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ORIGINAL ARTICLE

Inhibition of G Protein-Coupled Receptor 81 (GPR81) Protects Against Ischemic Brain Injury

Zhe Shen,[1] Lei Jiang,[1] Yang Yuan,[1] Tian Deng,[1] Yan-Rong Zheng,[1] Yan-Yan Zhao,[1] Wen-Lu Li,[1] Jia-Ying Wu,[2] Jian-Qing Gao,[3] Wei-Wei Hu,[1,4] Xiang-Nan Zhang[1,4] & Zhong Chen[1,4]

1 Department of Pharmacology, Key Laboratory of Medical Neurobiology of The Ministry of Health of China, Zhejiang Province Key Laboratory of Neurobiology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China
2 Department of Pharmacy, First Affiliated Hospital, Zhejiang University, Hangzhou, China
3 Institute of Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China
4 Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, Hangzhou, China

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Correspondence
Xiang-Nan Zhang, Ph.D. and Zhong Chen, Ph.D., Department of Pharmacology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China.
Tel.: +86-571-8820-8228;
Fax: +86-571-8820-8228;
E-mails: xiangnan_zhang@zju.edu.cn; chenzhong@zju.edu.cn
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SUMMARY

Aim: Lactates accumulate in ischemic brains. G protein-coupled receptor 81 (GPR81) is an endogenous receptor for lactate. We aimed to explore whether lactate is involved in ischemic injury *via* activating GPR81. **Methods:** N2A cells were transfected with GFP-GPR81 plasmids 24 h previously, and then treated with GPR81 antagonist 3-hydroxy-butyrate (3-OBA) alone or cotreated with agonists lactate or 3, 5-dihydroxybenzoic acid (3, 5-DHBA) during 3 h of oxygen–glucose deprivation (OGD). Adult male C57BL/6J mice and primary cultured cortical neurons were treated with 3-OBA at the onset of middle cerebral artery occlusion (MCAO) or OGD, respectively. **Results:** The GPR81 overexpression increased the cell vulnerability to ischemic injury. And GPR81 antagonism by 3-OBA significantly prevented cell death and brain injury after OGD and MCAO, respectively. Furthermore, inhibition of GPR81 reversed ischemia-induced apoptosis and extracellular signal-regulated kinase (ERK) signaling may be involved in the neuroprotection. **Conclusions:** G protein-coupled receptor 81 (GPR81) inhibition attenuated ischemic neuronal death. Lactate may aggravate ischemic brain injury by activating GPR81. GPR81 antagonism might be a novel therapeutic strategy for the treatment of cerebral ischemia.

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Introduction

Ischemic stroke is one of the leading causes of death and disability. Many efforts have been made to find effective neuroprotective agents, however, few has been successfully translated into clinical application from basic research. Due to the complexity of the pathological events after ischemia, the mechanisms underlying ischemic stroke remain not fully elucidated.

Lactic acid accumulated by anaerobic glycolysis is the principal component responsible for brain acidification during ischemia [1]. Interestingly, recent studies suggested that lactate, as a ligand, can regulate glycolytic and oxidative pathways by activating the lactate responsive G protein-coupled receptor GPR81 [2,3]. The lactate selective receptor GPR81 is also known as hydroxy-carboxylic acid receptor 1 that is highly expressed in adipose tissue [4]. The *in situ* hybridization showed widespread distribution of *GPR81* mRNA in brains, predominantly in neurons within the cortex, hippocampus, and cerebellum [5]. Some evidence implies that GPR81 activation associates with neuronal activity and cerebral energy metabolism [6]. Moreover, activation of GPR81 down-regulates the cAMP and inhibits protein kinase A [7], thereby inhibiting lipolysis and promoting energy-rich metabolites storage [8]. Therefore, GPR81 may play an important role in mediating lactate signaling in both intact and ischemic brains.

Intracerebral pH decreasing from 7.0 to 6.6 due to lactate accumulation during focal cerebral ischemia [9] evokes inward currents characteristic of acid-sensing ion channels or triggers glutamate release [10] and thereby contributes to ischemic injury [11–14]. Lactic acidosis leads to great infarct, either by impairing neurons or by exacerbating hypoxic loss of astrocytes [15], and the underlying mechanisms may be attributable to severe oxidative stress injury and neuronal apoptosis [16]. Lactate also induces tumor necrosis factor-alpha, interleukin-6 and interleukin-1 beta release [17], which further exacerbate neuronal injury [18]. In contrast, some studies suggested that lactic acidosis may protect against ischemic injury directly [19]. There is evidence indicating that administration of lactate reduces ischemic injury by supplying energy [20,21]. Overexpression of monocarboxylate transporters,

which enhances the uptake of lactate for neurons, reduces infarct brain volumes [22]. Our previous studies also showed that acidic preconditioning and postconditioning protect against the neuronal death in a variety of ischemia models [23,24]. Therefore, the contributions of lactic acid to ischemic brains are controversial and it is unknown whether GPR81 activation by accumulated lactate during ischemia is involved in ischemic injury.

Materials and Methods

Animals

Male adult C57BL/6 mice weighing 22–25 g were used. For primary cortical neuronal culture, pregnant Sprague–Dawley rats with embryonic (E18) fetuses were used. All experiments were approved by and carried out in accordance with the ethical guidelines of the Zhejiang University Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize any pain or discomfort, and the minimum number of animals was used.

