REVIEW

Short-period hypoxia increases mouse embryonic stem cell proliferation through cooperation of arachidonic acid and PI3K/Akt signalling pathways

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Abstract. Hypoxia plays important roles in some early stages of mammalian embryonic development and in various physiological functions. This study examined the effect of arachidonic acid on short-period hypoxia-induced regulation of G₁ phase cell-cycle progression and inter-relationships among possible signalling molecules in mouse embryonic stem cells. Hypoxia increased the level of hypoxia-inducible factor-1 α (HIF-1 α) expression and H₂O₂ generation in a time-dependent manner. In addition, hypoxia increased the levels of cell-cycle regulatory proteins (cyclin D₁, cyclin E, cyclin-dependent kinase 2 (CDK2) and CDK4). Maximum increases in the level of these proteins and retinoblastoma phosphorylation were observed after 12–24 h of exposure to hypoxic conditions, and then decreased. Alternatively, the level of the CDK inhibitors, p21^{Cip1} and p27^{Kip1} were decreased. These results were consistent with the results of [³H]-thymidine incorporation and cell counting. Hypoxia also increased the level of [³H]-arachidonic acid release and inhibition of cPLA₂ reduced hypoxia-induced increase in levels of the cell-cycle regulatory proteins and [³H]-thymidine incorporation. The level of cyclooxygenase-2 (COX-2) was also increased by hypoxia and inhibition of COX-2 decreased the levels of cell-cycle regulatory proteins and [³H]-thymidine incorporation. Indeed, the percentage of cells in S phase, levels of cell cycle regulatory proteins, and [³H]-thymidine incorporation were further increased in hypoxic conditions with arachidonic acid treatment compared to normoxic conditions. Hypoxia-induced Akt and mitogen-activated protein kinase (MAPK) phosphorylation was inhibited by vitamin C (antioxidant, 10⁻³ M). In addition, hypoxia-induced increase of cell-cycle regulatory protein expression and [³H]-thymidine incorporation were attenuated by LY294002 (PI3K inhibitor, 10⁻⁶ M), Akt inhibitor (10⁻⁶ M), rapamycin (mTOR inhibitor, 10⁻⁹ M), PD98059 (p44/42 inhibitor, 10⁻⁵ M), and SB203580 (p38 MAPK inhibitor, 10⁻⁶ M). Furthermore, hypoxia-induced increase of [³H]-arachidonic acid release was blocked by PD98059 or SB203580, but not by LY294002 or Akt inhibitor. In conclusion,

arachidonic acid up-regulates short time-period hypoxia-induced G₁ phase cyclins D₁ and E, and CDK 2 and 4, in mouse embryonic stem cells through the cooperation of PI3K/Akt/mTOR, MAPK and cPLA₂-mediated signal pathways.

INTRODUCTION

Oxygen is a potent signalling molecule that has received increasing recognition for its ability to affect the fundamental characteristics of various types of stem cell (Csete 2005). Oxidative stress is also an important physiological and pathological regulator that might influence a cell's whole lifespan, from embryogenesis and development to the maintenance of normal function, dysfunction and ageing (Agarwal *et al.* 2005; Martin & Grotewiel 2006). Consistent with this, lowering oxygen concentration in the gaseous phase during embryonic cell culture, from atmospheric levels to more physiological levels, has been associated with improved embryonic development, in terms of blastocyst development rate and number of embryonic cells, in several species (Fischer & Bavister 1993). Previous results clearly demonstrate that low-oxygen (2%) culture conditions are indispensable for robust recovery of human embryonic stem cell (ESC) clones and may reduce numbers of chromosomal abnormalities (Forsyth *et al.* 2006). In addition, low oxygen associated with hypoxia has been found to promote survival of human embryonic stem cells (Ezashi *et al.* 2005). In mice, increased blastocyst development rates and/or cell numbers have been reported after culturing in 7% (Gardner & Lane 1996) or 5% oxygen (Umaoka *et al.* 1992; Orsi & Leese 2001) in both F1 hybrid and outbreed strains. Hypoxic conditions also regulate the number of inner cell mass cells in bovine blastocysts (Harvey *et al.* 2004) and enhance haematopoietic progenitor development (O'Neill *et al.* 2005). It is generally assumed that living organisms respond to reduced oxygen concentrations in a cell type- and tissue-specific manner. Production of reactive oxygen species (ROS) is an inevitable consequence of low-oxygen culture. ROS generation at low concentrations in cells may play roles as signalling molecules within signal transduction cascades triggered by growth factors, cytokines and hormones (Prata *et al.* 2004). Oxidative stress of tissue and cells is widely recognized as a key factor in the development of embryos (Pérez-Crespo *et al.* 2005). However, high concentrations of ROS can significantly inhibit cell growth and induce apoptosis (Holbrook & Ikeyama 2002; Juranek & Bezek 2005).

