h$^{-1}$) | 6 | before 108 ± 5.6 | 105.7 ± 6.4 | 35.1 ± 1.0 | 7.34 ± 0.01 |
|            |     | after 110 ± 8.9   | 110.6 ± 9.6 | 30.1 ± 1.9 | 7.47 ± 0.02 |
| Glycerin   | (10 mL·kg$^{-1}$·h$^{-1}$) | 6 | before 105 ± 6.2 | 100.7 ± 6.4 | 36.3 ± 2.3 | 7.37 ± 0.01 |
|            |     | after 118 ± 10.8  | 120.6 ± 9.6 | 26.5 ± 2.8 | 7.43 ± 0.02 |

MABP: mean arterial blood pressure.
significance \((P<0.01)\). Glycerin failed to demonstrate significant changes.

**rCBF**

The effect of BHB on rCBF changes before and after BLCL is shown in Fig. 8. Immediate fall of rCBF by about 40% from its baseline was observed after BLCL. rCBF in BHB-treated rats demonstrated a tendency to be higher than that in saline-treated rats throughout the experiment, but this was not statistically significant.
Effects of 3 h-delayed treatment of BHB or Glycerin on cerebral water contents are shown in Fig. 9. Six hours after BLCL, delayed treatment of saline showed a significant ($P<0.01$) increase in cerebral water content as compared to sham-operated rats. BHB and Glycerin showed significant ($P<0.01$) decreases in cerebral water contents as compared to saline even though continuous infusion of BHB or Glycerin started 3 h after BLCL.

**DISCUSSION**

The brain is known to be the most vulnerable tissue to noxious stimuli, as hypoxia, anoxia, ischemia and the like. Under normal conditions, the brain mainly utilizes glucose as its primary source of energy, which is then completely metabolized through the glycolytic pathway to finally produce high-energy substrates. Hence, maintenance of blood glucose level is crucial for the brain’s integrity, however under cerebral ischemia, the importance of blood glucose levels takes different meaning. Epidemiological (1)–(3) and animal studies (4–6) indicate that elevated blood glucose level as observed in diabetes mellitus is a definite risk factor in the management of cerebral ischemia. Aggravation of ischemic brain damage by hyperglycemia is mainly attributed to the excessive accumulation of tissue lactate, which is produced by enhanced glucose anaerobic metabolism under the hypoxic condition (6–9). Moreover, hyperglycemic rats pretreated with glucose died within 12 h after the induction of cerebral ischemia, while normoglycemic rats survived longer (4). For these reasons, glucose or glucose-containing solution can not be used for cerebral ischemia treatments, although improvement of cerebral energy metabolism is of great importance for the treatment of cerebrovascular diseases. The present study attempts to examine the effect of BHB as an alternative energy substrate in the brain during hypoxia, anoxia and ischemia.

Firstly, we examined the protective effect of BHB on 1) N$_2$-induced hypoxia and 2) KCN-induced anoxia in mice and rats as compared to that of glycerin, a hypertonic agent. These two experimental models are generally used to screen drugs for cerebrovascular diseases; such drugs include activators of cerebral energy metabolism (14, 15), cerebral vasodilators (15–17), CNS depressants (18, 19) and activators of the CNS cholinergic system (20) or acetylcholine esterase inhibitors (21, 22). In the two models, BHB significantly prolonged the survival time at the dose of 50 mg·kg$^{-1}$·h$^{-1}$ or more, while glycerin failed in all models.

Secondly, we also attempted to define the plausible BHB mechanisms that are involved in prolonging the survival time in the above-mentioned models. To achieve that end, we used rats that had BLCL-induced global ischemia, in which we observed cerebral edema formation and deranged cerebral energy metabolism that were marked with dramatic decreases in rCBF. The rats used in this study were Wistar strain rats purchased from SLC. The SLC Wistar strain rats are known to be similar to the Fischer-344 strain rats (23), which exhibit apparent cerebral ischemia only by BLCL. These rats are reported to have a defect in Willis’s circle, probably due to its narrow posterior communicating anastomosis, resulting in less irrigation from other normal cerebral arteries to the ischemic area as compared to other strain rats, when they are subjected to BLCL (24, 25). Thus, blood supply to the brain in Fischer-344 and SLC Wistar strain rats are mainly maintained by bilateral common carotid arteries.

