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Oxygen tension controls the expression of the monocarboxylate transporter MCT4 in cultured mouse cortical astrocytes via a Hypoxia-Inducible Factor-1α-mediated transcriptional regulation

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ABSTRACT

The monocarboxylate transporter MCT4 is a high capacity carrier important for lactate release from highly glycolytic cells. In the central nervous system, MCT4 is predominantly expressed by astrocytes. Surprisingly, MCT4 expression in cultured astrocytes is low, suggesting that a physiological characteristic, not met in culture conditions, is necessary. Here we demonstrate that reducing oxygen concentration from 21% to either 1% or 0% restored in a concentration-dependent manner the expression of MCT4 at the mRNA and protein levels in cultured astrocytes. This effect was specific for MCT4 since the expression of MCT1, the other astrocytic monocarboxylate transporter present *in vitro*, was not altered in such conditions. MCT4 expression was shown to be controlled by the transcription factor Hypoxia-Inducible Factor-1α (HIF-1α) since under low oxygen levels, transfecting astrocyte cultures with a siRNA targeting HIF-1α largely prevented MCT4 induction. Moreover, the prolyl hydroxylase inhibitor dimethyloxaloylglycine (DMOG) induced MCT4 expression in astrocytes cultured in presence of 21% oxygen. In parallel, glycolytic activity was enhanced by exposure to 1% oxygen as demonstrated by the increased lactate release, an effect dependent on MCT4 expression. Finally, MCT4 expression was found to be necessary for astrocyte survival when exposed for a prolonged period to 1% oxygen. These data suggest that a major determinant of astrocyte MCT4 expression *in vivo* is likely the oxygen tension. This could be relevant in areas of high neuronal activity and oxygen consumption, favouring astrocytic lactate supply to neurons. Moreover, it could also play an important role for neuronal recovery after an ischemic episode.

Keywords : energy metabolism; HIF-1α; lactate; MCT1; glycolysis

INTRODUCTION

Astrocytes constitutively exhibit an important glycolytic activity, metabolizing a large quantity of the glucose available to lactate which is released into the medium (Bouzier-Sore et al. 2006; Itoh et al. 2003). Moreover, astrocytes possess a large reserve in glycolytic capacity that can be mobilized in certain situations (Pellerin and Magistretti 1994; Walz and Mukerji 1990). This metabolic phenotype confers to astrocytes a high resistance to pathological conditions such as ischemia/hypoxia in which energy supply from oxidative phosphorylation is compromised, leading to a strong enhancement of glycolysis (Marrif and Juurlink 1999; Vega et al. 2006). In this context, large amounts of lactate are produced and lactate release from the cell becomes critical to avoid lactic acidosis and cell death (Callahan et al. 1990; Kelleher et al. 1993; Swanson et al. 1995). Thus, the presence of a high capacity lactate transporter is likely essential for astrocytes under both physiological and pathological conditions.

Cellular lactate transport is ensured by a family of proton-linked carriers named monocarboxylate transporters that belong to the larger SLC16A solute carrier family (Halestrap and Meredith 2004). Astrocytes have been reported to express two isoforms of such lactate carriers characterized by their distinct affinity for lactate: MCT4 (SLC16A3) exhibits a low affinity for lactate (Km ~34 mM) as compared to MCT1 (SLC16A1; Km ~5 mM). Such a kinetic characteristic prevents saturation at high lactate concentrations and thus renders MCT4 well-adapted to export large amounts of lactate from highly glycolytic cells (Dimmer et al. 2000). Indeed, MCT4 is specifically expressed by highly glycolytic cells such as the so-called “white fibers” of skeletal muscle (Wilson et al. 1998) and in cancer cells (Brahimi-Horn et al. 2011) where it is responsible for lactic acid efflux.

In this study, we show that although MCT4 is specifically expressed by astrocytes *in vivo*, as previously reported (Pellerin et al. 2005; Rafiki et al. 2003), its expression is almost undetectable in primary cultures of mouse cortical astrocytes maintained in classical culture conditions. However, exposing cultured astrocytes to a low oxygen tension (1 % and 0 % oxygen), restores MCT4 expression via a transcriptional activation involving the transcriptional factor HIF-1α, allowing important lactate release and ultimately preventing cell death. We suggest that maintaining cultured astrocytes under low oxygen tension may

better reflect the physiological situation *in vivo*, and reveal a putative adaptive mechanism in astrocytes to avoid cell death in conditions leading to intense glycolytic activity.

MATERIAL AND METHODS

Primary cultures of mouse cortical astrocytes

Astrocyte-enriched cultures were prepared from OF1:SWISS mice (Janvier, Le Genest-Saint-Isle, France). Astrocytes were prepared from 1-2 day-old neonatal mouse cortex as previously described (Sorg and Magistretti 1991; Yu et al. 1993). Mice were decapitated, the forebrain was removed aseptically from the skull, meninges were excised carefully, and the neocortex dissected (hippocampus and white matter were removed from cortices). The cells were mechanically dissociated by passage through needles of decreasing gauges (1.2 x 40 mm, 0.8 x 40 mm, and 0.5 x 16 mm) with a 5 ml syringe. Cells were then seeded on 6-well uncoated plates or on 24-well plates containing glass coverslips coated with poly-L-ornithine (15 µg/ml; Sigma, Buchs, Switzerland) at a density of 50'000 cells/ml and maintained in Dulbecco's Modified Eagle's Medium-high glucose (DMEM7777; 4500 mg/L glucose, L-glutamine, and sodium pyruvate; Sigma, Buchs, Switzerland) supplemented with 0.01% antibiotic antimycotic solution 100X stabilized (10000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B/ml; #A5955, Sigma, Buchs, Switzerland) and 10 % fetal calf serum in water-saturated atmosphere at 37°C with 5% CO_2:95% air. Medium was renewed 4 days after seeding and then twice a week. Microglial cells were detached and removed from the astrocyte cultures by shaking plates when changing the medium. Cells were used 3 weeks after seeding when the cultures became confluent.

siRNA transfection

For RNAi experiments, the culture medium was replaced by DMEM with 10 % fetal calf serum but without antibiotics. The transfection preparation containing the small interfering RNA (siRNA) against MCT4 (200 nM; Qiagen, Hombrechtikon, Switzerland), MCT1 (300 nM; Qiagen, Hombrechtikon, Switzerland) or HIF-1α (100 nM, #1027423, Invitrogen, Basel, Switzerland) and the Lipofectamine™ 2000 reagent (#11668027, Invitrogen, Basel, Switzerland) was prepared in Opti-MEM/GlutaMax (#51985-034, Invitrogen, Basel, Switzerland) according to the manufacturer's instructions. After 5 hours of incubation, the transfection medium was replaced with DMEM with 10 % FCS and antibiotics. Astrocytes

were then used for hypoxia experiments 24 hours after the beginning of transfection. A non-silencing siRNA with no homology to any known mammalian gene was used as negative control (AllStars Negative Control siRNA, #1027281, Qiagen, Hombrechtikon, Switzerland). Sense and antisense MCT4 (sense-strand RNA sequence, 5'-r(GAAGCAUUAUCCAGAUCUA)dTdT-3'; antisense-strand RNA sequence, 5'-r(UAGAUCUGGAUAAUGCUUC)dTdG-3') oligoribonucleotides correspond to positions 324 to 344 of the mouse MCT4 mRNA sequence (target sequence: 5'-CAGAAGCATTATCCAGATCTA-3', Embl NM_030696.3). Sense and antisense MCT1 (sense-strand RNA sequence, 5'-r(GGAAUUCAUCUACACUUAA)dTdT-3'; antisense-strand RNA sequence, 5'-r(UUAAGUGUAGAUGAAUUCC)dTdG-3') oligoribonucleotides correspond to positions 183 to 203 of the mouse MCT1 mRNA sequence (target sequence: 5'-TTGGAATTCATCTACACTTAA-3', Embl NM_009196.3**).** Sense and antisense HIF-1α (sense-strand RNA sequence, 5'-r(CAAGCAGCAGGAAUUGGAACAUUAU)dTdT-3'; antisense-strand RNA sequence, 5'-r(AUAAUGUUCCAAUUCCUGCUGCUUG)dTdG-3') oligoribonucleotides correspond to positions 2641 to 2665 of the mouse HIF-1α mRNA sequence (target sequence: 5'-CAAGCAGCAGGAATTGGAACATTAT-3', Embl NM_010431.2).

