PKC and MAPKs Pathways Mediate EGF-induced Stimulation of 2-Deoxyglucose Uptake in Mouse Embryonic Stem Cells

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Key Words

Embryonic stem (ES) cell • 2-DG uptake • PKC • MAPKs • EGF

Abstract

It has been reported that epidermal growth factor (EGF) and EGF receptor were highly expressed in embryo, suggesting that the EGF system is related to early embryo development in an autocrine and/or paracrine manner. Glucose becomes the preimplantation exogenous energy substrate and enters the blastocyst via glucose transporters. Thus, the effect of EGF on [³H]-2-deoxyglucose (2-DG) uptake and its related signaling pathways were examined in mouse embryonic stem (ES) cells. EGF significantly increased 2-DG uptake in time- and concentration- dependent manner (>12 hr, >10 ng/ml) and increased mRNA and protein level of glucose transporter 1 (GLUT1) compared to control, respectively. Actinomycin D and cycloheximide completely blocked the effect of EGF on 2-DG uptake. EGF-induced increase of 2-DG uptake was blocked by AG1478 (EGF receptor tyrosine kinase blocker), genistein or herbimycin (tyrosine kinase inhibitors). In addition, EGF effect was blocked by neomycin and

U 73122 [phospholipase C (PLC) inhibitors] as well as staurosporine and bisindolylmaleimide I [protein kinase C (PKC) inhibitors]. EGF was also observed to increase inositol phosphates (IPs) formation and activate a PKC translocation from the cytosolic to membrane fraction, suggesting a role of PLC and PKC. SB 203580 [p38 mitogen activated protein kinase (MAPK) inhibitor] or PD 98059 (p44/42 MAPKs inhibitor) blocked EGF-induced increase of 2-DG uptake. EGF also increased phosphorylation of p38 MAPK and p44/42 MAPKs, which was blocked by genistein or bisindolylmaleimide I, respectively. In conclusion, EGF partially increased 2-DG uptake via PKC, p38 MAPK, and p44/42 MAPKs in mouse ES cells.

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Introduction

Mouse preimplantation embryos preferentially metabolize pyruvate until the late morula/early blastocyst stage, at which time glucose becomes the predominant energy substrate [1]. Glucose, however, is required earlier than the blastocyst stage for optimal development [2, 3].

Glucose deprivation at this critical time of glucose need may be responsible for the later problems these embryos experience. Glucose can be transported across membranes by two different mechanisms: a Na⁺-coupled active carrier system (SGLT 1-3), and a growing family of structurally related Na⁺-independent glucose transporter glycoproteins (GLUT 1-9) [4, 5]. These are expressed in a tissue-specific manner and under the control of specific hormones and growth factors. It has been reported that epidermal growth factor (EGF) and EGF receptor were highly expressed in embryo, suggesting that the EGF system is related to early embryo development in an autocrine and/or paracrine manner [6-10]. Furthermore, Lee and Fukui [11] reported that supplementing bovine embryo culture medium with EGF increased total cell number [inner cell mass, that contains ES cells, and trophectoderm cells] in blastocysts. The response is elicited by EGF receptor that triggers at least three intracellular signaling pathways involving the mitogen-activated protein kinases ERK-1/2, phospholipase C γ (PLC γ), and phosphatidylinositol-3-kinase [12]. Thus, more basic studies are needed to elucidate whether EGF is physiological regulator of glucose uptake in mouse ES cells.

GLUT1 is believed to play a key role in maintaining basal glucose uptake for metabolism in many cell types [13-16]. Its expression is detectable throughout preimplantation development from the oocyte through the blastocyst stage [17-19] and it increases 11-fold in developing embryos from the two-cell stage to the blastocyst [19]. This may reflect in part, the finding that GLUT1 is the major glucose transporter in the inner cell mass (ES cells) of the preimplantation embryo. Actually the inner cell mass also relies on GLUT1 for provision of glucose from the blastocoel. Recruitment of GLUT1 from an intracellular compartment to the plasma membrane provides a mechanism for increased glucose transport in response to growth factors. Lyer and colleagues [20] reported gene encoding GLUT1 protein in J1 ES cells. In addition, microarray analysis showed that glucose transporter 1 (Glut1/Slc2a1) was expressed in rat embryonic multipotent neural stem cells [21]. However, effect of EGF on glucose transporter of embryonic stem cells is still unknown although glucose is an important energy substrate for ES cells and GLUT1 is one of major glucose transporters in the ES cells.