Focal Cerebral Ischemia and Drugs Administration

Mice were anesthetized with an intraperitoneal injection of choral hydrate (350 mg/kg). Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) as described previously [25]. Briefly, a 6–0 nylon monofilament suture, blunted at the tip and coated with 1% poly-L-lysine, was inserted 10 mm into the internal carotid to occlude the origin of the MCA. Achievement of ischemia was confirmed by monitoring regional cerebral blood flow by laser Doppler flowmetry (Model Moor VMS-LDF2; Moor Instruments Ltd., Devon, UK). Animals with <80% reduction in CBF in the core of the MCA area were excluded from the study. Body temperature was maintained at 37°C by a heat lamp (FHC, Bowdoinham, ME, USA) during surgery. Mice were given an intracerebroventricular injection of 2 μL 3-hydroxy-butyrate (3-OBA; Sigma, St. Louis, MO, USA) at the onset of MCAO. 3-OBA was dissolved in normal saline before injection. Control mice were injected with the same volume of saline.

Infarct volume was determined at 24 h after surgery by 2, 3, 5 -triphenyltetrazolium hydrochloride (TTC; 0.25%) staining, and the extents of the normal and infarct areas were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and determined by a indirect method, which corrects for edema. The percentage of the corrected infarct volume was calculated by dividing the infarct volume by the total contralateral hemispheric volume, and this ratio was then multiplied by 100.

Cell Culture, OGD Procedures, and Cell Viability Determination

For primary cortical neuronal culture, E18 rats were used. Briefly, the dissected cortex was treated with 0.125% trypsin in Hank's buffer (in mmol/L: 137 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 0.34 $Na_2HPO_4 \cdot 7H_2O$, 10 glucose, and 10 HEPES) for 10 min at 37°C and dissociated by repeated passage through a series of fire-polished Pasteur pipettes. Approximately 2×10^5 cells/cm^2 were seeded onto poly-L-lysine (10 μg/mL)-coated plates and dishes. The neurons were cultured in serum-free neurobasal medium (Invitrogen, Grand Island, NY, USA) supplemented with 2% B27 (Invitrogen), 10 U/mL penicillin, 10 U/mL streptomycin, and 0.5 mmol/L glutamine at 37°C in a humidified atmosphere with 5% CO_2. Cultures were maintained for 7 days before further treatment and were routinely observed under a phase-contrast inverted microscope. To certify the neuron percentage in our cultures, we stained the neurons by immunostaining against NeuN and we found that the neuron percentage is approximated 85–90%.

Mouse neuroblastoma neuro-2a (N2A) cells were routinely cultured in DMEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco), 10 U/mL penicillin, and 10 mg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

For OGD treatment, cells were rinsed once with warm glucose-free DMEM (Gibco), and then refreshed with glucose-free DMEM prebalanced in an O_2-free chamber at 37°C. Cells were then immediately placed into a sealed chamber (MIC-101; Billups-Rothenburg, Del Mar, CA, USA) loaded with mixed gas containing 5% CO_2 and 95% N_2 for 5 min at 20 L/min. Primary cultured neurons were kept in chambers for 4 h and N2A cells for 3 h. The indicated concentrations of 3-OBA, lactate (Sigma), and 3, 5-dihydroxybenzoic acid (3,5-DHBA; Sigma) were dissolved in glucose-free DMEM medium and added to the cells during OGD. Control cells were given equal refreshment but were incubated in glucose-containing DMEM at 37°C in an atmosphere of 5% CO_2, respectively. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay after OGD.

Plasmids and Transfection

The mice *GPR81* cDNA was amplified from a mouse fat cDNA library by PCR with the forward primer: 5′-GGA AGA TCT TCC ATG CCA GTC CTC TCT CCA AC-3′and the reverse primer: 5′- CCG GAA TTC CGT CAA CAC ACT TGG AGA TCC C-3′. The PCR product was inserted into the *BglII* and *EcoRI* site of pEGFP-C1 plasmid to construct the pEGFP-GPR81 plasmid. N2A cells were transfected using jetPRIME reagent (Polyplus transfection) according to the manufacturer's instructions, and cells were subjected to further experiments 24 h after transfection.

Apoptosis Assay

Neuronal apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay (TUNEL) using a cell death detection kit (Roche Diagnostics Corporation, Mannheim, Germany), according to the manufacturer's protocol. Images of nine random fields were captured from each sample. The results were expressed as percentage of TUNEL-positive cells to total DAPI-stained cells. Cell death was detected using combined staining with the chromatin dye, Hoechst 33342, and propidium iodide (PI). Hoechst 33342 (10 μg/mL) was added to the culture medium for 10 min at 37°C before cells were incubated with PI (10 μg/mL) for a further 10 min at 4°C. After three washes with phosphate-buffered saline (PBS), cells were fixed with 4% paraformaldehyde in PBS for 15 min at room tempera-

