High-Glucose-Induced Prostaglandin E2 and Peroxisome Proliferator-Activated Receptor δ Promote Mouse Embryonic Stem Cell Proliferation

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Abstract

Peroxisome proliferator-activated receptor is a nuclear receptor that has been implicated in blastocyst implantation, cell cycle, and pathogenesis of diabetes. However, the signal cascades underlying this effect are largely unknown in embryonic stem cells. This study examined whether or not there is an association between the reactive oxygen species-mediated prostaglandin E2 (PGE2)/peroxisome proliferator-activated receptor (PPAR) δ and the growth response to high glucose levels in mouse ESCs. A high concentration of glucose (25 mM) significantly increased the level of [3H]thymidine incorporation, the level of 5-bromo-2′-deoxyuridine incorporation, and the number of cells. Moreover, 25 mM glucose increased the intracellular reactive oxygen species, phosphorylation of the cytosolic phospholipase A2 (cPLA2), and the release of [3H]arachidonic acid ([3H]AA). In addition, 25 mM glucose also increased the level of cyclooxygenase-2 (COX-2) protein expression, which stimulated the synthesis of PGE2. Subsequently, high glucose-induced PGE2 stimulated PPARδ expression directly or through Akt phosphorylation indirectly through the ε type prostaglandin receptor receptors. The PPARδ antagonist inhibited the 25 mM glucose-induced DNA synthesis. Moreover, transfection with a pool of PPARδ-specific small interfering RNA inhibited the 25 mM glucose-induced DNA synthesis and G1/S phase progression. Twenty-five millimolar glucose also increased the level of the cell cycle regulatory proteins (cyclin E/cyclin-dependent kinase [CDK] 2 and cyclin D1/CDK 4) and decreased the phosphorylation of the cytosolic phospholipase A2, COX-2, and PPARδ pathways. In conclusion, high glucose promotes mouse ESC growth in part through the cPLA2-mediated PGE2 synthesis and in part through PPARδ pathways.

Disclosure of potential conflicts of interest is found at the end of this article.

Introduction

Glucose provides more than just a source of combustible energy. It also produces a wide variety of cellular signals. In this way, glucose provides a mechanism through which the broadly defined outside “environment” can communicate directly with the specific cell/tissues in response to either glucose or excess glucose. These cellular responses to glucose uptake can also be a source of embryopathy, including the relationship between glucose uptake and diabetes [1]. There are wider implications regarding the effect of glucose concentration on the differentiation of ESCs into other cell types, because early human and mouse embryo development in vitro has been shown to be enhanced in a medium lacking glucose [2, 3]. However, the protocols for examining ESC differentiation mainly used media containing high glucose concentrations [4]; their use is presumably based on the use of high-glucose media for maintaining the ESCs. Therefore, there is some debate as to the necessity of using media with a high glucose level for an ESC culture.

Recent work has established peroxisome proliferator-activated receptors (PPARs) as one mechanism through which glucose-driven transcriptional regulation can occur [5]. Three PPAR isotypes, PPARα, PPARβ/δ, and PPARγ, have been identified [6]. PPARα is expressed mainly in the liver, heart, kidney, brown adipose tissue, and stomach mucosa [7, 8]. PPARβ is found primarily in the adipose tissue [9]; PPARβ/δ is the most ubiquitously expressed, even though its physiological and pathophysiological roles are less clear, particularly in human tissue [10]. PPARδ is the only PPAR isoform expressed during rat early embryo organogenesis [11]. In addition, PPARδ has been linked to the proliferation of colon cancer, preadipocyte proliferation, and embryo implantation. Therefore, we hypothesize that PPARδ plays an important role in the proliferation of ESCs. A previous study demonstrated that both prostaglandin E2 (PGE2) and arachidonic acid (AA) supplementation show in vivo and in vitro protection against diabetic malformations [12]. Moreover, in various cell types, PGE2 production was increased, whereas it was decreased by cyclooxygenase-2 (COX-2) inhibitor, when cells were exposed to high concentration of glucose [13]. Moreover, the high-glucose-induced increase in PGE2 production requires activation of protein kinase C (PKC) and mitogen-activated protein kinase pathways [14], as well as PKC-induced phospholipase A2 (PLA2) activation and the release of arachidonic acid [15]. Although several studies have shown that PPARδ plays an important role in the lipid metabolism and that specific agonists might be effective in improving the metabolic syndrome, the

physiological implications of the endogenous AA metabolism in the activation of PPAR in ESCs and the functions of PPAR6 in mouse ESC proliferation exposed to high glucose levels are largely unknown.