The effects of hypoxia on progression of ESCs through the cell cycle are unknown, even though it has been demonstrated that hypoxia causes an increase in proliferation of ESCs derived from blastocysts (Ezashi *et al.* 2005). A family of cyclins, in complex with partner cyclin-dependent kinases (CDK), co-operatively regulate mammalian cell-cycle progression. Cell-cycle progression and the G₁/S transition are regulated by CDKs, whose activity is inhibited by CDK inhibitors. Expression of cyclin D₁, and CDK4/6 in G₁ of the cell cycle acts as primary sensor of both positive and negative environmental signals (Sherr & Roberts 1999). Alternatively, up-regulation of CDK inhibitors, such as p21^{Cip1/Waf1} and p27^{Kip1}, are commonly responsible for inhibiting G₁ cell-cycle progression, and for a cell's withdrawal from the cycle (Sherr *et al.* 1999). p27^{Kip1} can bind to CDK4 and inhibit catalytic activity of the CDK4/cyclin D₁ enzymes (Vlach *et al.* 1997). In addition, p21^{Cip1/Waf1} interferes with CDK/cyclin complexes and blocks DNA replication (Brown *et al.* 1997). It has been hypothesized that short time-period hypoxia may induce a cell proliferation-signalling pathway and overcome cell death-signalling pathways. Several research groups have proposed that the MAPKs, PI3K/Akt and cPLA₂ pathways are involved in hypoxic signal transduction (Ottino *et al.* 2004; Risbud *et al.* 2005). Release of free

fatty acids, due to activation of phospholipases and lipases, is one of the first pathological events to occur following ischaemic injury and evidence indicates that hydrolysis of membrane phospholipids may be the main source of liberated free fatty acids (Sun *et al.* 2004). PLA₂ (which may be the primary enzyme responsible for phospholipid hydrolysis associated with ischaemic injury) catalyses cleavage of sn-2 fatty acyl ester bonds of glycerophospholipids, resulting in release of free arachidonic acid and lysophospholipids. It is believed that cPLA₂ is important for rapid arachidonic acid release in cells and for modulating a number of intracellular processes, including cell proliferation (Hammamieh *et al.* 2007). However, little or no information exists regarding interaction among the signalling pathways involved in shorter time-period exposure to hypoxia, although exposure to hypoxia for long periods leads to several signalling pathways involved in cell death. In addition, the role of signalling molecules in cell-cycle progression may be cell type and/or stimulus specific. Therefore, further examination of molecular events underlying the proliferation of ESCs are needed to advance our understanding of *in vitro* culture parameters that can maintain their stem cell phenotype and multipotent capabilities during expansion.

In vitro studies using stem cell populations from mammalian blastocysts have provided a powerful tool for examining the effects of hypoxia on cellular functions. Use of stem cells *in vitro* has provided better understanding of the complex molecular pathways that facilitate these events (Han *et al.* 2006; Heo & Han 2006; Lee *et al.* 2006). The survival of ESCs is not only of utmost importance for normal development *in vivo* but also for safe manipulation of cells under stressful conditions. Therefore, this model will be helpful for better understanding the effects of low-oxygen environments on mouse ESCs, particularly relationships between signal molecules under low-oxygen tension. This study has examined the effect of arachidonic acid on hypoxia-induced control of cell-cycle regulatory proteins and inter-relationships among possible signalling molecules in mouse ESCs.

MATERIALS AND METHODS

Materials

Mouse ESCs were obtained from American Type Culture Collection (ES-E14TG2a or ES-R1; Manassas, VA, USA). Experiments were conducted with E14TG2a cells, except in studies indicated. Foetal bovine serum was purchased from Biowhittaker (Walkersville, MD, USA) and PD98059 or ethyleneglycoltetraacetic acid was supplied by Sigma Chemical Company (St. Louis, MO, USA). Anti-cyclin D₁, anti-cyclin E, anti-CDK2 and anti-CDK4 were acquired from Santa Cruz Biotechnology (Delaware, CA, USA). p-p44/42 and p44/42, p-Akt and Akt, p-mTOR, p-p70S6K1 and Oct-4 antibodies were acquired from New England Biolabs (Herts, UK). Goat antirabbit immunoglobulin G (IgG) was purchased from Jackson Immunoresearch (West Grove, PA, USA) and Liquiscint was obtained from National Diagnostics (Parsippany, NY, USA).

ES cell culture

Mouse ESCs were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA) supplemented with 3.7 g/L sodium bicarbonate, 1% penicillin and streptomycin, 1.7 mM L-glutamine, 0.1 mM β-mercaptoethanol, 5 ng/mL mouse leukaemia inhibitory factor (LIF), and 15% foetal bovine serum with or without a feeder layer, and were cultured for 5 days in a standard medium containing LIF. 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 ESCs

The ESCs cultured in 35 mm or 60 mm culture dishes were washed twice with phosphate-buffered saline (PBS). The media was then exchanged with fresh DMEM supplemented with 5% fetal bovine serum but without the LIF. The experiments were performed in a modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) at 37 °C under either normoxic conditions (92.3% air and 5.5% CO₂) or under hypoxic conditions (2.2% O₂, 5.5% CO₂ and 92.3% N₂) at a flow rate of 20 litres/min for 30 min. The chamber was purged with gas, sealed and placed in a conventional incubator at 37 °C.