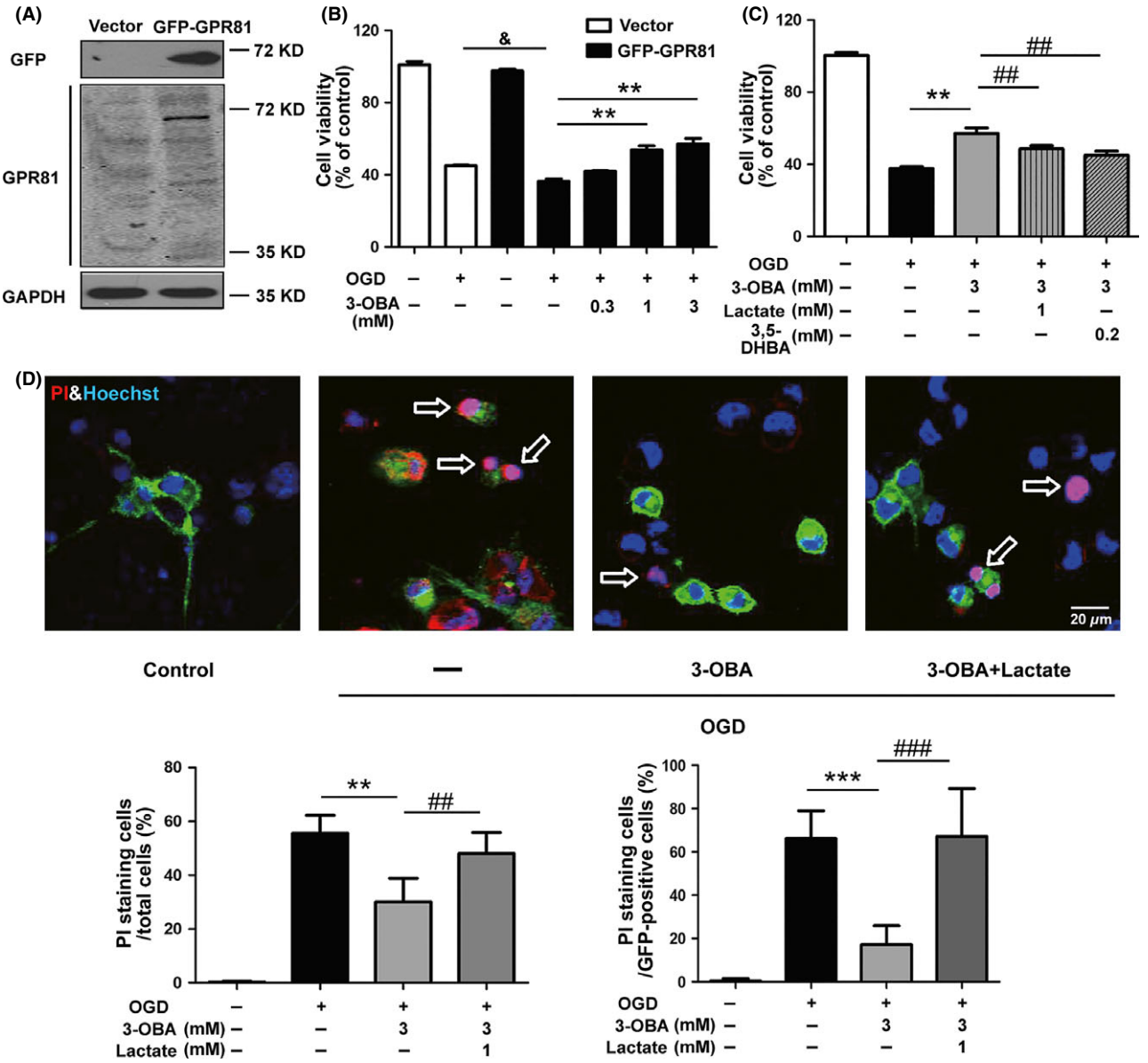

Figure 1 G protein-coupled receptor 81 (GPR81) is involved in oxygen–glucose deprivation-induced neuronal cell death. N2A cells were transfected with vector or GFP-GPR81 plasmids 24 h previously. Cells were treated with the compounds at indicated concentrations for 3 h during the oxygen–glucose deprivation (OGD). The cell viability was determined after the OGD exposure. (**A**) Transfection effects were confirmed by Western blot detecting GFP and GPR81. (**B**) The effects of GPR81 antagonist 3-hydroxy-butyrate (3-OBA) on cell viability were determined in GFP-GPR81-transfected cells. (**C**) The effects of GPR81 agonists, lactate (1 mmol/L) and 3, 5-DHBA (0.2 mmol/L) on cell viability were determined in GFP-GPR81-transfected cells. (**D**) Cell death was determined by double-staining with Hoechst 33342 (blue) and propidium iodide (PI, red) in GFP-GPR81 transfected cells (green). The arrows showed the PI-positive cells. Scale bars, 20 μm. Three independent experiments were included and conducted in triplicate wells. Statistical comparisons were performed with one-way ANOVA followed by Dunnett's *t*-test. $^{\&}P < 0.05$ versus nontransfected group. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus OGD treatment group. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus 3-OBA treatment group.

ture. Cells were viewed under a fluorescence microscope (BX-51; Olympus, Tokyo, Japan).

Western Blot Analysis

Cultured N2A cells were lysed after 3 h of OGD in ice-cold RIPA buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton-X100, 0.5% sodium deoxycholate, 1 mmol/L PMSF, 1 mmol/L Na_3VO_4, 1 mmol/L aprotinin, and 10 μg/mL leupeptin). For cytosolic and mitochondrial proteins extraction, an isolation kit (Beyotime, C3601) was used according to the manufacturer's instructions. In brief, the cells were rinsed with PBS and gently lysed with a hypotonic buffer on ice. The lysate was centrifuged at 1000 × *g* for 10 min at 4°C, the supernatant

was further centrifuged 3500 × *g* for 10 min at 4°C, and the pellet was collected as the mitochondria-enriched fraction. The remaining supernatant was then centrifuged 12,000 × *g* for 20 min at 4°C, and the supernatant was considered as cytosolic protein fraction. Protein samples were separated using SDS-polyacrylamide gel electrophoresis and transferred to the nitrocellulose membrane. After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies against Cytochrome C (1:800; CST, Beverly, MA, USA), Cleaved-caspase3 (1:1000; CST), Bax (1:1000; CST), Bcl-xl (1:1000; CST), GPR81 (1:500; Santa Cruz, CA, USA), p-ERK (1:1000; CST), ERK (1:1000; CST) or GAPDH (1:3000; KangChen, Shanghai, China) at 4°C overnight. After repeated washes, the membranes were reacted with antibodies against rabbit IgG (IRDye 800-coupled, 1:10,000) or mouse IgG (IRDye 700-coupled, 1:5000) for 2 h at room temperature. Images were acquired with the Odyssey infrared imaging system (LI-COR Biosciences, 9120, Lincoln, NE, USA) and analyzed using the Odyssey software [26]. The relative optical density was obtained by comparing the measured values with the mean values from the control group.