Hypoxia experiments

Hypoxia experiments were performed in commercial hypoxic chambers (#29829; Billups-Rothenberg hypoxic chamber, StemCell Technologies, Basel, Switzerland). Astrocyte cultures received fresh DMEM medium supplemented with 10 % FCS prior to be introduced in the hypoxic chamber. Humidity inside the chamber was maintained by placing a Petri dish filled with deionized sterile water at the bottom of the chamber. The hypoxic chamber was then flushed with a gas mixture containing either 1 % O_2/5 % CO_2/94 % N_2 or 5 % CO_2/95 % N_2 (Carbagaz, Lausanne, Switzerland) at a flow rate of 10 L/min during 15 minutes regulated by a flow meter (#27311; StemCell Technologies, Basel, Switzerland) (Yu et al. 1989). For the control ("normoxic") condition, the chamber was flushed with a gas mixture containing 21 % O_2/5 % CO_2/74 % N_2. The chamber was then placed in an incubator at 37°C for the time required and it was reflushed with the same gas mixture every 24 hours to maintain the imposed environmental condition. The oxygen level in the chamber was measured with an oxymeter (StemCell Technologies, Basel, Switzerland); the pH in the medium was measured by a *SevenGo DUO PRO pH/ION* oxymeter (*#51302611*, Mettler Toledo, Bussigny-près-Lausanne, Switerland). For the reoxygenation experiment, the medium was not changed after

hypoxia to strictly assess the effect of reintroducing normoxic conditions without reperfusion. The reduced L-glutathione (#G6013, Sigma, Buchs, Switzerland) experiment was performed in the D7777 medium. The cultures were incubated during 5 hours prior to perform hypoxia in order to let the compound enter into the cells and to re-stabilize the pH.

Glutathione, AICAR and sodium azide experiments

Glutathione reduced ethyl ester (#G1404, Sigma, Buchs, Switzerland), 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) (#A9978, Sigma, Buchs, Switzerland) and sodium azide (#S8032, Sigma, Buchs, Switzerland) treatments were performed in a DMEM medium at pH 7.2 (#D5030, Sigma, Buchs, Switzerland) supplemented with Antibiotic Antimycotic Solution (#A5955, Sigma, Buchs, Switzerland), 44 mM $NaHCO_3$ and 25 mM D-Glucose during 24 hours. For control conditions, phosphate-buffered saline (PBS) was added as vehicle (PBS 10X; Laboratorium Dr.Bichsel AG, Interlaken, Switzerland).

Immunochemistry

For immunofluorescence analysis, astrocytes were washed with PBS and directly fixed 20 minutes in 4 % paraformaldehyde at room temperature. Fixed cells were washed again with PBS and nonspecific sites were blocked with 0.5 % casein for 1 hour at room temperature. Double immunostainings were carried out overnight at 4°C in PBS containing 0.3 % Triton X-100 and 0.25 % bovine serum albumin with the different antibodies: rabbit anti-mouse MCT4 (1:250 dilution; #sc-50329; SantaCruz, Heidelberg, Germany) or rabbit anti-MCT1 (1:500 dilution; Pierre et al. 2000) and mouse anti-Glial Fibrillary Acidic protein (GFAP) (1:500 dilution; #Z0334, DakoCytomation, Baar, Switzerland). After washing three times with PBS, cells were incubated in a PBS solution containing a donkey Cy3-conjugated anti-rabbit (1:500 diltution; #711-165-152, Jackson Immunoresearch, Baltimore, MD, USA) and a donkey FITC-conjugated anti-mouse (1:500 dilution; #715-095-150, Jackson Immunoresearch, Baltimore, MD, USA) for 2 hours at room temperature. After washing twice in PBS, cells were incubated during 5 minutes with DAPI (#D9542, Sigma, Buchs, Switzerland) dissolved in PBS (1/100000), rinsed again twice 10 minutes in PBS and then mounted with Mowiol mounting medium (#81381, Sigma, Buchs, Switzerland). Preparations were then maintained at 4°C until observation with a Zeiss LSM 710 Quasar Confocal Microscope (Zeiss, Feldbach, Switzerland).

Lactate release assay

For the lactate release assay, astrocytes were taken out from the hypoxic chamber and the medium was replaced with DMEM medium pH 7.2 (#D5030, Sigma, Buchs, Switzerland) supplemented with Antibiotic Antimycotic Solution (#A5955, Sigma, Buchs, Switzerland), 44 mM $NaHCO_3$ and 5 mM D-Glucose. Then, cells were put in an incubator at 37°C under "normoxic" conditions. After 20 minutes of incubation, the medium was harvested for lactate measurement while protein content was determined on the cell extract. Lactate level in the medium was assessed according to the protocol of Rosenberg and Rush (Rosenberg and Rush 1966). The culture medium was mixed with a 0.33 M glycine-semicarbazide buffer supplemented just before starting the incubation with 0.015 M nicotinamide adenine dinucleotide (#12342857; Roche, Basel, Switzerland) and 70 U/ml L-lactate dehydrogenase (#10127876001; Roche, Basel, Switzerland). The quantification was performed by measuring the NADH produced via the L-lactate dehydrogenase-catalyzed enzymatic reaction with a microplate reader (SynergyMx; BioTek, Luzern, Switzerland) at 340 nm. The released lactate values were normalized to the total protein content for each well; finally lactate release was expressed in nmoles/mg protein.

Quantitative real-time PCR analysis

Total RNA was isolated using RNeasy Protect Mini Kit (#74106, Qiagen, Hombrechtikon, Switzerland). For cDNA synthesis, 200 ng of total RNA were reverse transcribed using Taqman Reverse Transcription Reagents kit (N808-0234, Applied Biosystems, Luzern, Switzerland) with random hexamers according to the manufacturer's instructions in a total volume of 50 µl. Then, 1 µl of cDNA was mixed with the suitable primer (0.3 µM) and the SYBR Green PCR master mix (Applied Biosystems, Luzern, Switzerland) to perform the PCR reaction in a total reaction volume of 10 µl. Each sample was tested in duplicate. Quantitative determination of MCT1 (For, 5'-AATGCTGCCCTGTCCTCCTA-3'; Rev, 5'-CCCAGTACGTGTATTTGTAGTCTCCAT-3' (Embl NM_009196.3); Microsynth, Balgach, Switzerland), MCT4 (For, 5'-GGCTGTTTTATCATCACGGGTT-3'; Rev, 5'-GTGTCGCTGTAGCCAATCCC-3'(Embl NM_030696.3); Microsynth, Balgach, Switzerland) and HIF-1α (For, 5'- CCC AAA GAC AAT AGC TTC GCA -3'; Rev, 5'- ACA GTC ACC TGG TTG CTG CAA -3'(Embl NM_010431.2); Microsynth, Balgach, Switzerland) mRNA expression levels was performed with the StepOnePlus™ Real-Time PCR System or the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Luzern, Switzerland) with β-2-microglobulin (For, 5'- CCC CAC TGA GAC TGA TAC ATA CG-3';

Rev, 5'-CGA TCC CAG TAG ACG GTC TTG-3'(Embl NM_009735); Microsynth, Balgach, Switzerland) mRNA used as an endogenous control. For data analysis, the raw threshold cycle (CT) value was first normalized to the housekeeping gene for each sample to obtain the ΔCT value. The normalized ΔCT value was then calibrated to the control cell samples to obtain the ΔΔCT value.

Western blotting

Astrocytes were washed twice in ice-cold PBS, total proteins were extracted by cellular lysis in 40 µl of ice-cold buffer containing 50 mM Tris pH 7.5, 1 mM EDTA, 1 % SDS and a mixture of protease inhibitors (Complete 11257000; Roche, Basel, Switzerland). About 50 µg of proteins were denatured (95°C) during 5 minutes in SDS-PAGE sample buffer (60 mM Tris-HCl pH 6.8, 5 % SDS, 6.6 % glycerol, 5 mM EDTA, 5 % β-mercaptoethanol and 0.1 % bromophenol blue). Samples were separated on a 10 % acrylamide running gel and a 4 % acrylamide stacking gel using an Electrophoresis Unit (Bio-Rad, Cressier, Switzerland). Proteins were then electroblotted onto nitrocellulose membranes (0.45 µm; #162-0115, Bio-Rad, Cressier, Switzerland) using an Electrophoresis Unit (Bio-Rad, Cressier, Switzerland). Nonspecific binding sites were blocked for 2 hours at room temperature with a solution of Tris-Buffered-Saline (TBS-T; 50 mM Tris-HCl pH 7.5, 150mM NaCl) supplemented with 0.1 % Tween-20 and containing 10 % (wt/vol) of skim milk. Blots were then incubated overnight at 4°C with specific primary antibodies in TBS-T 0.1 % containing 1 % skim milk: rabbit anti-mouse MCT4 (1:250 dilution; #sc-50329; SantaCruz, Heidelberg, Germany), rabbit anti-MCT1 (1:1000 dilution; Pierre et al. 2000), rabbit anti-HIF-1α (1:500 dilution; #NB100-449, Novus Biologicals, Cambridge, United Kingdom) and rabbit anti β-tubulin (1:1000 dilution; #2128, Cell Signaling, Berverly, MA, USA). Blots were washed three times in TBS-T 0.1% and they were subsequently incubated 2 hours at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG (#NA9340V, 1:10.000 dilution; GE Healthcare, Glattbrugg, Switzerland) and/or horseradish peroxidase-conjugated goat anti-mouse IgG (#1858413, 1:10.000 dilution; Thermo Fisher Scientific, Pierce, Lausanne, Switzerland). After being washed three times in TBS-T 0.1%, blots were processed using Immun-Star™WesternC™ Chemiluminescent Kit (#170-5070, Bio-Rad, Cressier, Switzerland). Chemiluminescence detection was performed with the ChemiDoc™ XRS System (#170-8070, Bio-Rad, Cressier, Switzerland) and quantification was made with the ImageLab 3.0 software (Bio-Rad, Cressier, Switzerland).