Alkaline phosphatase staining

Approximately 70% confluent mouse ESCs were washed twice with PBS and were fixed for about 15 min in 4% formaldehyde (in PBS) at room temperature. After washing with PBS, alkaline phosphatase substrate solution (200 µg/mL naphthol AS-MX phosphate, 2% N,N-dimethylformamide, 0.1 M Tris and 1 mg/mL fast red TR salt) was added and after incubation for 10–15 min at room temperature; if stained, the preparation washed with PBS and photography was carried out.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was extracted from mouse ESCs using STAT-60, monophasic solution of phenol and guanidine isothiocyanate from Tel-Test Inc. (Friendwood, TX, USA). Reverse transcription (RT) was conducted with 3 µg RNA using a reverse transcription system kit (AccuPower® RT PreMix, Bioneer, Daejeon, South Korea) with oligo-dT18 primers. Then, 5 µL of RT products were amplified with polymerase chain reaction (PCR) kit (AccuPower® PCR PreMix) followed by: denaturation at 94 °C for 5 min and 30 cycles at 94 °C for 45 s, 58 °C for 30 s and 72 °C for 30 s followed by 5 min extension at 72 °C. Primers for this were 5'-CGTGAGACTTT-GCAGCCTGA-3' (sense), 5'-GGCGATGTAAGTGATCTGCTG-3' (antisense) for Oct-4 (519 bp); 5'-GTGGAATTT GTCCGAGAC-3' (sense), 5'-TGGAGTGGGAGGAGGAGGTAAC-3' (antisense) for SOX-2 (550 bp); and 5'-TCTTACATCGCGCTCATCAC-3' (sense), 5'-TCTTGAC-GAAGC AGTCGTTG-3' (antisense) for FOX-D3 (171 bp). PCR of β-actin was also performed as control for quantity of RNA.

Measurements of intracellular H₂O₂ levels

For detection of intracellular H₂O₂, we used CM-H₂DCFDA (DCF-DA), which acts as an H₂O₂-sensitive fluorophore. Cells plated on the gelatin-coated dishes were incubated in hypoxic conditions. They were then moved to the dark and treated with 10 µM DCF-DA for 30 min at room temperature under normoxia. Cells were imaged by laser confocal microscopy (×400, fluoview 300, Olympus, Tokyo, Japan), fluorescence was excited at 488 nm, and emitted light was observed at 515 nm. To quantify intracellular H₂O₂ levels, cells treated with DCF-DA were rinsed twice with ice-cold PBS, and were scraped. A 100-µL cell suspension was loaded into a 96-well plate and examined by luminometer (Victor3, PerkinElmer, Turku, Finland) with a fluorescent plate reader at excitation and emission wavelengths of 485 and 535 nm, respectively.

Arachidonic acid release

[³H]-Arachidonic acid release experiments were performed using a modification of the method reported by Xing *et al.* (1997). ESC cultures were incubated for 24 h in DMEM containing [³H]-arachidonic acid 0.5 µCi/mL. They were then washed three times with DMEM (pH 7.4) and were incubated at 37 °C, in hypoxic conditions for 0–48 h in incubation medium. At the end of this

period, incubation medium was transferred to ice-cold tubes containing 100 μL of 55 mM ethyleneglycoltetraacetic acid and ethylenediaminetetraacetic acid (final concentration, 5 mM each). Culture medium was then centrifuged at 12 000 g to eliminate cell debris. Level of radioactivity in the supernatant was determined by placing samples in scintillation vials containing scintillation fluid, and radioactivity was measured using a liquid scintillation counter (LS6500, Beckman Instruments, Fullerton, CA, USA). Cells that remained attached to the plate were scraped into 1 mL of 0.1% sodium dodecyl sulfate. Nine hundred microlitres of the resulting cell lysate was used for scintillation counting. The remaining 100 μL was used for protein determination. For each condition, the quantity of [^3H]-arachidonic acid released (determined as described previously) was first standardized with respect to the protein level. This standardized level of [^3H]-arachidonic acid released was compared as a percentage of the total level (%) of [^3H]-arachidonic acid that had been incorporated into the cells at the beginning of the incubation period (or total released radioactivity plus the total cell – associated radioactivity at the end of the stimulation period).

[^3H]-Thymidine incorporation and cell counting

[^3H]-Thymidine incorporation experiments were carried out using the methodology described by Brett *et al.* (1993). Zhang *et al.* (2005) reported that most ESCs could be arrested in the G_0/G_1 phase using a serum deprivation culture, and furthermore, the synchronized ESCs could successfully re-enter a normal cell cycle after re-supplying serum. In this study, cells were cultured in a single well until they reached 50% confluence. They were then washed twice with PBS and were maintained in serum-free DMEM, including all supplements. After 24 h incubation, cells were washed twice with PBS, and were incubated with fresh serum-free DMEM, containing all supplements and agents indicated. 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 1 h at 37 °C. The cells were washed twice with PBS, fixed in 10% trichloroacetic acid at 23 °C for 15 min, and then were washed twice with 5% trichloroacetic acid. Acid-insoluble material was dissolved in 2 N NaOH for 12 h at 23 °C. Aliquots were removed to measure radioactivity using a liquid scintillation counter. All values are reported as means ($\pm$ standard error) of triplicate experiments. Values were converted from absolute counts to a percentage of the control in order to allow a comparison between experiments.

