Effect of Hypoxia on 2-Deoxyglucose Uptake and Cell Cycle Regulatory Protein Expression of Mouse Embryonic Stem Cells: Involvement of Ca^{2+} /PKC, MAPKs and HIF-1 α

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Key Words

Embryonic Stem (ES) Cell • Hypoxia • 2-DG Uptake • Cell Cycle Regulatory Proteins • PKC • p44/42 MAPKs

Abstract

This study investigated the signal molecules linking the alteration in 2-dexoyglucose (2-DG) uptake and DNA synthesis in mouse embryonic stem (ES) cells under hypoxia. Hypoxia increased the 2-DG uptake and GLUT-1 protein expression level while the undifferentiated state of ES cells and cell viability were not affected by the hypoxia (1 - 48h). Subsequently, [^3H] thymidine incorporation was significantly increased at 12 hours of hypoxic exposure. Hypoxia increased the Ca^{2+} uptake and PKC β , ϵ , and ζ translocation from the cytosol to the membrane fraction. Moreover, hypoxia increased the level of p44/42 mitogen-activated protein kinases (MAPKs) phosphorylation and hypoxia inducible factor-1 α (HIF-1 α) in a time-dependent manner. On the other hand, inhibition of these pathways blocked the hypoxia-induced increase in the 2-DG uptake and GLUT-1 protein expression level. Under hypoxia, cell cycle regulatory protein expression [cyclin D1, cyclin E, cyclin-dependent kinase (CDK) 2, and CDK 4] were increased in a time-dependent manner, which were blocked by PD

98059. pRB protein was also increased in a time-dependent manner. In conclusion, under hypoxia, there might be a parallel relationship between the expression of GLUT1 and DNA synthesis, which is mediated by the Ca^{2+} /PKC, MAPK, and the HIF-1 α signal pathways in mouse ES cells.

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Introduction

Preimplantation embryos develop *in vivo* under conditions of low oxygen. Uterine oxygen concentration decreases to around 3-5% at the time of implantation in the hamster and rabbit [1]. Consistent with this, lowering the oxygen concentration in the gaseous phase during embryo culture, from atmospheric levels to more physiological levels, has been associated with improved embryo development, in terms of blastocyst development rate and embryo cell number, in a number of species [2]. Previous results clearly demonstrate that physiological oxygen (2%) culture conditions are indispensable for robust human ES cells clone recovery and may reduce chromosomal abnormalities [3]. In addition, low O_2 associated with hypoxia has been found to promote the survival of human

embryonic stem cells [4]. In the mouse, increased blastocyst development rates and/or cell numbers have been reported following culture under 7% [5] or 5% oxygen [2, 6] which compared to 20% oxygen, in both F1 hybrid and outbreed strains. Hypoxia also regulates the number of inner cell mass cells in bovine blastocysts and enhances hematopoietic progenitor development [1]. Embryos are exceedingly sensitive to oxidative damage, and blastocysts produced under lower O₂ have significantly more inner cell mass cells than those produced under higher O₂ tensions. The primary transcriptional regulators of both cellular and systemic hypoxic adaptation in mammals are hypoxia-inducible factors (HIF), which consists of an α - and β -subunit [7-9]. Although both HIF-1 subunits are constitutively expressed, HIF-1 α is rapidly degraded in the presence of cellular O₂ via the ubiquitin-mediated proteosome pathway [10]. HIFs regulates the expression of at least 180 genes involved in metabolism, cell survival, erythropoiesis, and vascular remodeling by binding cis-acting hypoxia response elements located in the enhancers and/or promoters of these genes [11]. GLUT1 gene expression and glucose transporter are also stimulated in a variety of cells under hypoxias, a response that is mediated by the transcription factor HIF-1 [12, 13].

The ability to transport and metabolize glucose is central to the function of the early embryo prior to implantation. Adequate supply of nutrients is not only essential to growth and development of the preimplantation embryo but may also be linked to metabolic programming events that may influence later stages fetal development [14]. Glucose transport in the preimplantation embryo is mediated by a family of sodium independent facilitative transport proteins known as GLUTs which have been implicated in key roles in early development [15]. The ubiquitous GLUT1 is expressed throughout development and is thought to provide the embryo with its basal glucose requirements. One explanation for the high glucose consumption of developing embryos is their glycolytic production of energy, consuming much more glucose compared to cells producing energy by oxidative phosphorylation. On the other hand, the high proliferation rates of embryonic cells are thought to be energy-consuming, contributing to the increased glucose consumption. Therefore, oxygen tension might regulate GLUT1 expression as well, and any correlative study should consider this fact which is frequently ignored. However, no *in vitro* study investigating the relationship between GLUT1 and proliferation is available in mouse ES cells. Thus, it was hypothesized that hypoxia will alter the GLUT 1 levels in ES cells in an attempt to maintain the

energy production necessary to fuel the cellular oxidative metabolism of ES cells and transiently protect them against cellular apoptosis. However, the molecular components that are involved in glucose transport and proliferation response to hypoxia are poorly understood in embryonic stem cells.