Statistical analysis

All results are presented as mean ± SEM from triplicate determinations. All experiments were performed at least three times with similar results. Statistical analysis was performed using a one-way or two-way ANOVA followed by either a Dunnett's or a Tukey's post hoc test where appropriate, unless indicated otherwise. A level of $P < 0.05$ was considered statistically significant in all cases; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. All statistical analyses and graphical representations were performed with Prism software (version 5.0. GraphPad, San Diego, CA, USA).

RESULTS

Expression of MCT4, but not MCT1, is induced in presence of low oxygen levels in primary cultures of mouse cortical astrocytes

MCT4 immunostaining in the adult mouse brain is characterized by an important cell-specific labeling throughout the hippocampus and the cortex. Cells expressing the glial fibrillary acidic protein (GFAP) were found to be labeled for MCT4 (Figure 1, first three columns), which confirms previous observations that MCT4 is found predominantly on astrocytes in the central nervous system (Pierre and Pellerin 2005; Rafiki et al. 2003). However, primary cultures of mouse cortical astrocytes exhibited almost undetectable basal MCT4 expression (Figure 1, last column) as previously described (Marcillac et al. 2011). A putative difference between *in vivo* and *in vitro* conditions that might explain such a contrasting level of MCT4 expression by astrocytes could be the degree of oxygenation. To assess the effect of oxygen tension on astrocytic MCT4 expression, cultured cortical astrocytes were maintained in a modular chamber at 1 % or 0 % oxygen (instead of 21 % as in the control condition) during various periods of time. Reduced oxygen tension up to ten days led to a highly significant increase in MCT4 mRNA expression compared to the control condition. The maximal expression was observed between 24 and 48 hours, for both oxygen concentrations tested (Figure 2A). Moreover, a significant difference was observed between 0 and 1 % oxygen at each time point, highlighting a concentration-dependent effect on MCT4 expression. Western blot analysis revealed a highly significant increase of MCT4 protein expression also up to ten days of incubation with reduced oxygen tension compared to the control condition (Figure 2B). MCT4 protein expression became already detectable after 24

hours of incubation and continued to rise over the ten days of incubation for both oxygen concentrations (Figure 2B). Immunolabellings on cultured astrocytes clearly showed a widespread induction of MCT4 expression in all astrocytes, with a more intense expression observed under 0 % oxygen compared to 1 % oxygen (Figure 2C). At the subcellular level, labeling could be observed over the entire cell body as well as associated with small processes extending from it.

Similar investigations were performed for MCT1, the other monocarboxylate transporter expressed by astrocytes *in vitro*. Compared to MCT4, MCT1 is already strongly expressed in primary cultures of mouse cortical astrocytes incubated in presence of 21 % oxygen, both at the mRNA and protein levels (Figure 3). Interestingly, no significant effect of reduced oxygen tension was observed on MCT1 mRNA levels compared to the control condition (Figure 3A). In parallel, no significant effect on MCT1 protein expression at both oxygen concentrations could be detected compared to the control condition (Figure 3B). MCT1 immunolabelings confirmed a similar expression in most astrocytes, but in contrast to MCT4, it was present already in the control condition and was not increased by reducing oxygen tension (Figure 3C).

Induction of MCT4 expression by low oxygen tension is dependent on Hypoxia-Inducible Factor-1α.

The transcription factor Hypoxia-Inducible Factor-1α (HIF-1α) has been described as a key cellular oxygen sensor that can coordinate the metabolic response to lowering oxygen concentrations. Thus, HIF-1*α* expression profile was analyzed in cultured astrocytes exposed to either 1 or 0 % O_2 during different periods of time. HIF-1*α* protein expression increased with time compared to the control condition and reached a maximum after 72 hours, although this increase was significant after 48 hours for cultures exposed to both 1 and 0 % O_2 (Figure 4A). Moreover, a concentration-dependent effect on HIF-1α protein expression could be evidenced between 1 % and 0 % oxygen, being statistically significant between 72 hours and 7 days of incubation (Figure 4A). In contrast, no change in HIF-1α mRNA levels could be detected under similar conditions (data not shown). Immunostainings for HIF-1*α* showed both cytoplasmic and nuclear expression in almost all cultured astrocytes when exposed to low oxygen tension (data not shown). The possible implication of HIF-1α in the transcriptional effect of low oxygen tension on MCT4 mRNA expression was evaluated first by knocking

down HIF-1α via siRNA interference. Astrocyte cultures were transfected with a siRNA targeting HIF-1α or with a universal non-coding siRNA (siscramble) and then maintained at 1 % O_2 during 48 hours. Induction of MCT4 expression was largely prevented at both mRNA and protein levels with the siRNA targeting HIF-1α while no significant effect was observed with the control siRNA (Figure 4B, C). Analysis of HIF-1α mRNA expression confirmed that the siRNA succeeded in downregulating HIF-1α by almost 80% (data not shown). Similarly to low oxygen tension, the chemical agent DMOG (dimethyloxalylglycine; a cell permeable prolyl-4-hydroxylase inhibitor and thus HIF-1α stabilizer) led to a significant enhancement of HIF-1α protein expression when used at concentrations between 1 and 5 mM (Figure 4D), without affecting HIF-1α mRNA levels (data not shown). In parallel, a significant induction of MCT4 expression at both mRNA and protein levels could be observed with DMOG concentrations of 1 mM and above (Figure 4E, F).

Induction of MCT4 expression by low oxygen tension is not dependent on the production of ROS, and can not be mimicked by blockade of oxidative phosphorylation nor by AMPK activation.

Reduction in the efficacy of the respiratory chain takes place when oxygen availability is compromised. This process leads to an increased production of reactive oxygen species (ROS) which may be responsible for HIF-1α protein stabilization and observed increased MCT4 expression. In order to assess this possibility, astrocytes were treated with the cellular antioxidant glutathione (reduced, cell permeable form) at different concentrations ranging from 100 μM to 10 mM prior to incubation at 21 or 1 % oxygen during 48 hours. No significant difference could be detected between control and treated conditions, indicating that the treatment with reduced glutathione could not prevent MCT4 induction, both at the mRNA and protein levels (Figure 5A, B). Similarly, when astrocyte cultures were treated with sodium azide (an inhibitor of the electron transport chain complex IV) at concentrations up to 10 mM during 24 hours, no enhancement in MCT4 mRNA expression was observed (as could have been expected since glycolytic activity in astrocytes increases dramatically under such a condition) but rather a non-significant decrease was obtained at the highest sodium azide concentrations (Figure 5C). Since it was previously demonstrated that an activation of the AMP-activated protein kinase (AMPK), an enzyme that play a central role in cellular energy homeostasis, is responsible for the NO-induced enhancement of glycolysis in astrocytes (Almeida et al. 2004), the possible implication of AMPK in the regulation of MCT4 expression was investigated in our conditions. For this purpose, cultured astrocytes were

treated with the AMPK activator 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) at various concentrations up to 300 μM during 24 hours. No significant effect could be observed at any concentration tested (Figure 5D), despite a strong activation of AMPK as demonstrated by its phosphorylated level upon AICAR stimulation (data not shown).

MCT4 protein turnover is slow upon return to standard oxygen concentration in culture

The persistence of MCT4 expression after its induction by a transient exposure to low oxygen tension and upon reoxygenation has been evaluated. Cultured astrocytes were maintained at 1 % O_2 during 48 hours, then they were reoxygenated with 21% oxygen during various periods of time. Interestingly, mRNA levels decreased significantly in the next hour following the beginning of the reoxygenation period, and after 6 hours it had returned almost to the control level (Figure 6A). In contrast, the levels of the protein were much more stable, since it remained elevated for several days to finally come back near its initial expression level only after six days of reoxygenation (Figure 6B). In order to distinguish between slow protein degradation or a persistent translational activity, the protein synthesis inhibitor cycloheximide was used. First, cultured astrocytes were maintained at 1 % oxygen during 48 hours, then treated or not with cycloheximide (10 μmol/L) just prior to the reoxygenation period of 24 hours. Cycloheximide treatment in this case had no effect on MCT4 protein expression upon reoxygenation. However, cycloheximide treatment prior to exposure to 1 % oxygen prevented the enhancement in MCT4 protein expression, thus confirming the efficacy of the drug on protein synthesis (Figure 6C).

Enhanced MCT4 expression leads to larger lactate release and is essential for long-term astrocyte survival under low oxygen tension

In order to evaluate the functional consequences of enhanced MCT4 expression, lactate release was determined in cultured astrocytes after exposure to low oxygen tension. After incubation at 1 % oxygen for different periods of time, lactate accumulation in the extracellular medium was assessed over a period of 30 minutes under 21 % oxygen. A time-dependent effect was observed on lactate release which was already significant after 24 hours of incubation at 1 % oxygen compared to the control and increased by sevenfold after ten days (Figure 7A). While treatment with a control siRNA or a siRNA against MCT1 did not prevent the enhancement of lactate release upon incubation of astrocytes at 1 % O_2 for 5 days, the specific siRNA against MCT4 almost abolished this effect after a similar incubation period (Figure 7B). Interestingly, extensive cell death occurred abruptly after 6 days of

incubation with 1 % oxygen when astrocytes were treated with the siRNA against MCT4, while little effect on the viability was observed when astrocytes where transfected with a control siRNA or a siRNA targeting MCT1 for the same period (Figure 7C).