Cells were washed twice with PBS and were trypsinized from culture dishes. The cell suspension was mixed with a 0.4% (w/v) trypan blue solution and the number of living cells was determined using a haemocytometer.

Western blot analysis

Cell homogenates (20 μg protein) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes. After the blots had been washed with TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], membranes were blocked with 5% skimmed milk for 1 h and were incubated with the appropriate primary antibodies at dilutions recommended by the manufacturer. The membrane was then washed, and primary antibodies were detected using goat antirabbit IgG or goat antimouse IgG, conjugated to horseradish peroxidase. Bands were then visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK).

Fluorescence activated cell sorting analysis

Cells were incubated under hypoxic conditions for 12 h. They were then dissociated in trypsin/EDTA, pelleted by centrifugation, and re-suspended at approximately 10^6 cells/mL in PBS

containing 0.1% bovine serum albumin. Cells were then fixed in 70% ice-cold ethanol, followed by incubation in freshly prepared nucleus staining buffer (250 µg/mL propidium iodide and 100 µg/mL RNase) for 30 min at 37 °C. Cell cycle histograms were generated after analysing propidium iodide-stained cells by fluorescence activated cell sorting (Beckman Coulter, Fullerton, CA, USA). At least 10^4 events were recorded for each sample. Samples were analysed using CXP software (Beckman Coulter).

Statistical analysis

Results are expressed as mean $\pm$ standard error. All experiments were analysed by ANOVA. In some procedures, this was followed by comparison of treatment means with control values using the Bonnferroni-Dunn test. A *P*-value of < 0.05 was considered significant.

RESULTS

Effect of hypoxia on cell-cycle regulatory proteins

To confirm undifferentiated states of mouse ESCs used in the present study, alkaline phosphatase activity was measured as a representative marker for lack of differentiation (Fig. 1a). In addition, RT-PCR for Oct-4, SOX-2, FOX-D3 and Western blot analysis for Oct-4 were conducted to validate non-differentiated states of ESCs (Fig. 1b,c). These results suggested that mouse ESCs maintained an undifferentiated state under the experimental conditions used here.

Figure 1. Effect of hypoxia or arachidonic acid on characterization of mouse embryonic stem cells. Cells were treated with arachidonic acid (AA) (10^{-7} M) for 30 min prior to being exposed to hypoxia for 48 h. (a) Alkaline phosphatase enzyme activity was measured as a representative marker of undifferentiated cells, as described in the Materials and Methods section. (b) Oct-4 (519 bp), SOX-2 (550 bp), FOX-D3 (171 bp) and β-actin (350 bp) mRNA expression level under the normoxic, hypoxic, AA alone, or hypoxia treated with AA. (c) Oct-4 protein expression level under normoxic, hypoxic, AA alone, or hypoxia and treated with AA conditions. The example shown is a representative of five independent experiments. Bands represent 50–60 kDa of Oct-4 and 41 kDa of β-actin, respectively.

Figure 2. Effect of hypoxia on HIF-1 α expression and reactive oxygen species (ROS) generation. (a) Cells were exposed to hypoxia for 0–360 min and HIF-1 α was detected by Western blot as described in the Materials and Methods section. Each example shown is a representative of four experiments. (b) Cellular level of H₂O₂ was measured after the mouse embryonic stem cells were incubated under hypoxia for 0–30 min. Values represent means $\pm$ SE of three independent experiments with triplicate dishes. * P < 0.05 versus control. (c) Cells were incubated under hypoxia for 0–30 min and then dichlorofluorescein (DCF)-sensitive cellular ROS was observed by confocal microscopy. The example shown is a representative of three independent experiments.

To ensure that hypoxia used in this study induces typical cell responses to hypoxic stress, the HIF-1 α expression level and ROS generation were first observed. As shown in Fig. 2a–c, the significant increase in HIF-1 α and ROS appeared from 5 min of hypoxic exposure. However, hypoxia did not affect cell viability and lactate dehydrogenase release (which is a marker of structural damage) under experimental conditions used (data not shown). In experiments to determine whether hypoxia increased levels of cell-cycle regulatory proteins (cyclin D₁, cyclin E, CDK2 and CDK4), maximum increase in level of these proteins and retinoblastoma phosphorylation was observed after 12–24 h of exposure to hypoxic conditions, and then it decreased (Fig. 3a). On the other hand, levels of CDK inhibitors, p21^{Cip1} and p27^{Kip1} decreased after hypoxic exposure. Effect of hypoxia on the level of [³H]-thymidine incorporation was examined by incubating the mouse ESCs under the same hypoxic conditions. As shown in Fig. 3b, maximum increase in the level of [³H]-thymidine incorporation was observed after 12 h of exposure to hypoxic conditions (60% increase versus control; * P < 0.05); this is consistent with the increase of cyclins and CDKs. Subsequently, there was a significant increase in number of cells after 12 h incubation of hypoxia (Fig. 3c). To examine whether the effect of hypoxia would be present in other mouse ESCs, the ES-R1 cell line was used. As shown in Fig. 3d–f, these results are consistent with those of E14TG2a.