Determination of ATP Levels

The level of ATP in primary cultured neurons and transfected N2A cells were determined using the ATP assay kit (Beyotime Institute of Biotechnology, JiangSu, China), according to the manufacturer's instruction. Briefly, harvested cultured cells after OGD were lysed with a lysis buffer, followed by centrifugation at 12,000 × *g* for 10 min at 4°C. The level of ATP was determined by mixing 50 μL of the supernatant with 50 μL of luciferase reagent, which catalyzed the light production from ATP and luciferin. The emitted light was linearly related to the ATP concentration and measured using a microplate luminometer (Varioskan Flash, 5250040; Thermo, Waltham, MA, USA).

Immunofluorescence and Confocal Microscopy

After fixation with 4% paraformaldehyde for 15 min, cells were permeabilized with 0.3% Triton X-100 in PBS and blocked with 5% normal donkey serum for 2 h. Slices were incubated overnight at 4°C with primary antibodies: anti-Cytochrome C (1:200; CST), or anti-Tom20 (1:200; Anbo Biotechnology, San Francisco, CA, USA). Samples were washed with PBS for three times and incubated in secondary antibodies for 2 h at room temperature. Coverslips were observed under a confocal microscope (Fluoview FV1000; Olympus). Mander's overlap efficiency was measured and analyzed as previous described by Image Pro-Plus software [27].

Statistical Analysis

All data were collected and analyzed in a blind fashion. Data are presented as mean ± SD. One-way ANOVA (analysis of variance) with Dunnett's T3 *post hoc* test was applied for multiple comparisons. $P < 0.05$ was considered statistically significant.

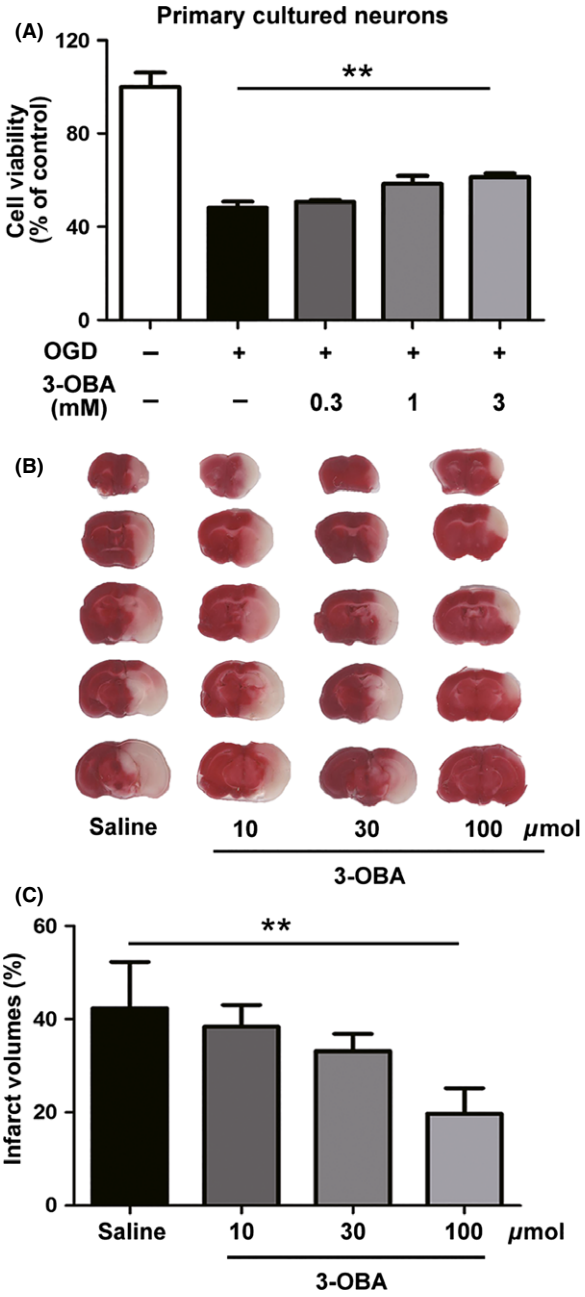

Figure 2 Inhibition of GPR81 protected against neuronal injury *in vitro* and *in vivo*. (**A**) Primary cultured rat cortical neurons were treated with 3-OBA at the beginning of oxygen–glucose deprivation (OGD). The cell viability was assessed by the MTT assay after 4-h OGD. (**B** and **C**) Mice were given an intracerebroventricular injection of 3-OBA at the onset of middle cerebral artery occlusion (MCAO). Infarct volumes were quantified by TTC staining 24 h after MCAO. Data were expressed as mean ± SD n = 6 for each group. Statistical comparisons were performed with one-way ANOVA followed by Dunnett's *t*-test; $^{**}P < 0.01$ versus indicated groups.

