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ORIGINAL ARTICLE

Protection of hypoglycemia-induced neuronal death by β-hydroxybutyrate involves the preservation of energy levels and decreased production of reactive oxygen species

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Glucose is the main energy substrate in brain but in certain circumstances such as prolonged fasting and the suckling period alternative substrates can be used such as the ketone bodies (KB), beta-hydroxybutyrate (BHB), and acetoacetate. It has been shown that KB prevent neuronal death induced during energy limiting conditions and excitotoxicity. The protective effect of KB has been mainly attributed to the improvement of mitochondrial function. In the present study, we have investigated the protective effect of D-BHB against neuronal death induced by severe noncoma hypoglycemia in the rat *in vivo* and by glucose deprivation (GD) in cortical cultures. Results show that systemic administration of D-BHB reduces reactive oxygen species (ROS) production in distinct cortical areas and subregions of the hippocampus and efficiently prevents neuronal death in the cortex of hypoglycemic animals. *In vitro* results show that D-BHB stimulates ATP production and reduces ROS levels, while the nonphysiologic isomer of BHB, L-BHB, has no effect on energy production but reduces ROS levels. Data suggest that protection by BHB, not only results from its metabolic action but is also related to its capability to reduce ROS, rendering this KB as a suitable candidate for the treatment of ischemic and traumatic injury.

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INTRODUCTION

Glucose is the main energy source in brain. However, under certain conditions other energy substrates such as the ketone bodies (KB), acetoacetate (AcAc), and β-hydroxybutyrate (BHB) can be used by brain, including the suckling period,[1,2] prolonged fasting,[3] and the ketogenic diet;[4] all these situations are associated with increased KB levels in blood. Several studies have shown that KB can protect the brain against damage associated with diverse neurotoxic insults such as hypoxia,[5] ischemia,[6–8] hypoglycemia,[9,10] and excitotoxicity.[11,12] In addition, the ketogenic diet has been used for a long time for the treatment of refractory epilepsy.[4,13,14] The protective effect of KB has been mostly attributed to their conversion to AcetylCoA improving mitochondrial metabolism and preserving energy levels.[15–17] However, studies suggest that besides providing energy to neurons, KB reduce the production of reactive oxygen species (ROS) contributing to their protective effect. It has been observed *in vitro* that AcAc reduces ROS levels induced by glutamate exposure and glycolysis inhibition in cultured neurons,[12,16] and previous *in vivo* studies have shown that BHB decreases hypoglycemia and glutamate-mediated lipoperoxidation in the rat brain.[10,18] The mechanism underlying the reduction of ROS by BHB has not been elucidated but we have previously reported that KB display a scavenging action of ROS, in particular of the hydoxyl radical (•OH).[10] The physiologic and the nonphysiologic isomers of BHB (D- and L-BHB, respectively) scavenge •OH in a free-cell system and reduce neuronal death and ROS production induced by glycolysis inhibition in cultured hippocampal neurons and hypoglycemia-induced lipoperoxidation *in vivo*.[10] These observations support the potential of KB to prevent oxidative stress and cell death induced during energy limiting conditions or overexcitation. Two suitable experimental conditions to further investigate the mechanisms underlying the protective action of KB are the *in vivo* model of insulin-induced hypoglycemia and the *in vitro* model of glucose deprivation (GD). These conditions are associated with decreased glucose supply to neurons favoring the consumption of other energy substrates. It is known that hypoglycemia and GD lead to the generation of ROS and oxidative stress, which contributes to the death of neurons.[10,19–21] In a previous study, we have showed that prolonged hypoglycemia not leading to coma causes neuronal death in the rat cerebral cortex and increases lipoperoxidation in cortex, hippocampus, and striatum.[22] The selective vulnerability to hypoglycemic neuronal death has been well described in animals experiencing the hypoglycemic coma.[23] However, the distribution of ROS in distinct regions of the hypoglycemic rat brain has not been described nor its relation to selective neuronal death. Using the oxidation sensitive fluorescent marker, dihydroethidium (DHE), we have now investigated the distribution of ROS producing cells in the cortex and the hippocampus of hypoglycemic rats not

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subjected to coma. In addition, we aimed to investigate whether BHB reduces ROS generation in distinct cortical areas (frontal, parietal, temporal, and piriform) and hippocampal subregions (CA1, CA3, dentate gyrus, DG), and whether this effect correlates with a decrease in the number of degenerating neurons as assessed by Fluorojade B (FJB) staining. Results show that prolonged noncoma hypoglycemia followed by glucose reperfusion (GR) significantly increases ROS production in all cortical and hippocampal areas, while neuronal death is induced mainly in the cortex. The systemic administration of BHB effectively and significantly reduces ROS levels in all cortical and hippocampal regions and completely prevents cell death in the cortex. Results from cortical cultures show that D-BHB, the physiologic isomer of BHB, efficiently preserves ATP production, reduces ROS generation, and notably prevents neuronal death induced by GD, while the nonphysiologic isomer, L-BHB also reduces ROS production and partially prevents neuronal death, but has no effect on ATP levels. D- and L-BHB also reduced DHE oxidation in cells exposed to xanthine/xanthine oxidase (Xa/Xo), a superoxide producing system, and to H_2O_2 further suggesting the antioxidant action of BHB. These results support the protective action of BHB against neuronal damage induced by energy failure, and suggest that the preservation of energy levels in combination with reduced ROS production accounts for the protective action of BHB.

MATERIALS AND METHODS

Animal Treatment

Three-month-old Male Wistar rats (320 to 380 g) from the animal house of the Instituto de Fisiología Celular (Universidad Nacional Autónoma de México, UNAM) were used throughout the study. They were handled according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80-23 revised 1996), with the approval of the Animal Care Committee (CICUAL) of the Instituto de Fisiología Celular, UNAM. All efforts were made to optimize the number of animals used and minimize their suffering. Rats were housed under control conditions of light and temperature with food and water *ad libitum*. One week before the induction of hypoglycemia, animals were implanted with epidural electrodes under 1.5% to 2.0% isofluorane anesthesia for electroencephalogram (EEG) recording and were randomly distributed among the different experimental and control group. Rats were fasted overnight and hypoglycemia was induced following the methodology described.[22] Briefly, rats were intraperitoneally injected with 24 U/kg bovine insulin (Sigma, St Louis MO, USA) and blood samples were obtained from the tail to monitor glucose levels before (time 0) and at different intervals after insulin administration (0.5, 6, 7, and 24 hours) with a glucometer (Abbott Laboratories, Bedford, MA, USA). Interhemispheric EEG was recorded 30 minutes before the administration of insulin, during the hypoglycemic period and during glucose infusion until normal electrical activity completely recovered. Between 2 and 3 hours after insulin injection EEG showed an increase in the amplitude and the slowing of the theta (4 to 8 Hz) and delta (1 to 4 Hz) waves; the animals were drowsy and lost their righting reflex (RR) (when the animals lose the ability to recover the horizontal position when turned upside down), a behavior preceding the onset of isoelectricity (no EGG activity or flat EEG) (Figure 2). At this moment, a group of animals with severe hypoglycemia (HS) was rescued with glucose alone by an intraperitoneal administration of 0.2 mL of 50% glucose followed by an intravenous infusion (25% glucose in Krebs-Henseleit buffer at 1.5 mL/h during 3 hours) through the femoral vein using a perfusion pump (Harvard Apparatus 22, South Natik, MA, USA). An additional glucose intraperitoneal injection (1 mL 25% glucose) was administered if blood glucose levels remained below 20 mg/dL at the end of the 3-hour infusion. None of the animals from this group showed isoelectricity. A second group of animals (HS Iso) was identically treated but hypoglycemia was left to progress until the animals showed isolectricity approximately 3 hours after insulin administration. These animals exhibited periods of isolectricity of variable duration (3 to 7 minutes) and normal brain activity was recovered 1 hour after the onset of glucose infusion (Figure 2).