In vitro studies using stem cell populations with mammalian blastocysts have provided a powerful tool for analyzing the hypoxic effects on cellular functions. The use of stem cells *in vitro* has allowed the elucidation of the complex molecule pathways that facilitate these events [16]. The survival of ES cells is not only of utmost importance for normal development *in vivo* but also for the safe manipulation in cells under stressful conditions. Therefore, this model will be helpful for understanding the effects of a physiological low-oxygen environment in the mouse ES cells, particularly for investigating the relationships between HIF-1 α , glucose transporter, and cell proliferation under low oxygen tension. Therefore, this study investigated the signal molecules linking the alteration in 2-deoxyglucose (2-DG) uptake and DNA synthesis observed in mouse embryonic stem (ES) cells under hypoxia.

Materials and Methods

Materials

The mouse ES cells were obtained from American Type Culture Collection (ES-E14TG2a). The fetal bovine serum was purchased from Biowhittaker (Walkersville, MD, USA). PD 98059, A23187, BAPTA-AM, EGTA, 2-methoxyestradiol (2ME2) and the trypan blue was obtained from Sigma Chemical Company (St. Louis, MO, USA). The bisindolylmaleimide I and staurosporine were purchased from Calbiochem (La Jolla, CA, USA). The anti-Pan PKC was obtained from upstate biotechnology (Charlottesville, VA, USA). The anti-PKC- β , ϵ , ζ , anti-GLUT-1, HIF-1 α , cyclin D1, cyclin E, CDK 2, CDK 4, SSEA-1, Oct-4, and pRB were purchased from Santa Cruz Biotechnology (Delaware, CA, USA). The phospho-p44/42 and p44/42 antibodies were acquired from New England Biolabs (Herts, UK). The goat anti-rabbit IgG was purchased from Jackson Immunoresearch (West Grove, PA, USA). The liquiscint was obtained from National Diagnostics (Parsippany, NY, USA). All other reagents were of the highest purity commercially available.

ES cell culture

Mouse ES cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Gibco-BRL, Gaithersburg, MD) supplemented with 3.7 g/L sodium bicarbonate, 1% penicillin and streptomycin, 1.7 mM L-glutamine, 0.1mM β -mercaptoethanol, 5 ng/mL mouse leukemia inhibitory factor, and 15% fetal bovine serum (FBS) with or without a feeder layer and cultured for five

days in standard medium plus LIF. The cells were grown on either gelatinized 12-well plates or a 60 mm culture dish in an incubator maintained at 37°C in an atmosphere containing 5% CO₂ and air.

Hypoxic treatment of ES cells

The ES cells cultured in 35 mm or 60 mm culture dishes were washed twice with phosphate-buffered saline (PBS). Subsequently, the media was changed to fresh DMEM supplemented with 5% fetal calf serum but without LIF [17]. The experiments were performed in an incubator at 37°C under normoxic conditions by maintaining the cells in either 92.3% air and 5.5% CO₂ or under hypoxias by incubating the cells in a modular incubator chamber gassed with 2.2% O₂, 5.5% CO₂, and 92.3% N₂ (Billups-Rotheberg Inc, CA, USA) at a flow rate of 20 liters/min for 30 min. After the chamber had been purged with gas, it was sealed and placed in conventional incubator at 37°C [18].

Trypan blue exclusion assay

The ES cells were incubated under normoxic or hypoxias as described above and washed twice with PBS. The cells were then detached from the culture dishes utilizing a 0.05% trypsin-0.5 mM EDTA solution, and the action was quenched with a soybean trypsin inhibitor (0.05 mg/ml). Subsequently, a 0.4% (w/v) trypan blue solution (500 µl) was added to the cell suspension and the cells were counted using a hemocytometer under optical microscopy, while keeping a separate count of the blue cells. Cells failing to exclude the dye were considered to be non-viable and the data is expressed as the percentage of viable cells. Cell injury was assessed using the LDH activity. The level of LDH activity in the medium was measured using a LDH assay kit (Iatron Lab, Tokyo, Japan). The level of LDH released is expressed as percentage of the control (normoxia).

[³H] thymidine incorporation

The [³H] thymidine incorporation experiments were carried out as described by [19, 20]. Zhang et al. reported that most ES cells could be arrested in the G0/G1 phase using a serum deprivation culture [21]. Furthermore, the synchronized ES cells could successfully reenter a normal cell cycle after resupplying the serum. In this study, the cells were cultured in one well until they reached 50% confluence, washed twice with PBS and maintained in serum free DMEM including all supplements. After 24h incubation, the cells were washed twice with PBS, and incubated with fresh serum free DMEM including all the supplements and indicated agents. After the indicated incubation period, 1 µCi of [methyl-³H] thymidine (specific activity: 74 GBq/mmol, 2.0 Ci/mmol; Amersham Biosciences, Buckinghamshire, UK) was added to the cultures. Incubation with [³H] thymidine continued for 1h at 37°C. The cells were then washed twice with PBS, fixed in 10% trichloroacetic acid (TCA) at 23°C for 15 min, and then washed twice with 5% TCA. The acid-insoluble material was dissolved in 2 N NaOH for 12h at 23°C. Aliquots were removed to determine radioactivity using a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA). All values are means (± S.E.) of triplicate experiments. Values were converted from absolute counts to a per-

centage of the control to allow for comparison between experiments.