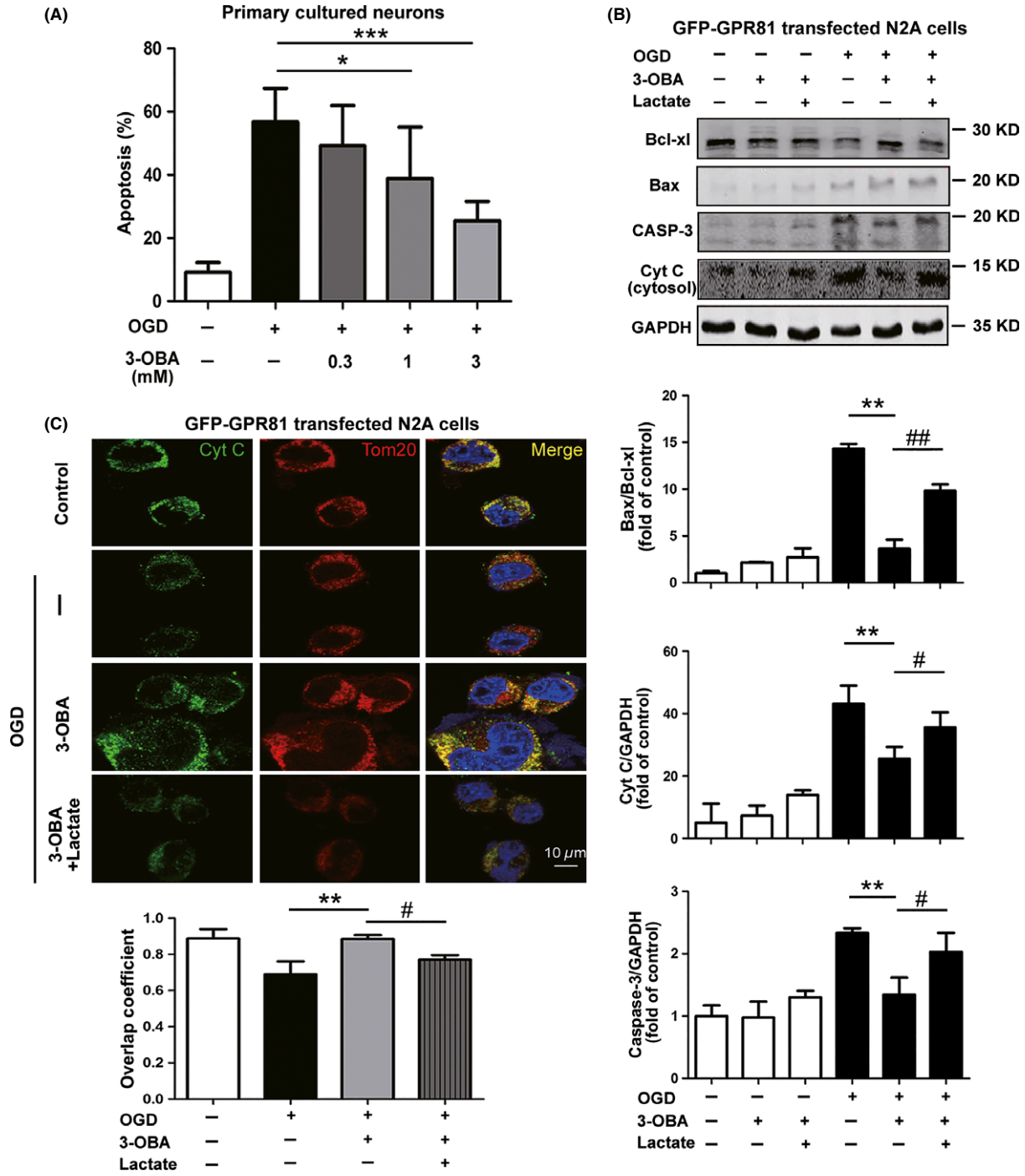

Figure 3 Inhibition of GPR81 reduced oxygen–glucose deprivation (OGD)-induced apoptosis. (**A**) Primary cultured rat cortical neurons were treated with 3-OBA at the beginning of OGD. After OGD, the apoptosis was determined using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL) staining, while the cell population was visualized by DAPI staining. The TUNEL-positive ratio in each section was calculated from nine random fields. Data are expressed as mean ± SD n = 3 for each group. GFP-GPR81-transfected N2A cells were subjected to OGD for 3 h in the presence of 3 mmol/L 3-hydroxy-butyrate (3-OBA) alone or with lactate (1 mmol/L). (**B**) In GFP-GPR81 transfected N2A cells, Bax, Bcl-xl, cytosolic Cyt C, and caspase-3 were detected by Western blot at the end of OGD. Semi-quantitative analysis of indicated proteins normalized to GAPDH was shown in the bar chart (mean ± SD, n = 3). (**C**) GFP-GPR81 transfected N2A cells were immunostained with Cyt C (green) and Tom20 (red). Nuclei were stained with DAPI. Scale bars, 10 μm. Columns represent the overlap coefficient. At least 20 cells from three independent experiments were included for each group. The data were expressed as mean ± SD. Statistical comparisons were performed with one-way ANOVA followed by Dunnett's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus OGD treatment group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus 3-OBA treatment group.