Another group of animals was treated with D-BHB (HS+D-BHB) and received one intraperitoneal administration of 250 mg/kg of D-BHB 1 hour after insulin administration. None of these rats showed isoelectricity after 3 hours of insulin injection, thus they were rescued with glucose infusion as described above plus a second intraperitoneal administration of D-BHB (250 mg/kg) at the time of the loss of the RR. Control animals received vehicle solution instead of insulin (0.1% acetic acid) and were treated in parallel with the hypoglycemic animals. All rats were anesthetized and transcardially perfused 24 hours after vehicle or insulin administration for brain extraction (see below). In each experiment, one animal from each group was included (control, HS, HS+D-BHB, HS Iso). Animals showing seizures were eliminated from the study.

Determination of Reactive Oxygen Species in Brain Sections

Reactive oxygen species production in the brain of hypoglycemic rats was monitored using the oxidation sensitive fluorescent marker DHE (Invitrogene, Molecular Probes, Eugene, OR, USA). Dihydroethidium was solubilized in 50% DMSO in 10 mmol/L phosphate buffer and all animal groups received two intraperitoneal DHE administrations (6 mg/kg each), 1 hour and 2.5 hours after insulin or vehicle injection. It is preferentially oxidized by superoxide, although it is also sensitive to other reactive species providing an index of ROS production. After its oxidation, DHE produces the fluorescent compounds ethidium (Et) and 2-hydroxyethidium.[24]

Twenty-four hours after insulin injection, animals were anesthetized and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer. Brains were left in fixative solution for 24 hours and then transferred to a 20% to 30% sucrose gradient (24 and 48 hours, respectively) and 25 μm coronal sections were cut in a cryostat from +1.70 to −4.20 from Bregma according to Paxinos and Watson.[25] Sections were mounted on slides and counterstained with the nuclear dye Hoechst (0.001% in phosphate-buffered saline for 15 minutes) to facilitate the identification of Et-positive cells. Sections were rinsed and covered with fluoromount-G (EMS, Hatfield, PA USA), and were observed under an epifluorescence Olympus microscope using U-MNG2 (528 to 605) and U-MNU2 (355 to 465 nm) filters for Et and Hoechst, respectively.

Six coronal sections taken at different levels from bregma (1.2, 0.2, −0.26, −3.14, −3.8, and −4.3) were analyzed per animal. Images from different areas of the cerebral cortex (frontal, temporal, parietal, and piriform) were captured: three fields per section from the frontal cortex; six fields from the parietal and temporal cortices, and one field per section for the piriform cortex. Three sections were taken at the level of the striatum (1.2 mm, 0.2 mm, and −0.26 mm) and five fields per section were captured from the dorsolateral striatum. At the level of the hippocampus, three sections were taken (−3.14, −3.8, and −4.3) and four fields per section were analyzed (CA1, CA3, DG, and hilus). The area of each one of the fields was the same and corresponded to 0.48 mm^2. The Et fluorescence was quantified from the images by means of the Image J (NIH, Bethesda, USA; http//:imagej.nih.gov/ij/) program by an investigator blind to the different experimental conditions. Background fluorescence was adjusted to zero and the signal from bright fluorescent cells was measured. Data are expressed as means ± s.e.m. (standard error of the mean) of Et fluorescence per unit area (Et fluorescence/mm^2).

FluoroJade Staining

Fluorojade B staining was performed as described previously.[22] One drop of a 1% NaOH solution diluted in 80% ethanol was added to brain sections mounted on slides. After 2 minutes it was replaced by 70% ethanol solution. Slides were covered with 0.06% potassium permanganate for 10 minutes and then sections were washed and incubated for 20 minutes with 0.0004% FJB solution prepared in 0.1% acetic acid. Finally, the sections were washed, dried at 50°C, rinsed with xylol for 5 minutes and covered with permount. Sections were observed under an epifluorescence Olympus microscope using a U-MNB2 filter (395 to 590 nm) and total FJB-positive cells were manually counted in each cortical and hippocampal area using the Image J program in 18 brain sections (from 3.20 to −4.20 mm from Bregma) from each rat by an investigator blind to the different treatments. In addition, the total number of FJB-positive cells located in the superficial and medium (II to IV) and the deep layers (V to VI) of the cortex were counted separately.

Determination of D-Beta-Hydroxybutyrate Levels in Blood

The D-BHB concentration was determined in plasma of control and insulin-treated animals by an enzymatic assay as previously reported.[10] Briefly, blood was collected in heparinized tubes 20 minutes after one administration of 250 mg/kg D-BHB in intact and fasted rats or after the second dose of D-BHB at the time of the loss of the RR in insulin-treated animals.

An equal volume of 10% perchloric acid was added, samples were centrifuged at 3,400 × *g* for 10 minutes and the supernatant collected. D-beta-hydroxybutyrate was determined following the method previously described.[10,11]

Cell Culturing

Cortical primary cultures were prepared from Wistar rat embryos of 17 to 18 days of gestation as previously described.[20] Cells were suspended in Neurobasal (Gibco Life Technologies, Grand Island, NY, USA) culture medium supplemented with 1% B27/1% B27 Minus AO (Gibco Life Technologies), 0.5 mmol/L L-glutamine, and 20 μg/mL gentamicin (Gibco Life Technologies), and plated at a density of $2.2 \times 10^5/cm^2$ precoated with poly-L-lysine (5 μg/mL, Sigma-Aldrich, St Louis, MO, USA). Cells were cultured for 8 days *in vitro* (DIV) at 37°C in a humidified 5% CO_2/95% air atmosphere. Four days after plating, glucose (5 mmol/L), cytosine arabinoside (0.8 μmol/L, Sigma-Aldrich), and 200 μL of fresh Neurobasal medium (containing 2% B27 Minus AO) was added.

Cell Treatments

After 8 DIV, medium was withdrawn from cells plated on 12-well plates and neurons exposed to glucose-free (GD) medium (Dulbecco's Modified Eagle Medium, DMEM, Gibco Life Technologies) for 1.5 hours. After this period, the GD medium was changed for the Neurobasal glucose-containing medium previously withdrawn (glucose reperfusion, GR). We determined that the glucose concentration in Neurobasal medium after 8 DIV ranges between 10 and 13 mmol/L. Cell survival was monitored after 18 hours recovery by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (Sigma-Aldrich) reduction assay and the lactic acid dehydrogenase release assay, as previously described.[20] Briefly, after 18 hours of GR cells were incubated with MTT (150 μmol/L) for 1 hour at 37°C in 5%CO_2/95% air atmosphere; the medium was aspirated and the precipitated formazan salts were solubilized in acidic isopropanol. Absorbance was monitored spectrophotometrically at a wavelength of 570 nm. Cell viability is expressed as percentage of MTT reduction relative to control. Lactate dehydrogenase (LDH) activity was assessed in the culture medium by measuring the decrease in the optical density resulting from the oxidation of NADH at 340 nm using pyruvate as a substrate. After treatment, culture medium was added to potassium phosphate buffer (0.05 mol/L, pH 7.5) with NADH (9.4 mmol/L, Sigma RBI, St Louis, MO, USA). The reaction was started with the addition of pyruvate (20 mmol/L, Sigma RBI) to the mixture, and the change in optical density was monitored after 5 minutes in a spectrophotometer. Data are expressed as percent LDH release relative to control. When the effect of BHB was tested, cultures were treated either with D-BHB or with L-BHB. Dose–response curves were performed to establish the concentrations of D- and L-BHB eliciting protection (Supplementary Figure 1). A 10-mmol/L concentration was chosen for incubation during the GD or the GR period and a combination of 10 mmol/L (GD)+5 mmol/L (GR) was elicited when KB were added during both periods. The effect of KB was also compared with that of pyruvate and lactate tested at the same concentrations (Supplementary Figure 1). Cell survival was monitored after the end of the GR period as described above. In a different series of experiments, cultures were recovered in new Neurobasal medium containing different concentrations of added glucose (5, 10, and 20 mmol/L) either in the presence or in the absence of 10 mmol/L D-BHB. The effect of isomers was also tested when added alone to the recovery medium in the absence of glucose (Supplementary Figure 2).