Immunofluorescence staining with SSEA-1 and pRB

Cells were fixed and treated with monoclonal antibody against mouse SSEA-1 (1:50, Santa Cruz Biotechnology, Inc, Santa Cruz, CA) or rabbit pRB (1:50, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), and then incubated for 30 min with fluorescein isothiocyanate-conjugated (FITC-conjugated) second antibody raised in rabbit against mouse IgM (1:100) or goat against rabbit IgM (1:100). Fluorescence images were visualized with a fluorescence microscope (Fluoview 300, Olympus, Japan).

Bromodeoxyuridine incorporation

The level of 5-bromo-2'-deoxyuridine (BrdU) (a thymidine analog) incorporation was measured in order to determine the level of DNA synthesis. The ES cells were serum-starved for 24h prior to hypoxic exposure. The ES cells were then exposed to hypoxia for 12h. Fifteen µM BrdU was then added, and incubation was continued for an additional 1h. After several washes with PBS, the cells were fixed with methanol [10% (vol/vol) for 10 min at 4°C], followed by incubation in 1 N HCl for 30 min at room temperature. The cells were then washed and incubated with 0.1 M sodium tetraborate for 15 min. Alexa Fluor 488-conjugate Mouse anti-BrdU mAb (diluted 1:200, Molecular Probes, OR, USA) in 2% BSA-PBS was incubated overnight at 4°C. After washing in PBS, coverslips were mounted onto glass slides with a Dako Fluorescent mounting medium using gelvatol and examined under an optical microscope (fluoview 300, Olympus).

For the double-labeling experiments, the cells were fixed in acid alcohol and processed for Oct-4 staining, followed by BrdU staining. The fixed cells were incubated with the rabbit anti-Oct-4 antibody (1:100, Santa Cruz Biotechnology, Delaware, CA, USA) for 1h at room temperature and Alexa Fluor 555 anti-rabbit IgG (1:100, Molecular Probes, Eugene, OR) for 1h at room temperature. This was followed by incubation in 1 N HCl, neutralization with 0.1 M sodium tetraborate, and incubation with Alexa Fluor 488-conjugate Mouse anti-BrdU mAb for 1h at room temperature. After washing with PBS, the BrdU/Oct-4 stained cells were examined under confocal microscopy (fluoview 300, Olympus).

⁴⁵Ca²⁺ uptake experiment

The Ca²⁺ uptake experiments were carried out using a slight modification of the methodology reported by [22]. To measure the Ca²⁺ uptake, the culture medium was removed by aspiration and the cells were gently washed twice with the uptake buffer (140 mM NaCl, 2 mM KCl, 1 mM KH₂PO₄, 10 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, 5 mM L-alanine, 5 µM indomethacin, and 10 mM HEPES/Tris, pH 7.4). After washing, the cells were incubated in an uptake buffer containing 1 µCi/ml ⁴⁵Ca²⁺ at 37°C for 30 min. At the end of the incubation period, the cells were washed three times with ice-cold uptake buffer, and digested in 1 ml 0.1% SDS. The level of ⁴⁵Ca²⁺ incorporated intracellularly was determined by removing 900 µl of each sample and counting the radioactivity using a liquid scin-

tillation counter (LS6500; Beckman Instruments, Fullerton, CA). The remainder of each sample was used to determine the protein level [23]. The radioactivity in each sample was normalized to the protein level and corrected for the zero-time uptake per mg protein. All uptake measurements were carried out in triplicate.

HIF-1 α small interfering ribonucleic acid transfection

Cells were grown to 75% confluence in each dish and were transfected for 24h with either a SMARTpool of small interfering RNAs specific for HIF-1 α (100 nmol/L) or a non-targeting small interfering RNA (as negative control; 100 nmol/L; Dharmacon, Inc., Lafayette, CO, USA) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2-DG uptake experiment

The 2-DG uptake experiments were carried out using the methodology reported by [22, 24]. The 2-DG uptake was examined by removing the culture medium by aspiration and gently washing the cells twice with the uptake buffer (140 mM NaCl, 2 mM KCl, 1 mM KH₂PO₄, 10 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, 5 mM L-alanine, 5 μ M indomethacin, and 10 mM HEPES/Tris, pH 7.4). After the washing, the cells were incubated in an uptake buffer containing 1 μ Ci/ml 2-DG at 37°C for 30 min. At the end of the incubation period, the cells were again washed three times with ice-cold uptake buffer, and the cells were digested in 1 ml 0.1% SDS. The intracellular 2-DG uptake was determined by removing 900 μ l of each sample and counting the radioactivity using a liquid scintillation counter. The remainder of each sample was used for protein determination [23]. The radioactivity counts for each sample were then normalized to the protein level, and were corrected for the zero-time uptake per mg protein. All the uptake measurements were performed in triplicate.

Preparation of cytosolic and total membrane fractions

Preparation of cytosolic and total membrane fractions was performed using a slight modification of the methodology reported by [25]. DMEM of the mouse ES cells was exchanged 12h before the experiments. The medium was then removed and the cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation and resuspended in a buffer A [137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 μ g/ml leupeptin (pH 7.5)]. The resuspended cells were then mechanically lysed on ice by trituration using a 21.1-gauge needle. The lysates were first centrifuged at 1,000g for 10 min at 4°C. The supernatants were then centrifuged at 100,000g for 1h at 4°C to prepare the cytosolic and total particulate fractions. The supernatants (cytosolic fraction) were then precipitated with 5 vol. of acetone, incubated on ice for 5 min and centrifuged at 20,000g for 20 min at 4°C. The resulting pellet was resuspended in buffer A containing 1% (v/v) Triton X-100. The particulate fractions containing the membrane fraction were washed twice and resuspended in buffer A containing 1% (v/v) Triton X-100.