ESCs are pluripotent cell lines that are derived from the blastocyst stage of early mammalian embryos. These unique cells are characterized by their capacity for prolonged undifferentiated proliferation in tissue culture, as well as by their ability to maintain their potential to differentiate into derivatives of all three germ layers [16]. Therefore, mouse ESCs might be regarded as a versatile biological system, and their use has led to major advances in cell and developmental biology. The growth of mouse ESCs in culture is likely to require the increased uptake of glucose and other substrates [1]. Therefore, mouse ESCs might be useful for examining the effect of high glucose levels on the proliferation of the inner cell mass in early embryos. This study was designed to determine whether there is an association between the PGE2/PPAR6 mediated by reactive oxygen species and the growth response to high glucose in mouse ESCs.

**Materials and Methods**

**Materials**

The mouse ESC line was obtained from American Type Culture Collection (Manassas, VA, http://www.atcc.org) (ES-E14TG2a). Fetal bovine serum was purchased from BioWhittaker (Walkersville, MD, http://www.cambrex.com). The D-glucose, manniitol, LY294002, N-acetylcysteine (NAC), ascorbic acid, arachidonyltrifluoro-methyl ketone, mecaprine, indomethacin, PGE2, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM, and β-actin were obtained from Sigma-Aldrich (St. Louis, http://www.sigmaaldrich.com). The Akt inhibitor GW9662 and L-165041 were purchased from Calbiochem (La Jolla, CA, http://www.emdbiosciences.com). [3H]Thymidine was purchased from NEN (Cambrex). Fluoro 3-AM was supplied by Molecular Probes (Eugene, OR, http://probes.invitrogen.com). The phospho-cytosolic phospholipase A2 (phospho-cPLA2), total cPLA2, phospho-Akt (Thr406, Ser473), total Akt, PPAR6, cyclin D1, cyclin E, cyclin-dependent kinase (CDK) 2, and CDK 4 antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, http://www.scbt.com). Goat anti-rabbit IgG was acquired from Jackson Immunoresearch Laboratories (West Grove, PA, http://www.j immortal.com). Liquiscent was obtained from National Diagnostics (Parsippany, NJ, https://www.nationaldiagnostics.com). All other reagents were of the highest purity commercially available.

**ESC Culture**

The mouse ESCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-BRL, Gaithersburg, MD, http://www.gibcobrl.com) 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 leukemia inhibitory factor (LIF), and 15% fetal bovine serum (FBS) without a feeder layer and cultured for 15 days in standard medium plus LIF. The cells were grown on gelatinized 12-well plates or a 60-mm culture dish in an incubator maintained at 37°C in an atmosphere of 5% CO2 in air. The medium was changed to serum-free DMEM with LIF before experiments. After that, the cells were washed twice with phosphate-buffered saline (PBS) and then maintained in a serum-free medium (5 mM glucose) including all supplements and indicated agents.

**Alkaline Phosphatase Staining**

Approximately 70% confluent mouse ESCs were washed twice with PBS and fixed with 4% formaldehyde (in PBS) for approximately 15 minutes at room temperature. The cells were washed with PBS and incubated using an alkaline phosphatase substrate solution (200 μg/ml naphthol AS-MX phosphate [3-Hydroxy-2-naphthoic Acid 2, 4-Dimethyl-anilide Phosphate], 2% N,N-dimethylformamide, 0.1 M Tris [pH 8.2], and 1 mg/ml Fast Red TR salt [4-chloro-2-methylbenzenediazoyium salt; zinc chloride]) for 10 minutes at room temperature. After being washed with PBS, the cells were photographed.

**Immunofluorescence Staining with SSEA-1**

The cells were fixed and treated with the monoclonal antibody against mouse SSEA-1 (1:50; Santa Cruz Biotechnology) and incubated for 30 minutes with the FITC-conjugated secondary antibody (raised in rabbit against mouse IgG (1:100). The fluorescence images were visualized using a fluorescence microscope (Fluoview 300; Olympus, Tokyo, http://www.olympus-global.com).

**[3H]Thymidine Incorporation**

The [3H]thymidine incorporation experiments were carried out using the methodology reported by Brett et al. [17], Zhang et al. [18] reported that most ESCs could be arrested in the G0/G1 phase using a serum deprivation culture. Furthermore, the synchronized ESCs could successfully reenter a normal cell cycle after being resupplied with the serum. The cells were washed twice with PBS and incubated with fresh serum-free DMEM (5 mM glucose) 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, Little Chalfont, U.K., http://www.amersham.com) was added to the cultures. The incubation with [3H]thymidine was continued for an additional 1 hour at 37°C. The cells were then washed twice with PBS, fixed in 10% trichloroacetic acid (TCA) at 23°C for 15 minutes, and then washed twice with 5% TCA. The acid-insoluble material was dissolved in 0.2 N NaOH for 12 hours at 23°C. Aliquots were removed to determine the level of radioactivity using a liquid scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA, http://www beckman.com). All values are reported as the mean (± SE) of triplicate experiments. The values were converted from absolute counts to a percentage of the control to allow for a comparison between experiments.