Embryonic stem cells are pluripotent cell lines that are derived from the blastocyst-stage early mammalian embryo. Therefore, mouse ES cells can be regarded as a versatile biological system that has led to major advances

in cells and developmental biology [22-24]. These unique cells are characterized by their capacity for prolonged undifferentiated proliferation in culture maintaining the potential to differentiate into derivatives of all three germ layers [25]. The growth of mouse ES cells in culture is also likely to require increased uptake of glucose and other substrates [26]. GLUT1 responsiveness to growth factors is, therefore, extremely important as a mechanism for providing increased substrate to cope with increased cellular demand arising from growth, proliferation, and stress [27]. To date there is little information concerning the effect of EGF on the GLUT1 in mouse ES cells, and the mode of GLUT1 regulation by EGF. Thus, we investigated the effect of EGF on glucose uptake and its related signal cascades in the mouse ES cells.

Materials and Methods

Materials

Mouse ES cell was obtained from American Type Culture Collection (ES-E14TG2a). Fetal bovine serum was purchased from Biowhittaker (Walkersville, MD, USA). EGF, staurosporine, actinomycin D, cycloheximide, SB 203580, 2-deoxy-D-glucose, Coomassie Brilliant Blue G, fluorescence isothiocyanate-conjugated (FITC-conjugated) goat-anti mouse IgM, and β -actin were obtained from Sigma Chemical Company (St. Louis, MO, USA). Bisindolylmaleimide I, PD 98059, herbimycin A, genistein, neomycin, U 73122, and AG1478 were purchased from Calbiochem (La Jolla, CA, USA). [³H]-2-deoxyglucose, [³H] inositol phosphates, [¹⁴C]-fructose, [³H]-L-alanine, and [³H]-L-arginine were purchased from Dupont/NEN (Boston, MA, USA). Anti-Pan Protein Kinase C was obtained from upstate biotechnology (Charlottesville, VA, USA). Rabbit anti-GLUT-1 was purchased from Santa Cruz Biotechnology (Delaware, CA, USA). Phospho-EGFR, EGFR, anti-phospho-p44/42 MAP Kinase (Thr202/Tyr204) antibody, anti-p44/42 MAP Kinase polyclonal antibody, anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody, and anti-p38 MAP kinase antibody were purchased from Cell Signaling Technology (Herts, UK). Goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA, USA). All other reagents were of the highest purity commercially available. Liquiscint was obtained from National Diagnostics (Parsippany, NY, USA).

ES cell culture

The procedures for mouse ES cell culture have been described by Smith and Hooper [28]. ES cells were grown in the Dulbecco's Modified Eagle Media (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, 10 ng/ml mouse leukemia inhibitory factor (LIF), and 15% fetal bovine serum (FBS) without a feeder layer and cultured for five days in standard medium plus LIF.

The feeder free ES cells were grown on gelatinized 12-well plates or 60 mm culture dish in an incubator maintained at 37 °C in an atmosphere of 5% CO₂ in air. The medium was removed and replaced with serum free DMEM including all supplements contained LIF for 12 hr prior to the experiments. After that, the cells were washed twice with Phosphate Buffered Saline (PBS) and then maintained in a serum free DMEM including all supplements and indicated agents.

Alkaline phosphatase staining

Cells were washed twice with PBS and fixed for about 15 min with 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 poured and after incubation for about 15 min at room temperature, if stained, washed with PBS and photographed.

