Dual Properties of Lactate in Müller Cells: The Effect of GPR81 Activation

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PURPOSE. Besides being actively metabolized, lactate may also function as a signaling molecule by activation of the G-protein–coupled receptor 81 (GPR81). Thus, we aimed to characterize the metabolic effects of GPR81 activation in Müller cells.

METHOD. Primary Müller cells from mice were treated with and without 10 mM L-lactate in the presence or absence of 6 mM glucose. The effects of lactate receptor GPR81 activation were evaluated by the addition of 5 mM 3,5-DHBA (3,5-dihydroxybenzoic acid), a GPR81 agonist. Western blot analyses were used to determine protein expression of GPR81. Cell survival was assessed through 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assays. Lactate release was quantified by commercially available lactate kits. ^{13}C-labeling studies via mass spectroscopy and Seahorse analyses were performed to evaluate metabolism of lactate and glucose, and mitochondrial function. Finally, Müller cell function was evaluated by measuring glutamate uptake.

RESULTS. The lactate receptor, GPR81, was upregulated during glucose deprivation. Treatment with a GPR81 agonist did not affect Müller cell survival. However, GPR81 activation diminished lactate release allowing lactate to be metabolized intracellularly. Furthermore, GPR81 activation increased metabolism of glucose and mitochondrial function. Finally, maximal glutamate uptake decreased in response to GPR81 activation during glucose deprivation.

CONCLUSIONS. The present study revealed dual properties of lactate via functioning as an active metabolic energy substrate and a regulatory molecule by activation of the GPR81 receptor in primary Müller cells. Thus, combinational therapy of lactate and GPR81 agonists may be of future interest in maintaining Müller cell survival, ultimately leading to increased resistance toward retinal neurodegeneration.

Keywords: lactate, glucose, Müller cells, metabolism, glutamate uptake

Müller cells are the main retinal glial cell. Among multiple essential functions, Müller cells are key components in buffering molecules in the extracellular compartments of the inner retina[1,2] and aid in neuroprotection by removing excessive glutamate from the synapse between bipolar cells and retinal ganglion cells (RGCs) to prevent excitotoxicity.[3–5] To facilitate these functions, Müller cells rely heavily on a steady energy production. Müller cell energy metabolism is therefore essential in sustaining RGC survival, thereby reducing the risk of inner retinal conditions, such as glaucoma and diabetic retinopathy.[2,4,5] Previous studies have suggested that Müller cells are predominantly glycolytic[6–8] but shift to mitochondrial metabolism during excessive stress, such as sparse glucose availability and accumulation of reactive oxygen species (ROS).[9–11] With this in mind, lactate should be crucial during physiological conditions in the retina since retinal lactate is produced at a higher rate than pyruvate as the end product of glycolysis. Moreover, retinal tissue expresses high amounts of various monocarboxylate transporters (MCTs), which facilitate lactate uptake. Additionally, especially Müller cells are known to have a substantial expression of MCT-1 and MCT-4.[12]

We recently revealed that lactate is taken up and metabolized prior to glucose by the human Müller cell line MIO-M1,[13] consequently enhancing overall function and survival of the Müller cells. Similar protective effects of lactate have been seen in the brain, wherein cultured brain slices were able to maintain synaptic function by the use of lactate as the single energy source, and lactate, not glucose, was needed for neurons to recover from hypoxic conditions.[14–16]

Besides being an active metabolite, lactate has also been shown to play a potential role in neuronal signaling by the activation of G-protein–coupled receptor 81 (GPR81), also known as hydroxycarboxylic acid receptor 1 (HCAR1).[17,18] GPR81 activates the Gi-mediated pathway, by which adenylate cyclase is inhibited, resulting in a decrease of the second messenger cyclic AMP (cAMP), thereby altering numerous intracellular mechanisms. In line with this, a study performed in GPR81-silenced BxPC3 cells led to reduced mitochondrial activity and survival in specific cancer cells.[19]

The presence of lactate receptor GPR81 was recently revealed in high abundance in the retina, especially in RGCs and Müller cells.[20] Thus, it is tempting to suggest that lactate-mediated protection occurs through the activation of GPR81.

We recently showed that exposure to 10 mM L-lactate facilitated MIO-M1 cell function and survival during sufficient and insufficient glucose supply, mimicking the healthy and diseased retina, respectively. Since the retinal condition glaucoma is associated with periods of hypo- and hyperocular perfusion[21–23] it is likely that glucose is taken up during normal perfusion and is metabolized to lactate during hypoperfusion. Lactate may therefore serve as an energy substrate and mediator of neuroprotection during fluctuating energy availability. We therefore aimed to investigate the metabolic implications of treatment with 10 mM L-lactate in glucose-supplemented and glucose-deprived primary Müller cells from mice. Furthermore, treatment with 5 mM of a GPR81 agonist (3,5-dihydroxybenzoic acid) was used to examine whether the lactate-linked effects were due to direct metabolism of L-lactate or through GPR81 activation.