Effect of arachidonic acid on hypoxia-induced increase of cell-cycle regulatory proteins and [³H]-thymidine incorporation

Relationships between hypoxia-induced arachidonic acid, cell-cycle regulatory proteins and [³H]-thymidine incorporation was examined. Hypoxia increased the level of [³H]-arachidonic

Figure 3. Effect of hypoxia on the expression level of the cell-cycle regulatory proteins. (a) The mouse ES-E14TG2a cells were exposed to hypoxia for various times (0–48 h) and total proteins were then subjected to SDS-PAGE followed by blotting with the CDK2, cyclin E, CDK4, cyclin D₁, p-RB, p21 and p27 antibodies. Each example shown is a representative of four experiments. (b) Mouse embryonic stem cells were exposed to hypoxia for 0–48 h and pulsed with 1 μ Ci of [³H]-thymidine for 1 h prior to counting. (c) The cells were exposed to hypoxia for 0–48 h and then were counted using a haemocytometer. Values are reported as a mean $\pm$ SE of three independent experiments with triplicate dishes. **P* < 0.05 versus control. (d) The mouse ES-R1 cells were exposed to hypoxia for various times (0–48 h) and total proteins were then subjected to SDS-PAGE then blotting with the CDK2, cyclin E, CDK4, cyclin D₁, p-RB, p21 and p27 antibodies. Each example shown is a representative of four experiments. (e) The cells were exposed to hypoxia for 0–48 h and were pulsed with 1 μ Ci of [³H]-thymidine for 1 h prior to counting. (f) The cells were exposed to hypoxia for 0–48 h and then were counted using a haemocytometer. The values are reported as a mean $\pm$ SE of three independent experiments with triplicate dishes. **P* < 0.05 versus control.

acid releases in a time-dependent manner (Fig. 4a). As shown in Fig. 4b, inhibition of cPLA₂ reduced hypoxia-induced increase in levels of cell-cycle regulatory proteins. This was consistent with the result of [³H]-thymidine incorporation (Fig. 4c). As shown in Fig. 4d, hypoxia increased expression of cyclooxygenase-2 (COX-2) in a time-dependent manner (0–24 h). However, hypoxia had no effect on COX-1. Furthermore, hypoxia-induced increase of COX-2 expression level was inhibited by AACOCF₃ or mepacrine (phospholipase A₂ inhibitors) (10⁻⁵ M) (Fig. 4e). In addition, indomethacin (a COX inhibitor, 10⁻⁷ M) inhibited the hypoxia-induced increase in cell-cycle regulatory protein levels (Fig. 4f). These results were consistent with those for [³H]-thymidine incorporation (Fig. 4g). Indeed, hypoxia increased the percentage of cell population in S phase to 51.6% compared to the control level (24.5%). In addition, arachidonic acid increased cell population in S phase to 67.5%. The percentage of cells in S phase was further increased with hypoxia and arachidonic acid (78.2%) (Fig. 5a). In experiments to determine whether arachidonic acid had an influence in hypoxia-induced increase of cell-cycle regulatory proteins (cyclin D₁, cyclin E, CDK2, CDK4 and phosphor retinoblastoma proteins) and [³H]-thymidine incorporation, additive increase in levels of cell-cycle regulatory proteins and [³H]-thymidine incorporation was observed when arachidonic acid treatment and hypoxia were present concurrently (Fig. 5b,c).

Interrelationship between arachidonic acid and PI3K/Akt or MAPKs in hypoxia-induced regulation of cell-cycle regulatory proteins