Results

GPR81 is Involved in Oxygen–Glucose Deprivation-Induced Neuronal Cell Death

To determine the role of GPR81 in ischemia, GFP-GPR81 was transfected into mouse neuroblastoma neuro-2a (N2A) cells 24 h before. The transfection efficiency was confirmed by Western blot detecting of GFP and GPR81, respectively (Figure 1A). Our data showed that GFP-GPR81 transfection aggravated OGD-induced cell death from 45.06 ± 0.27 to 36.43 ± 1.22% (Figure 1B, $P < 0.05$). The transfected cells were then exposed to 3-hydroxy-butyrate (3-OBA), the antagonist of GPR81 [28]. We found that in the transfected N2A cells, 3-OBA treatment significantly prevented the cell viability reduction in a concentration-dependent manner (Figure 1B, $P < 0.01$). Furthermore, lactate, the agonist of GPR81, reversed the protection of 3-OBA in a dose-dependent manner (Figure S1). Similarly, 3, 5-dihydroxybenzoic acid [29] (3, 5-DHBA), another agonist of GPR81, reversed the protection from 57.19 ± 2.95% to 45.09 ± 2.28% (Figure 1C, $P < 0.01$). Using combined Hoechst 33342 and PI staining, we found that 3-h OGD increased both apoptotic and necrotic N2A cells, while 3-OBA treatment reduced PI and Hoechst positive-staining from 55.57 ± 1.78% to 30.11 ± 2.77% (Figure 1D, $P < 0.01$). In GFP positive-staining cells, the cell death decreased from 66.11 ± 3.46% to 17.11 ± 2.79% after the treatment of 3-OBA (Figure 1D, $P < 0.001$), and this reduction was more prominent. In addition, lactate significantly reversed the protection of 3-OBA (Figure 1D, $P < 0.001$). These data suggested that GPR81 is involved in ischemic neuronal injury.

Inhibition of GPR81 Protected Against Ischemic Neuronal Injury Both *In Vitro* and *In Vivo*

Constitutive GPR81 expression in C57BL/6 mice brain was detected (Figure S2A). The expression of GPR81 in the cortex after indicated duration of middle cerebral artery occlusion (MCAO) was assessed by Western blot. The results revealed that GPR81 was transiently upregulated and peaked at 1 h after ischemia (Figure S2B). To further determine the involvement of GPR81 in ischemic injury, primary cultured neurons were exposed to the indicate concentration of 3-OBA at the onset of OGD. It was shown that 3-OBA increased cell viability in a concentration-dependent manner (Figure 2A, $P < 0.01$). To confirm the neuroprotection from GPR81 antagonism against ischemic brain injury, mice were given an intracerebroventricular injection of indicate dosage of 3-OBA. It was shown that 3-OBA decreased infarct volumes in a dose-dependent manner (Figure. 2B,C, $P < 0.01$). These data showed that inhibition of GPR81 protected against brain ischemic injury *in vivo* and *in vitro*.

Inhibition of GPR81 Reduced OGD-Induced Apoptosis

Apoptosis is well-accepted responsible for the cell demise after ischemia. And we found that 3-OBA reduced PI and Hoechst positive-staining. Therefore we postulated that GPR81 antagonism may release ischemia-induced apoptosis. To this end, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL) staining was carried out to test the apoptotic cell ratio with 3-OBA treatment. The results showed that 4-h OGD in primary cultured neurons significantly increased the apoptosis ratio, whereas treatment with 3-OBA reversed the cell apoptosis (Figure 3A, $P < 0.05$). The antiapoptosis protein Bcl-xl and pro-apoptosis protein Bax have been proved closely associated with apoptosis [30]. Our results showed that following 3-h OGD Bax/Bcl-xl ratio was significantly increased in GFP-GPR81 transfected N2A cells, whereas it was decreased with 3-OBA treatment (Figure 3B, $P < 0.01$). In addition, the Bax/Bcl-xl ratio reduction by 3-OBA was partly reversed by cotreatment with GPR81 agonist lactate ($P < 0.01$). After ischemia, Cytochrome C (Cyt C) releases from mitochondria into cytosol and thus activates caspase family proteins leading apoptotic cell death. Caspase-3, another marker of apoptosis, gave results similar to Bax/Bcl-xl ratio (Figure 3B, $P < 0.01$). Cyt C releasing into cytosol induced by 3-h OGD was significantly down-regulated by 3-OBA treatment (Figure 3B, $P < 0.01$). Similarly, cotreatment with lactate increased the cytosolic Cyt C level ($P < 0.05$). To verify their effects on Cyt C release, mitochondria and Cyt C were visualized

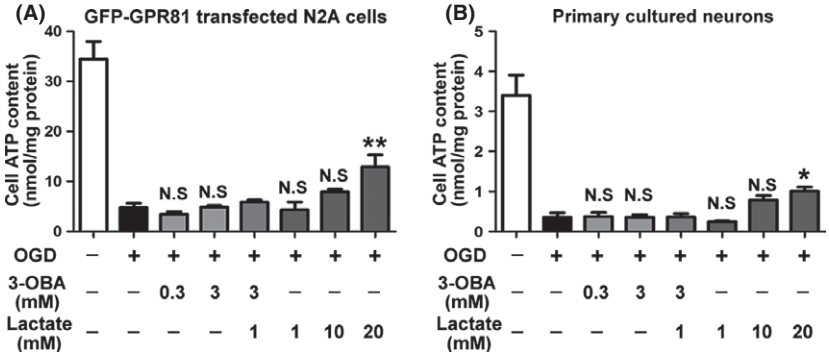

Figure 4 The impact of GPR81 agonist and antagonist on ATP level. (**A**) GFP-GPR81 transfected cells treated with indicated compounds during 3 h oxygen–glucose deprivation (OGD). (**B**) Primary cultured neurons treated with indicated compounds during 4-h OGD. ATP level in cellular supernatant was determined at the end of OGD. The data were expressed as mean ± SD n = 3 for each groups. Statistical comparisons were performed with one-way ANOVA followed by Dunnett's *t*-test. *$P < 0.05$ and **$P < 0.01$ versus OGD alone group. N.S, not statistically significant.

with fluorescent, and the overlaps between mitochondria and Cyt C were quantified. In control cells, the mitochondria marker Tom20 was almost colocalized with Cyt C. However, less overlap, suggesting Cyt C release, was observed in OGD cells, which could be reversed by 3-OBA incubation. Similarly, this overlap can be partly counteracted with lactate treatment (Figure 3C). Overall, these data suggested inhibition of GPR81 reduced ischemia-induced apoptosis.