METHODS

Cell Cultures

Primary Müller cells were cultured from dissected retinas of neonatal mice (C57Bl/6JRj; Janvier, Le Genest-Saint-Isle, France) at postnatal days 7 or 8. The mice were euthanized by cervical dislocation and the eyes were taken out and transferred to a tube with Dulbecco's phosphate buffered saline (D-PBS). Retinas were dissected under a microscope (Leica S4E; Brønshøj, Denmark). The purification of Müller cells was obtained according to the method previously described.[5,13] For some experiments Müller cells were purified simultaneously with RGCs. In these purifications, cell suspension containing Müller cells, after respectively negative selection for macrophages and positive selection for RGCs, were seeded out directly in the wells/flasks.[5] In other cases, the dissected retinas were incubated for 30 minutes in Dulbecco's modified Eagle's medium, DMEM (21885; Gibco, Life Technologies, Thermo Fisher, Hvidovre, Denmark) supplemented with collagenase (65 U/mL) and trypsin (0.25%). Afterward, the cells were rinsed thrice in DMEM containing 10% fetal bovine serum (Gibco, Life Technologies) to terminate the digestion. The digested retinas were mechanically dissociated by gentle trituration through a 16- to 18-gauge needle, producing a cell suspension of tissue microaggregates and single cells. Cells were seeded in tissue culture plates and flasks (TPP Switzerland, Trasadingen) at a density of 0.7 to 2 retina/cm^2 depending on the particular plates and/or flasks (96 well, 24 well, 6 well, etc.). Cells were maintained at 37°C in a humidified atmosphere with 5% CO$_2$ cultured in DMEM containing glucose (1 g/L), pyruvate (1 mM), L-alanyl-glutamine (3.97 mM), supplemented with penicillin (90 U/mL) and streptomycin (90 μg/mL), and 10% fetal bovine serum (Gibco, Life Technologies). The Müller cells were rinsed vigorously three or four times 48 hours after seeding to prevent the attachment of other cells to the plates. The cells were kept in culture until confluency reached 80% to 90%. The culture medium was changed twice a week. Aggregates and debris were removed by forcible resuspension of medium onto the cell monolayer. Cell cultures of approximately 80% to 90% confluency were used for experiments. Cell culture medium was changed 1 day prior to all experiments. Figure 1 shows a representative image of the Müller cell cultures. Research animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
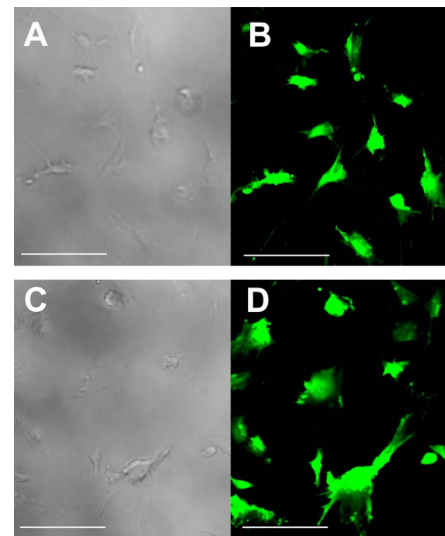
FIGURE 1. Primary murine Müller cell cultures. Light microscopy of the Müller cell cultures (**A, C**). The Müller cells positively expressed the Müller cell–specific marker, glutamine synthetase (GS) (**B**). Additionally, Müller cells expressed the glial cell–specific glutamate transporter EAAT1 (**D**). All images were acquired through a ×20/0.5 objective. *Scale bar*: 100 μm.

Western Blot Analysis

Primary mouse Müller cell cultures were scraped off after treatment for 2 hours with or without 6 mM glucose (DMEM A1443001; Gibco, Life Technologies). The cells were centrifuged at 8000*g* for 10 minutes at 4°C. Supernatants were discarded and cells were lysed in 30 μL radioimmune precipitation assay (RIPA) buffer (Sigma-Aldrich, Søborg, Denmark), which also included protease cocktail inhibitors 1 and 2 (Sigma-Aldrich). Protein lysates were centrifuged at 8000*g* for 10 minutes at 4°C. The samples containing 22 μg proteins were loaded onto gels to investigate protein expression of GPR81 by the use of a GPR81 antibody (cat. no. SAB1300090; Sigma-Aldrich). The blots were preincubated for 1 hour with Tris-buffered saline (TBS) (20 mM Tris-HCl, 150 mM NaCl) containing 5% nonfat dry milk. Afterward, the blots were incubated with a primary antibody against GPR81 (dilution 1:200) in TBS 1% nonfat dry milk overnight at 4°C. The blots were washed in TBS and incubated with the secondary antibody, goat anti-rabbit IgG alkaline phosphatase–conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA), followed by visualization using BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate-nitro blue tetrazolium) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). To ensure that equal amounts of protein were loaded in each lane, the membranes were incubated with beta-actin antibody at dilution 1:1000 (Cell Signaling Technologies, Danvers, MA, USA). Bands were quantified by densitometry through the use of Fiji, ImageJ software (National Institutes of Health, Bethesda, MD, USA). The density of each band was normalized to its own beta-actin band.

MTT Viability Assay

Müller cell survival was determined by the colorimetric method, MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide), measuring the ability of viable cells to reduce MTT. During treatment, the primary mouse Müller cell cultures were incubated in DMEM (A1443001; Gibco, Life Technologies) with the presence or absence of glucose

combined with the presence and absence of 10 mM L-lactate and/or 5 mM of the GPR81 agonist, 3,5-dihydroxybenzoic acid (3,5-DHBA) (Sigma-Aldrich) for 2 hours. Afterward, the cells were incubated with 12 mM MTT solution for 1 hour at 37°C and the cells were solubilized in 10% sodium dodecyl sulfate (SDS) and 0.01 M HCl. Eighteen hours later, the absorbance was measured at 560 nm. The background readings (blank wells with medium, MTT solution, and solubilizing buffer) were subtracted from the absorbance readings of the treated wells to obtain an adjusted absorbance reading representing cell viability. Finally, the readings were divided by the adjusted absorbance readings of control cells (6 mM glucose) to obtain a percentage of cell survival.

Lactate Assay

Primary Müller cells were treated with DMEM (A1443001; Gibco, Life Technologies) with or without the presence of 6 mM glucose for 2 hours at 37°C. The media was transferred to a 10-KDa spin filter (Amicon Ultra4, UFC801024; Merck, Søborg, Denmark) for 15 minutes at 4000g at 4°C to remove lactate dehydrogenase. The cells were afterward scraped off and centrifuged at 20,000g at 4°C for 20 minutes. The pellet was reconstituted in 100 μL 1 M potassium hydroxide (KOH) and protein content was measured by BCA protein assay (Sigma-Aldrich). The lactate measurements of the media were performed by using colorimetric detection through the Lactate Assay Kit (MAK064-1KT; Sigma-Aldrich) according to the manufacturer's protocol. Absorbance was read at 560 nm.

Metabolic Labeling Studies

To examine lactate metabolism in the primary Müller cells, the cells were incubated for 2 hours at 37°C in basic DMEM (A1443001; Gibco, Life Technologies) in the presence (6 mM) or absence (0 mM) of unlabeled D-glucose. At the start of incubation (Time = 0 hours), the cells were spiked with an aliquot of sodium L-[U-^{13}C] lactate (98% [^{13}C] enriched; CLM-1579-PK; Cambridge Isotopes Laboratories, Inc., Tewksbury, MA, USA) to a final concentration of 10 mM L-lactate. Other conditions required incubation with 6 mM labeled D-glucose (D-[^{13}C] glucose (99%) [^{13}C] enriched; CLM-1396-10; Cambridge Isotopes Laboratories, Inc.) with and without spiking of unlabeled sodium lactate to a final concentration of 10 mM or spiking with a GPR81 agonist to a final concentration of 5 mM. After the 2-hour incubation, the medium was collected and cells were rinsed twice with ice-cold PBS. Subsequently, the cells were extracted in 70% ethanol and centrifuged (20,000g and 20 minutes at 4°C) to separate the soluble extract (supernatant) from insoluble components (pellet). Protein content was determined in the pellets via the BCA protein assay kit (Sigma-Aldrich) using BSA as standard. Media and cell extracts were both lyophilized and reconstituted in water for further biochemical analysis by gas chromatography coupled to mass spectrometry (GC-MS). Spectrometric analyses were performed as previously described.[24] Briefly, extract and media samples were adjusted to pH 1–2 with HCl and evaporated to dryness under nitrogen flow. Analytes were extracted into an organic phase (96% ethanol/benzene) followed by derivatization with 14 % DMF/86% MTBSTFA with a modified procedure from Mawhinney et al.[25] Standards containing unlabeled metabolites of interest and cell extracts were separated and analyzed in a gas chromatograph (7820A chromatograph, J&W GC column HP-5MS, parts no. 19091S-433; Agilent Technologies, Glostrup, Denmark) coupled to a mass spectrometer (5977E; Agilent Technologies). The isotopic enrichment of the metabolites of interest was corrected for natural abundance of ^{13}C using the unlabeled standards and calculated according to