Hypoxia induced phosphorylation of Akt as well as mTOR and p70S6K1, which was inhibited by vitamin C (antioxidant, 10⁻³ M), LY294002 (PI3K inhibitor, 10⁻⁶ M), and Akt inhibitor (10⁻⁶ M) (Fig. 6a,b). Levels of hypoxia-induced cell-cycle regulatory proteins were also inhibited by PI3K or the Akt inhibitor (Fig. 6c). This result is consistent with that of [³H]-thymidine incorporation (Fig. 6d). In addition, as shown in Fig. 7a, hypoxia induced phosphorylation of p38 MAPK or p44/42 MAPKs, which was inhibited by vitamin C (antioxidant, 10⁻³ M). Moreover, PD98059 (p44/42 MAPKs inhibitor, 10⁻⁵ M) or SB203580 (p38 MAPK inhibitor, 10⁻⁶ M) inhibited hypoxia-induced increase in cyclin D₁, cyclin E, CDK2 and CDK4 (Fig. 7b). Hypoxia-induced [³H]-thymidine incorporation was also blocked by inhibiting p44/42 MAPKs and the p38 MAPK pathways (Fig. 7c). However, hypoxia-induced Akt or mTOR phosphorylation was not influenced by p44/42 MAPKs nor p38 MAPK inhibitor (Fig. 8a) and p44/42 MAPKs or p38 MAPK activation was not affected by inhibition of PI3K/Akt (Fig. 8b). Inhibition of p38 MAPK or p44/42 MAPKs blocked hypoxia-induced increase in the level of [³H]-arachidonic acid release (Fig. 8d), whereas increase in the level of [³H]-arachidonic acid release was not affected by inhibition of PI3K/Akt (Fig. 8c). Moreover, hypoxia-induced activation of COX-2 was unaffected by the PD98059, but SB203580 inhibited hypoxia-induced increase in COX-2 expression (Fig. 8e).

DISCUSSION

This study has demonstrated that arachidonic acid up-regulates short time-period hypoxia-induced G₁ phase cyclins D₁ and E, and CDK 2 and 4 through cooperation of the PI3K/Akt/mTOR/p70S6K1 and MAPKs/cPLA₂-mediated signalling pathways in mouse ESCs. There is evidence to suggest that D-type cyclins play an important role in cell-cycle progression. Over-expression of cyclin D₁ in rat fibroblasts causes more rapid entry into the S phase (Jiang *et al.* 1993; Quelle *et al.* 1993). In addition, cyclin E works as a cofactor for CDK2 in late G₁ phase (Koff *et al.* 1991). The cyclin-CDK complex plays a critical role in cell-cycle regulation by

Figure 4. Effect of hypoxia-induced arachidonic acid (AA) release on cell-cycle progression. (a) Cells were incubated with $[^3\text{H}]\text{-AA}$ (0.5 $\mu\text{Ci}/\text{mL}$) for 24 h and then exposed to hypoxia for various times (0–48 h) prior to counting. Values are represented as a mean $\pm$ SE of three independent experiments with triplicate dishes. $*P < 0.05$ versus control. (b) Cells were pre-treated with mepacrine or AACOCF₃ (phospholipase A₂ inhibitor (10^{-5} M)) for 30 min prior to hypoxic exposure for 12 h. Total lysates were then subjected to SDS-PAGE and blotting with the CDK2, cyclin E or CDK4, cyclin D₁, antibodies. (c) Cells were pre-treated with AACOCF₃ or with mepacrine for 30 min prior to hypoxic exposure for 12 h and then with 1 μCi of $[^3\text{H}]\text{-thymidine}$ for 1 h prior to counting. Values are represented as a mean $\pm$ SE of four independent experiments. $*P < 0.05$ versus control, $**P < 0.05$ versus hypoxia alone. (d) Cells were exposed to hypoxia for 0–24 h and expression of COX-1 and -2 were detected as described in the Materials and Methods section. (e) Cells were pre-treated with AACOCF₃ or mepacrine for 30 min prior to hypoxic exposure for 2 h. Total lysates were then subjected to SDS-PAGE and blotting with COX-2 antibody. Bars in the lower panels of the figure indicate mean $\pm$ SE of four experiments for each condition determined from densitometry relative to β -actin. $*P < 0.05$ versus control, $**P < 0.05$ versus hypoxia alone. (f) The cells were pre-treated with indomethacin (COX inhibitor, 10^{-7} M) for 30 min prior to hypoxic exposure for 12 h. Total lysates were then subjected to SDS-PAGE and blotting with the CDK2, cyclin E or CDK4, cyclin D₁ antibodies. (g) The cells were pre-treated with indomethacin for 30 min prior to hypoxic exposure for 12 h and then were incubated with 1 μCi of $[^3\text{H}]\text{-thymidine}$ for 1 h prior to counting. Values are represented as a mean $\pm$ SE of four independent experiments. $*P < 0.05$ versus control, $**P < 0.05$ versus hypoxia alone.

Figure 5. Effect of hypoxia-induced arachidonic acid on DNA synthesis. The cells were treated with arachidonic acid (AA) (10^{-7} M) for 30 min prior to being exposed to hypoxia for 12 h. (a) They were then washed with PBS, fixed, stained and analysed by flow cytometry. Gates were configured manually to determine the percentage of cells at G₁, S and G₂/M phases based on DNA content. The percentage of cells at the G₀/G₁, S and G₂/M phases was obtained from three independent experiments. (b) Cells were treated with arachidonic acid for 30 min prior to being exposed to hypoxia for 12 h. Total lysates were then subjected to SDS-PAGE and blotting with the CDK2, cyclin E or CDK4, cyclin D₁ and p-RB antibodies. Each example shown is a representative of four experiments. (c) Cells were treated with arachidonic acid for 30 min prior to hypoxic incubation for 0–48 h, and then were pulsed with 1 μ Ci of [³H]-thymidine for 1 h prior to counting (* P < 0.05 versus control). Values are represented as a mean $\pm$ SE of three independent experiments with triplicate dishes. * P < 0.05 versus control, ** P < 0.05 versus hypoxia alone.

controlling phase transition of cell-cycle progression, in which cyclin is the regulatory unit and CDK is the catalytic component of the complex (Tanner *et al.* 2000; Sandal 2002). In the present study, short time-period hypoxic exposure of ESCs significantly increased cyclin D₁, cyclin E, CDK4 and CDK2 expression. These results indicate that CDK expression and cyclin expression is also strikingly affected by hypoxia (Lee *et al.* 2007).