Determination of Reactive Oxygen Species in Cultures

Cells were plated on 12-well plates and ROS levels were determined immediately after 1.5 hours GD or 1 hour after GR using DHE. Dihydroethidium (2.3 μmol/L) was added to the medium (DMEM) during GD and added again during GR. Cells were washed with Lockey medium (mmol/L): (NaCl 154, KCL 5.6, $NaHCO_3$ 3.6, $CaCl_2$ 2.6, HEPES 5.0, pH 7.4), fixed with formaldehyde 3.7% on ice for 5 minutes and observed by epifluorescence microscopy (Nikon Diaphot, Tokyo, Japan) using the DM 400 filter. Images from six fields per well were captured and Et fluorescence intensity was quantified by an investigator blind to the different experimental conditions. D-beta-hydroxybutyrate or L-BHB was incubated only during GD (10 mmol/L), or during GD (10 mmol/L) and GR (5 mmol/L). Data are expressed as mean Et fluorescence intensity ± s.e.m. The effect of KB on the oxidation of DHE was also tested in cells exposed to Xa/Xo to induce the production of the superoxide radical. Cultures were preincubated with Xa (100 μmol/L) for 30 minutes and then Xo (45 mU/mL) was added for 1.5 hours in the presence or the absence of D- or L-BHB plus DHE (2.3 μmol/L). Cells were also exposed to H_2O_2 (200 μmol/L) in the presence or the absence of D- or L-BHB (10 mmol/L) plus DHE during 3 hours. At the end of the exposure, cultures were washed and Et fluorescence determined as described above.

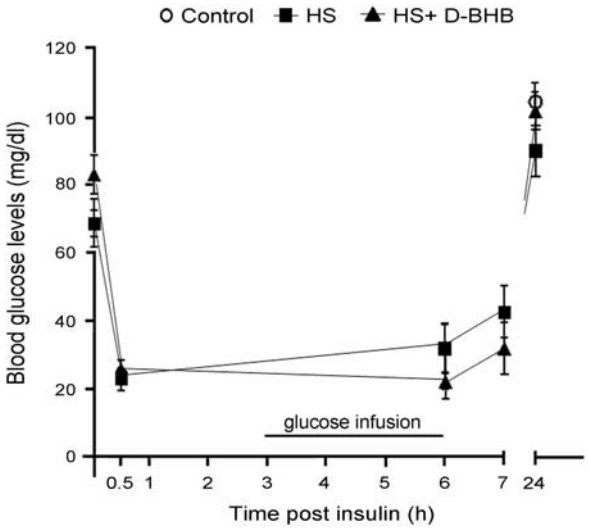

Figure 1. Changes in blood glucose induced by severe hypoglycemia (HS). Blood glucose concentration in control and hypoglycemic animals treated (HS+BHB) and nontreated (HS) with D-BHB before (time 0) and after insulin administration, and after glucose reperfusion. At the time of the loss of the righting reflex (RR) (2 to 3 hours after insulin) animals received 25% glucose (intraperitoneally) followed by a continuous 3-hour intravenous infusion of 25% glucose. Control animals were injected with vehicle solution. Blood glucose levels were monitored at the indicated times. Data represent means ± s.e.m. ($n = 3$ to 7). BHB, beta-hydroxybutyrate.

ATP Determination

Cells were plated on 12-well plates for 8 DIV. ATP concentration was determined immediately after 1.5 hours GD or after 3 hours GR. ATP levels were determined by means of a luminometer through the luceferin-luciferase quimioluminescent kit (Molecular Probes, Eugene, OR, USA), as previously described[20] and ATP concentrations were calculated from readings obtained from an ATP standard curve (from 6.5 to 250 pmol). Aliquots of cell homogenates were kept for protein determination by the Lowry's method [26] and data are expressed as pmol/μg of protein.

Statistics

All data are expressed as means ± s.e.m. and were analyzed by one-way ANOVA followed by a Fisher's least multiple comparison test.

RESULTS

Glucose Concentrations and Electroencephalogram Recording

Basal blood glucose concentrations ranged between 68 and 83 mg/dL and no statistical differences between the groups were observed. The two groups of insulin-treated rats showed glucose concentrations of 22.9 (HS) and 25.7 mg/dL (severe hypoglycemia, HS+D-BHB) 30 minutes after insulin administration. At the end of glucose infusion, animals slowly recovered glucose levels and at 24 hours all groups achieved normal glucose concentrations. The recovery of glucose levels in HS+D-BHB animals tended to be slower, but no statistical differences between the groups were observed (Figure 1). Figure 2 shows the EEG recordings obtained before and during insulin administration and after glucose infusion in a representative animal. Between 2 and 3 hours after insulin administration, the EEG showed the previously reported characteristic changes of hypoglycemia,[22,27] which consist in the

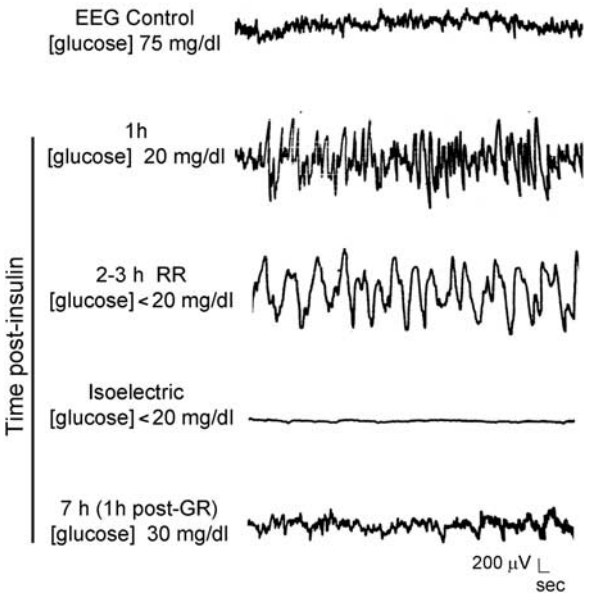

Figure 2. Changes in brain electrical activity (electroencephalogram, EEG) induced by severe hypoglycemia (HS). Representative EEG recording showing the changes in brain electrical activity in one insulin-injected animal before (EEG control) and during HS (1 to 3 hours after insulin), during isoelectricity and after the hypoglycemic episode (1 hour after glucose reperfusion, GR). The recording at the bottom shows the recovery of brain electrical activity 1 hour after the end of glucose infusion (7 hours after insulin).

slowing of the electrical brain activity and the predominance of the low frequency theta (4 to 8 Hz) and delta (1 to 4 Hz) waves. If animals were left to progress to the isoelectric period, then electrical brain activity was completely suppressed showing a flat EEG; 1 hour after the end of glucose infusion (7 hours after insulin) brain activity recovered (Figure 2). Animals receiving no D-BHB were separated into two groups; the first group was rescued with glucose at the time of the loss of the RR (HS) while the second group of animals was left to progress to the coma state (HS Iso) (isoelectricity) for a short period of variable duration (3 to 7 minutes) and was rescued with glucose alone after this period. None of the D-BHB-treated animals (HS+D-BHB) fell into the coma state 3 hours after insulin administration and thus they were rescued with glucose plus a second intraperitoneal administration of D-BHB at this time.