Biemann et al.[26] Data are presented as labeling (%) of M + X, where M is the mass of the unlabeled molecule and X is the number of labeled C-atoms in a given metabolite.

Bioenergetic Measurements Using the Seahorse Analyzer

Mitochondrial respiratory function was measured using real-time assessment of the oxygen consumption rate (OCR) with the Seahorse XF^{e}96 Extracellular Flux Analyzer (Agilent Technologies). Primary mouse Müller cells were cultured in a Seahorse 96-well cell culture microplate at a density corresponding to 0.19 retina/well for 1 week. On the day of the assay, cells were preincubated 2 hours before the analysis under the different experimental conditions, namely, medium with or without 6 mM glucose and/or 10 mM lactate in the absence or presence of 5 mM 3,5-DHBA. Prior to the assay, culture media were changed to unbuffered DMEM (pH 7.4) supplemented with the different conditions and the cells were equilibrated for 10 minutes at 37°C in a CO$_2$-free incubator. Time of calibration inside the Seahorse instrument was set for a further 15 minutes. Each OCR measurement cycle consisted of 3-minute mix and 3-minute measurement of the oxygen level. The analysis of mitochondrial function was initiated by three baseline OCR measurement cycles. These were followed by the sequential injection of oligomycin, an ATP synthase (complex V) inhibitor (1 μM final concentration), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP, 2 μM), an uncoupling agent that collapses the proton gradient, and a final injection of a mixture of rotenone (1 μM), a complex I inhibitor, and antimycin A (1 μM), a complex III inhibitor. The latter combination shut down the mitochondrial respiration and enabled the estimation of nonmitochondrial oxygen consumption. Two OCR measurement cycles were taken in between each injection and three final measurement cycles. The pH of the reagents used to test mitochondrial function was adjusted to 7.4. The OCR was recorded and calculated by the Seahorse XF^{e}96 software, Wave (Agilent Technologies). To normalize the data after the Seahorse analysis, the protein content for each well was measured using the Pierce BCA assay with BSA as standard (Thermo Fisher Scientific, Hvidovre, Denmark). Mitochondrial respiration parameters were calculated from the OCR measurements as follows. Basal respiration, nonmitochondrial respiration (minimum measurement after rotenone/antimycin injection), was subtracted from the last measurement obtained before oligomycin injection; oxygen consumption associated with ATP production, measurement after oligomycin injection, was subtracted from the last measurement before oligomycin injection; maximal respiration, measurement obtained after FCCP injection; spare respiratory capacity, basal respiration subtracted from maximal respiration. Results are expressed as mean values ± standard error of the mean (SEM) of three to six different cell batches, at least 10 wells/condition in each plate.

Glutamate Uptake Assay

The kinetic characterization of glutamate transport into the primary mouse Müller cells was performed as described in our previous studies.[13] The primary mouse Müller cells were cultured in 24-well plates with a density of 0.9 retina/mL and cells were treated with the previously mentioned conditions for 2 hours. The cells were preincubated for 3 minutes at 37°C in HBSS (HEPES buffered saline solution; 142 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM Na$_2$HPO$_4$, and 10 mM HEPES) containing different concentrations of L-glutamate

A

GPR81
~ 73 kDa

Beta-actin
~ 45 kDa

B
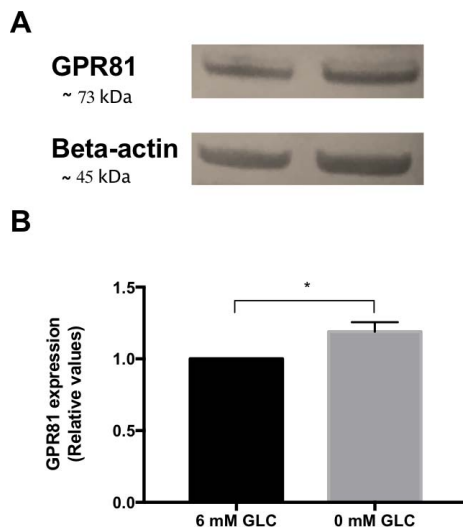
FIGURE 2. Increased GPR81 protein expression in primary Müller cells in the absence of glucose. (A) A representative Western blot of GRP81 expression in primary mouse Müller cells treated for 2 hours in the presence and absence of 6 mM glucose (GLC) (B) Relative expression of GRP81 in conditions with 6 mM GLC (*black column*) and 0 mM GLC (*gray column*). Values are presented as means ± SEM of fold basal change in three experiments. *$P < 0.05$. Statistics: unpaired *t*-test.

(range, 2–50 μM). Subsequently, the cells were incubated for an additional 3 minutes in HBSS including trace amounts (1: 200) of L-[3,4-3H]-glutamate (4 μCi/mL; Perkin-Elmer, Waltham, MA, USA). The cellular content of radioactivity was determined in cells extracted in KOH and analyzed by liquid scintillation counting in a Liquid Scintillation Analyzer, Tri-Carb 2900TR (Perkin-Elmer). Uptake rates were corrected for protein concentrations, which were measured in the cell extracts using BCA protein assay kit (Sigma-Aldrich). Unspecific uptake was determined by incubation at 0°C. Uptake kinetics were assumed to follow the normal Michaelis–Menten kinetics. The maximal uptake rate, V_{max}, was determined using a nonlinear regression in GraphPad Prism 7 ($y = V_{max} \cdot x/(K_m + x)$).