It has previously been reported that in many tissues, hypoxia leads to an increase in release of fatty acids, including arachidonic acid (Michiels *et al.* 2002; Lambert *et al.* 2006). The

Figure 6. Involvement of PI3K/Akt and mTOR/p70S6K1 on hypoxia-induced cell-cycle progression. (a) Cells were pre-treated with vitamin C (antioxidant, 10^{-3} M) for 30 min prior to 2 h of hypoxia exposure and were analysed by Western blot (p-Akt 308, 473). (b) They were pre-treated with LY294002 (PI3K inhibitor, 10^{-7} M), or Akt inhibitor (10^{-7} M) for 30 min prior to 2 h of hypoxic exposure and analysed by Western blot (p-Akt, p-mTOR, p-p70S6K1). (c) Cells were pre-treated with LY294002, Akt inhibitor, and rapamycin for 30 min prior to hypoxic exposure for 12 h and total lysates were subjected to SDS-PAGE and blotting with the CDK2, cyclin E or CDK4, cyclin D₁ antibodies. Each example shown is a representative of four experiments. (d) Cells were pre-treated with LY294002 or Akt inhibitor for 30 min prior to hypoxic exposure for 12 h and then with 1 μ Ci of [³H]-thymidine for 1 h prior to counting. Values are represented as a mean $\pm$ SE of four independent experiments. * $P < 0.05$ versus control, ** $P < 0.05$ versus hypoxia alone.

present study has also demonstrated that hypoxia promotes release of arachidonic acid, by mouse ESCs from hypoxic conditions. Exposing mouse ESCs to hypoxia did not alter expression of COX-1, but induced expression of COX-2. Induction of COX-2 by hypoxia was also partly dependent on activation of p38 MAPK, which is consistent with previous results (Cui *et al.* 2006; Panel *et al.* 2006). Mitogenic effects derived from arachidonic acid release and COX-2 induction are linked primarily to excessive synthesis of PGE₂ (Saed *et al.* 2005). In the previous study, arachidonic acid and PGE₂ have been shown to amplify PGE₂ production by inducing COX-2 expression (Bagga *et al.* 2003; Yang *et al.* 2004). Thus, arachidonic acid and PGE₂-dependent amplification of COX-2 are hypothesized to be important parts of sustained proliferative conditions in mouse ESCs. Overall, these results suggest that hypoxic induction of arachidonic acid release and COX-2 plays a role in cell-cycle progression of mouse ESCs.

Numerous reports indicate that the PI3K/Akt pathway is involved in regulation of HIF-1 α expression and is known to play an important role in cell population growth and survival (Dugourd *et al.* 2003). In this study, the level of Akt phosphorylation was increased after exposing

Figure 7. Involvement of MAPKs on hypoxia-induced cell-cycle progression. (a) Cells were pre-treated with vitamin C (10^{-3} M) for 30 min prior to 90 min hypoxia exposure and were analysed by Western blot (p-p38 MAPK, p-p44/42 MAPKs). (b) Cells were pre-treated with PD98059 (p44/42 MAPKs inhibitor, 10^{-5} M), SB203580 (p38 MAPK inhibitor, 10^{-6} M) for 30 min prior to hypoxic exposure for 12 h. Total lysates were then subjected to SDS-PAGE and blotting with CDK2, cyclin E or CDK4, cyclin D₁ antibodies. (c) Cells were pre-treated with PD98059 or SB203580 for 30 min prior to hypoxic exposure for 12 h and then were incubated with 1 μ Ci of [³H]-thymidine for 1 h prior to counting. Values are represented as a mean $\pm$ SE of four independent experiments. * P < 0.05 versus control, ** P < 0.05 versus hypoxia alone.