The Impact of GPR81 Agonist and Antagonist on ATP Level

3-OBA and lactate are considered as the energy substrates for neurons [31,32]. To test whether 3-OBA and lactate provided energy during ischemia, we examined the intracellular ATP level both in GFP-GPR81 transfected N2A cells and primary cultured neurons with OGD treatment. The results showed that OGD significantly decreased the ATP level in two cell types (Figure 4A,B). Neither 3 mmol/L 3-OBA treatment nor 1 mmol/L lactate (the dose used in this study) could reverse the ATP decline, but higher level of lactate (20 mmol/L) significantly increased the concentration of ATP in primary cultured neurons (Figure 4B, $P < 0.05$), as well as transfected N2A cells (Figure 4A, $P < 0.01$). These data suggested that energy supplying may not responsible for the neuroprotection of 3-OBA.

Extracellular Signal-Regulated Kinase Signaling Pathway is Involved in the Protection of GPR81 Antagonism

The extracellular signal-regulated kinase (ERK) signaling pathway played an antiapoptotic role in ischemia-induced apoptosis [33]. To determine the involvement of ERK pathway in the protection of GPR81 antagonism, we detected the phosphorylated ERK by Western blot. The results showed that OGD treatment decreased the levels of phosphorylated ERK, which was reversed by 3-OBA incubation in both primary cultured neurons (Figure 5A, $P < 0.05$) and GFP-GPR81 transfected N2A cells (Figure S3A, $P < 0.01$). ERK dephosphorylation was confirmed by a specific MEK inhibitor U0126 in Western blot analysis. Furthermore, we found that U0216 reversed the protective effects of 3-OBA from $72.18 \pm 3.78\%$ to $39.68 \pm 13.47\%$ in neurons (Figure 5B, $P < 0.01$). Similarly, U0216 counteracted the protection in transfected N2A cells (Figure S3B, $P < 0.01$). To reveal whether GPR81-mediated apoptosis is related to ERK dephosphorylation, caspase-3 level was determined in the absence and presence of U0126. Our data showed that U0126 counteracted the 3-OBA-conferred caspase-3 reduction in primary cultured neurons (Figure 5A, $P < 0.05$), as well as in GFP-GPR81 transfected N2A cells (Figure S3A, $P < 0.01$). These data suggested that ERK signaling pathway was involved in the protection of GPR81 antagonism.

Discussion

Lactate accumulation is an important event after cerebral ischemia [1,34,35]. However, the contributions of lactate to ischemic brains are still controversial [36]. Previous investigations on lac-