**5-Bromo-2′-Deoxyuridine Incorporation**

The level of 5-bromo-2′-deoxyuridine (BrdU) (a thymidine analog) incorporation was measured to determine the level of DNA synthesis. The ESCs were serum-starved for 24 hours before being stimulated with 25 mM glucose. The ESCs were then treated with 25 mM glucose for 12 hours. Fifteen micromolar BrdU was added during the final 16 hours of incubation. After several washes with PBS, the cells were fixed with methanol (10% [vol/vol] for 10 minutes at 4°C), followed by incubation in 1 N HCl for 30 minutes at room temperature. The cells were washed and then incubated for 15 minutes with 0.1 M sodium tetraborate, Alexa Fluor 488-conjugated mouse anti-BrdU monoclonal antibody (mAb) (diluted 1:200; Molecular Probes) in 2% bovine serum albumin (BSA)-PBS was incubated overnight at 4°C. After being washed in PBS, coverslips were mounted onto glass slides with a Dako Fluorescent mounting medium (Dako, Glostrup, Denmark, http://www.dako.com) using gelvatol and examined by optical microscopy (Fluoview 300; Olympus). The mean ± SE number of BrdU-positive cells per field of vision was determined. Ten fields of vision per coverslip were counted.

For the double-labeling experiments, the cells were fixed in acidified alcohol and processed for Oct-4 staining, which was followed by BrdU staining. The fixed cells were incubated with the rabbit anti-Oct-4 antibody (1:100; Santa Cruz Biotechnology) for 1 hour at room temperature and Alexa Fluor 555 anti-rabbit IgG (1:100; Molecular Probes) for 1 hour at room temperature. This was followed by incubation in 1 N HCl and neutralization with 0.1 M sodium tetraborate and then with Alexa Fluor 488-conjugated mouse anti-BrdU mAb for 1 hour at room temperature. After being washed with PBS, the BrdU/Oct-4-stained cells were examined by confocal microscopy (Fluoview 300; Olympus).

**Cell Proliferation Assay**

To determine the number of cells, the cells were washed twice with PBS and trypsinized from the culture dishes. The cell suspension was mixed with a 0.4% (w/v) trypan blue solution, and the

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number of live cells was determined using a hemocytometer. Cells failing to exclude the dye were considered nonviable.

$H_2O_2$ Release
The $H_2O_2$ levels were determined using a modification of the method reported by Zhou et al. [19]. The cells were washed twice with ice-cold PBS, harvested by microcentrifugation, and resuspended in Krebs-Ringer phosphate solution (KRP) (145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl$_2$, 1.22 mM MgSO$_4$, 5.5 mM glucose; [pH 7.35]). One hundred microliters of the reaction mixture (50 $\mu$l Amplex Red reagent containing 0.1 U/ml horseradish peroxidase in KRP) was added to each microplate well that had been prewarmed to 37°C for 10 minutes. The reaction was started by adding 20 $\mu$l of KRP to the resuspended cells. The fluorescence readings became stable within 30 minutes of starting the reaction, and the absorbance at $\lambda$ 560 nm was then measured using a fluorescence microplate reader (Multi-skran; Thermo Labsystems Inc., Franklin, MA, http://www.thermo- com).

Measurement of Lipid Peroxides
The lipid peroxide (LPO) levels in the mouse ESCs were determined by measuring the malondialdehyde content using the method reported by Ohkawa et al. [20]. One hundred microliters of the sonicated cells was mixed with 100 $\mu$l of an 8% sodium dodecyl sulfate (SDS) solution, 200 $\mu$l of a 0.8% 2-thiobarbituric acid solution, and 200 $\mu$l of a 20% acetic acid solution. The mixture was heated to 95°C for 60 minutes. After incubation, the mixture was cooled in ice-cold water. The nonspecific red pigment was extracted by adding 1 ml of an n-butanol-pyridine mixture (15:1 [vol/vol]) and centrifuging the sample at 1,550g for 10 minutes. The organic supernatant was measured by spectrophotometry at emission and excitation wavelengths of 553 and 515 nm, respectively. 1,1,3,3-Tetraethoxypropane was used as the standard, and the LPO values of the samples are expressed as nmol/mg protein.

Assay of Cellular Reactive Oxygen Species
The intracellular production of reactive oxygen species (ROS) was measured using confocal microscopy according to the method reported by Lee et al. [21]. Generation of ROS was assessed using the fluorescence indicator 5-(and-6)-chloromethyl-2,7-dichlorodihydro-fluorescein diacetate (CM-H$_2$DCF-DA) (Molecular Probes), which becomes highly fluorescent upon oxidation by intracellular $H_2O_2$ [22, 23]. To confirm involvement of ROS in 25 mM glucose-induced cell proliferation, mouse ESCs were treated with NAC (10$^{-5}$ M) before being treated with either 25 mM glucose or $H_2O_2$ (5 $\mu$M) for 1 hour. The cells were washed with Dulbecco’s PBS and incubated for 15 minutes in Krebs-Ringer solution containing 5 $\mu$M CM-H$_2$DCF-DA. The ROS generation was detected (excitation, 488 nm; emission, 515–540 nm) using a fluorescent microscope (Fluoview 300; Olympus).