The protein in each fraction was quantified using the Bradford procedure [23].

Western blot analysis

The cell homogenates (20 μ g protein) were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. After the blots had been washed with TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], the membranes were blocked with 5% skim milk for 1h and incubated with the appropriate primary antibodies at the dilutions recommended by the supplier. The membrane was then washed, and the primary antibodies were detected using goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. The bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK).

RNA isolation and RT-PCR

Total RNA was extracted from mouse ES cells using STAT-60, monophasic solution of phenol and guanidine isothiocyanate from Tel-Test, Inc. (Friendwood, Tex., USA). Reverse transcription was conducted with 3 μ g RNA using reverse transcription system kit (AccuPower® RT PreMix, Korea) with oligo-dT18 primers. After that, 5 μ l of RT products was amplified with PCR kit (AccuPower® PCR PreMix, Korea) followed by: denaturation at 94°C for 5 min and 30 cycles at 94°C for 45s, 58°C for 30 min and 72°C for 30 min followed by 5 min extension at 72°C. The primers were and 5'-CGT GAG ACT TTG CAG CCT GA-3' (sense), 5'-GGC GAT GTA AGT GAT CTG CTG-3' (antisense) for Oct-4 (519 bp), 5'-GTG GAA TTT GTC CGA GAC-3' (sense), 5'-TGG AGT GGG AGG AGG AGG TAA C-3' (antisense) for SOX-2 (550bp), 5'-TCT TAC ATC GCG CTC ATC AC-3' (sense), 5'-TCT TGA CGA AGC AGT CGT TG-3' (antisense) for FOX-D3 (171bp). PCR of β -actin was also performed as control for quantity of RNA.

Fluorescence activated cell sorter (FACS) analysis

The ES cells were serum-starved for 24h prior to hypoxic exposure and then cells were dissociated in trypsin/EDTA, pelleted by centrifugation, resuspended at approximately 106 cells/ml in in PBS containing 0.1% BSA. When required (for Oct 4 staining), cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. The cells were labeled with the rabbit anti-Oct 4 (1: 50) or the mouse anti-SSEA 1 antibody (1:50, Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and then incubated with fluorescein isothiocyanate-conjugated (FITC-conjugated) second antibodies (1:50). The cells were washed and resuspended in PBS and then read by flow cytometry (Beckman Coulter, CA). Samples were analyzed using CXP software (Beckman Coulter, CA).

Statistical analysis

The results are expressed as the mean $\pm$ the standard error (S.E.). All the experiments were analyzed by ANOVA, which were followed in some experiments by a comparison of the treatment means with the control using the Bonnferroni-Dunn test. A P value < 0.05 was considered significant.

Fig. 1. Effect of hypoxia on the characterization of mouse ES cells. (A) Mouse ES cells were exposed to hypoxia for 0 to 360 min and HIF-1 α was detected by western blotting as described in Materials and Methods. (B, C) The cell viability was determined under hypoxia (0–72 h) using a trypan blue assay. (C) The LDH release was measured under hypoxia (0–72h) as described in Materials and Methods. (D) Mouse ES cells were exposed to hypoxia for 12h and then Flow cytometry analysis to monitor the percentage of Oct-4 and SSEA-1 positive in cells under normoxic or hypoxia. The examples are representative of 3 independent experiments. (E) Mouse ES cells were exposed to hypoxia for 12h and then immunofluorescence stained with Oct-4 and SSEA-1 antibody. Scale bars represent 20 μ m (magnification $\times$ 100). (F) Oct-4 (519 bp), SOX-2 (550 bp), FOXD3 (171 bp), and β -actin (350 bp) mRNA expression levels under normoxic or hypoxia. The example shown is a representative of 5 independent experiments.

Results

Effect of hypoxia on 2-DG uptake and DNA synthesis

In order to ensure that the hypoxia used in this study induces typical cell responses to hypoxic stress, the alteration of the HIF-1 α expression level at different times (0–360 min) was first observed. As shown in Fig. 1A, the significant increase in HIF-1 α appeared from 5 min to 60 min of hypoxic exposure. Moreover, the cell viability and amount of LDH release, which is a marker of structural damage, were also measured. As shown in Fig. 1B and C, hypoxia did not affect the cell viability and LDH release up to 48h. The undifferentiated state of the mouse ES cells used in this experiment was confirmed by examining the expression of the undifferentiated stem cells markers, including the Oct-4 and stage-specific embry-

onic antigen (SSEA-1). Based upon FACS analysis, Oct-4 and SSEA-1 expression of the mouse ES cells was not changed in both normoxia and hypoxia (Fig. 1D). Immunofluorescence staining also showed that mouse ES cells expressed SSEA-1 or Oct-4 that is characteristic of undifferentiated mouse ES cells (Fig. 1E). In addition, RT-PCR for Oct-4, SOX-2 and FOXD3 were conducted to validate undifferentiated states of ES cells (Fig. 1F). These results suggested that mouse ES cells maintained an undifferentiated state under the experimental conditions used in this study.