Statistics

Data were analyzed using GraphPad software (GraphPad Prism version 7.0, San Diego, CA, USA). Statistical comparisons between two groups were analyzed by unpaired 2-tailed *t*-test. Statistical comparisons between several groups were analyzed by 1-way ANOVA followed by either Tukey's multiple comparison post test or Dunnett's multiple comparison post test to evaluate statistical differences between experimental groups or compared to a common control, respectively. Survival data, GC-MS data, and cellular bioenergetics were analyzed using 2-way ANOVA (Tukey's multiple comparisons post hoc test). In all analyses $P < 0.05$ was considered to be statistically significant.
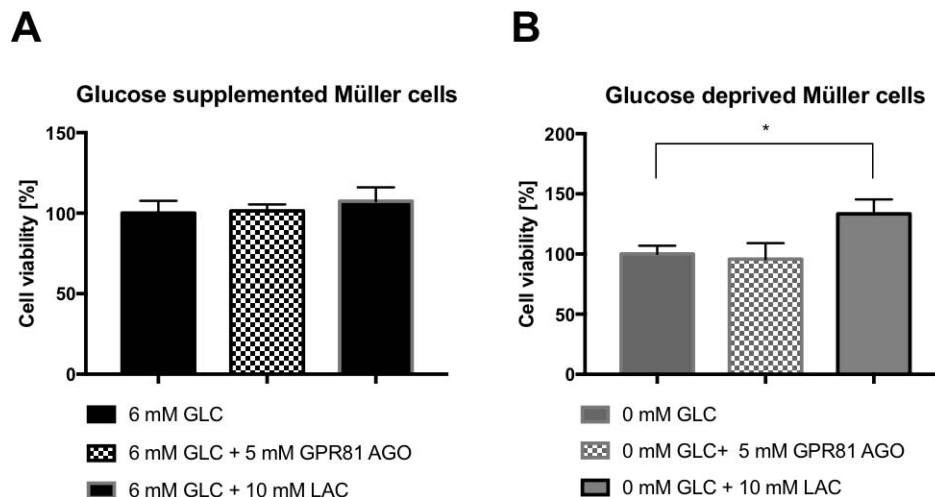
RESULTS

GPR81 Protein Expression Increases During Glucose Deprivation in Primary Müller Cells

To evaluate a potential regulation of lactate-mediated pathways in response to glucose deprivation, we determined the protein expression of the lactate receptor, GPR81. Protein expression of GPR81 was upregulated 1.43 ± 0.26 during glucose deprivation for 2 hours ($P < 0.05$) (Fig. 2).

Primary Müller Cell Survival Is Unaffected by GPR81 Activation, Whereas Lactate Exposure Increases Survival During Glucose Deprivation

An MTT assay was used to investigate whether GPR81 was toxic or compensatorily protective for the primary Müller cells.

Glucose deprivation for 2 hours was cytotoxic and decreased primary Müller cell viability to 40.6 ± 5.3% ($P < 0.001$). However, GPR81 did not affect survival, either in glucose-deprived or glucose-supplemented cells. Exposure to 10 mM L-lactate increased survival of glucose-deprived cells by 33.4% ($P < 0.05$) (Fig. 3).

A

Glucose supplemented Müller cells

■ 6 mM GLC
▨ 6 mM GLC + 5 mM GPR81 AGO
■ 6 mM GLC + 10 mM LAC

B

Glucose deprived Müller cells

■ 0 mM GLC
▨ 0 mM GLC+ 5 mM GPR81 AGO
■ 0 mM GLC + 10 mM LAC

FIGURE 3. Unaffected primary Müller cell survival in response to GPR81 activation in the presence (**A**) and absence of glucose (**B**). Primary mouse Müller cells were treated for 2 hours with (*dotted columns*) or without 5 mM of the GPR81 agonist, 3,5-DHBA, in the presence (*black columns*) and absence of 6 mM glucose (*gray columns*). An MTT assay was used to quantify relative cell viability. Treatment with the GPR81 agonist did not have any effect on cell viability, whereas the addition of 10 mM L-lactate during glucose deprivation increased cell viability. Values are presented as means ± SEM of three to six experiments. *$P < 0.05$. Statistics: 1-way ANOVA (Tukey's multiple comparisons test).
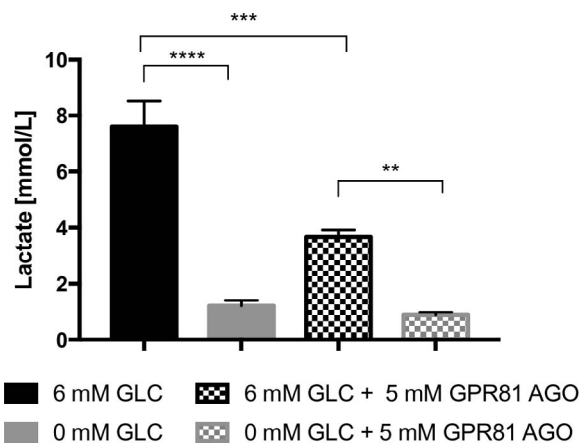
FIGURE 4. Decreased lactate release in primary Müller cells in response to GPR81 activation in the presence of glucose. Primary mouse Müller cells were treated for 2 hours with (*dotted columns*) or without 5 mM of the GPR81 agonist, 3,5-DHBA, in the presence (*black columns*) and absence of 6 mM glucose (*gray columns*). A commercial lactate assay kit was used to quantify the amounts of lactate released to the media. Treatment with the GPR81 agonist resulted in reduced amounts of released lactate. Values are presented as means ± SEM of four experiments. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Statistics: 1-way ANOVA (Tukey's multiple comparisons test).

## Reduced Lactate Release in Primary Müller Cells in Response to GPR81 Activation

Primary mouse Müller cells were treated for 2 hours with or without 5 mM of the GPR81 agonist, 3,5-DHBA, in the presence and absence of 6 mM glucose mimicking physiological and subphysiological settings, respectively. Lactate was released to the extracellular environment in all four conditions. Within 2 hours, 8 ± 1 mM lactate was released in the presence of glucose, whereas only 1.2 ± 0.2 mM was released during glucose deprivation ($P < 0.0001$). Treatment with the GPR81 agonist decreased extracellularly released lactate amounts to 4 ± 0.3 mM in the presence of glucose ($P < 0.001$). Extracellular lactate amount remained low in the absence of glucose, and GPR81 agonist treatment had no significant effect (Fig. 4).