The concentration of D-BHB in blood was determined in the different groups of animals and is shown in Table 1. In fasted rats, D-BHB blood levels increased to 0.37 mmol/L relative to in intact animals, which showed levels of 0.056 mmol/L. Twenty minutes after one D-BHB (250 mg/kg) injection levels increased to 0.69 mmol/L in control (fasted) animals, while insulin-treated animals showed 0.19 mmol/L. In hypoglycemic animals receiving two doses of 250 mg/kg D-BHB, levels increased to 0.41 mmol/L at the time of the loss of the RR. These results suggest that D-BHB-treated animals show mild ketonemia and that the KB is used as an alternative substrate to glucose during hypoglycemia, since D-BHB decreases from 0.69 to 0.41 mmol/L at the time of the loss of the RR.

Neuronal Death Induced by Severe Hypoglycemia and Its Protection by D-Beta-Hydroxybutyrate

Figure 3A shows the number of FJB-positive degenerating cells as observed 24 hours after insulin administration in different cortical areas of hypoglycemic animals not showing isoelectricity (HS).

Table 1. Plasma levels of D-β-hydroxybutyrate (D-BHB) in rats with the different treatments

Treatment	*D-BHB [mmol/L]*
Intact	0.056 ± 0.021
Fasted	0.376 ± 0.010^{a}
Fasted+D-BHB 1 dose	$0.698 \pm 0.048^{a,b}$
Insulin	0.194 ± 0.025
Insulin+D-BHB 2 doses	$0.410 \pm 0.058^{a,c}$

The levels of D-BHB were determined in plasma by an enzymatic assay in intact, fasted and hypoglycemic rats treated or nontreated with D-BHB. Animals were killed 20 minutes after the first D-BHB administration or at the time of the loss of the righting reflex in animals receiving two administrations of D-BHB (250 mg/kg). Data represent means ± s.e.m. from at least 3 to 6 independent animals. $^{a}P < 0.05$ relative to intact. $^{b}P < 0.05$ relative to fasted. $^{c}P < 0.05$ relative to insulin.

Only a few FJ-positive cells were observed in control animals in the frontal, parietal, and temporal cortices. The number of FJB-positive cells significantly increased in the frontal and the parietal cortical areas of hypoglycemic animals. The increase in the parietal cortex was higher and significantly different from the that in the frontal ($P < 0.05$), temporal, and piriform cortices ($P < 0.005$) (Figure 3A). Animals treated with D-BHB (HS+D-BHB) showed a notable reduction in the number in FJB-positive cells in all cortical areas. This reduction is significantly different in the frontal and parietal cortices ($P < 0.05$) (Figure 3A). As expected, in animals subjected to a short period of isoelectricity the number of FJB-positive cells augmented in all cortical areas except for the piriform cortex. The parietal cortex showed the largest increase, which is statistically different relative to the frontal and the temporal cortices (Figure 3A).

Most of the degenerating cells were observed in the superficial and medium (II to IV) layers in all cortices rather than the deep layers (V and VI), and animals treated with D-BHB showed a significant reduction in the number of FJB-positive cells in all layers (Figure 3B). In rats exposed to isoelectricity the number of degenerating cells augmented mainly in layers II to IV while in layers V and VI this number remained unchanged (Figure 3B). The effect of D-BHB on cell death induced by coma was not determined because none of the D-BHB-treated rats showed isoelectricity. In Figure 3C, FJB-positive cells are shown in brain sections from representative severe hypoglycemic animals treated and nontreated with D-BHB, as well as hypoglycemic rats showing isoelectricity. As can be observed, FJB-positive cells are present in the frontal, parietal, and temporal cortices in hypoglycemic rats (HS), while D-BHB-treated rats show basically no degenerating cells (HS+D-BHB). More FJB-positive cells are visible in cortical areas from animals showing a short isoelectric period (HS Iso). From these animals only one showed FJB-positive cells in the crest of the dentate gyrus (377 cells) while in the HS group without coma only two rats showed degenerating cells (50 and 167, respectively) in this region. No FJB-positive cells were observed in other subregions of the hippocampus in any group of animals.

Reactive Oxygen Species Production Induced by Severe Hypoglycemia and Its Reduction by D-Beta-Hydroxybutyrate

Figure 4A shows Et fluorescence data in distinct cortical areas of animals of the control, HS, and HS+D-BHB groups. As can be observed, the frontal and the piriform cortices showed a higher basal Et signal in control animals as compared with the temporal and parietal cortices. In HS animals, the Et fluorescent signal significantly augmented in all cortical areas. Increases of 2.0-, 2.8-, 8.0-, and 10.0-fold were observed in the piriform, frontal, parietal, and temporal cortices, respectively. Et-positive cells were mainly