As shown in Fig. 2A, 2-DG uptake was peaked in 12h of hypoxia (100% increase vs. control, $P < 0.05$) and then sustained to 48h. GLUT-1 protein expression level (79% increase vs. control, $P < 0.05$) and [3 H] thymidine incorporation (80% increase vs. control; $P < 0.05$) were also increased after 12h of hypoxic exposure (Fig. 2B

Fig. 2. Effect of hypoxia on 2-DG uptake, GLUT-1 protein expression levels, and [^3H] thymidine/BrdU incorporation. (A) 2-DG uptake was assessed after 0–72h of hypoxic exposure as described in Materials and Methods. The values are reported as the mean $\pm$ S.E. of 3 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control (normoxia), # $P < 0.01$ vs. Control (normoxia). (B) GLUT1 protein expression level was determined by Western blotting with plasma membrane protein as described in Materials and Methods. Bands represent 50–60 kDa of

GLUT1, and 41 kDa of β -actin, respectively. The example shown is a representative of 4 independent experiments. The lower panel of B depicting the bars denotes the mean $\pm$ S.E. of 4 experiments for each condition determined from densitometry relative to β -actin. * $P < 0.05$ vs. Control (normoxia). (C) Mouse ES cells were exposed to hypoxia for 12h and then pulsed with $1\mu\text{Ci}$ of [^3H] thymidine for 1 hour prior to counting. (D) Mouse ES cells were exposed to hypoxia for 12h and double labeled with Oct-4 and BrdU antibody simultaneously. Scale bars represent $20\mu\text{m}$ (magnification $\times 100$).

and C). In order to validate these results, double labeling for Oct-4 and BrdU expression was performed to determine if hypoxia exerts its growth-promoting effect on undifferentiated ES cells. In these experiments, the ES cell population contained more than 90% of undifferentiated (Oct-4 positive) cells. The observed effects reflect the role of hypoxia in the undifferentiated ES cells, not in the spontaneously differentiated progeny (Fig. 2D).

Involvement of $[\text{Ca}^{2+}]_i$ /PKC on hypoxia-induced increase of 2-DG uptake

In order to determine if intracellular Ca^{2+} is involved in the hypoxia-induced increase in 2-DG uptake, Ca^{2+} uptake was examined under hypoxia. As shown in Fig. 3A, pretreating the cells with EGTA ($4 \times 10^{-3}\text{M}$, extracel-

lular Ca^{2+} chelator) or BAPTA-AM (10^{-6}M , intracellular Ca^{2+} chelator) inhibited the hypoxia-induced increase in Ca^{2+} uptake (155% increase vs. control, $P < 0.05$). A23187 (calcium ionophore, 10^{-6}M) was used as positive control. Moreover, pretreating the cells with A23187 prior to hypoxia significantly increased the 2-DG uptake (105% increase vs. control, $P < 0.05$) and GLUT-1 protein expression levels (65% increase vs. control, $P < 0.05$). On the other hand, pretreating the cells with either EGTA or BAPTA-AM inhibited the hypoxia-induced increase in 2-DG uptake and the GLUT-1 protein expression levels (Fig. 3B, C).

In order to determine if the PKC pathway is involved in the hypoxia-induced increase in 2-DG uptake, this study initially investigated whether or not hypoxia induced PKC

Fig. 3. Effect of $[Ca^{2+}]_i$ on hypoxia-induced increase in 2-DG uptake. (A) Mouse ES cells were pretreated with A 23187 (10^{-6} M), EGTA (4×10^{-3} M), or BAPTA-AM (10^{-6} M) for 30 min prior to 12h of normoxic and hypoxic exposure and then the Ca^{2+} uptake was measured. * $P < 0.05$ vs. Control (normoxia), ** $P < 0.05$ vs. Hypoxia. The values are reported as the mean $\pm$ S.E. of 4 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control (normoxia), ** $P < 0.05$ vs. Hypoxia. (B) Mouse ES cells were pretreated with A 23187 (10^{-6} M), EGTA (4×10^{-3} M), or BAPTA-AM (10^{-6} M) for 30 min prior to 12h of hypoxic exposure and then the 2-DG uptake was assessed. The values are reported as the mean $\pm$ S.E. of 3 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control (normoxia), ** $P < 0.05$ vs. Hypoxia. (C) Mouse ES cells were pretreated with A 23187, EGTA, or BAPTA-AM for 30 min prior to hypoxic exposure for 12h, and then the plasma membrane protein was extracted and blotted with the antibody against GLUT-1. The example shown is a representative of 4 independent experiments. The bands represent 50~60 KDa of GLUT-1 and 41 KDa of β -actin, respectively. The lower panel of C depicting the bars denotes the mean $\pm$ S.E. of 4 experiments for each condition determined from densitometry relative to β -actin. * $P < 0.05$ vs. Control (normoxia), ** $P < 0.05$ vs. Hypoxia.

β_p , ϵ , and ζ translocation from the cytosol to the membrane compartment (Fig. 4A). In the next step, pretreating the cells with EGTA, BAPTA-AM, or bisindolylmaleimide I resulted in the inhibition of the hypoxia-induced pan PKC translocation (Fig. 4B). The effect of staurosporine or bisindolylmaleimide I (PKC inhibitors, 10^{-7} M) on the hypoxia-induced increase in the 2-DG uptake and GLUT-1 protein expression levels was also examined. As shown in Fig. 4C and D, these inhibitors blocked the hypoxia-induced increase in 2-DG uptake and GLUT-1 protein expression levels.