Fluorescence-Activated Cell Sorting Analysis
Cells were incubated with 25 mM glucose for 24 hours, and the cells were dissociated in trypsin/EDTA, pelleted by centrifugation, and resuspended at approximately 10$^5$ cells per milliliter in PBS containing 0.1% BSA. The cells were fixed in 70% ice-cold ethanol, followed by incubation in a freshly prepared nucleus-staining buffer (250 $\mu$g/ml propidium iodide [PI] and 100 $\mu$g/ml RNase) for 30 minutes at 37°C. The cell cycle histograms were generated after analyzing the PI-stained cells by fluorescence-activated cell sorting (Beckman Coulter). At least 10$^4$ events per sample were recorded. The samples were analyzed using CXP software (Beckman Coulter).

AA Release
To quantitate AA release by modification of the method of Xing et al. [24], confluent mouse ESCs were incubated for 24 hours (0.5 $\mu$Ci/ml $^{3}$H[AA] in serum-free DMEM including LIF). The monolayers were then washed and incubated for 1 hour (37°C; DMEM). At the end of the incubation, the medium was transferred to ice-cold tubes containing 55 mM EGTA and 5 mM EDTA and centrifuged

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PPARδ Small Interfering RNA
The cells were grown in each dish until they reached 75% confluency. They were then transfected for 24 hours with either a SMARTpool of the small interfering RNAs specific to PPARδ (200 pmol/l) or a nontargeting small interfering RNA (as negative control: 200 pmol/l; Dharmacon, Inc., Lafayette, CO, http://www.dharmacon.com) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Preparation of Cytosolic and Total Membrane Fractions
The preparation of the cytosolic and total membrane fractions was performed using a modification of the method reported by Muckman et al. [25]. The cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation, and resuspended in buffer A (137 mM NaCl, 8.1 mM Na$_2$HPO$_4$, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml leupeptin; [pH 7.5]). The resuspended cells were then lysed mechanically on ice by trituration with a 21.1-gauge needle. The lysates were first centrifuged at 1,000g for 10 minutes at 4°C. The supernatants were centrifuged at 100,000g for 1 hour at 4°C to prepare the cytosolic and total particulate fractions. The supernatants (cytosolic fraction) were then precipitated with 5 volumes of acetone, incubated for 5 minutes on ice, and centrifuged at 20,000g for 20 minutes at 4°C. The resulting pellet was resuspended in buffer A containing 1% (vol/vol) Triton X-100. The particulate fractions, which contained the membrane fraction, were washed twice and resuspended in buffer A containing 1% (vol/vol) Triton X-100. The protein in each fraction was quantified using the Bradford procedure [26].

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction
Total RNA was extracted from mouse ESCs using STAT-60 monophasic solution of phenol and guanidinium isothiocyanate from Tel-Test (Friendswood, TX, http://www.isotexdiagnostics.com). Reverse transcription was conducted with 3 $\mu$g of RNA using a reverse transcription system kit (AccuPower PCR PreMix, Bioneer, Daejeon, Republic of Korea, http://www.bioneer.com) with oligo(dT)$_{18}$ primers. After that, 5 $\mu$l of reverse transcription (RT) products was amplified with a polymerase chain reaction (PCR) kit (AccuPower PCR PreMix, Korea), followed by denaturation at 94°C for 5 minutes and 30 cycles at 94°C for 15 seconds, 55°C for 1 minute, and 72°C for 45 seconds, followed by a 5-minute extension at 72°C. Amplifications of Oct4, FOXD3, SOX2, EP (1, 2, 3, 4), and PPAR (α, δ, γ) cDNAs were performed in mouse ESCs using primers described in Table 1. PCR of β-actin was also performed as control for quantity of RNA.

Real-Time RT-PCR
The cells were treated with 25 mM glucose for 1 hour before extraction of the total RNA. Real-time quantification of the RNA targets was performed in a Rotor-Gene 2000 real-time thermal cycling system (Corbett Research, Sydney, New South Wales, Australia, http://www.corbett lifescience.com) using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, http://www1.qiagen.com). The reaction mixture (20 $\mu$l) contained 200 ng of the total RNA, 0.5 $\mu$M of each primer, an appropriate amount of enzymes and fluorescent dyes, as recommended by the supplier.