DISCUSSION

MCT4 is a low affinity, high capacity lactate transporter that is present in cells exhibiting important glycolytic activity (Halestrap and Meredith 2004). In the central nervous system, this is the case for astrocytes, at least *in vivo* as indicated by our results and previous studies (Pellerin et al. 2005; Rafiki et al. 2003). MCT4 is purported to play an important role in favoring lactate release from glycolytic cells (Dimmer et al. 2000). Expression of MCT4 was previously shown to be upregulated, together with many glycolytic enzymes, in several tissues and cell types under so-called hypoxic conditions both *in vitro* (Kay et al. 2007; Perez de Heredia et al. 2010; Ullah et al. 2006) and *in vivo* (Balmaceda-Aguilera et al. 2012; McClelland and Brooks 2002; Ngan and Wang 2009; Py et al. 2005). Furthermore, it was demonstrated that the enhancement of MCT4 expression under such conditions is dependent on the transcription factor HIF-1α and that the promoter region of the MCT4 gene contains hypoxia response elements (HREs) to which HIF-1α can bind and activate transcription (Ullah et al. 2006). This effect was specific for MCT4 as MCT1 expression was unaffected in the same conditions (Ullah et al. 2006). Our data clearly demonstrate that an O_2-dependent regulation of MCT4 (but not of MCT1) expression via HIF-1α also takes place in cultured astrocytes. Interestingly, it was previously shown that MCT4 expression could be induced by nitric oxide in primary cultures of mouse cortical astrocytes maintained in classical culture conditions (21 % oxygen) (Marcillac et al. 2011) via a mechanism involving also HIF-1α (Brix et al. 2012). Thus, it appears that astrocytes can undergo HIF-1α-mediated increases in MCT4 expression by both O_2-dependent and O_2-independent mechanisms.

HIF-1α is a major oxygen sensor in eukaryotic cells that is responsible for coordinating responses to altered oxygen levels including metabolic adaptations such as enhancement of glycolysis (Semenza 2009). Two O_2-dependent mechanisms for HIF-1α activation have been described. The first one takes place usually upon chronic hypoxia and involves a reduction in O_2 available for hydroxylation of HIF-1α at proline residues by prolyl hydroxylases (PHDs), preventing its ubiquitination and eventual degradation by the

proteasome. The second one is classically observed with intermittent hypoxia and involves the formation of reactive oxygen species (ROS) that directly inhibit PHDs (by oxidation of a ferrous ion in the catalytic site) which leads to HIF-1α stabilization. Failure to prevent low oxygen level-induced, HIF-1α–dependent enhancement of MCT4 expression in cultured astrocytes by the cell-permeable reducing agent glutathione ethyl ester, or to mimic the effect of low oxygen levels by using the respiratory chain inhibitor sodium azide (known to favor ROS production), argues against the implication of ROS in this process. This conclusion is reinforced by the observation that exposure of cultured astrocytes to hydrogen peroxide (one form of ROS) did not lead to enhanced MCT4 expression (Marcillac et al. 2011). It was also previously reported that activation of AMPK, a central cellular energy sensor, was necessary for stimulating HIF-1α dependent gene expression under hypoxic conditions in some cell lines (Lee et al. 2003; Neurath et al. 2006). Moreover, the stimulation of glycolytic activity by nitric oxide in cultured astrocytes has been shown to involve an activation of AMPK (Almeida et al. 2004). However, our results do not support the hypothesis that AMPK activation is sufficient to cause the HIF-1α–dependent enhancement of MCT4 expression, as direct activation of AMPK by the chemical agent AICAR was without effect. Thus, the most likely explanation for the impact of lowering oxygen levels on MCT4 expression in astrocytes is the reduced availability of oxygen for the hydroxylation reaction catalyzed by PHDs, leading to stabilization of HIF-1α.

In order to provide an adequate interpretation of our data, some important points must be clarified. Which partial oxygen pressure (pO_2) can be considered normoxia for brain cells? Below which pO_2 values hypoxia begins? What are the true correlates in cultured conditions of *in vivo* situations? These questions were very nicely addressed recently by Carreau et al. (Carreau et al. 2011). First of all, pO_2 in various areas of the brain has been measured by different techniques and was found to be heterogeneous (Ndubuizu and LaManna 2007). On average, values fall usually within the range 23.8-33.3 mmHg (which corresponds to O_2 concentrations of 3.1-4.4 %) and follow a gradient from the deepest layers to the surface of the brain. Because these values are well below the atmospheric pO_2 (160 mmHg or 21 %) which is often considered as normoxia, the authors have defined a new concept called "physioxia". Physioxia represents the physiological range of pO_2 found *in situ* in a specific tissue. Of course, physioxia depends not only of the particular area of tissue considered but also of its state (resting or activated), as the local pO_2 depends on both O_2 delivery and

consumption. In these conditions, how can one mimic *in vitro* the situation *in vivo*? It is clear that classical cultured conditions using a concentration of 21 % O_2 cannot be considered physiological, as far as brain cells are concerned. Although choosing a value around 3-4 % O_2 might be a good average estimate of the "resting" state, it does not give an idea of the dynamic capacity. Although we acknowledge that switching from a supraphysiological concentration (21 % O_2) down to concentrations (1 % and 0 % O_2) on the verge of severe hypoxia is somewhat extreme, it simply serves to illustrate the principle that transcriptional regulation of the MCT4 gene via HIF-1α by altering oxygen tension not only occurs in astrocytes, but represents a graded response most likely covering both the "physioxic" and the hypoxic range. Indeed, a previous study has described a less intense but similar increase in glycolysis and lactate production together with enhanced expression of glycolytic enzymes and transporters in cultured astrocytes exposed to 5% O_2 for 24 hours, although MCT4 was not specifically investigated in this case (Vega et al. 2006). From these considerations, it appears likely that a certain degree of MCT4 expression would be constitutive in astrocytes *in vivo* at resting pO_2 values (as shown here and in previous studies) and that such expression could vary if local oxygen tension was modified as could occur under pathological circumstances (e.g. ischemia).

Expression of MCT4 appears essential to allow appropriate lactate release to occur with augmented glycolytic activity. Preventing MCT4 expression has deleterious consequences on cell survival as demonstrated here on cultured astrocytes exposed to a low oxygen tension. A possible explanation could be the consequence of intracellular lactic acidosis likely to occur if lactate release is limited by downregulation of MCT4. Indeed, it was previously shown that intracellular acidification induced by exposure to lactic acid causes astrocyte cell death *in vitro* (Goldman et al. 1989). In addition, it was also demonstrated that a combination of intracellular acidosis and hypoxia in cultured astrocytes produced more cell death than each condition alone (Bondarenko and Chesler 2001). Interestingly, downregulation of MCT4 expression in tumor cells (characterized by high glycolytic activity) was able to reduce proliferation and tumor growth (Le Floch et al. 2011). Based on this finding, targeting glycolytic activity and the associated capacity to export lactate via MCT4 has become a new anti-cancer therapeutic strategy (Chiche et al. 2013). These observations confirm the critical role played by MCT4 in highly glycolytic cells, as a reduction in its expression can lead under specific circumstances to cell death. The capacity of astrocytes to upregulate MCT4 expression, particularly in pathological conditions involving a reduction in

pO_2 such as ischemia, might be essential not only for astrocytes survival, but also could be protective for neighboring neurons. It was previously shown both in slices and in the intact brain that the lactate produced by glial cells during an ischemic episode is necessary for neurons upon reoxygenation to recover from the ischemic insult (Berthet et al. 2009; Schurr et al. 1997; Schurr et al. 2001). Long-lasting elevation of MCT4 protein expression together with enhanced glycolytic activity in astrocytes following a transient hypoxic episode, as demonstrated in this study, might be important for neurons in the recovery period following the ischemic episode to provide them a sustain supply of lactate. This mechanism may participate in the metabolic adaptations offering neuroprotection in such pathological situations.

In conclusion, our results establish that the level of expression of the monocarboxylate transporter MCT4 by astrocytes is controlled by oxygen tension via a HIF-1α-dependent mechanism. Such a finding provides an explanation for the observed difference between *in vitro* and *in vivo* expression by astrocytes. Moreover, such a regulation could be important under physiological conditions to provide the appropriate metabolic environment to sustain neuronal activity, and in pathological situations such as ischemia to favor neuronal recovery after an insult.

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REFERENCES

Almeida A, Moncada S, Bolanos JP. 2004. Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. Nat Cell Biol 6:45-51.

Balmaceda-Aguilera C, Cortes-Campos C, Cifuentes M, Peruzzo B, Mack L, Tapia JC, Oyarce K, Garcia MA, Nualart F. 2012. Glucose Transporter 1 and Monocarboxylate Transporters 1, 2, and 4 Localization within the Glial Cells of Shark Blood-Brain-Barriers. PLoS One 7:e32409.

Berthet C, Lei H, Thevenet J, Gruetter R, Magistretti PJ, Hirt L. 2009. Neuroprotective role of lactate after cerebral ischemia. J Cereb Blood Flow Metab 29:1780-9.

Bondarenko A, Chesler M. 2001. Rapid astrocyte death induced by transient hypoxia, acidosis, and extracellular ion shifts. Glia 34:134-42.