Involvement of MAPKs in hypoxia-induced increase of 2-DG uptake

The phosphorylation of p44/42 MAPKs was examined in order to determine if the hypoxia-induced increase in 2-DG uptake is involved in the MAPKs pathways. As shown in Fig. 5A, hypoxic exposure induced the phosphorylation of p44/42 MAPKs in a time-dependent manner from 15 min of hypoxic exposure. Moreover, staurosporine (10^{-7} M) blocked the phosphorylation of the p44/42 MAPKs (Fig. 5B). The cells were pretreated with PD 98059 (MEK inhibitor, 10^{-5} M) prior to hypoxic exposure. As shown in Fig. 5C and D, PD 98059 inhibited the hypoxia-induced increase in 2-DG uptake and GLUT-1 protein expression levels, respectively ($P < 0.05$). In addition, pretreating the cells with staurosporine, PD 98059, and 2-methoxyestradiol (2ME2, HIF-1 α inhibitor, 10^{-4} M) inhibited the hypoxia-induced increase in HIF-1 α (Fig.

6A and B). As shown in Fig. 6 C and E, 2ME2 inhibited the hypoxia-induced increase in the 2-DG uptake and GLUT-1 protein expression level. Cells were transfected with a pool of HIF-1 α specific siRNAs (100 nmol/L) or non-targeting siRNAs (100 nmol/L). In this experiment, HIF-1 α siRNA inhibited the hypoxia-induced increase in the 2-DG uptake and GLUT-1 protein expression level (Fig. 6 D and F).

Fig. 4. Effect of PKC on hypoxia-induced increase of 2-DG uptake. (A) The PKC $\beta 1$, ϵ , and ζ isoforms present in either the cytosolic compartment or membrane compartment was detected by western blotting, as described in Materials and Methods. The bands represent the 80 - 90 kDa of PKC $\beta 1$, ϵ , and ζ and 41 kDa of β -actin. Each example shown is a representative of 3 experiments. (B) Mouse ES cells were pretreated with EGTA, BAPTA-AM, or bisindolylmaleimide I (10^{-7} M) for 30 min prior to hypoxic exposure for 30 min and then Pan-PKC was detected by western blotting, as described in Materials and Methods. The bands represent the 80 kDa of Pan-PKC. Each example shown is a representative of 4 experiments. (C, D) Mouse ES cells were pretreated with staurosporine or bisindolylmaleimide I (10^{-7} M) for 30 min prior to 12h of hypoxic exposure and the 2-DG uptake or western blotting (GLUT-1) was assessed. The values are reported as the mean $\pm$ S.E. of 4 independent experiments with triplicate dishes. The example shown is a representative of 4 independent experiments. The bands represent 50~60 KDa of GLUT-1 and 41 KDa of β -actin, respectively. The lower panels of B and D depicts the bars showing the mean $\pm$ S.E. of 4 experiments for each condition determined from densitometry relative to β -actin. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. Hypoxia.

Involvement of MAPKs in hypoxia-induced DNA synthesis

In an experiment to determine if the hypoxia stimulates cell proliferation, the levels of cell cycle regulatory protein expression (cyclin D1, cyclin E, CDK 2, and CDK 4) were increased in a time-dependent manner (Fig. 7A, B). In addition, mouse ES cells in both normoxia and hypoxia expressed pRB and hypoxia increased pRB pro-

tein levels in a time-dependent manner (Fig. 7C, D). In order to examine the hypoxia-induced increase in $[^3\text{H}]$ thymidine incorporation and cell cycle regulatory protein expression are involved in MAPKs pathways, the cells were pretreated with PD 98059 (MEK inhibitor, 10^{-5} M) prior to hypoxic exposure. The inhibitor decreased both the hypoxia-induced increase in the levels of cell cycle regulatory protein expression (Fig. 8A, B, C, and D) and

Fig. 5. Effect of MAPKs on hypoxia-induced increase of 2-DG uptake. (A) Mouse ES cells were exposed to hypoxia for 0 to 120 min and the phosphorylated p44/42 MAPKs were detected as described in Materials and Methods. (B) Mouse ES cells were pretreated with staurosporine (10^{-7} M) for 30 min prior to 30 min of hypoxic exposure and western blotting (p44/42 MAPKs) was performed. (C, D) Mouse ES cells were pretreated with PD 98059 for 30 min prior to hypoxic exposure for 12h and then 2-DG uptake and western blotting (GLUT-1) was assessed. The values are reported as the mean $\pm$ S.E. of 3 independent experiments with triplicate dishes. The example shown is representative of 3 experiments. The bands represent 50–60 KDa of GLUT-1 and 41 KDa of β -actin, respectively. The lower panels of D depicts the bars showing the mean $\pm$ S.E. of 3 experiments for each condition determined from densitometry relative to β -actin. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. Hypoxia.

[3 H] thymidine incorporation (Fig. 8E).