The Rotor-Gene 2000 cycler was programmed as follows: 30 minutes at 50°C for reverse transcription; 15 minutes at 95°C for DNA polymerase activation; 15 seconds at 95°C for denaturing; and 45 cycles of 15 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C. The data were collected during the extension step (30 seconds at 72°C). The PCR was followed by melting curve analysis to

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confirm the specificity and identity of the RT-PCR products, which can distinguish between specific PCR products and the nonspecific PCR products resulting from primer-dimer formation. The temperature of the PCR products was increased from 65°C to 99°C at a rate of 1°C every 5 seconds, and the resulting data were analyzed using the software provided by the manufacturer.

**PGE2 Assay**

Mouse ESCs plated on 60-mm culture plates were grown in an FBS-free medium for 24 hours and divided into groups according to the experimental protocol. The PGE2 concentration in the culture medium was measured using an enzyme-linked immunosorbent assay with a PGE2 High Sensitivity Immunoassay kit (R&D Systems Inc., Minneapolis, http://www.rndsystems.com).

**Western Blot Analysis**

The cell homogenates (containing 20 μg of protein) were separated by electrophoresis through 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The blots were then washed with H2O, blocked for 1 hour with 5% skim milk powder in Tris-buffered saline/Tween 20 (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween 20), and incubated with the appropriate primary antibody at the dilutions recommended by the supplier. The nitrocellulose membrane was washed, and the primary antibodies were detected with either goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase. The bands were visualized by enhanced chemiluminescence (Amersham Biosciences U.K., Little Chalfont, Buckinghamshire, U.K., http://www.amershambiosciences.com).

**Statistical Analysis**

The results are expressed as the mean ± SE. The difference between two mean values was analyzed using a Student t test. A p value <.05 was considered significant.

### RESULTS

**Effect of High Glucose on Mouse ESC Proliferation**

The undifferentiated state of the mouse ESCs used in this experiment was confirmed by examining the expression of the undifferentiated stem cells markers, including Oct4, FOXD3, and SOX2 expression levels in the presence or absence of 25 mM glucose. The PGE2 concentration in the culture medium was measured using an enzyme-linked immunosorbent assay with a PGE2 High Sensitivity Immunoassay kit (R&D Systems Inc., Minneapolis, http://www.rndsystems.com).

**Table 1. Primers used for polymerase chain reaction**

<table>
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<tr>
<th>Gene</th>
<th>Identification</th>
<th>Primer sequence, 5’–3’</th>
<th>Product size (bp)</th>
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<td>Oct-4</td>
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<td></td>
<td>Antisense</td>
<td>GGGATGTAAGTGACTGCTG</td>
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<td>FOXD3</td>
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<td></td>
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Abbreviation: bp, base pairs.

**Figure 1.** Effect of high glucose levels on the characterization of mouse embryonic stem (ES) cells. (A): Oct4, FOXD3, SOX2, and β-actin mRNA expression levels in the presence or absence of 25 mM glucose. (B): Oct4 and β-actin protein expression levels in the presence or absence of 25 mM glucose. The bands represent 50–60 kDa of Oct4 and 41 kDa of β-actin. (C): The alkaline phosphatase enzyme activity in the cells was measured in the presence or absence of 25 mM glucose, as described in Materials and Methods. (D): Immunofluorescence staining of mouse ES cells with mouse stage-specific embryonic antigen-1-specific antibody.
incubating the cells with 25 mM glucose for 12 hours [27], the present study examined the effect of various glucose concentrations over this period. The effects of glucose concentrations of 3 mM or less are not observed (supplemental online data). As shown in Figure 2A, a high glucose concentration increased the level of \([\text{3H}]\)thymidine incorporation in a dose-dependent manner. Moreover, a treatment with 25 mM glucose significantly increased the level of \([\text{3H}]\)thymidine incorporation (48% increase vs. 5 mM glucose; \(p < .05\)). On the other hand, 25 mM mannitol had no such effect. There was an increase in the number of BrdU-labeled cells in response to 25 mM glucose, which is consistent with the observed increase in the level of \([\text{3H}]\)thymidine incorporation (Fig. 2B). Double labeling for Oct4 and BrdU expression was performed to determine whether 25 mM glucose exerts its growth-promoting effect on undifferentiated ESCs. In these experiments, the ESC population contained more than 90% undifferentiated (Oct4-positive) cells. This highlights the role of 25 mM glucose in the undifferentiated ESCs but not in a spontaneously differentiated progeny (Fig. 2C). Finally, there was a significant increase in the number of cells observed after incubation with 25 mM glucose for 12 hours (Fig. 2D).