Immunofluorescence staining with SSEA-1

Cells were fixed and treated with monoclonal antibody against mouse SSEA-1 [stage-specific embryonic antigen-1 (undifferentiated stem cell marker), 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). Fluorescence images were visualized with a fluorescence microscope (Fluoview 300, Olympus, Japan).

Uptake experiment

[³H]-2-deoxyglucose (2-DG) uptake experiments were conducted as modification of method described by Henriksen et al [29]. To study 2-DG uptake, the culture medium was removed by aspiration, 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 L-alanine, and 10 mM HEPES/Tris, pH 7.4). After the washing, it was incubated at 37°C for 30 min in an uptake buffer that contained 10 µM unlabeled 2-DG and 1 µCi/ml [³H]-2-DG. At the end of the incubation period, the cells were again washed three times with ice-cold uptake buffer, and the cells were solubilized in 1 ml 0.1% Sodium Dodecyl Sulfate (SDS). To determine the 2-DG uptake incorporated intracellularly, 900 µl of each sample was removed and counted in a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA). The remainder of each sample was used for protein determination [30]. The radioactivity counts in each sample were then normalized with respect to protein and were corrected for zero-time uptake per mg protein. All uptake measurements were made in triplicate. Fructose, L-alanine, or L-arginine uptake experiments were conducted as described by the method of Corpe et al [31], Nishida et al [32], or Acevedo et al [33], respectively.

Inositol phosphates formation assay

The assay was modified from Berridge et al [34]. Cell cultures were labeled with myo-[³H]-inositol (2.5 µCi/ml, 2 ml final) for 24 hr and followed by the addition of 10 mM LiCl for 15 min and was treated with the appropriate agent. Medium was removed, cells were scraped off the dish in 1.2 ml H₂O and

extracted in 1.8 ml chloroform/methanol (1:2, v/v), and the upper phase was applied to Bio-Rad AG 1-X8 columns (Bio-Rad Laboratories, Hercules, CA, USA). Following washes with 5 mM inositol and H₂O, the fraction containing the [³H]-inositol phosphates (IP₁, IP₂, and IP₃) was eluted with 1 M ammonium formate and 0.1 N formic acid.

Preparation of cytosolic and membrane fractions

Preparation of cytosolic and plasma membrane fractions was performed by a modification of the method of Mackman et al [35]. DMEM of mouse ES cells was exchanged at 48 hr before experiment. 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 µg/ml leupeptin (pH 7.5)]. The resuspended cells were then mechanically lysed on ice by trituration with a 21.1-gauge needle. The lysates were first centrifuged at 1,000g for 10 min at 4°C. The supernatants were centrifuged at 100,000g for 1 hr at 4°C to prepare cytosolic and particulate fractions (plasma membrane). The supernatants (cytosolic fraction) were then precipitated with 5 vol. of acetone, incubated for 5 min on ice and centrifuged at 20,000g for 20 min at 4°C. The resultant pellet was resuspended in buffer A containing 1% (v/v) Triton X-100. The particulate fractions, which contained 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 with a Bradford procedure [30].

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). Two micrograms of purified RNA were synthesized into cDNA using avian leukemia virus reverse transcriptase with oligo-dT(18)-primers. After that, 5 µl of RT products was amplified using Taq DNA polymerase followed by: denaturation at 94°C for 5 min and 40 cycles at 94°C for 45 sec, 55°C for 1 min and 72°C for 1 min followed by 5 min extension at 72°C. The primers were 5'-AGCGTCATCTTCATCCCAGC-3' (sense), 5'-CCACAATGCTCA GGTAGGAC-3' (antisense) for GLUT-1 (544 bp), 5'-AAAGAGGGGGAAGAAGCATC-3' (sense), 5'-GGCACAACAAACATCCCAC-3' (antisense) for GLUT-2 (309 bp), 5'-ATGGCAGTGGCTGGTTGTTTC-3' (sense), 5'-TCCTGAGCTGAAGAGAATGTCC-3' (antisense) for GLUT-3 (508 bp), 5'-CTCTTCTTCCCCATTACTTCCAG-3' (sense), 5'-GAAGGCTGAGCAAGAGGACTGAG-3' (antisense) for GLUT-4 (496 bp), CATCTCCATCATCGTCCTCA-3' (sense), 5'-GTAGATGGTGGTGAGGAGAC-3' (antisense) for GLUT-5 (531 bp), 5'-CGTGAGACTTTGCAGCCTGA-3' (sense), 5'-GGCGATGTAGTGATCTGCTG-3' (antisense) for Oct-4 (519 bp), and 5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3' (sense), 5'-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3' (antisense) for β-actin (350 bp). Also, PCR of β-actin was performed as control for quantity of RNA.