Discussion

The present results firstly show that the PKC-dependent activation of p44/42 MAPKs and HIF-1 α are an important part of the 2-DG uptake and cell cycle regulatory mechanisms in mouse ES cells under low oxygen tension. In the present study, low-oxygen conditions increase the expression of GLUT1, which is compatible to the adaptive response that enhances glucose uptake during periods of oxygen deficiency. Based on these results, it is possible that enhanced embryonic cell proliferation is responsible for both high glucose utilization and over expression of GLUT1. Some studies have shown a parallel

increase in the expression of proliferation markers and GLUT1 [26, 27]. Moreover, the expression of GLUT1 in fibroblasts is higher in proliferating cells when compared to contact-inhibited ones [28, 29]. This possible compensation provides a window in time during which the introduction of opportune interventions targeted at the cellular metabolism can potentially reverse the trajectory set in motion by the initial insult.

Studies on hypoxic injuries have already shown that there is a link between a lack of oxygen, an energy deficiency and a disturbance in the Ca^{2+} homeostasis, leading to several biochemical tissue alterations [30]. Therefore, the Ca^{2+} uptake in mouse ES cells was examined in the presence or in the absence of different inhibitors in order to determine the regulatory mechanisms that might contribute to this hypoxia-induced increase in $[\text{Ca}^{2+}]_i$.

Fig. 6. Effect of HIF-1 α on hypoxia-induced increase of 2-DG uptake. (A, B) Mouse ES cells were pretreated with staurosporine, PD 98059, or 2ME2 for 30 min prior to hypoxic exposure for 1h and then HIF-1 α was detected by western blotting. (C) Mouse ES cells were pretreated with 2ME2 for 30 min prior to hypoxic exposure for 12h and the 2-DG uptake was assessed. (D) Mouse ES cells were transfected for 24h with either a SMARTpool of HIF-1 α siRNAs (100 nmol/L) or a non-targeting control siRNA (100 nmol/L) using LipofectAMINE 2000 prior to hypoxic exposure for 12h and the 2-DG uptake was assessed. (E) Mouse ES cells were pretreated with 2ME2 for 30 min prior to hypoxic exposure for 12h and the western blotting (GLUT-1) was assessed. (F) Mouse ES cells were transfected for 24h with either a SMARTpool of HIF-1 α siRNAs (100 nmol/L) or a non-targeting control siRNA (100 nmol/L) using LipofectAMINE 2000 prior to hypoxic exposure for 12h and the western blotting (GLUT-1) was assessed. The values are reported as the mean $\pm$ S.E. of 4 independent experiments with triplicate dishes. The example shown is representative of 4 experiments. The bands represent 120 KDa of HIF-1 α , 50~60 KDa of GLUT-1 and 41 KDa of β -actin, respectively. The lower panels of A, B, and D depicts the bars showing the mean $\pm$ S.E. of 4 experiments for each condition determined from densitometry relative to β -actin. * P <0.05 vs. Control, ** P <0.05 vs. Hypoxia.

Hypoxia was found to increase the Ca^{2+} uptake in mouse ES cells. An increase in Ca^{2+} uptake resulting from an energy deficiency has been proposed by several authors to be one of the causes for cell mortality and tissue necrosis during ischemia-reperfusion or hypoxia-reoxygenation [30, 31]. On the other hand, if the hypoxia and the incubation time are limited, as in the model studied here, the increase in $[\text{Ca}^{2+}]_i$ is not sufficient to induce cell death but is sufficient to activate the cell [30]. However, the

nature of the transporter responsible for this process was not examined in this study.

The involvement of PKC in the glucose transport stimulation was clearly demonstrated, as shown by the lack of up-regulation of 2-DG uptake in the presence of the PKC inhibitors as well as by the translocation of the isoform from the cytosol to the membrane fraction of the tissue lysate (a decrease in the cytosolic levels of the protein and a corresponding increase in the membrane

Fig. 7. Effect of hypoxia on cell cycle regulatory protein expression. Mouse ES cells were exposed to hypoxia for various times (0 - 48h) and then the total lysates were then subjected to SDS-PAGE and blotted with the cyclin D1, cyclin E, CDK 2, CDK 4, or pRB antibody (A, B, D). (C) Mouse ES cells were immunofluorescence stained with pRB antibody. Scale bars represent 20 μ m (magnification x 400). Each example shown is a representative of 4 experiments.

levels). In agreement with these results, the stimulation of PKC by various phorbol esters has been reported to increase the rate of glucose transport and GLUT4 translocation in a rat heart [32]. Of the PKC isoforms involved in the activation of glucose transport by stimulated hypoxia, the results suggest that the conventional (β 1), the novel (ϵ), and atypical (ζ) PKC isoforms are involved in the increase in GLUT1 activity in mouse ES cells. PKC ϵ plays an important role in the cardiomyocyte physiology [33], particularly in response to ischemic or hypoxic stress, and they reported that it is strongly affected by hypoxia in HL-1 cells [34]. It is not known whether the differences in dependency on the different PKC isoforms reflect the variances in the cell model and/or the injuries used in these experiments. The underlying mechanisms of the PKC regulation of GLUT1 are not known but may involve the phosphorylation of the protein, either by the PKCs or by a kinase regulated by the PKCs. Standard sequence analysis software (e.g. PROSITE) predicts a number of putative kinase target sites within the intracellular loops of GLUT1. Some of these sites are conserved in human GLUT1 and rat GLUT1, which indicates functional relevance.