Bouzier-Sore AK, Voisin P, Bouchaud V, Bezancon E, Franconi JM, Pellerin L. 2006. Competition between glucose and lactate as oxidative energy substrates in both neurons and astrocytes: a comparative NMR study. Eur J Neurosci 24:1687-94.

Brahimi-Horn MC, Bellot G, Pouysségur J. 2011. Hypoxia and energetic tumour metabolism. Curr Opin Genet Dev 21:67-72.

Brix B, Mesters JR, Pellerin L, Johren O. 2012. Endothelial cell-derived nitric oxide enhances aerobic glycolysis in astrocytes via HIF-1alpha-mediated target gene activation. J Neurosci 32:9727-35.

Callahan DJ, Engle MJ, Volpe JJ. 1990. Hypoxic injury to developing glial cells: protective effect of high glucose. Pediatr Res 27:186-90.

Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, Kieda C. 2011. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. J Cell Mol Med 15:1239-53.

Chiche J, Ricci JE, Pouyssegur J. 2013. Tumor hypoxia and metabolism -- towards novel anticancer approaches. Ann Endocrinol (Paris) 74:111-4.

Dimmer KS, Friedrich B, Lang F, Deitmer JW, Broer S. 2000. The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem J 350 Pt 1:219-27.

Goldman SA, Pulsinelli WA, Clarke WY, Kraig RP, Plum F. 1989. The effects of extracellular acidosis on neurons and glia in vitro. J Cereb Blood Flow Metab 9:471-7.

Halestrap AP, Meredith D. 2004. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflugers Arch 447:619-28.

Itoh Y, Esaki T, Shimoji K, Cook M, Law MJ, Kaufman E, Sokoloff L. 2003. Dichloroacetate effects on glucose and lactate oxidation by neurons and astroglia in vitro and on glucose utilization by brain in vivo. Proc Natl Acad Sci U S A 100:4879-84.

Kay HH, Zhu S, Tsoi S. 2007. Hypoxia and lactate production in trophoblast cells. Placenta 28:854-60.

Kelleher JA, Chan PH, Chan TY, Gregory GA. 1993. Modification of hypoxia-induced injury in cultured rat astrocytes by high levels of glucose. Stroke 24:855-63.

Le Floch R, Chiche J, Marchiq I, Naiken T, Ilc K, Murray CM, Critchlow SE, Roux D, Simon MP, Pouyssegur J. 2011. CD147 subunit of lactate/H+ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors. Proc Natl Acad Sci U S A 108:16663-8.

Lee M, Hwang JT, Lee HJ, Jung SN, Kang I, Chi SG, Kim SS, Ha J. 2003. AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. J Biol Chem 278:39653-61.

Marcillac F, Brix B, Repond C, Johren O, Pellerin L. 2011. Nitric oxide induces the expression of the monocarboxylate transporter MCT4 in cultured astrocytes by a cGMP-independent transcriptional activation. Glia 59:1987-95.

Marrif H, Juurlink BH. 1999. Astrocytes respond to hypoxia by increasing glycolytic capacity. J Neurosci Res 57:255-60.

McClelland GB, Brooks GA. 2002. Changes in MCT 1, MCT 4, and LDH expression are tissue specific in rats after long-term hypobaric hypoxia. J Appl Physiol 92:1573-84.

Ndubuizu O, LaManna JC. 2007. Brain tissue oxygen concentration measurements. Antioxid Redox Signal 9:1207-19.

Neurath KM, Keough MP, Mikkelsen T, Claffey KP. 2006. AMP-dependent protein kinase alpha 2 isoform promotes hypoxia-induced VEGF expression in human glioblastoma. Glia 53:733-43.

Ngan AK, Wang YS. 2009. Tissue-specific transcriptional regulation of monocarboxylate transporters (MCTs) during short-term hypoxia in zebrafish (Danio rerio). Comp Biochem Physiol B Biochem Mol Biol 154:396-405.

Pellerin L, Bergersen LH, Halestrap AP, Pierre K. 2005. Cellular and subcellular distribution of monocarboxylate transporters in cultured brain cells and in the adult brain. J Neurosci Res 79:55-64.

Pellerin L, Magistretti PJ. 1994. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci U S A 91:10625-9.

Perez de Heredia F, Wood IS, Trayhurn P. 2010. Hypoxia stimulates lactate release and modulates monocarboxylate transporter (MCT1, MCT2, and MCT4) expression in human adipocytes. Pflugers Arch 459:509-18.

Pierre K, Pellerin L. 2005. Monocarboxylate transporters in the central nervous system: distribution, regulation and function. J Neurochem 94:1-14.

Pierre K, Pellerin L, Debernardi R, Riederer BM, Magistretti PJ. 2000. Cell-specific localization of monocarboxylate transporters, MCT1 and MCT2, in the adult mouse brain revealed by double immunohistochemical labeling and confocal microscopy. Neuroscience 100:617-27.

Py G, Eydoux N, Lambert K, Chapot R, Koulmann N, Sanchez H, Bahi L, Peinnequin A, Mercier J, Bigard AX. 2005. Role of hypoxia-induced anorexia and right ventricular hypertrophy on lactate transport and MCT expression in rat muscle. Metabolism 54:634-44.

Rafiki A, Boulland JL, Halestrap AP, Ottersen OP, Bergersen L. 2003. Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain. Neuroscience 122:677-88.

Rosenberg JC, Rush BF. 1966. An enzymatic-spectrophotometric determination of pyruvic and lactic acid in blood. Methodologic aspects. Clin Chem 12:299-307.

Schurr A, Payne RS, Miller JJ, Rigor BM. 1997. Glia are the main source of lactate utilized by neurons for recovery of function posthypoxia. Brain Res 774:221-4.

Schurr A, Payne RS, Miller JJ, Tseng MT. 2001. Preischemic hyperglycemia-aggravated damage: evidence that lactate utilization is beneficial and glucose-induced corticosterone release is detrimental. J Neurosci Res 66:782-9.

Semenza GL. 2009. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda) 24:97-106.

Sorg O, Magistretti PJ. 1991. Characterization of the glycogenolysis elicited by vasoactive intestinal peptide, noradrenaline and adenosine in primary cultures of mouse cerebral cortical astrocytes. Brain Res 563:227-33.

Swanson RA, Farrell K, Simon RP. 1995. Acidosis causes failure of astrocyte glutamate uptake during hypoxia. J Cereb Blood Flow Metab 15:417-24.

Ullah MS, Davies AJ, Halestrap AP. 2006. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem 281:9030-7.

Vega C, R. Sachleben L J, Gozal D, Gozal E. 2006. Differential metabolic adaptation to acute and long-term hypoxia in rat primary cortical astrocytes. J Neurochem 97:872-83.

Walz W, Mukerji S. 1990. Simulation of aspects of ischemia in cell culture: changes in lactate compartmentation. Glia 3:522-8.

Wilson MC, Jackson VN, Heddle C, Price NT, Pilegaard H, Juel C, Bonen A, Montgomery I, Hutter OF, Halestrap AP. 1998. Lactic acid efflux from white skeletal muscle is catalyzed by the monocarboxylate transporter isoform MCT3. J Biol Chem 273:15920-6.

Yu N, Martin JL, Stella N, Magistretti PJ. 1993. Arachidonic acid stimulates glucose uptake in cerebral cortical astrocytes. Proc Natl Acad Sci U S A 90:4042-6.

FIGURE LEGENDS

Figure 1. Immunocytochemical characterization of MCT4 expression by astrocytes *in vivo* and *in vitro*. Fluorescent immunolabelings for MCT4 (in red; first and third rows) and GFAP (in green; second and third rows). Colocalization appears in yellow (Merge; third row). Nuclei were labeled with DAPI (in blue). (First column) Double immunolabelings in the mouse hippocampus observed at low magnification. (Second column) Double immunolabelings of a single astrocyte in the mouse cortex observed at high magnification. (Third column) 3D reconstruction images of the double labeled astrocyte shown in the second column. (Fourth column) Double immunolabelings in primary cultures of mouse cortical astrocytes. Images obtained by confocal microscopy. Calibration bar = 20 μm (first and fourth columns) or 5 μm (second and third columns).

Figure 2. Effect of reduced oxygen levels on MCT4 mRNA and protein expression in primary cultures of mouse cortical astrocytes. (A) Time-dependent changes in MCT4 mRNA expression by astrocytes exposed to either 1 % or 0 % oxygen. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two- ways ANOVA followed by Tukey's post hoc test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Representative experiment repeated twice with similar results. (B) Time-dependent changes in MCT4 protein expression by astrocytes exposed to either 1 % or 0 % oxygen. Upper panels show typical Western blots for the MCT4 protein. β-tubulin was used as reference. Graph shows western blot quantification. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Tukey's post hoc test. $*P < 0.05$; $***P < 0.001$. Representative experiment repeated twice with similar results. (C) Fluorescent immunolabelings for MCT4 (in red; top and bottom rows) and GFAP (in green; bottom row) on cultured astrocytes exposed to different oxygen levels for 7 days. Nuclei were labeled with DAPI (in blue). Images obtained by epifluorescence microscopy. Calibration bar = 20 μm.