Table 1. Effect of EGF on 2-DG, fructose, L-alanine, and L-arginine uptake. Mouse ES cells were treated with EGF (10 ng/ml) for 24 hr. Then 2-DG, fructose, L-arginine, and L-alanine uptake were conducted as described in Materials and Methods. Values are the means $\pm$ S.E. of 3 independent experiments with triplicate dishes. * $P < 0.05$ vs. control.

	Control	EGF
	(pmol/mg protein/min)	
2-DG	320 $\pm$ 3.17	398 $\pm$ 3.24*
fructose	220 $\pm$ 4.88	227 $\pm$ 5.24
L-alanine	80 $\pm$ 5.21	88.8 $\pm$ 3.47
L-arginine	92 $\pm$ 3.66	101 $\pm$ 6.71

Western blot analysis

Cells were harvested, washed twice with PBS, and lysed with a buffer [20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride, 0.5 mM sodium orthovanadate] for 30 min on ice. The lysates were then cleared by centrifugation (10 min at 15,000 r.p.m., 4°C). Protein concentration was determined following the Bradford procedure [30]. Equal amounts of protein (20 μ g) were resolved by electrophoresis on 10% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to nitrocellulose. After the blots were 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 hr and incubated with the appropriate primary antibody at dilutions recommended by the supplier. Then the membrane was washed, and primary antibodies were detected with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase, and the bands were then visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK).

Statistical analysis

Results were expressed as means $\pm$ standard errors (S.E.). The difference between two mean values was analyzed by the nonparametric Wilcoxon sign test or ANOVA. The difference was considered statistically significant when $P < 0.05$.

Results

Characterization of mouse ES cells

To confirm undifferentiated state of mouse ES cell used in present studies, alkaline phosphatase activity was measured as a representative undifferentiated marker. Mouse ES cells were stained into violet color in presence or absence of EGF (10 ng/ml). Immunofluorescence staining also showed that mouse ES cells expressed a cell-surface marker protein, a characteristic of undifferentiated mouse ES cells, stage-specific embryonic antigen-1 (SSEA-1) (Fig. 1A). In addition, Oct-4 (POU domain transcription factor), which is other undifferentiated ES cell marker was assessed by RT-PCR and western blotting. A fragment of amplified Oct-4 DNA of 519 bp was detected from mouse ES cells but not from mouse embryonic fibroblasts (MEF) cells (Fig. 1B). Oct-4 protein expression was also identified (Fig. 1C).

Therefore, mouse ES cells used in present studies were grown in an undifferentiated state. In addition, under the treatment of 10 ng/ml EGF for 24 hr, mouse ES cells still maintained an undifferentiated state.