The link between PKC and p44/42 MAPKs was investigated. p44/42 MAPKs activation appeared to be downstream of PKC. This is because the PKC inhibitors block the phosphorylation of the p44/42 MAPKs during hypoxia. It is also interesting that PKC, which is closely related to the 2-DG uptake in our experimental model, may activate the p44/42 MAPKs, even if this pathway is not the only pathway capable of converging to this sub-family of MAPKs. In contrast, Tong et al. [35] reported that ischemic preconditioning enhanced the glucose uptake by a p38 MAPK-mediated mechanism in Langendorff-perfused rat hearts. Hypoxia increased Ca^{2+} uptake, which is critical molecule to initiate various survival signals. Previous study showed that in hypoxic neurons, moderate increases in $[\text{Ca}^{2+}]_i$ trigger important survival signals, including phosphorylation of the antiapoptotic factor Akt and the p44/42 MAPKs [36]. One possible explanation for this difference might be the different cell types or experimental conditions used. These results demonstrate that HIF-1 α can be directly phosphorylated by p44/42 MAPKs. Activation of the p44/42 MAPKs pathway leads to an increase in the transcriptional activity of the HIF-1 α complex and downstream gene activation.

Fig. 8. Effect of p44/42 MAPKs on hypoxia-induced increase of cell cycle regulatory protein expression and $[^3\text{H}]$ thymidine incorporation. Mouse ES cells were pretreated with PD 98059 (10^{-5} M) for 30 min prior to hypoxic exposure and then western blotting (cyclin D1, cyclin E, CDK 2, and CDK 4) (A, B, C, or D) and $[^3\text{H}]$ thymidine incorporation (E) were performed. The values are represented as a mean $\pm$ S.E. of 4 independent experiments. The lower panels of A, B, C, or D depicts the bars showing mean $\pm$ S.E. of 4 experiments for each condition determined from densitometry relative to β -actin. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. Hypoxia.

This is just one of the most important signal transduction pathways during the hypoxic injury of mouse ES cells. Previous studies have clearly demonstrated that hypoxia-inducible GLUT1 expression is dependent on the binding of a nuclear protein, HIF-1, to the hypoxia-inducible DNA element upstream of the GLUT1 gene [37]. While the specific p44/42 MAPKs phosphorylation sites on HIF-1 α have yet to be determined, a recent study reported that the phosphorylation of threonine 796 enhances the transcriptional response in hypoxia and prevents the hydroxylation of Asn-803 by FIH [38]. Alternatively, [39] demonstrated that p44/42 MAPKs can phosphorylate the p300/CBP co-activator, leading to increased HIF-1 α transcriptional activity.

There is evidence showing that hypoxia enhances the expression of the cell cycle regulator proteins. In fact, hypoxia contributes to the self-renewal of murine and human normal hematopoietic stem cells, whereas if cul-

tured in 20% O_2 , the cells stopped proliferation and differentiated [40, 41]. In this study, the control of the cell cycle regulatory protein expression (cyclin D1, cyclin E, CDK 2, and CDK 4) were dependent on the p44/42 MAPKs pathways. According to the present results of FACS analysis, immunostaining, and RT-PCR for undifferentiated markers, ES cells still expressed these markers highly under our experimental conditions, despite hypoxia-induced extracellular signal-regulated kinase (ERK) activation. Consistent with our results, basic fibroblast growth factor contributes at least in part to the maintenance of human ES cells through the MAPK kinase 1 (MEK1)/ERK pathway [42]. In other reports, ERK1/2 signaling pathway plays an important role in the mitogenic/survival effect on CNS stem cells by PDGF-AA, but not for the initial steps of neuronal differentiation [43]. Moreover, MEK kinase 1 (MEKK1) plays a key role in the self-renewal and signal transducer and

activator of transcription 3 (STAT3) activation in Bcr-Abl-transformed ES cells [44]. These results contrast with other reports showing that ERK signaling stimulates differentiation [45, 46]. Based upon present results, although the contribution of MAPK to ES cells needs to be elucidated in further research, we suggest that MAPK signaling is critical for the regulation of self-renewal and propagation of mouse ES cells under the conditions used in this study. The p44/42 MAPKs have been shown to phosphorylate HIF-1 α and activate transcription of HIF-1 α target genes, which are particularly relevant to proliferation/survival factors, glucose transporters and glycolytic enzymes [47, 48]. Therefore, HIF-1 α contributes to autocrine signaling pathways that are crucial for mouse ES cell proliferation and glucose transport. Indeed, the increased glycolysis is a normal response to proliferation. The intermediary metabolites of the glycolytic pathway provide the precursors for synthesis of glycine, serine, purine, pyrimidine, and phospholipids, all of which are essential for cell growth and maintenance of cells under stress [49]. Therefore, these data provide the initial connection between GLUT1 and cell cycle regulatory proteins under hypoxia and novel insight into the regulation of stem cells. These results suggest that suitable hypoxia

can promote proliferation and modify the cell fate of ES cells *in vitro*, which could be a useful tool for expansion of ES cells for ex vivo cell therapy and for a mechanism study of embryo development. Further investigations will be needed to clarify the molecular mechanisms linked between GLUT1 and proliferation under hypoxia. In conclusion, these results indicate that up-regulation of glucose uptake is strongly related to the stimulation of DNA synthesis induced by hypoxia in mouse embryonic stem cells and that Ca²⁺/PKC, MAPKs, and HIF-1 α are partially involved in the signaling pathways mediating this process.

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