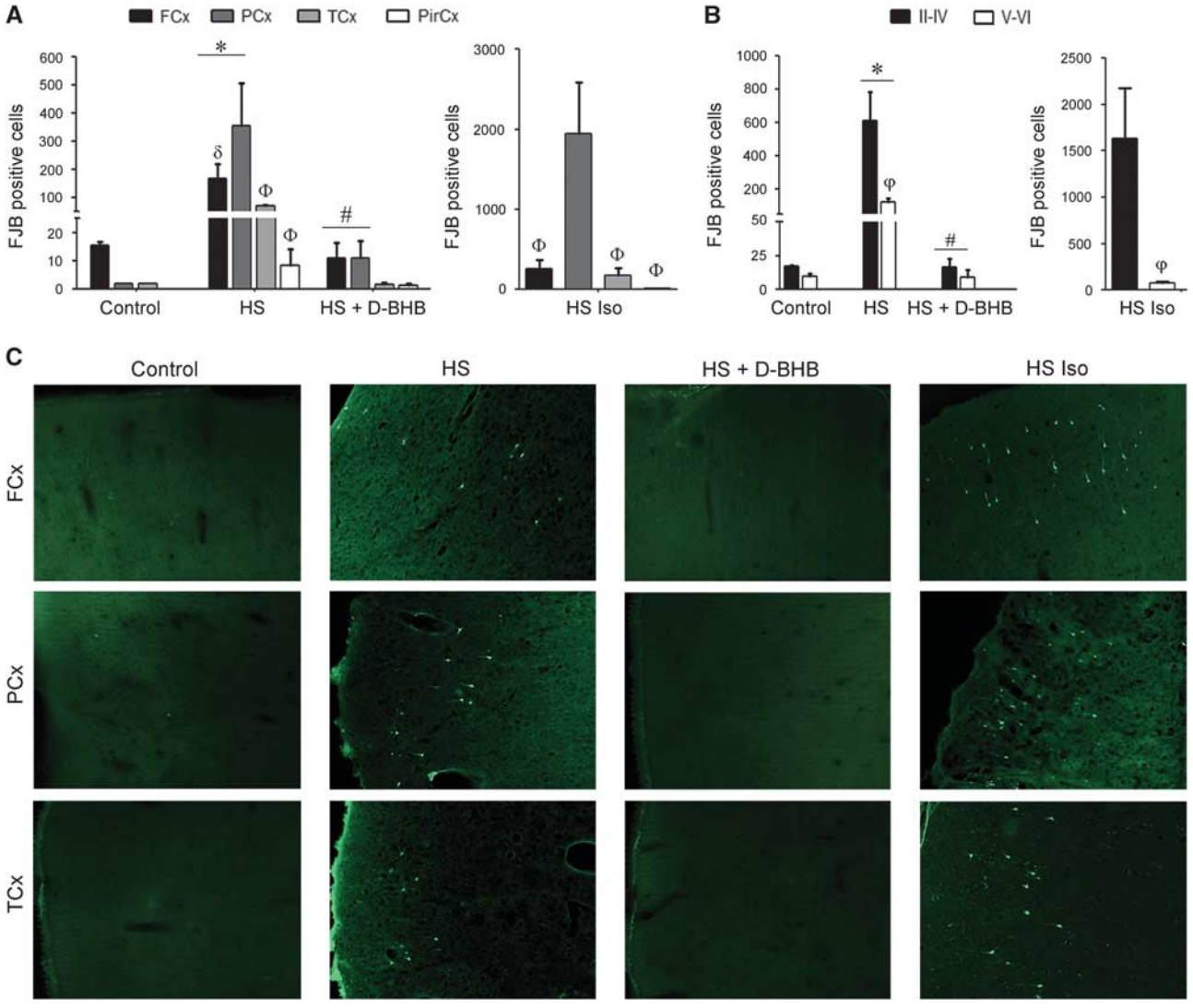

Figure 3. Number of degenerating Fluorojade B (FJB)-positive cells in different cortical areas of control and hypoglycemic animals treated (HS +D-BHB) and nontreated (HS) with D-BHB and of hypoglycemic animals exposed to isoelectricity (HS Iso) (**A**). The total number of FJB-positive cells in the superficial and medium (II to IV) and deep (V and VI) cortical layers in the different groups of animals is also shown (**B**). Data are expressed as ± s.e.m. Control ($n = 3$), HS ($n = 4$), HS+DBHB ($n = 6$), HS Iso ($n = 5$). $^{*}P < 0.05$ relative to control; $^{\delta}P < 0.05$ relative to parietal cortex, $^{\Phi}P < 0.005$ relative to parietal cortex, $^{\#}P < 0.05$ relative to HS, $^{\varphi}P < 0.05$ relative to layers II to IV. Images from brain sections of representative animals of each group showing FJB-positive cells in different cortical areas are shown (**C**). HS, severe hypoglycemia; BHB, beta-hydroxybutyrate.

observed in the middle layers (III and IV) in all cortical areas. In animals treated with D-BHB the Et fluorescent signal significantly diminished in all areas, recovering its control value. Figure 4B shows representative images of Et-positive cells in the parietal and the frontal cortex of animals from the different groups.

The changes in Et fluorescence induced by hypoglycemia were also investigated in the hippocampus. As shown in Figure 5A, the hilus and CA3 showed higher basal levels of the Et signal as compared with CA1 and the DG in the control group. In HS rats, Et fluorescence increased in all the hippocampal subregions as compared with control animals. The most important changes relative to control levels were observed in CA1 and the DG showing increases of 11.2- and 17.2-fold, respectively, while in the hilus and CA3 Et fluorescence increased 4.3- and 2.7-fold, respectively. In animals treated with D-BHB Et fluorescence did not increase and remained close to control levels. Figure 5B shows representative images of the different hippocampal regions of animals exposed to the different treatments. As can be observed Et-positive cells are visible in all hippocampal neuronal layers, while in control and D-BHB-treated animals the Et signal is notably lower.

Effect of D-Beta-Hydroxybutyrate on Neuronal Death and ATP Levels in Cultured Cortical Neurons Exposed to Glucose Deprivation

The effect of D-BHB against neuronal death was also investigated in cortical cultured neurons exposed to 1.5 hours of GD followed by 18 hours recovery in glucose-containing medium. Neuronal damage was evaluated by the MTT reduction and the LDH release assays. As observed in Figure 6A, GD caused a 54.8% release of LDH and a 67.0% decrease in MTT reduction. The protective effect of D-BHB against GD-induced neuronal death was tested after its incubation during GD or during GR, or during both periods. According to dose–response curves the maximum protective effect of D-BHB was observed at 10 mmol/L when incubated either

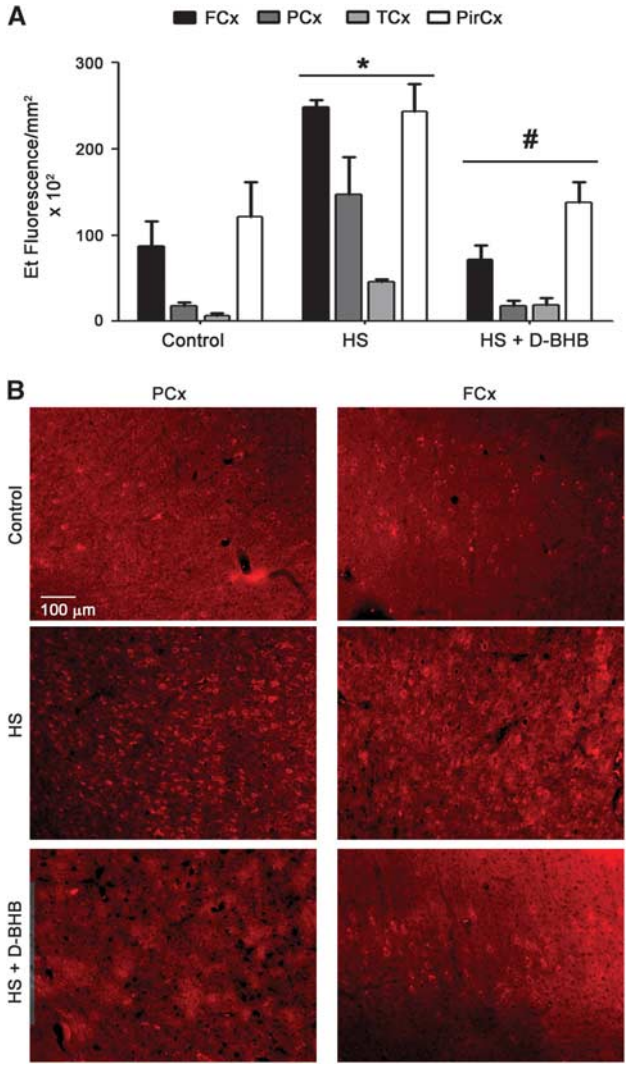

Figure 4. Et fluorescence units per area (Et fluorescence/mm^2) in different cortical areas of control and hypoglycemic animals treated (HS+D-BHB) and nontreated (HS) with D-BHB (**A**). Images from different areas of the cerebral cortex (temporal, parietal, frontal, and piriform) were captured and the Et fluorescent signal measured. Data are expressed as means ± s.e.m. Control ($n = 3$), HS ($n = 4$), HS +D-BHB ($n = 6$). $^*P < 0.05$ relative to control; $^{\#}P < 0.05$ relative to HS. (**B**) Images taken from brain sections of representative animals of each group showing Et-positive cells in PCx and the FCx. HS, severe hypoglycemia; BHB, beta-hydroxybutyrate.

during or after GD (Supplementary Figure 1A and B). When it was added during both periods, the combination showing maximum protection was 10 mmol/L (GD)+5 mmol/L (GR) (data not shown). The LDH release assay indicates that neuronal death was significantly reduced to 20.9% and 17.1%, when D-BHB was added during GD or during GR, respectively, while it was reduced to 8.4% when D-BHB was incubated during both periods (Figure 6A). No statistical difference was observed between the three different protocols of D-BHB administration. Similar results were found when survival was monitored by the MTT reduction assay (Figure 6B). When D-BHB was added only during GD neuronal survival was significantly stimulated as indicated by the increase in MTT reduction to 61.4% of control values. Incubation of D-BHB during GR also increased significantly cell survival to 48.9% of control levels. Incubation of D-BHB during GD and GR increased cell survival to 75.2% of control. No statistical difference was observed between the three different treatments.