mouse ESCs to hypoxia, which is consistent with the stimulatory effect of hypoxia on PI3K activity. These results also show that inhibition of PI3K activity using LY294002 decreased the level of DNA synthesis. These data are consistent with previous studies, suggesting that PI3K is essential for cell proliferation in different cell types, such as neutrophils, endothelial cells and breast cancer cells (Kanda *et al.* 1997; Kanayasu-Toyoda *et al.* 2002; Gulati *et al.* 2006). Moreover, cyclin D₁ expression is controlled post-transcriptionally through the PI3K/Akt-dependent pathway (Muisse-Helmericks *et al.* 1998). The underlying mechanisms through which Akt may be involved in cell proliferation include Akt-dependent phosphorylation, inactivation and subcellular redistribution of p21 and p27 proteins, which inhibit cell-cycle progression, as well as the GSK-3-mediated accumulation of cyclin D₁. mTOR has also been shown to play a key role in regulating cell population growth, homeostasis and cell proliferation (Humar *et al.* 2002; Pervin *et al.* 2007). These results demonstrate that mTOR is a critical target of hypoxia, and is important in mediating hypoxia-induced proliferative responses in mouse ESCs. It was found that hypoxia significantly increased phosphorylation of mTOR and its downstream target,

Figure 8. Relationships between signalling molecules activated by hypoxia. (a) The cells were pre-treated with PD98059 or SB203580 for 30 min prior to hypoxic exposure for 2 h. Total lysates were then subjected to SDS-PAGE and blotting with p-Akt and p-mTOR antibodies. (b) Cells were pre-treated with LY294002 or Akt inhibitor for 30 min prior to hypoxic exposure for 90 min. Total lysates were then subjected to SDS-PAGE and blotting with p-p38 or p-p44/42 MAPK antibodies. Cells were incubated with [3 H]-AA for 24 h, washed three times with DMEM medium and then pre-treated with LY294002 or Akt inhibitor (c) and PD98059 or SB203580 (d) for 30 min prior to incubation under hypoxic conditions for 12 h prior to counting. Values are represented as mean $\pm$ SE of three independent experiments with triplicate dishes. * P < 0.05 versus control, ** P < 0.05 versus hypoxia alone. (e) Cells were pre-treated with PD98059 or SB203580 for 30 min prior to hypoxic exposure for 2 h. Total lysates were then subjected to SDS-PAGE and blotting with COX-2 antibody. Bars in the lower panels show the means $\pm$ SE of four experiments for each condition, determined from densitometry relative to β -actin. * P < 0.05 versus control, ** P < 0.05 versus hypoxia alone.

p70S6K1. Therefore, hypoxia directly activates PI3K signalling in these cells, and it ultimately leads to mTOR activation.

There is evidence to show that the significant role played by members of the MAPK families in the effects of hypoxia. Modulation of MAPK signalling by hypoxia varies according to cell type and acute or chronic exposure (Haddad 2004). It was previously reported that exposure to hypoxia results in early and transient activation of the different signalling pathways, including MAPK and PI3K/Akt, in a different cell contexts (Kwon *et al.* 2006). Consistent with these observations, we also observed a transient and early increase in phospho-p44/42 MAPKs and p38 MAPK, or phospho-Akt levels, under hypoxic conditions. In addition, pre-treatment with PI3K-specific inhibitors and MEK1/2-specific inhibitor significantly inhibited hypoxia-dependent increase in accumulation of cell-cycle regulatory proteins. In this regard, it should also be noted that hypoxia induces PI3K-independent activation of ERK1/2 or arachidonic acid release to support the proliferative response. Similar interactions between PI3K and ERK1/2 pathways have been reported in vascular cells and in hepatocytes in response to other mitogenic stimuli (Wilden *et al.* 1998; Coutant *et al.* 2002). However, intriguing questions remain concerning exactly how hypoxia initiates activation of individual signalling molecules in cells capable of

Figure 9. Hypothesized model for signalling pathways involved short-time hypoxia-induced proliferation of mouse embryonic stem cells. Hypoxia increases intracellular reactive oxygen species, which both activates p38 MAPK, which stimulates cPLA₂ to induce arachidonic acid (AA) release, and activates Akt, mTOR and p70S6K1. These increased cell-cycle regulatory proteins, cyclin D/E and CDK2/4, which may induce mouse embryonic stem cell proliferation.

proliferation, under conditions of short time-period hypoxia. An essential role for ROS as mediators of stimulation of arachidonic acid release or PI3K has been proposed for various cell types, which can be regulated through G proteins (Schumacker 2003). It will be of interest to test the possibility that G proteins can be activated in response to hypoxia in a ligand- and/or receptor-independent manner (Das *et al.* 2001). To our knowledge, this study is the first to elucidate an interrelationship between PI3K/Akt, MAPKs and cPLA₂ signalling molecules and expression of cell-cycle regulatory proteins by short time-period hypoxia in mouse ESCs (Fig. 9). Here, it was hypothesized that low-temporal hypoxic conditions would be beneficial to mouse embryonic stem cell proliferation, and maintenance of an undifferentiated phenotype, during monolayer culture. However, further studies will be needed to provide insight into the considerable influence of oxygen concentration on embryonic stem cell proliferation and to determine whether other signalling pathways would also be involved in hypoxia-induced DNA synthesis. In conclusion, arachidonic acid up-regulates short time-period hypoxia-induced G₁ phase cyclins D₁ and E, and CDK and 4 in mouse embryonic stem cells, through the co-operation of the PI3K/Akt/mTOR/p70S6K1 and MAPK/cPLA₂-mediated signal pathways.

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