Involvement of Oxidative Stress and cPLA2 in High-Glucose-Induced Cell Growth

The effect of 25 mM glucose on \(\text{H}_2\text{O}_2\) and LPO formation was first measured to determine the relationship between oxidative stress and the glucose level. Consistent with our previous results [27], Figure 3A and 3B shows that 25 mM glucose increased the level of \(\text{H}_2\text{O}_2\) (25 mM glucose, 0.99 ± 0.03; control, 0.59 ± 0.07 \(\mu\)mol/mg protein) and LPO formation (25 mM glucose, 0.62 ± 0.02; control, 0.47 ± 0.01 nmol/mg protein), respectively (\(p < .05\)). In addition, 25 mM glucose increased the level of dichlorofluorescein-sensitive cellular ROS, which was blocked by NAC (Fig. 3C). The level of cPLA2 phosphorylation was also increased from the 10-minute treatment with 25 mM glucose (Fig. 3D), which was blocked by NAC (10 \(\mu\)M) and ascorbic acid (10 \(\mu\)M) (Fig. 3E). Indeed, as shown in Figure 3F, NAC (10 \(\mu\)M), ascorbic acid (10 \(\mu\)M), AACOCF3 (10 \(\mu\)M), and mepacrine (10 \(\mu\)M) significantly blocked the 25 mM glucose-induced increase of \([\text{3H}]\)AA release. The cells were pretreated with NAC, ascorbic acid, AACOCF3, and mepacrine before addition of 25 mM glucose to examine the role of oxidative stress and the cPLA2 pathway in 25 mM glucose-induced cell proliferation. As shown in Figure 3G, NAC, ascorbic acid, AACOCF3, and mepacrine before addition of 25 mM glucose to examine the role of oxidative stress and the cPLA2 pathway in 25 mM glucose-induced cell proliferation.

Involvement of COX/PGE2 in High-Glucose-Induced Cell Proliferation

The COX (1 and 2) expression was examined as a function of the time to determine whether high glucose levels can induce the expression of the COX (1 and 2) proteins. The results showed that high glucose levels increased the level of COX-2 expression in a time-dependent manner but had no effect on COX-1 (Fig. 4A). Moreover, these increases were inhibited by either AACOCF3 or

Figure 2. Effect of high glucose levels on mouse ESCs proliferation. (A): The mouse ESCs were incubated with various concentrations of glucose or mannitol (5–50 mM) and pulsed with 1 \(\mu\)Ci of \([\text{3H}]\)thymidine for 1 hr. *, \(p < .05\) versus 5 mM glucose. (B): BrdU-positive cells in response to different glucose concentrations (5 or 25 mM) for 12 hr. The number of BrdU-positive cells per field of vision was determined. At least 10 fields of vision per coverslip were counted. *, \(p < .05\) versus 5 mM glucose. (C): The mouse ESCs were incubated with 25 mM glucose for 24 hr and double-labeled with the Oct4 and BrdU antibody. (D): The mouse ESCs were treated with glucose (5 or 25 mM) for 12 hr, and the cells were counted using a hemocytometer. The values represent the mean ± SE of four independent experiments with triplicate dishes. *, \(p < .05\) versus 5 mM glucose. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; hr, hours.
mepacrine (Fig. 4B). Indeed, as shown in Figure 4C, 25 mM glucose increased PGE2 production, which was blocked by AA-COCF3 (10^{-6} M) and indomethacin (10^{-6} M). As shown in Figure 4D, the mouse ESCs expressed the EP1, EP2, EP3, and EP4 receptors. Moreover, the results of real-time RT-PCR showed that the treatment with 25 mM glucose or PGE2 significantly increased the mRNA level of EP1 receptor, whereas those of other subtypes of EP receptors remained unchanged. The mouse ESCs were also incubated with 25 mM glucose and PGE2 for 12 hours. As shown in Figure 4E, 25 mM glucose or PGE2 (10^{-8} M) produced a higher level of [3H]thymidine incorporation than 5 mM glucose (52% and 47% increases, respectively). The level of Akt phosphorylation (Thr^{308}, Ser^{473}) was also increased by 25 mM glucose and PGE2 (Fig. 4F).

Effect of High Glucose Levels on PPARδ Activation

The expression of the PPAR isotypes was detected by RT-PCR to determine whether the PPARs are involved in the high-glucose-induced increase in [3H]thymidine incorporation. As shown in Figure 5A, 25 mM glucose increased PPARδ gene expression in mouse ESCs. On the other hand, PPARγ remained unchanged, whereas PPARγ was decreased. Indeed, 25 mM glucose increased the level of the PPARδ proteins in a time-dependent manner (0–24 hours) (Fig. 5B); the increase was blocked by NAC, AACOCF3, and mepacrine (Fig. 5C). The 25 mM glucose-induced stimulation of [3H]thymidine incorporation was also inhibited when the cells were transfected with the PPARδ-specific small interfering RNAs (siRNAs) (Fig. 5D). These results were confirmed by observ-
ing the mouse ESCs that had been treated with the L-165041 (PPARδ agonist) or GW9662 (PPARδ antagonist) (10^{-6} M) (Fig. 5E). The 25 mM glucose-induced increase in PPARδ expression was significantly blocked by either LY294002 (phosphoinositide 3-kinase [PI3K] inhibitor; 10^{-6} M) or the Akt inhibitor (10^{-5} M) (Fig. 5F). The example shown is a representative of four independent experiments. Abbreviations: hr, hours; PGE2, prostaglandin E2.