The effect of L-BHB, the nonphysiologic isomer of BHB, was also tested. Dose–response curves for L-BHB are shown in Supplementary Figure 1A and B. As shown in Figures 5A and 5B, incubation with 10 mmol/L L-BHB only during GD had no effect on neuronal survival according to LDH release and MTT data. In contrast, increased cell survival was observed when L-BHB was present during GD and GR and when it was added only during recovery suggesting that the protective action of L-BHB is exerted during GR. However, L-BHB was less effective and its effect statistically different from that of D-BHB (Figure 6A; Supplementary Figure 1). Images of cultured cells exposed to the different treatments are shown in Figure 6D. As can be observed, exposure to GD causes a decrease in the number of cells with a normal morphology and many of them appear shrunken with thin and damaged processes. In contrast, cells exposed to D-BHB during GD and GR or only during GR appear morphologically similar to cells present in control cultures, while a lower number of cells with a normal morphology can be observed in cultures treated with L-BHB. The effect of D-BHB was compared with that of other energy substrates, such as pyruvate and lactate. Pyruvate significantly prevented neuronal death restoring MTT reduction to 60% of the control when incubated during the GD period, and its effect was not statistically different from that of 5 and 10 mmol/L D-BHB (Supplementary Figure 1A). Lactate was ineffective when incubated during GD. In the case of posttreatment pyruvate elicited significant protection restoring MTT reduction to 51% and 63% at 2 and 10 mmol/L, respectively, and this effect was not different from that of 5 and 10 mmol/L D-BHB (Supplementary Figure 1B). Lactate increased neuronal survival at 2 mmol/L showing a smaller protective effect as compared with 5 and 10 mmol/L D-BHB (Supplementary Figure 1B).

To test whether the preservation of energy levels contributes to the protective action of D-BHB, ATP levels were determined immediately after GD or after 3 hours of recovery in glucose containing medium. As depicted in Figure 6C, ATP concentration significantly declined 68% of the control value immediately after GD (Figure 6C, left bars) and did not recover after 3 hours of GR (Figure 6C, middle). When D-BHB was present during GD, ATP levels declined only 30% of control levels (Figure 6C, left bars) and remained elevated 3 hours after GR (middle bars). When D-BHB was added during GD and GR, ATP levels were restored to 73% of control values 3 hours after GR (right bars). ATP concentrations were also examined when D-BHB was added only during the recovery period, and in this case ATP recovered to 78% of control values (control 11.86 ± 0.325; GD 1.78 ± 0.64; D-BHB 9.35 ± 1.83 $n = 3$). No statistical difference was found in the recovery of ATP levels observed after the different protocols of D-BHB administration.

In contrast to D-BHB, incubation with L-BHB during GD did not increase significantly ATP concentration as determined either immediately after GD or 3 hours after GR (Figure 6C, left and middle bars). When L-BHB was present during both GD and GR, ATP levels were neither restored. Similarly, L-BHB had no effect on ATP concentration when incubated only during recovery (control 11.86 ± 0.32; GD 1.78 ± 0.64; L-BHB 3.9, $n = 3$). As expected, these data suggest that the protective effect of L-BHB can not be attributed to a metabolic action.

In agreement with these results, we observed that 10 mmol/L D-BHB was able to rescue a small percentage (20.2%) of cells when added alone (no glucose) in the recovery medium (Supplementary Figure 2), suggesting that D-BHB can sustain energy metabolism to a limited extent in this conditions. In contrast, 10 mmol/L L-BHB was unable to prevent neuronal damage in the absence of glucose. When D-BHB was added to the recovery medium in combination with different concentrations of glucose, it

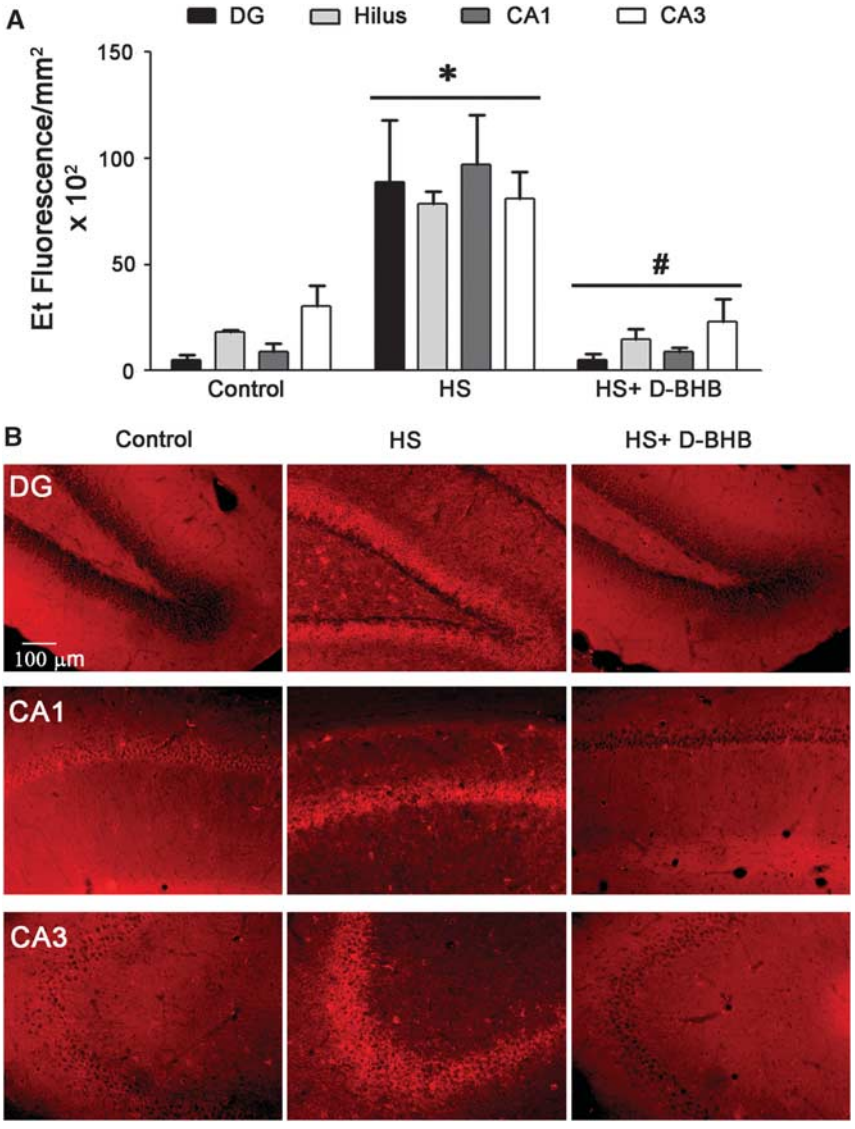

Figure 5. Et fluorescence units per unit area (Et fluorescence/mm^2) in different subregions of the hippocampus of control and hypoglycemic animals treated (HS+D-BHB) and nontreated (HS) with D-BHB (**A**). Images from different hippocampal subregions (CA1, CA3, dentate gyrus (DG), Hilus) were captured and the Et fluorescent signal measured. Data are expressed as means ± s.e.m. Control ($n = 3$), HS ($n = 4$), HS+D-BHB ($n = 6$). *$P < 0.05$ relative to control; #$P < 0.05$ relative to HS. (**B**) Images from brain sections of representative animals of each group show Et-positive cells in the different hippocampal subregions (**B**). HS, severe hypoglycemia; BHB, beta-hydroxybutyrate.

significantly increased cell survival at 5 and 10 mmol/L glucose, while this improvement was no longer significant when combined with 20 mmol/L glucose (Supplementary Figure 2). Protection by D-BHB in the presence of 10 mmol/L glucose agrees with the protective effect observed in the experiments shown in Figures 6A and 6B, in which the concentration of glucose in the recovery medium was 10 to 13 mmol/L. These results suggest that the improvement of cell survival by D-BHB requires the presence of glucose.