## Primary Müller Cells Prefer Lactate as a Metabolic Substrate Prior to Glucose

To investigate whether primary Müller cells are able to utilize lactate as energy substrate under glucose-supplemented and glucose-deprived conditions, cultures of primary Müller cells were incubated with [U-^{13}C] lactate in absence or presence of unlabeled glucose. GC-MS analyses were used to estimate the percentile ^{13}C-enrichment in lactate, alanine, and tricarboxylic acid (TCA) cycle metabolites and amino acids obtained from incubating the cells with [U-^{13}C]-labeled substrates. After the incubation with [U-^{13}C] lactate, labeling was found in lactate (M+3) (Fig. 5A), alanine, and metabolites produced after a first turn (M+2) (Fig. 5B) and second turn (M+4) (Fig. 5C) of the TCA cycle, demonstrating that lactate is readily taken up and metabolized in the primary Müller cells (Fig. 5). The presence of unlabeled glucose combined with labeled lactate increased metabolism of the labeled lactate by yielding increased intermediate TCA substrates, for example, alpha-ketoglutarate ($P < 0.01$), glutamate ($P < 0.01$), malate ($P < 0.01$), and aspartate ($P < 0.01$). To ensure a direct comparison among metabolized labeled glucose versus metabolized labeled lactate, an average value of the ^{13}C-enrichment arising from the metabolism of either ^{13}C-glucose or ^{13}C-lactate, molecular carbon labeling (MCL), was calculated. The MCL of metabolites was increased from ^{13}C-lactate compared with ^{13}C-glucose ($P < 0.05$) (Fig. 6).

GPR81 Activation Boosts Metabolism of Glucose in Primary Müller Cells

To further examine the substrate dependency, the metabolic interplay between lactate and glucose, and the effect of GPR81 activation in the primary Müller cells, cells were incubated with [U-^{13}C] glucose in absence and presence of unlabeled lactate or the GPR81 agonist, 3,5-DHBA. As expected, ^{13}C labeling was found in metabolites arising after the incubation with [U-^{13}C] glucose demonstrating the utilization of this substrate as an energy source in primary Müller cells (Fig. 7). However, a significant decrease in labeling was found in virtually all measured metabolites when unlabeled lactate was present. In the presence of the GPR81 agonist, the labeling patterns in first-turn TCA cycle metabolites following [U-^{13}C] glucose metabolism were slightly increased while the ^{13}C
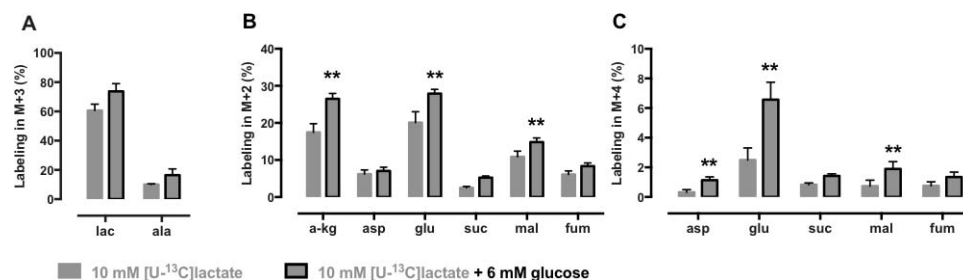
FIGURE 5. Lactate is metabolized in primary Müller cells. Cultures were incubated for 2 hours at 37°C in basic DMEM supplemented with 10 mM L-[U-^{13}C] lactate in absence (*gray columns*) or presence (*gray column* with *black border*) of unlabeled 6 mM glucose. At the end of the incubation, cell extracts were collected and analyzed using GC-MS for determination of the percentage distribution of ^{13}C-labeled metabolites presented by ^{13}C-labeling in M+3 for lactate and alanine (**A**) and in M+2 for metabolites obtained after a first run of the TCA cycle (**B**), as well as in the subsequent turn of the TCA cycle, M+4 (**C**). The incorporation of ^{13}C from [U-^{13}C] lactate in amino acids and metabolites indicates that lactate is taken up and used as substrate in primary Müller cells. No dilution of the labeling was obtained during combined labeled lactate and unlabeled glucose, indicating that lactate is preferentially utilized as an energy substrate in these cells. Data are presented as labeling (%) of M + X, where M equals the mass of the unlabeled molecule and X is the number of labeled C-atoms in a metabolite. Lac, lactate; ala, alanine; α-kg, alpha-ketoglutarate; asp, aspartate; glu, glutamate; suc, succinate; mal, malate; fum, fumarate. Values are presented as means ± SEM of four experiments. **$P < 0.01$. Statistics: 2-way ANOVA (Tukey's multiple comparisons test).
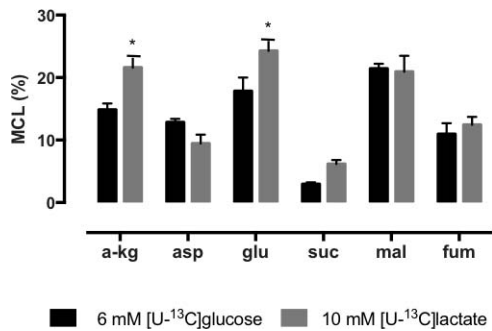
FIGURE 6. Lactate is a preferential energy source in primary Müller cells. Cultures were incubated for 2 hours at 37°C in basic DMEM (0 mM glucose) and supplemented with either 10 mM L-[U-^{13}C] lactate (*gray bars*) or 6 mM D-[U-^{13}C] glucose (*black bars*). At the end of the incubation, cell extracts were collected and analyzed using GC-MS for determination of the percentage distribution of ^{13}C-labeled metabolites. An average of the ^{13}C-enrichment in selected metabolites arising from metabolism of ^{13}C-lactate or ^{13}C-glucose is represented as the molecular carbon labeling (MCL). The MCL is calculated by multiplying the labeling (%) of the different isotopomers of a compound with the number of labeled atoms in that particular isotopomer, summing these products, and dividing them by the total number of carbon atoms in the relevant compound. The MCL of several metabolites obtained from ^{13}C-lactate was higher than the values calculated from ^{13}C-glucose. Values are presented as means ± SEM of four experiments. *P < 0.05. Statistics: 2-way ANOVA (Tukey's multiple comparisons test).

labeling in second-turn metabolites was significantly upregulated, suggesting an enhanced glucose metabolism induced by activation of GPR81.