Figure 3. Effect of reduced oxygen levels on MCT1 mRNA and protein expression in primary cultures of mouse cortical astrocytes (A) MCT1 mRNA expression by astrocytes exposed to either 1 % or 0 % oxygen as a function of time. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Tukey's post hoc test. ns, not significant vs. control; $*P < 0.05$. Representative experiment repeated twice with

similar results. (B) MCT1 protein expression by astrocytes exposed to either 1 % or 0 % oxygen as a function of time. Upper panels show typical Western blots for the MCT1 protein. β-tubulin was used as reference. Graph shows western blot quantification. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Tukey's post hoc test. ns, not significant vs. control; *$P < 0.05$. Representative experiment repeated twice with similar results. (C) Fluorescent immunolabelings for MCT1 (in red; top and bottom rows) and GFAP (in green; bottom row) on cultured astrocytes exposed to different oxygen levels for 7 days. Nuclei were labeled with DAPI (in blue). Images obtained by epifluorescence microscopy. Calibration bar = 20μm.

Figure 4. Involvement of the transcription factor HIF-1α in the oxygen level-dependent enhancement of MCT4 expression by cultured astrocytes (A) Time-dependent stabilization of the HIF1-α protein in astrocytes exposed to either 1 % or 0 % oxygen. Upper panels show typical Western blots for the HIF-1α protein. β-tubulin was used as reference. Graph shows western blot quantification. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Tukey's post hoc test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. control. Representative experiment repeated twice with similar results. (B) Effect of a siRNA against HIF-1α on the induction of MCT4 mRNA expression in cultured astrocytes by a low (1 %) oxygen level. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Tukey's post hoc test. ***$P < 0.001$. Representative experiment repeated twice with similar results. (C) Effect of a siRNA against HIF-1α on the induction of MCT4 protein expression in cultured astrocytes by a low (1 %) oxygen level. Upper panel shows a typical Western blot for the MCT4 protein. β-tubulin was used as reference. Graph shows western blot quantification. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Tukey's post hoc test. ***$P < 0.001$. Representative experiment repeated twice with similar results. (D) Concentration-dependent stabilization of the HIF-1α protein in astrocytes induced by the prolyl hydroxylase inhibitor DMOG. Upper panel shows a typical Western blot for the HIF-1α protein. β-tubulin was used as reference. Graph shows western blot quantification. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Dunnett's post hoc test. ***$P < 0.001$ vs. control. Representative experiment repeated twice with similar results. (E) Concentration-dependent effect of DMOG on MCT4 mRNA expression in cultured astrocytes. Each data point is the mean ± SEM, n = 3. Results

were analyzed with a two-way ANOVA followed by Dunnett's post hoc test. ***$P < 0.001$ vs. control. Representative experiment repeated twice with similar results. (F) Concentration-dependent effect of DMOG on MCT4 protein expression in cultured astrocytes. Upper panel shows a typical Western blot for the MCT4 protein. β-tubulin was used as reference. Graph shows western blot quantification. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Dunnett's post hoc test. **$P < 0.01$ or ***$P < 0.001$ vs. control. Representative experiment repeated twice with similar results.

Figure 5. Influence of an antioxidant, oxidative phosphorylation blockade and AMPK activation on MCT4 expression in primary cultures of mouse cortical astrocytes. (A) Effect of the cell-permeable reducing agent glutathione reduced ethyl ester tested at different concentrations on MCT4 mRNA expression in cultured astrocytes exposed to either 1 or 21 % oxygen. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Dunnett's post hoc test. ns, not significant vs. control. Representative experiment repeated twice with similar results. (B) Effect of the cell-permeable reducing agent glutathione reduced ethyl ester tested at different concentrations on MCT4 protein expression in cultured astrocytes exposed to either 1 or 21 % oxygen. Upper panel shows a typical Western blot for the MCT4 protein. β-tubulin was used as reference. Graph shows western blot quantification. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Dunnett's post hoc test. ns, not significant vs. control. Representative experiment repeated twice with similar results. (C) Effect of the cytochrome c oxidase inhibitor sodium azide tested at different concentrations on MCT4 mRNA expression in cultured astrocytes. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Dunnett's post hoc test. ns, not significant vs. control. Representative experiment repeated twice with similar results. (D) Effect of the AMPK activator AICAR tested at different concentrations on MCT4 mRNA expression in cultured astrocytes. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Dunnett's post hoc test. ns, not significant vs. control. Representative experiment repeated twice with similar results.

Figure 6. Long-lasting expression of MCT4 protein in cultured astrocytes upon reoxygenation after a transient exposure to 1 % oxygen (A) MCT4 mRNA expression in cultured astrocytes after exposure to 1 % oxygen for 48 hours and following various reoxygenation periods (at 21 % oxygen). Each data point is the mean ± SEM, n = 3. Results

were analyzed with two-way ANOVA followed by Dunnett's post hoc test. *$P < 0.05$ or ***$P < 0.001$ vs. 0 hour. Representative experiment repeated twice with similar results. (B) MCT4 protein expression in cultured astrocytes after exposure to 1 % oxygen for 48 hours and following various reoxygenation periods (at 21 % oxygen). Upper panel shows a typical Western blot for the MCT4 protein. β-tubulin was used as reference. Graph shows western blot quantification. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Dunnett's post hoc test. ***$P < 0.001$ vs. 0 day. Representative experiment repeated twice with similar results. (C) Effect of the protein synthesis inhibitor cycloheximide on MCT4 protein expression in cultured astrocytes induced by exposure to 1 % oxygen and submitted to reoxygenation with 21 % oxygen. Upper panel shows a typical Western blot for the MCT4 protein. β–tubulin was used as reference. Graph shows western blot quantification. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Tukey's post hoc test. ***$P < 0.001$; ns, non-significant. Representative experiment repeated twice with similar results.

Figure 7. Involvement of MCT4 in the enhancement of lactate release by cultured astrocytes and in long-term survival upon exposure to 1 % oxygen over several days. (A) Effect of low (1 %) oxygen level on lactate release by cultured astrocytes. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Dunnett's post hoc test. *$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$ vs. control for each oxygen concentration. Representative experiment repeated twice with similar results. (B) Effect of siRNAs against MCT1 and MCT4 on the induction of lactate release by low (1 %) oxygen levels in cultured astrocytes after 5 days of incubation. Each data point is the mean ± SEM, n = 3. Results were analyzed with a two-way ANOVA followed by Dunnett's post hoc test. ***$P < 0.001$. Representative experiment repeated twice with similar results. (C) Phase contrast microscopy illustrating the effect of MCT1 and MCT4 siRNAs upon incubation at low (1 %) oxygen levels during 6 days on cultured astrocyte survival. Scale bar = 40 μM.