Effect of Beta-Hydroxybutyrate on Reactive Oxygen Species Production in Cultured Cortical Neurons Exposed to GD+GR, Xa/Xo, and H_2O_2

In a previous study, we reported that both isomers of BHB efficiently prevent the production of ROS induced by glycolysis inhibition.[10] We have now investigated the effect of L- and D-BHB on ROS generated during and after GD. As shown in Figure 6E, the Et fluorescent signal increased at the end of GD relative to control values, and D-BHB significantly reduced this increase, while L-BHB had no effect. Cultures exposed to 1.5 hours of GD followed by 1 hour of GR showed a notable increase in Et fluorescence relative to cultures exposed only GD indicating that ROS are mainly produced during GR. Cultures exposed to L- or D-BHB during GD and GR significantly reduced ROS production. The effect of D-BHB was significantly different from that of L-BHB (Figure 6E).

These results suggest that protection by BHB can be attributed not only to a metabolic action but also to an antioxidant effect. To further test this hypothesis, we used two different conditions to stimulate ROS production that are not directly associated with energy failure. To stimulate the production of superoxide, cells were exposed to Xa/Xo in the presence of DHE and the Et fluorescent signal was quantitated. As observed in Figure 6F, both BHB isomers reduced Et fluorescence showing D-BHB a better effect. Similarly, the increase in the Et signal induced by

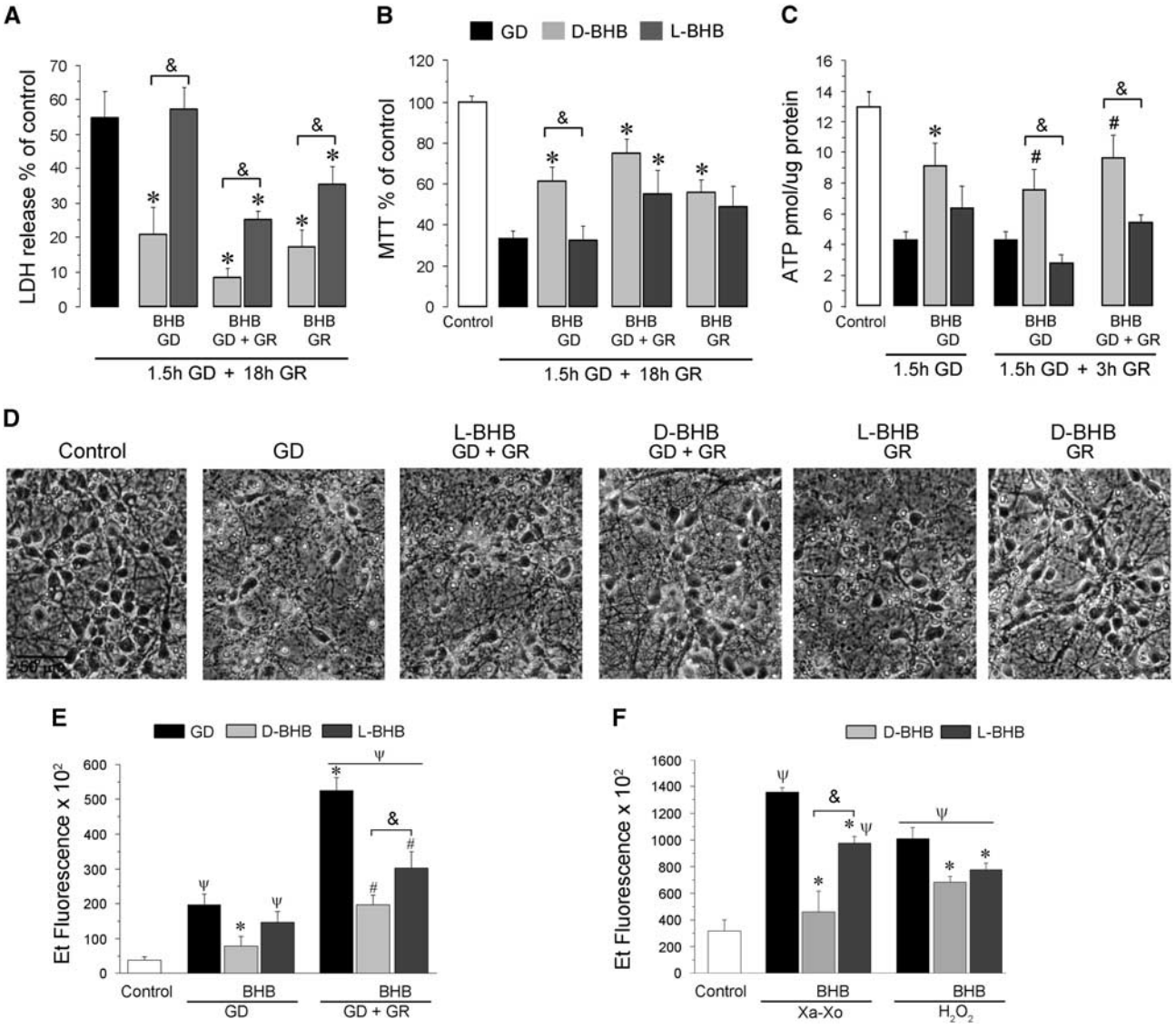

Figure 6. Cell survival monitored by lactate dehydrogenase (LDH) (**A**) and MTT (**B**) assays in cortical cultures exposed to GD+GR. D-BHB and L-BHB were incubated only during GD (10 mmol/L, left bars), during GD and GR (10 and 5 mmol/L, respectively, middle bars) and only during GR (10 mmol/L, right bars). Data are expressed as means ± s.e.m. ($n = 5$ to 10 independent experiments performed in duplicate). $^{*}P < 0.05$ relative to GD, $^{\&}P < 0.05$ relative to L-BHB. Changes in ATP levels are shown in (**C**). Cells were exposed to GD in the presence or the absence of D- and L-BHB (left bars) or exposed to GD and 3 hours of GR (middle and right bars). D- and L-BHB were added only during GD (right and middle bars) or during both GD and GR (right bars). Data are expressed as means ± s.e.m. ($n = 3$ to 7) independent experiments. $^{*}P < 0.05$ relative to GD, $^{\#}P < 0.05$ relative to GD+GR, $^{\&}P < 0.05$ relative to L-BHB. Bright field microscopy images of a representative experiment showing the morphologic changes elicited by the different treatments are shown in (**D**). The effect of D- and L-BHB on reactive oxygen species (ROS) production induced by GD+GR, by xanthine/xanthine oxidase (Xa/Xo) and H_2O_2 are shown in (**E**) and (**F**). Data are expressed as means ± s.e.m. ($n = 3$ to 4). $^{\Psi}P < 0.05$ relative to control, $^{*}P < 0.05$ relative to GD, Xa/Xo or H_2O_2, $^{\#}P < 0.05$ relative to GD+GR, $^{\&}P < 0.05$ relative to L-BHB. BHB, beta-hydroxybutyrate; GD, glucose deprivation.

incubation of cells in the presence of H_2O_2 was significantly reduced by BHB showing the two isomers similar effects.