GPR81 Activation Increases Mitochondrial Activity in Primary Müller Cells

Since GPR81 increased mitochondrial TCA cycle metabolite synthesis, we aimed to further investigate the effect of GPR81 activation on mitochondrial respiratory function in primary Müller cells. Oxygen consumption during mitochondrial respiration was simultaneously monitored under different conditions via the Seahorse XF^e instrument and the OCR was calculated in real time. Several compounds were employed to assess mitochondrial respiration including oligomycin that inhibits ATP synthase, FCCP that collapses the proton

gradient and induces a maximal consumption of oxygen and a mixture of rotenone and antimycin A, complex I and complex III inhibitors, respectively, that together shut down mitochondrial respiration. Figures 8A and 8B show kinetic traces of the changes in OCR throughout a typical mitochondrial function assay in primary Müller cells preincubated with different substrates and conditions. From those traces, the mitochondrial respiration parameters, including basal respiration, ATP production associated with oxygen consumption, maximal respiratory capacity, and spare respiratory capacity, were calculated. The OCR values obtained in the presence of lactate and/or glucose (Figs. 8C, 8D) were similar, indicating that both glucose and lactate are efficient substrates for mitochondrial respiration with lactate resulting in the highest maximal respiratory capacity. In the presence of glucose, mitochondrial respiration was increased after GP381 activation with 3,5-DHBA. Furthermore, the GPR81 agonist was able to upregulate mitochondrial respiration in the absence of glucose or lactate.

Glutamate Uptake in Primary Mouse Müller Cells Decreases in Response to GPR81 Activation

Kinetic [^{3}H]-glutamate uptake assays were performed on primary Müller cells to investigate whether 10 mM L-lactate exposure had a protective enhancing effect on the Müller cell function, and whether the effects of L-lactate exposure and GPR81 agonist exposure were the same in physiological and pathophysiological settings: glucose-supplemented and glucose-deprived cells, respectively. Maximal glutamate uptake in primary Müller cells compensatorily increased from 0.84 ± 0.23 nmol • min^{-1} • mg protein^{-1} to 1.48 ± 0.11 nmol • min^{-1} • mg protein^{-1} after 2 hours of glucose deprivation (P < 0.05) (Fig. 9A). GPR81 agonist treatment for 2 hours had no significant effect on glutamate uptake in glucose-supplemented cells (Fig. 9B). However, in glucose-deprived cells, GPR81 activation resulted in a decreased maximal uptake of 0.83 ± 0.27 nmol • min^{-1} • mg protein^{-1} after 2 hours (P < 0.05) (Fig. 9C). The Michaelis–Menten constant of the glutamate uptake did not change significantly in either of the mentioned conditions.
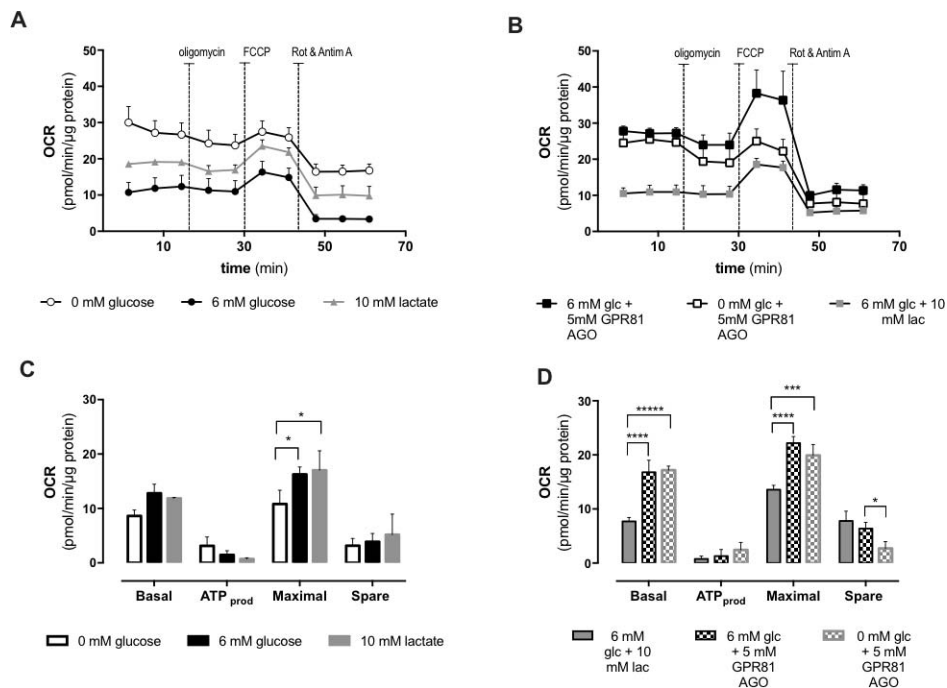
DISCUSSION

Lactate has often been overlooked as a metabolic reserve molecule, but recent studies have put more emphasis on
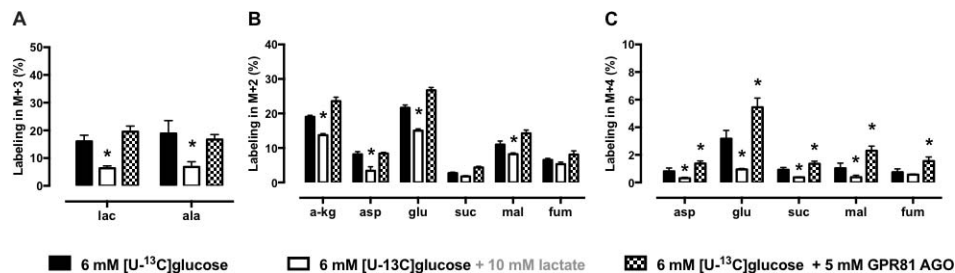
FIGURE 7. Increased metabolism of ^{13}C-glucose in primary Müller cells in response to GPR81 activation. Cultures were incubated for 2 hours at 37°C in basic DMEM-supplemented 6 mM D-[U-^{13}C] glucose in absence or presence of unlabeled 10 mM lactate, or the GPR81 agonist, 3,5-DHBA. At the end of the incubation, cell extracts were collected and analyzed using GC-MS for determination of the percentage distribution of ^{13}C-labeled metabolites presented by ^{13}C-labeling in M+3 for lactate and alanine (**A**) and in M+2 for metabolites obtained after a first run of the TCA cycle (**B**), as well as in the subsequent turn of the TCA cycle, M+4 (**C**). The incorporation of ^{13}C from [U-^{13}C] glucose in amino acids and metabolites indicates that glucose is taken up and used as substrate in primary Müller cells. However, lactate-induced decreased labeling in metabolites obtained from [U-^{13}C] glucose suggests that lactate is preferentially utilized as an energy substrate in these cells. The labeling in amino acids and metabolites following [U-^{13}C] glucose metabolism was increased in the presence of the GPR81 agonist, suggesting an enhanced glucose metabolism induced by activation of GPR81. Data are presented as labeling (%) of M + X, where M equals the mass of the unlabeled molecule and X is the number of labeled C-atoms in a metabolite. Lac, lactate; ala, alanine; α-kg, alpha-ketoglutarate; asp, aspartate; glu, glutamate; suc, succinate; mal, malate; fum, fumarate. Values are presented as means ± SEM of four experiments. *P < 0.05. Statistics: 2-way ANOVA (Tukey's multiple comparisons test).