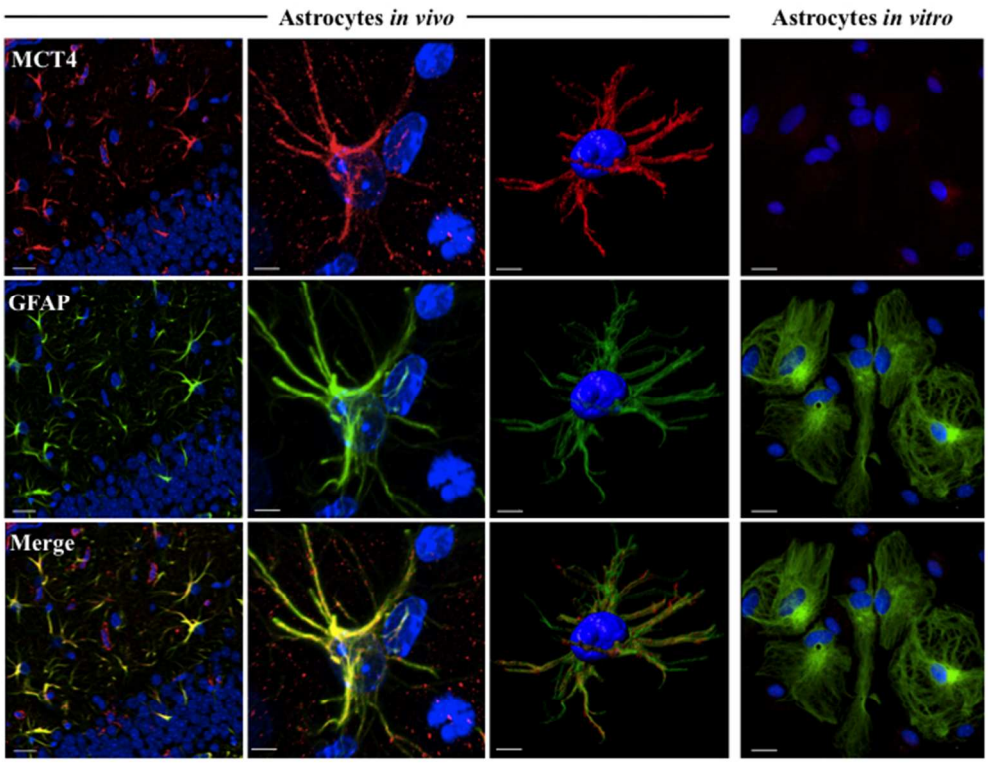

Figure 1
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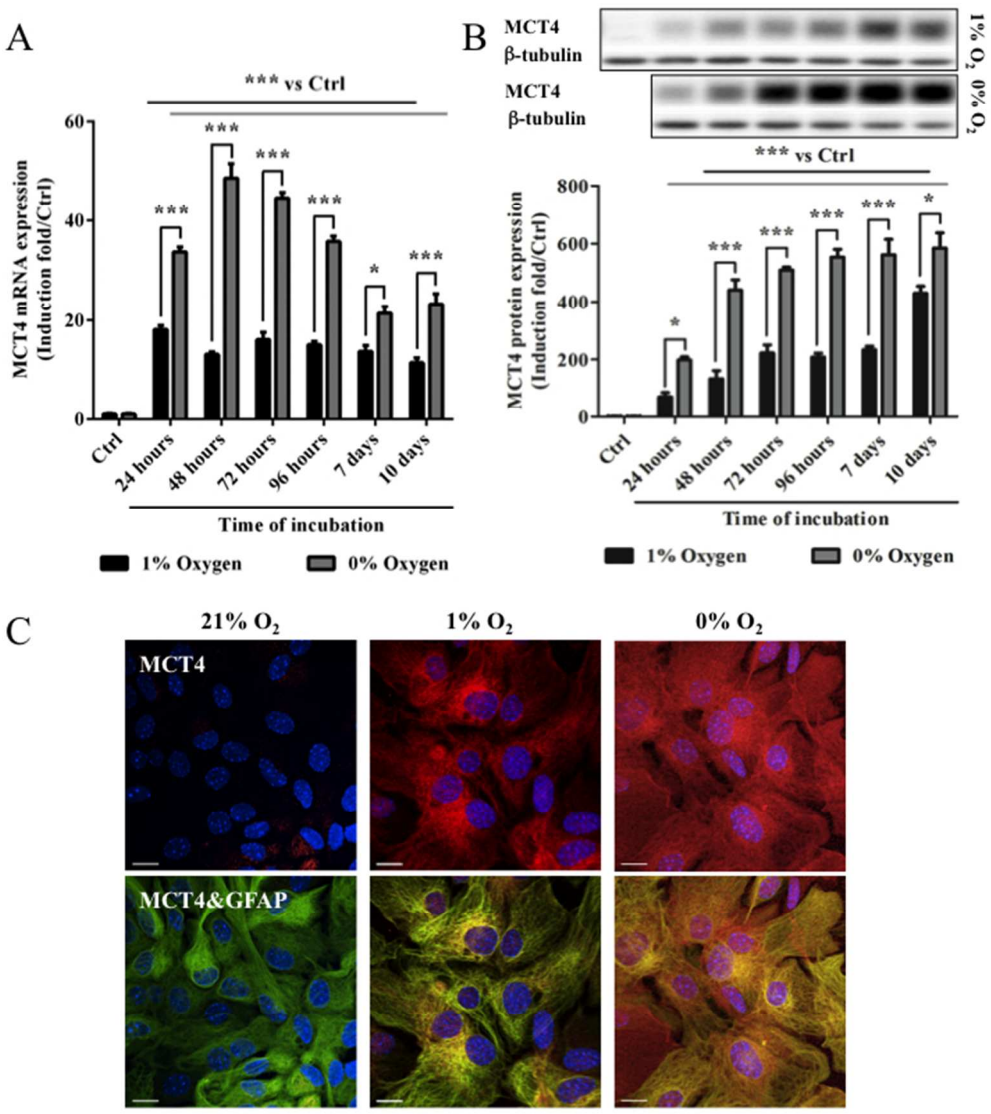

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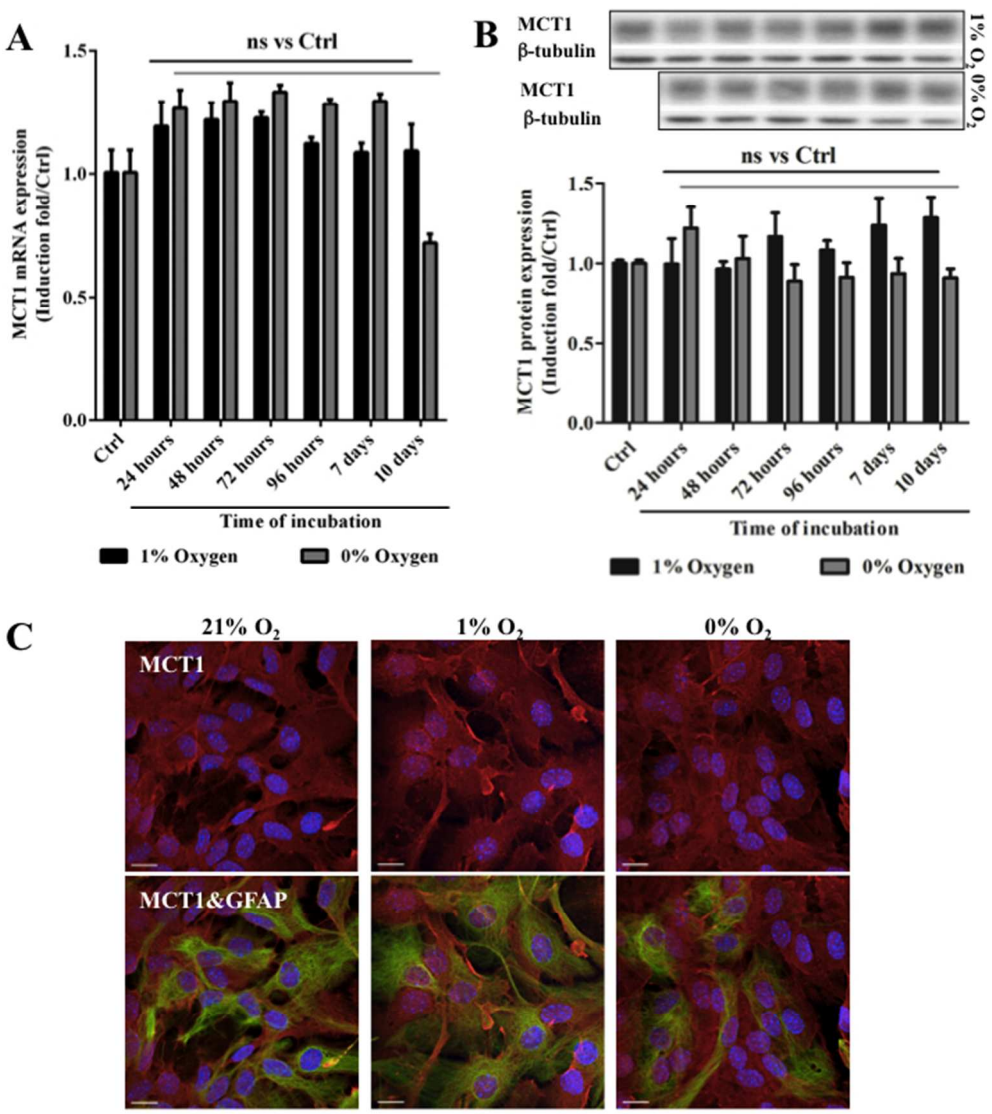

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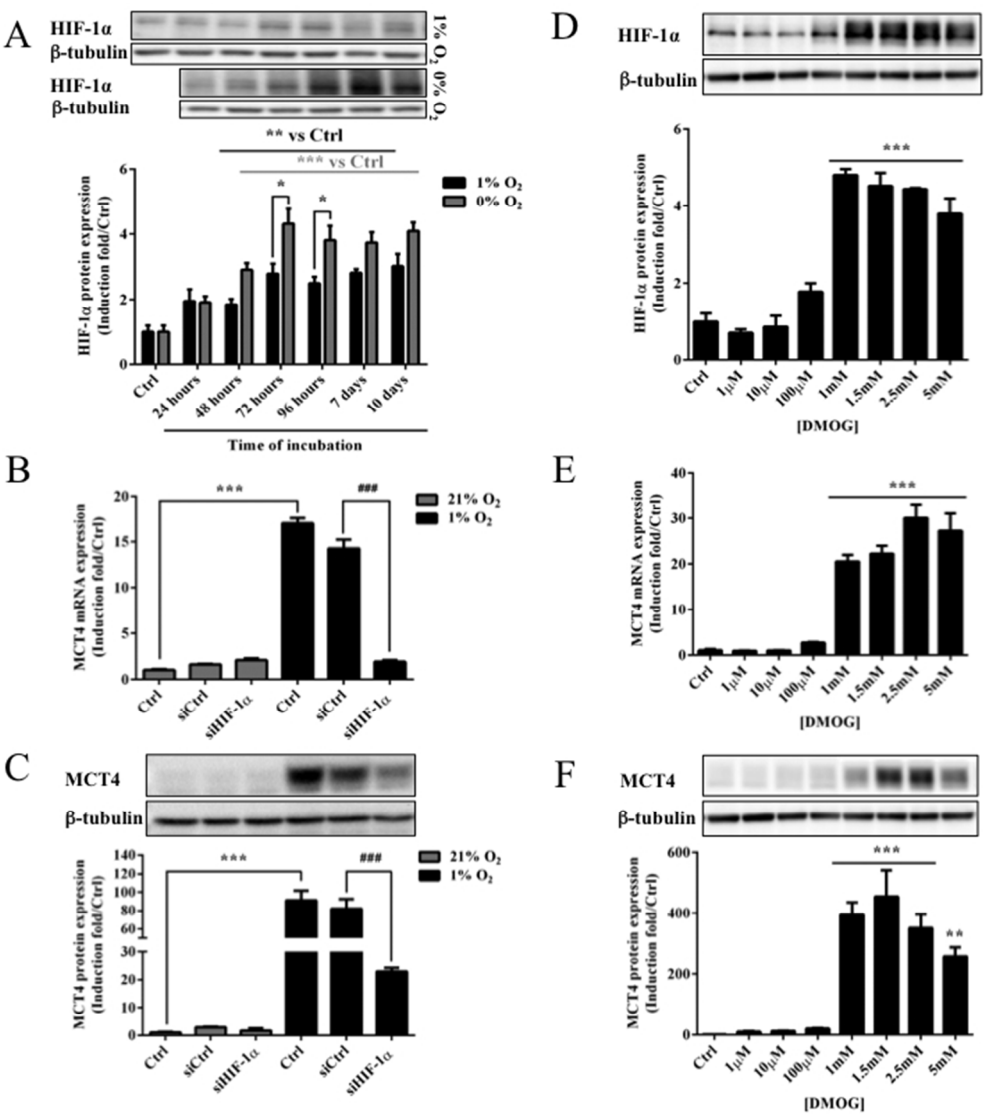