DISCUSSION

The present results agree with previous findings showing that prolonged noncoma hypoglycemia induces neuronal death mainly in the cerebral cortex[22,28,29] and add new information about the distribution of degenerating and ROS-producing cells. According to the present data, noncoma hypoglycemia leads to cell death in the parietal and frontal cortices, showing the parietal cortex the highest number of degenerating neurons. In contrast to previous studies,[28,29] we were unable to detect a significant number of FJB-positive neurons in the piriform cortex. This discrepancy, might be possibly attributed to some variations in the experimental protocols or to differences in strain vulnerability to hypoglycemic neuronal damage. The majority of FJB-positive cells are observed in the superficial and medium cortical layers (II to IV), and in animals experiencing a short period of isoelectricity, the number of FJB-positive cells increases mainly in these layers in all cortical areas except for the piriform cortex. Previous studies reported that animals experiencing a long period (60 minutes) of isoelectricity show most of degenerating neurons in layers II and III of the cerebral cortex.[23] In the present condition, degenerating neurons were also observed in layer IV suggesting that these cells are also vulnerable to prolonged noncoma hypoglycemia. In an

attempt to investigate the correlation between cell death and ROS production, the fluorescent signal emitted by Et-positive cells was measured in different cortical areas. Results indicate that the parietal cortex, which shows the highest number of FJB-positive cells, also substantially increases the Et signal (8-fold). In the case of the frontal cortex, the increase in Et fluorescence is more modest (2.8-fold), as it is also the increase in the number of degenerating neurons relative to the parietal cortex. The temporal cortex showed a large increase in Et fluorescence (10-fold) relative to its control value; however, the total fluorescent signal in this region is much lower than that in the parietal cortex, which correlates with the low number of FJB-positive cells present in this region. Finally, the piriform cortex shows only few FJB-positive cells and a modest increase in Et fluorescence (2-fold). These results suggest a correlation between the amount of fluorescence observed in the different cortical areas and the number of degenerating neurons. However, Et-positive neurons are present mainly in layers III and IV rather than superficial (II) and deep cortical layers (V and VI). Therefore, a good correlation was found between ROS production and cell death in layers III and IV but not in layer II where FJB-positive cells were found while no Et-positive cells were evident, at least 24 hours after insulin administration, suggesting that selective neuronal death cannot be completely attributed to oxidative stress.

However, in the hippocampus Et fluorescence was detected in all subregions being the DG and CA1 the areas with the highest increase in the Et signal. These two areas have been reported as the most vulnerable to cell death induced by the hypoglycemic coma.[23] However, in the present conditions only a few degenerating cells were observed in the crest of the DG in some of the animals, suggesting that despite increased ROS production, the hippocampus is more resistant than the cortex to oxidative damage and cell death induced by noncoma hypoglycemia, in agreement with previous studies showing more damage in the cortex than in the hippocampus of rats subjected to noncoma hypoglycemia.[22,29] They also agree with our recent observations in the brain of mice exposed to noncoma hypoglycemia, which show large increases in ROS production in the hippocampus but no cell death.[21] Therefore, in the present conditions no correlation was found between increased ROS production and cell death the case of the hippocampus.

In the present study we tested the potential of D-BHB to substitute for glucose as an energy substrate during glucose depletion and to prevent the subsequent death of neurons. *In vivo* results show that hypoglycemic animals rescued with glucose and treated with D-BHB showed decreased cell death in the cortex and ROS production in the cortex and the hippocampus as compared with animals rescued with glucose alone. We observed that D-BHB-treated animals did not show the EEG characteristic changes of the coma state after 3 hours of insulin injection. Similar results were reported in rat pups treated with BHB, which showed a delayed onset to the hypoglycemic coma.[30] This effect might be attributed to the utilization of D-BHB in the brain since we observed that blood levels of the KB decrease in insulin-treated rats at the time of the loss of the RR (0.4 mmol/L) as compared with noninsulin-treated animals receiving D-BHB (0.69 mmol/L). These results agree with recent reports suggesting that D-BHB can be used as an alternative substrate, as a decrease in the glucose metabolic rate is observed in the brain of animals subjected to the ketogenic diet.[31,32]

In vitro data show that D-BHB notably prevents neuronal death when incubated during GD and GR, but is also effective when incubated only during GD or only during GR. Consistently, ATP levels recover to the same extent when D-BHB is present only during GD, only during GR or during both periods. According to these data preserving ATP concentrations at levels 30% to 40% below control values is sufficient to significantly prevent neuronal death. These observations also indicate that D-BHB can stimulate ATP production during GD but also during GR likely through its metabolism by the tricarboxylic acid cycle. Even though, D-BHB was able to rescue a small percentage of neurons in the absence of glucose, the best recovery of cells was obtained with the combination of D-BHB plus 5 or 10 mmol/L glucose, suggesting that the improvement of cell survival by D-BHB requires the presence of glucose.

The present results agree with other studies showing protection by the ketogenic diet or BHB infusion against ischemic and traumatic brain injury.[6–8,33–36] The improvement of mitochondrial function and ATP production has been suggested by several studies as the main mechanism involved in the neuroprotective action of KB.[11,16,17,33] We also tested the protective effect of pyruvate and lactate, which can also be used by brain as alternative substrates to glucose through its metabolism by the tricarboxylic acid cycle. In agreement with previous studies,[37–39] we observed a reduction of neuronal death in the *in vitro* model by both substrates, while the effect of pyruvate was similar to that of D-BHB, lactate was less effective.

According to the present data, besides the stimulation of ATP production, D-BHB also reduces ROS levels. Similar results have been previously reported in cultured and isolated neurons[10–12,16] as well as in isolated mitochondria,[16] and this effect has been implicated in the protective action of KB against excitotoxic neuronal death.[10–12,16] In agreement, *in vivo* studies have shown that BHB reduces the increase in lipoperoxidation induced by brain ischemia and hypoglycemia.[6,10] An antioxidant action of KB can result from the increase in NADH oxidation and/or the improvement of mitochondrial function.[10,16] In addition, in a previous study we reported in a free cell system that both isomers of BHB can scavenge the hydroxyl radical with an IC50 of 2 to 3 mmol/L.[10] We also showed that both isomers reduce the number of Et-positive hippocampal cells during glycolysis inhibition, suggesting that they retain their capacity to scavenge ROS in a cellular system.[10] We now show that both BHB isomers reduce ROS production induced by the exposure to Xa/Xo, which produces superoxide and the hydroxyl radical after superoxide dismutation to H_2O_2 in the presence of Fe^{2+}. Similarly, D- and L-BHB reduce the Et fluorescence induced by cell exposure to H_2O_2. The ROS production in these conditions is not initiated by energy failure and therefore the effect of D-BHB would be more related to an antioxidant rather than a metabolic action. According to the present results, L-BHB reduces ROS generated during GR despite its lack of a metabolic action, suggesting that ROS production during this period not only results from mitochondrial dysfunction but additional sources might be involved. In agreement, it has been shown that ROS generated during GR mainly result from the activity of NADHP oxidase,[19] a superoxide producing enzyme, but other sources might also contribute.[20] The present data suggest that the protective action of L-BHB results from its capacity to reduce ROS, since it is effective only during posttreatment, when most ROS are produced.

Other mechanisms of protection by KB have been suggested such as the inhibition of ATP-sensitive potassium channels that induces membrane hyperpolarization and reduces neuronal firing, contributing to the control of seizures in epileptic patients,[40,41] and the blockade of chloride-dependent glutamate uptake into synaptic vesicles, inhibiting glutamate release.[42] These mechanisms might be involved in KB protection against ischemic and hypoglycemic injury, since the release of excitatory amino acids during these conditions initiates the excitotoxic cell death cascade.[43,44] In addition, it has recently been shown that the ketogenic diet and the infusion of BHB stabilize the hypoxia induced factor, which regulates the expression of genes involved in energy metabolism and antiapoptotic proteins such as Bcl-2.[7] It remains to be investigated whether these mechanisms are involved in the protective effect of BHB observed in the present experimental conditions.

In conclusion, the present observations suggest that D-BHB can substitute for glucose in an *in vivo* model of noncoma hypoglycemia and effectively prevent ROS generation and cell death in all affected cortical areas, regardless their differential production of ROS and vulnerability to cell death. *In vitro* results suggest that the metabolic activity of D-BHB in combination with its antioxidant action contributes to the effective protective action of this KB, and that these actions can take place during the recovery period in the presence of glucose. These observations support the therapeutic potential of D-BHB against ischemic and traumatic insults, which are associated with energy impairment and oxidative stress and which require treatments that can be effective when administered after the insult.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Journal of Cerebral Blood Flow & Metabolism website (http://www.nature.com/jcbfm)