In rats with cerebral ischemia by BLCL, there were no significant changes in physiological parameters, Pa$_{O_2}$, Pa$_{CO_2}$, pH and mean arterial blood pressure, among BHB-, Glycerin- and saline-treated groups. However, BHB at doses of 10, 30 and 100 mg·kg$^{-1}$·h$^{-1}$, suppressed the elevation of cerebral water and Na$^+$ contents at 6 h after BLCL. The suppression by BHB, however, had already reached the maximum at a dose of 30 mg·kg$^{-1}$·h$^{-1}$ and no dose-dependency was observed within the doses employed. Three-hour-delayed treatment of BHB at a dose of 30 mg·kg$^{-1}$·h$^{-1}$ also inhibited the extent of cerebral edema. The protective effects by BHB are considered to be direct because BHB failed to influence rCBF. On the other hand, glycerin (5 and 10 mL·kg$^{-1}$·h$^{-1}$) suppressed only the increment in cerebral water content.
The activated anaerobic glycolytic pathway is known to induce cerebral edema formation as a consequence of depletion of ATP and accumulation of lactate in cerebral tissues (6–9). Several studies suggest that the decrease in intracellular pH during ischemia has a direct correlation with accumulation of lactate (8, 9). The Na\(^+\)/H\(^+\) exchange transporter is activated by the decrease in intracellular pH during cerebral ischemia, resulting in leakage of intracellular H\(^+\) and uptake of extracellular Na\(^+\) together with water (9). Moreover, depressed activities of energy-dependent ion pumps such as Na\(^+\)/K\(^+\) ATPase under the ischemic condition accelerate cerebral edema formation (8). Namely, hypo-activity of Na\(^+\)/K\(^+\) ATPase leads to the failure of Na\(^+\) excretion from the intracellular space and finally to aggravation of edema formation. In our study, ATP reduction and lactate accumulation in ischemic brain tissues at 3 h after BLCL were attenuated by BHB, which could be distributed in the cerebral tissues even under the ischemic condition. We did not measure cerebral energy metabolites at either 6 h after BLCL or in an attempt in which BHB administration started 3 h after BLCL. However, since Na\(^+\) content was suppressed by BHB in these attempts, BHB would ameliorate cerebral edema formation by improving cerebral energy metabolism via the mechanisms mentioned above.

These evidences indicate that BHB is utilized as an energy substrate and may ameliorate the disruption of cerebral energy metabolism after hypoxia, anoxia and ischemia, where the anaerobic glycolytic pathway is activated. Phosphofructokinase is one of the major regulatory enzymes in the glycolytic pathway, and its activity increases under such anaerobic conditions, resulting in accumulation of lactate (26, 27). Thus, under these conditions administration of glucose only leads to the exacerbation of cerebral ischemic damage by the enhancement of the generation of lactate from pyruvate, while BHB does not.

BHB is metabolized to acetyl-CoA through pathways other than glycolysis before entering the TCA cycle. In the case of glucose, this substrate is firstly metabolized to pyruvate by glycolysis and then enters the TCA cycle. Previous studies have reported that BHB may inhibit pyruvate oxidation through the feedback inhibition by increased acetyl-CoA on the pyruvate dehydrogenase complex (28–30). Moreover, Newsholme et al. showed that the phosphofructokinase reaction was inhibited in the rat heart muscle when perfused with medium containing BHB (31). Thus BHB may inhibit lactate production from pyruvate by feedback inhibition via an increase in acetyl-CoA, which directly enters the TCA cycle without production of lactate. In contrast, Glycerin, one of the most useful hypertonic agents to reduce cerebral edema, had no effect on cerebral metabolite levels. Glycerin increases blood osmotic pressure and induces leakage of intracellular water in cerebral tissues into the vessels, resulting in alleviation of cerebral edema, by its physicochemical property, i.e., hypertonicity.

In summary, we showed that administration of BHB resulted in significant prolongation of the survival time in hypoxia and anoxia. Reduction in cerebral edema formation during BLCL-induced global cerebral ischemia suggests that BHB as an energy substrate substituted for glucose may exert its inhibitory effect on ischemic brain by potent maintenance of higher ATP levels and by inhibition of anaerobic lactate accumulation. These data provide the possibility that BHB has potential clinical utility as a therapeutic agent to treat patients in an acute phase of stroke, head trauma and other cerebrovascular disorders.

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