Effect of High-Glucose-Induced PPARδ on Cell Cycle Regulatory Proteins

On the basis of the previous study showing that high glucose increased the expression levels of the cyclin D1, cyclin E, CDK 4, and CDK 2 proteins, which are believed to be factors essential in the G1/S progression [27], the involvement of high-glucose-induced cPLA2 or PPARδ on the cell cycle regulatory proteins was examined. In this experiment, 25 mM glucose increased the levels of cyclin D1/CDK 4 and cyclin E/CDK 2 proteins, but decreased the levels of p21WalFplKp1 and p27Npc1, which were inhibited by AACOCF3, indomethacin, and GW9662, respectively (Fig. 6A). The 25 mM glucose-induced increase in the cyclin D1/CDK 4 and cyclin E/CDK 2 protein levels was inhibited when the cells were transfected with a pool of PPARδ-specific siRNAs (Fig. 6B). In addition, 25 mM glucose increased the percentage of the cell population in the S phase (control, 47.8%; 25 mM glucose, 75%). However, a pretreatment with GW9662 significantly decreased the level of 25 mM glucose-induced accumulation in the S phase (Fig. 6C).

DISCUSSION

This study examined whether or not there is an association between cPLA2 mediated by reactive oxygen species and the growth response of mouse ESCs to high glucose levels. Five millimolar d-glucose was used as the control because this is the normal concentration in a medium and is equivalent to the serum glucose concentration in normal individuals (90 mg/dl). Twenty-five millimolar d-glucose would be a very high serum level even for a diabetic patient, but it is commonly used to investigate the effects of high glucose under the conditions of in
ESCs with high glucose levels leads to the activation of cPLA2 and AA release. Upon activation through phosphorylation and membrane lipid remodeling, cPLA2 is translocated to cellular membranes, releasing AA from membrane phospholipids [31]. Recent studies have shown that ROS enhance AA release and intracellular calcium influx, cPLA2 is translocated to cellular membranes, releasing AA from membrane phospholipids [31].

The present result showed that the ROS level in mouse ESCs exposed to high d-glucose concentrations for 24 hours was higher than that in the control. This suggests that oxidative stress is a key factor in the etiology of the associated proliferative anomalies in cells cultured in high d-glucose concentrations. The origin of this increased ROS generation by high d-glucose is unclear, but a substrate overload in the mitochondrial electron transport chain might induce excessive generation of ROS [29]. Moreover, it has been reported that treatment of the cells with phorbol 12-myristate 13-acetate, a PKC activator, increased ROS [30]. Therefore, high glucose can induce cellular ROS through mitochondrial metabolism and intracellular signaling molecule, indicating PKC-dependent activation of NADPH oxidase [7, 8]. It was also observed that treating mouse ESCs with high glucose levels leads to the activation of cPLA2 and AA release. Upon activation through phosphorylation and intracellular calcium influx, cPLA2 is translocated to cellular membranes, releasing AA from membrane phospholipids [31]. Recent studies have shown that ROS enhance AA release and subsequent AA metabolism in macrophages [32]. In the present study, we provide the first direct evidence for an involvement of COX-2 in the stimulation of mouse ESC proliferation by high glucose. The elevation of glucose significantly

Figure 5. Effect of high glucose levels on PPAR gene expression. (A): Mouse ESCs were treated with 25 mM glucose for 12 hr, and the PPARα, δ, and γ gene expression levels were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). The PPAR α, δ, and γ gene expression levels were then analyzed using RT-PCR (Aa) and real-time RT-PCR (Ab). Each example shown is representative of three independent experiments. *p < .05 versus 5 mM glucose. (B): Mouse ESCs were incubated with 25 mM glucose for 0–24 hr and then harvested. The example shown is a representative of four independent experiments. *p < .05 versus 0 minutes. (C): Mouse ESCs were pretreated with NAC, AACOCF₃, and indomethacin for 30 minutes before being treated with 25 mM glucose for 12 hr. The example shown is a representative of four independent experiments. *p < .05 versus 0 minutes. (Aa): Mouse ESCs were transfected for 24 hr with either a SMARTpool of PPAR siRNAs (200 pmol/l) or a nontargeting control siRNA (200 pmol/l) using Lipofectamine 2000 prior to 25 mM glucose treatment for 24 hr. The cells were then pulsed with 1 μCi of [³H]thymidine for 1 hr. The values represent the mean ± SE of four independent experiments with triplicate dishes. *p < .05 versus 5 mM glucose; **p < .05 versus 25 mM glucose alone. (C): Mouse ESCs were pretreated with L-165041 (PPARδ agonist: 10⁻⁶ M), and GW9662 (PPARδ antagonist: 10⁻⁶ M) for 30 minutes prior to being treated with 25 mM glucose for 12 hr. The cells were then pulsed with 1 μCi of [³H]thymidine for 1 hr. The values represent the mean ± SE of four independent experiments with triplicate dishes. *p < .05 versus 5 mM glucose; **p < .05 versus 25 mM glucose alone. (F): The mouse ESCs were pretreated with LY294002 and the Akt inhibitor for 30 minutes before being treated with 25 mM glucose for 12 hr. The graph denotes the mean ± SE of four experiments for each condition determined from densitometry relative to each β-actin. *p < .05 versus 5 mM glucose; **p < .05 versus 25 mM glucose alone. Abbreviations: hr, hours; NAC, N-acetylcysteine; PPAR, peroxisome proliferator-activated receptor; siRNA, small interfering RNA.
increased the expression of COX-2 protein levels. In the present study, the concentration of PGE2 generated by the AA cascade through COX increased proliferation of mouse ESCs obtained under high glucose level. Previous work has shown that embryos from streptozotocin (STZ)-induced diabetic rats have a diminished PGE2 content, although they can produce PGE2 in large amounts [33]. It is interesting to speculate that AA might be depleted if PGE2 generation and release is increased in the diabetic embryo to maintain intracellular PGE2 levels [34]. Most interestingly, glucose-induced COX-2 upregulation was associated with a PPARδ [35]. The mechanisms by which high glucose increases the expression of PPARδ mRNA remain unclear, and further investigation is needed.