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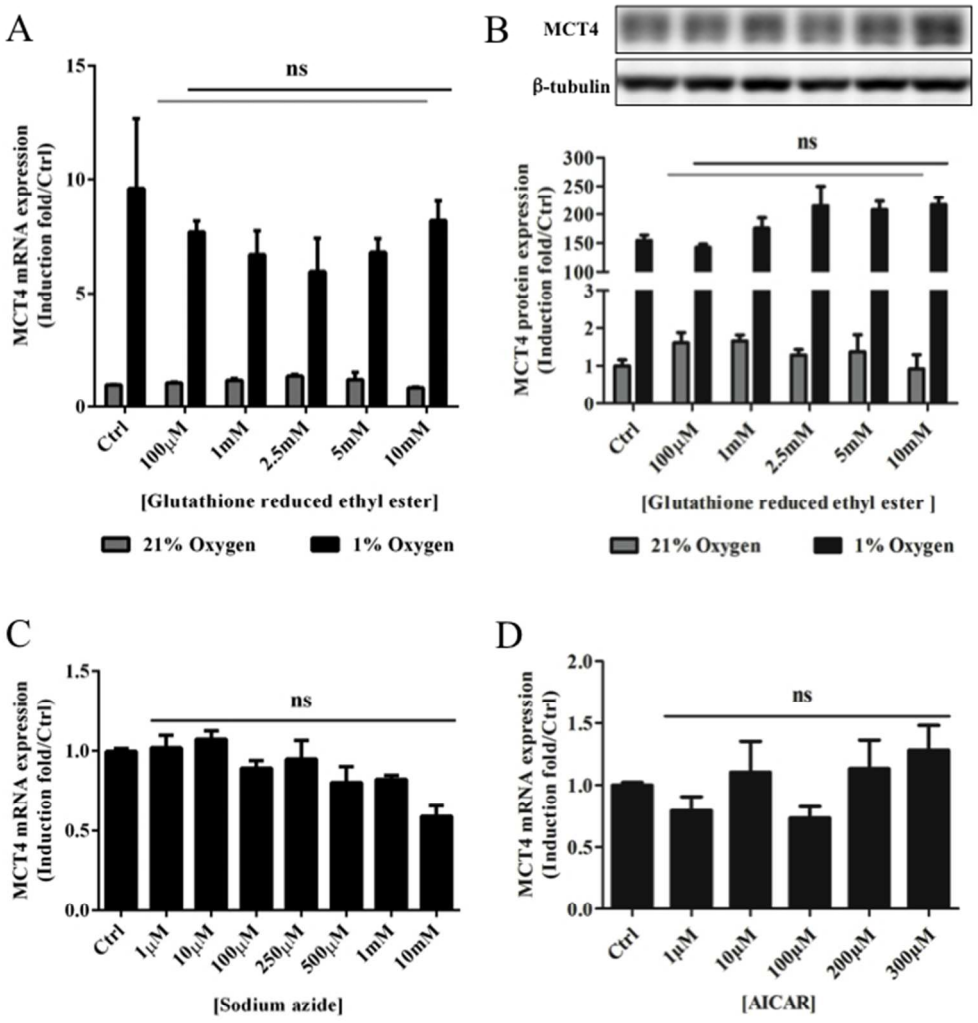

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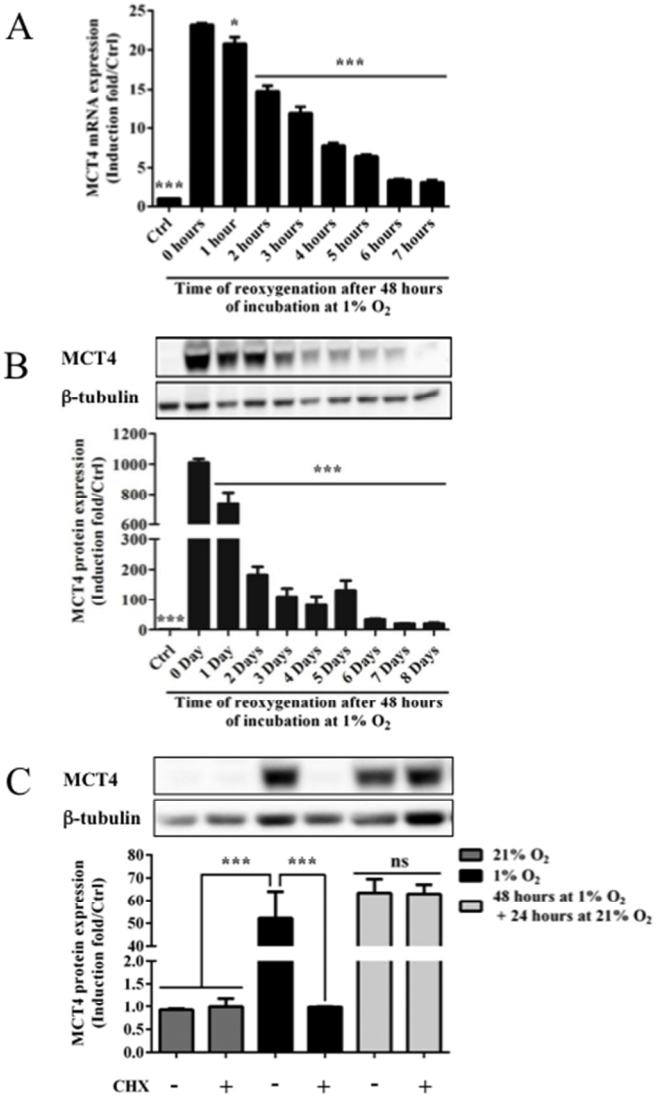

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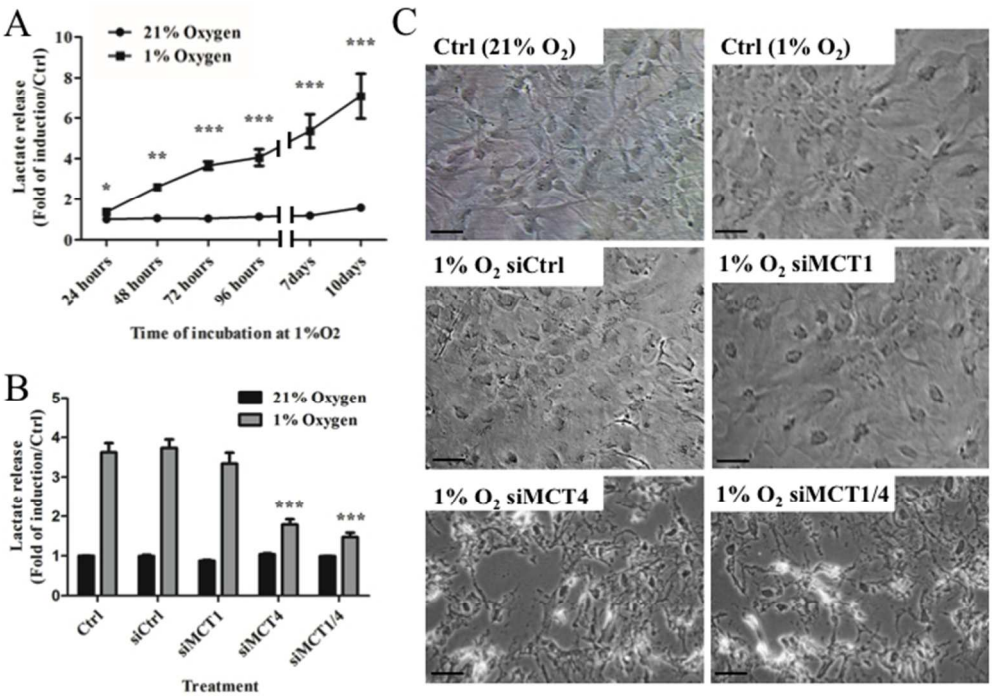

Figure 7
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