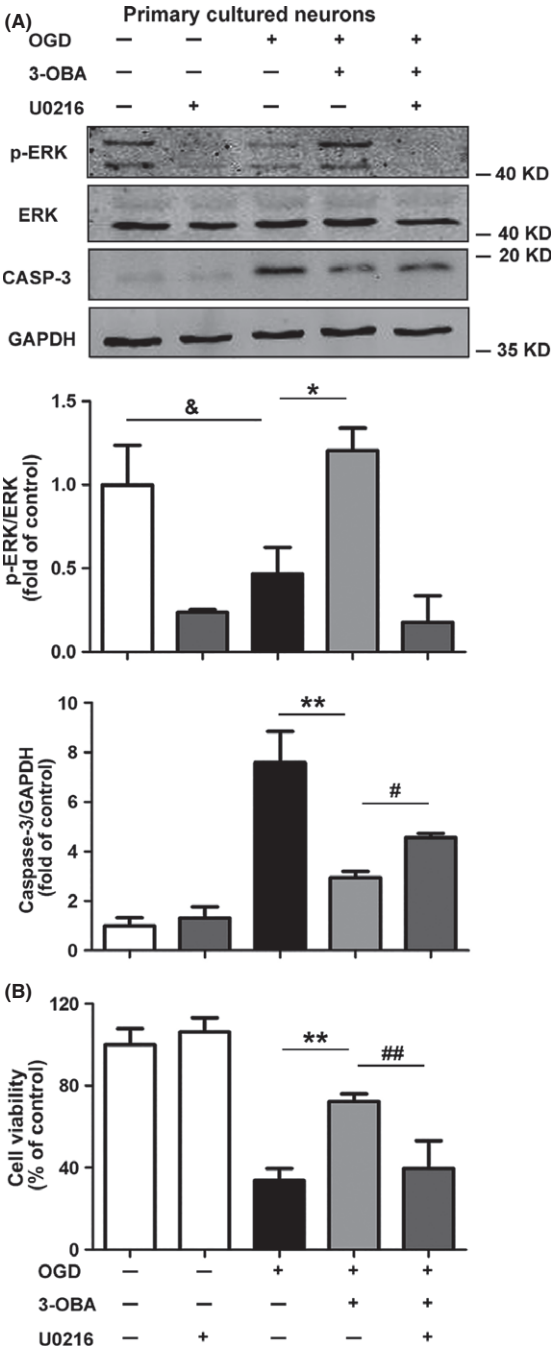

Figure 5 Extracellular signal-regulated kinase (ERK) signaling pathway involved in protection of GPR81 antagonism *in vitro*. Primary cultured neurons were treated with 3 mmol/L 3-hydroxy-butyrate (3-OBA) alone or with U0216 (5 μmol/L) for 3 h during oxygen–glucose deprivation (OGD). (**A**) The levels of phosphorylation ERK, total ERK, and caspase-3 were determined by Western blot and the semi-quantitative analyses normalized by GADPH were shown below. (**B**) Cell viability was determined by MTT assay after the OGD exposure. The data were expressed as mean ± SD n = 3 for each group. Statistical comparisons were performed with one-way ANOVA followed by Dunnett's *t*-test. $^{\&}P < 0.05$ versus control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus OGD treatment group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus 3-OBA treatment group.

tate principally focus on its role of as a pH regulator or an energy supplier. Interestingly, recent studies suggested that lactate acts as a signaling molecule to regulate neuronal activity via its endogenous receptor GPR81 [5,6,37]. However, little is known about GPR81 in lactate-involved ischemic brain injury. Here, we found that lactate receptor GPR81 was involved in cerebral ischemic injury. The GPR81 antagonism by 3-OBA showed significant neuroprotection either in oxygen–glucose deprivation (OGD) or middle cerebral artery occlusion (MCAO) model, and GPR81 prevented against ischemia-induced neuronal apoptosis partly by regulating ERK signaling.

We found that GPR81-transfected N2A cells, which are with higher transfection ratio versus primary cultured neurons, were more vulnerable to OGD insult compared with nontransfected ones (45.06 ± 0.27% vs. 36.43 ± 1.22% of control by MTT assay, respectively). Moreover, the GPR81 antagonism by 3-hydroxybutyrate (3-OBA) [28] conferred a dose-dependent protection in transfected N2A cells, which can be counteracted by the GPR81 agonists lactate [4] and 3, 5-dihydroxybenzoic acid (3, 5-DHBA) [29] (Figure 1). Also, we found that GPR81 mainly expressed in cortex and striatum which supposed to be the main damage regions in ischemic brain [9]. In addition, the level of GPR81 in cortex increased and peaked at 1 h and persisted until 12 h after ischemia. Since lactate accumulates during ischemia, our data suggested that lactate may aggravate ischemic injury by activating GPR81. To address the role of lactate as an energy supplier, we determined the intracellular ATP levels after treatment and found that only 20 mmol/L lactate partly rescued the ATP falling either in N2A cells or primary neurons (Figure 4). Indeed, lactate lower than 10 mmol/L is incompetent to compensate for energy crisis during brain ischemia [38,39], whereas it reversed the antagonism effect of 3-OBA. These data suggested that lactate may have dual effects on ischemic brain injury: Relative low concentration lactate (approximately 1–3 mmol/L) may contribute to neuronal injury through GPR81 receptor while high concentration offers its neuroprotection by supplying ATP. Although it was reported that high concentration 3-OBA can provide ATP [40], our results showed that 3 mmol/L 3-OBA offered its neuroprotection without increasing intracellular ATP. Thus, the 3-OBA may reduce ischemia-induced apoptosis *via* GPR81 rather than reversing ATP decline.

To further determine the neuroprotection of GPR81 antagonism on neurons and brains, the ischemic stroke models *in vitro* and *in vivo* were carried out. 3-OBA reduced OGD-induced primary cultured neuronal death in a concentration-dependent manner (Figure 2). Intracerebroventricular injection of 3-OBA at the onset of middle cerebral artery occlusion (MCAO) significantly alleviated ischemic brain injury. These data for the first time showed that GPR81 antagonism protect against ischemic brain injury.

Apoptosis is considered as a main death pattern of neuronal death in ischemic brains [41]; however, few investigations indicated that activating GPR81 can regulate apoptosis. We found that 3-OBA reduced both PI/Hoechst-positive and TUNEL-positive staining, indicating that 3-OBA ameliorated OGD-induced cell apoptosis. This assumption was further supported by the observation that 3-OBA treating reversed OGD-induced Bax/Bcl-xl ratio increasing and caspase-3 activation. Additionally, both Western blot and immunofluorescence showed that 3-OBA blocked Cyt C releasing into cytosol. These data provided further evidence that activating GPR81 may aggravate ischemic cell death. Furthermore, OGD exposure decreased the levels of phosphorylated ERK, which was rescued by 3-OBA treatment, suggesting that ERK might be involved in protection of GPR81 antagonism. U0126, a selective MEK inhibitor, counteracted the protection of 3-OBA, in terms of cell viability, caspase-3 ratio reduction. These data suggested that ERK may be an underlying signal responsible for the protection of GPR81 antagonism.

In conclusion, the current data indicate that lactate may aggravated ischemic brain injury by activating GPR81 and inhibition of GPR81 protects against ischemia-induced cell death *via* an antiapoptosis pathway. Therefore, GPR81 could serve as a potential therapeutic target for the treatment of cerebral ischemia.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. Lactate reverses the protection of 3-OBA in GFP-GPR81-transfected N2A cells.

Figure S2. The expression of GPR81 protein in physiological and pathological condition.

Figure S3. ERK signaling pathway is involved in protection of GPR81 antagonism in GFP-GPR81-transfected N2A cells.