FIGURE 8. Mitochondrial function is enhanced by GPR81 activation. Cultures of primary Müller cells were incubated for 2 hours at 37°C under the indicated experimental conditions. (A, B) Oxygen consumption rate (OCR) was monitored using a Seahorse XF^{e}96 Extracellular Flux Analyzer and a standard mitochondrial function assay. OCR measurements were taken before (basal) and after the addition of the inhibitor of ATP synthase, oligomycin (1 μM), the uncoupling agent FCCP (2 μM), and the inhibitors of mitochondrial complexes I and III, rotenone (Rot, 1 μM) and antimycin A (Antim A, 1 μM), respectively. (C, D) Mitochondrial respiration parameters including basal mitochondrial respiration (basal), oxygen consumption associated with ATP production (ATP production), maximal respiration (Maximal), and spare respiratory capacity (Spare) were calculated based on the OCR measurements shown in (A, B), as described in Methods. The table summarizes the statistical comparisons of all relevant conditions. Values are presented as means ± SEM of six independent experiments (at least 10 wells/condition per Seahorse microplate). *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, *****$P < 0.00001$. Statistics: 2-way ANOVA (Tukey's multiple comparisons test).

characterizing lactate as more than a metabolic waste product.[27–30] Hence, another beneficial effect of lactate is its possible neuroprotective property. In this context, lactate has recently been shown to promote neuronal survival in models of traumatic brain injury and ischemia.[31,32] Moreover, lactate has been linked to physiological restoration of long-term memory.[31–33] Similar advantageous properties of lactate have been identified in the in the retina, where lactate treatment has been shown to enhance Müller cell function and survival.[13]

Lactate has been demonstrated to exert its mechanism of action by uptake through MCTs.[12,13] However, in the early 2000s a new target for lactate was discovered, namely the receptor GPR81. Abundant GPR81 expression was recently identified in the inner retina,[20] indicating that activation of GPR81 may play a prominent role in inner retinal homeostasis. Thus, we aimed to elucidate whether the lactate-linked protective effects on Müller cells were due to direct metabolism of L-lactate and/or activation through GPR81.

Surprisingly, activation of GPR81 was shown to enhance endogenous metabolic features of the Müller cells while downregulating other functions, such as glutamate uptake, depending on the energy status of the cell, shedding light on the dual effects of lactate in Müller cells.

GPR81 protein expression has previously been verified in isolated murine RGCs and Müller cells by immunostaining. Moreover, mRNA expression levels of GPR81 have also been quantified in retinas obtained from mice.[20] However, in this study, we aimed to verify the presence of the receptor and possible regulations in response to altered levels of energy availability. Deprivation of glucose led to increased GPR81 expression, indicating an amplification of lactate-mediated effects through GPR81 activation. The presence of extracellular lactate has previously been shown to increase Müller cell survival through uptake of lactate by MCT transporters,[13] thus indicating that lactate uptake is crucial for Müller cell viability. Moreover, studies on various tumor cell lines have shown that
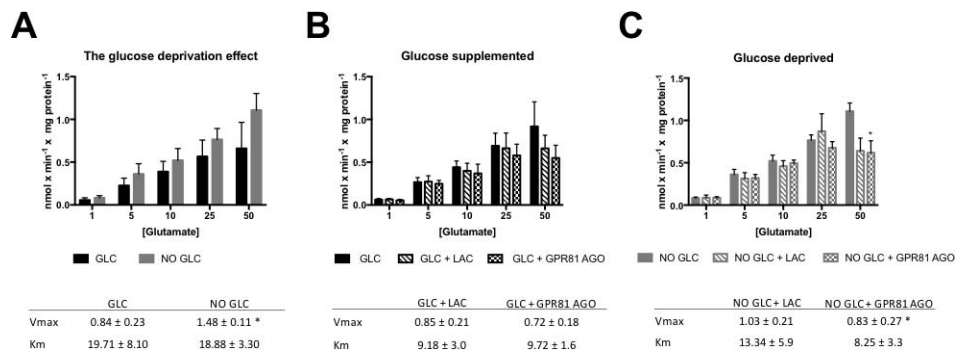
FIGURE 9. Decreased maximal glutamate uptake in response to GPR81 activation in the absence of glucose. Primary mouse Müller cells were treated for 2 hours with (*dotted columns*) or without 5 mM of the GPR81 agonist, 3,5-DHBA, in the presence (*black columns*) and absence of 6 mM glucose (*gray columns*). Kinetic study of [^{3}H]-glutamate uptake in the cells was performed. Nonlinear regression of the average uptake in the cells at extracellular glutamate concentrations from 2 to 50 μM was estimated by GraphPad Prism with a calculated Michaelis–Menten constant, K_m, and a maximal uptake, V_{max}, in glucose-supplemented cells compared to glucose-deprived cells (**A**) and glucose-supplemented cells (**B**) and glucose-deprived cells (**C**) compared to treatment with either lactate or the GPR81 agonist, respectively. Treatment with the GPR81 agonist in the absence of glucose resulted in reduced maximal glutamate uptake. Absence of glucose resulted in increased glutamate uptake compared to the condition with glucose (Student's *t*-test). Values are presented as means ± SEM of three or four experiments. Vmax is presented in nmol • min^{-1} • mg protein. K_m is presented in μM. *$P < 0.05$. Statistics: 1-way ANOVA (Dunnett's multiple comparisons test).

silencing GPR81 led to reduced survival,[19] which could support that lactate-mediated GPR81 activation is physiologically important in cell survival by activation of intracellular-mediated pathways. Thus, previous studies suggest that lactate uptake directly enhances cell survival, whereas lactate-mediated GPR81 activation may serve as a regulatory pathway that maintains the intracellular lactate homeostasis. To elucidate the role of GPR81 on primary mouse Müller cells, we investigated whether GPR81 activation would counteract the protective effects of lactate on cell survival. Surprisingly, the addition of 5 mM GPR81 agonist did not have any effect on survival, either cytotoxic or cytoprotective effects. To further investigate the link between GPR81 activation and lactate as a metabolite, lactate release and metabolism was explored. During physiological conditions, with sufficient amounts of glucose, lactate was released in high amounts corresponding nicely to the chosen lactate concentration of 10 mM in subsequent experiments with extracellular lactate exposure. During glucose deprivation, the extracellular concentration of lactate diminished significantly, thereby suggesting that lactate was metabolized internally instead of being released. Interestingly, GPR81 activation also resulted in decreased lactate release in the presence of glucose. In line with this, previous studies have shown that lactate-mediated activation of GPR81 on adipocytes inhibits lipolysis in vitro as well as in vivo,[34–36] and 3,5-DHBA has been verified as a specific agonist to facilitate this effect.[36] It has been proposed that GPR81 activation of adipocytes is to preserve energy as fat, especially during stress and short-term starvation.[37] During glucose deprivation in our study, we did not establish any significant effect on lactate release in response to GPR81 activation of the Müller cells. However, in the presence of physiological glucose levels, lactate release decreased significantly, thereby identifying that other metabolic pathways, besides fat metabolism, are affected in response to GPR81 activation. We furthermore elucidated the consequence of GPR81 activation on glucose metabolism and identified an increased glucose metabolism through oxidative phosphorylation in response to GRP81. Thus, GPR81 activation enhanced glucose metabolism through the TCA cycle while bypassing the production of lactate.