In the present study, we demonstrated a significant increase in PPARδ expression and a slight upregulation of PPARα in response to high glucose, whereas PPARγ was downregulated. Previous studies demonstrated that placentental PPARγ expression was decreased under mild hyperglycemia in gestational diabetic women or in STZ-induced diabetic rats [36, 37]. Thus, we hypothesize that overexpression of PPARδ in mouse ESCs is a sufficient condition to increase cell proliferation. In this study, we provided the first evidence for a role of PPARδ in the regulation of high-glucose-induced mouse ESC proliferation. We reproduced this observation using a PPARδ selective agonist, L-165041, to stimulate the growth of mouse ESCs and used a siRNA system to validate PPARδ as the mediator of these effects. The present result is consistent with a recent report that PPARδ promotes postconfluent cell proliferation in 3T3 fibroblasts [38]. The physiological and pharmacological roles of PPARδ are just beginning to emerge. It has recently become clear that PPARδ has a function in epithelial tissues, but inconsistent reports leave the situation controversial. Indeed, some reports suggest that ligand activation of PPARδ potentiates cell growth [39], whereas other reports suggest that ligand activation of PPARδ attenuates cell growth [40]. More recently, it was suggested that ligand activation of PPARδ induces expression of COX-2 [39], which could theoretically promote cell growth and inhibit apoptosis through mechanisms that involve the production of prostaglandins. These data raise the possibility of a
Effect of High Glucose on Mouse ESC Proliferation

The cell cycle is dysregulated under high-glucose conditions, and G1 phase cell cycle progression is believed to be responsible for the glucose-induced mouse ESC proliferation [27]. There is growing evidence that specific CDK inhibitors p21WAF1/Cip1 and p27Kip1 are critically involved in the G1 phase cell cycle progression in mouse ESCs exposed to a high glucose level [47]. Based upon these and previous results, it is hypothesized that PPARδ activation increases the level of binding of the Adenovirus E2 promoter-binding factor/DNA-binding heterodimerization partner protein heterodimers to their target genes, and the PPARδ ligands might increase the level of pRb phosphorylation and accelerate G1/S phase transition in mouse ESCs. In addition, PPARδ upregulates cyclin/CDK and mediates G1 cell cycle progression. Therefore, high-glucose treatment results in G1 cell cycle progression and increased proliferation. Figure 7 shows a hypothetical model of the signaling mechanisms involved in mediating the high-glucose-induced proliferation of mouse ESCs. Consistent with present results, overexpression of PPARδ in vascular smooth muscle cell increased cell proliferation by increasing the cyclin A and CDK 2, as well as decreasing p57Kip2 [39]. Although the proliferative properties as a result of inducing G1 cell cycle progression are associated with the activation of PPARδ activation by high glucose in various cell types, it appears that the effect of a high glucose level on cell cycle and proliferation depends on the cell cycle or conditions. The present study can be regarded as an extension of studies aimed at elucidating the functional role of a high concentration of glucose in mouse ESC expansion and demonstrating the value and the advantages of this factor in the ESC culture system. On the basis of these results, we suggest that the series of high-glucose experiments provide a fascinating insight into the role of a high concentration of glucose in the expansion of ESCs and the establishment of a stable culture system for ESCs associated with maintenance of self-renewal. In conclusion, these results demonstrate that mouse ESC growth was enhanced by high-glucose-induced PPARδ, which is mediated, at least in part, through the induction of COX-2 expression and PGE2 production.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.
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