When exploring lactate metabolism, we found incorporation of ^{13}C in metabolites after incubation with ^{13}C-labeled lactate, indicating that lactate is indeed metabolized in the Müller cells. Interestingly, the addition of unlabeled glucose in primary Müller cells increased metabolism of the labeled lactate, revealing novel interactions of glucose and lactate in Müller cells (Fig. 10). Thus, our findings suggest that the Müller cells prefer lactate over glucose as an energy substrate. This particular preference of energy substrates has also been seen in the human Müller cell line MIO-M1[13] and is further supported by the increase in MCL for selected metabolites from ^{13}C-lactate compared with ^{13}C-glucose, emphasizing the role of ^{13}C-lactate as a preferred energy substrate. Based on our results, we hypothesize that Müller cells may compartmentalize their metabolism of glucose and lactate. Glucose may thus be taken up and metabolized to lactate. In favor of this hypothesis, we showed that lactate is subsequently released to the extracellular environment. The increased amounts of extracellular lactate may potentially result in a positive feedback mechanism, followed by further increased lactate uptake, since lactate was identified as a preferable energy substrate (Fig. 10).

The combination of labeled glucose and 5 mM GPR81 agonist resulted in increased amounts of labeled metabolites,
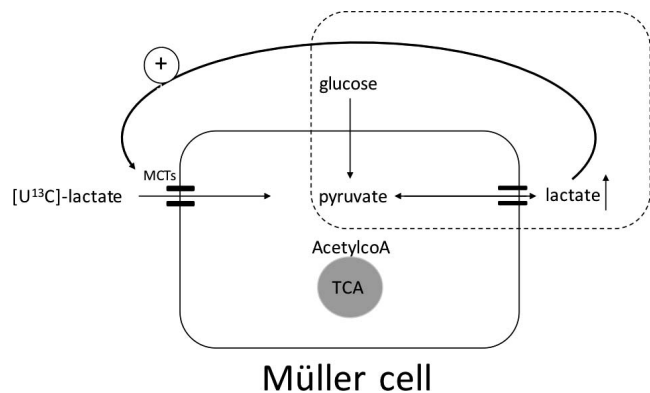
FIGURE 10. Glucose–lactate interaction hypothesis in Müller cells. Glucose is taken up by the Müller cell and metabolized to lactate, which is released to the extracellular environment. The increase in extracellular lactate concentrations may potentially result in a positive feedback mechanism, ultimately resulting in enhanced lactate uptake, since lactate was identified as a preferable energy substrate.

indicating that GPR81 activation boosts glucose metabolism. This also correlates with the identified decrease in lactate release during simultaneous glucose supplementation and GPR81 activation as well as the increased mitochondrial activity shown by Seahorse analysis.

In general, glucose deprivation has been shown to boost mitochondrial activity in Müller cells, suggesting a shift from glycolytic metabolism to mitochondrial oxidative phosphorylation during metabolic stress.[10,11] Since glucose deprivation combined with GPR81 agonist treatment resulted in increased mitochondrial function, it is tempting to suggest that GPR81 activation compensatorily increases metabolism of available metabolites to meet the energy requirements. Moreover, previous studies have identified that silencing of GPR81 in cultured pancreatic carcinoma cells led to a 50% reduction in mitochondrial activity within 24 hours,[19] further emphasizing that GPR81 is crucial in maintaining mitochondrial function.

To accommodate the need for increased resistance toward the pathological stress defined by glucose deprivation, Müller cells may thus upregulate GPR81 receptors to boost metabolism and mitochondrial function. Once activated, a Gi-mediated pathway will facilitate a decrease in cAMP.[18] Interestingly, the presence of cAMP has been shown to downregulate MCTs in various cell types such as cerebrovascular endothelial cells and intestinal epithelia.[38,39] In this context, an increase in GPR81 receptor expression and activation would thus hypothetically also result in an increased expression of MCTs followed by enhanced lactate uptake and utilization. Ultimately, this may explain why the cells do not need to take up glutamate as an alternative energy source,[9] since they have sufficient energy substrates in the form of lactate. Correspondingly, we showed a decrease in maximal glutamate uptake in response to GPR81 exposure. No significant changes in glutamate uptake was found in cells treated with 10 mM lactate.

In the human MIO-M1 cell line, we have previously shown that extracellular lactate availability increases the maximal glutamate uptake after 2 hours glucose deprivation.[13] These contradictory findings may be explained by differences in the energy status of the different cell types. Hence, MIO-M1 cells were more prone to die during glucose deprivation compared to primary Müller cells. Moreover, ATP production was significantly reduced in MIO-M1 cells over time, whereas primary Müller cells remained stable in their ATP production and consumption. Thus, the differences may be explained by their different thresholds for energetic crises. Consequently, Müller cells in general may compensatorily increase their glutamate uptake to meet the requirements for further energy substrates; however, once having reached the threshold of compensation, the cells require additional available energy substrates. This explanation corresponds with previous findings, which have suggested that glutamate uptake is indirectly dependent on glycolysis and lactate formation.[13,40,41] However, the cells may subside to mitochondrial energy production during accumulative stress to potentially sustain the gradient-driven transport of glutamate by indirectly maintaining the sodium–potassium pump.[11]

Nevertheless, the dual role of lactate as a metabolite and signaling molecule by GPR81 activation has revealed novel regulatory features of lactate, in which GPR81 activation is able to boost mitochondrial metabolism and function, ultimately leading to maintained survival of Müller cells. In summary, the present study reveals novel properties of lactate as an active metabolic energy substrate and as a regulatory molecule that activates GPR81 receptors in primary Müller cells. The dual properties of lactate and the intricate details of lactate metabolism and signaling still remain to be explored.

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