Effect of EGF on glucose, fructose, L-alanine, and L-arginine uptake of mouse ES cells

In experiments to examine effects of the EGF on glucose, fructose, L-alanine, or L-arginine uptake, EGF increased 2-DG uptake whereas did not affect in fructose, L-alanine, and L-arginine uptake (Table 1). To determine effective concentration and treatment time of EGF, the time-dependent effect of EGF on 2-DG uptake was firstly examined in mouse ES cells (Fig. 2A). ES cells were incubated with 10 ng/ml EGF for different times (0 to 48 hr). As shown in Fig. 2A, EGF increased 2-DG uptake from 12 hr ($P < 0.05$). The maximal increased effect of EGF was observed at 24 hr, and the increased effect was maintained after more prolonged incubations with EGF for up to 48 hr without further increase. Next, the dose dependency of EGF was examined. ES cells were incubated for 24 hr with EGF of various concentrations (0 to 1,000 ng/ml). EGF over 1 ng/ml significantly increased 2-DG uptake (Fig. 2B) ($P < 0.05$) and the maximum response appeared at the concentration of 100 ng/ml EGF. In this study, 10 ng/ml EGF treatment for 24 hr was used. We also observed whether EGF affects either the affinity of 2-DG for the uptake process (K_m) or the maximal velocity (V_{max}) by using the Lineweaver-Burk plot. As shown in Fig. 3, the kinetic analysis of uptake data indicated the 56 % increase of V_{max} for 2-DG uptake in EGF-treated cells compared to control, whereas there was no change of K_m value.

To examine whether the EGF-induced increase of 2-DG uptake is dependent on gene transcription and protein synthesis, firstly, the expressions of GLUT isotypes were detected by RT-PCR. As shown in Fig. 4A, GLUT 1 was most highly expressed compared to GLUT 2, 3, 4, and 5 in mouse ES cells. In further experiments, as shown in Fig. 4B, EGF-induced increase of 2-DG uptake was prevented by actinomycin D (transcription inhibitor) and cycloheximide (translation inhibitor). Actinomycin D and

Fig. 1. Characterization of mouse ES cells. (A) Alkaline phosphatase (AP) and SSEA-1 expression was measured as a representative undifferentiated marker in presence or absence of EGF (10 ng/ml). Scale bars represent 20 μ m (magnification $\times 400$). (B) Analysis of the expression of genes for Oct-4 and β -actin by EGF in mouse ES cells and mouse embryonic fibroblast (MEF) as negative control. (C) Effect of EGF on Oct-4 and β -actin protein levels. Bands represent 50-60 kDa of Oct-4 and 41 kDa of β -actin, respectively.

Fig. 2. Time course (A) and dose response (B) of EGF effect on 2-DG uptake. ES cells were treated with EGF (10 ng/ml) for various time points (0 to 48 hr) or with different doses of EGF (0 to 1,000 ng/ml) for 24 hr before uptake experiment. Values are means $\pm$ S.E. of 4 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control.

cycloheximide alone had no effect. Thus, we determined effect of EGF on GLUT1 mRNA and protein expression level. In the present study, EGF significantly increased GLUT1 mRNA level (22% increase vs. control; $P < 0.05$) and GLUT1 protein expression level in membrane fraction (59% increase vs. control; $P < 0.05$) (Fig. 4C, D).

Effect of an EGF receptor tyrosine kinase inhibitor and tyrosine kinase inhibitors on 2-DG uptake

To determine if intact tyrosine kinase activity of the EGF receptor was necessary to elicit the EGF-induced increase of 2-DG uptake, cells were preincubated either

with AG1478, EGF receptor tyrosine kinase inhibitor, or genistein or herbimycin A, tyrosine kinase inhibitors, respectively. Figure 5A demonstrated EGF increased 2-DG uptake to 67% of that seen in the control. Moreover, AG 1478, herbimycin A, or genistein completely prevented EGF-induced stimulation of 2-DG uptake, respectively. Next, to identify involvement of EGF receptor and tyrosine kinase activity of EGF receptor in EGF-induced stimulation of 2-DG uptake, Western blotting analysis using antisera specific to total-EGF receptor and phosphorylated EGF receptor was employed. As shown in Fig. 5B, EGF increased phosphorylation of EGF receptor without altering the total-EGF receptor content.

Fig. 3. Effect of EGF on the kinetic parameters of 2-DG uptake. Mouse ES cells were incubated for 24 hr in the presence of 10 ng/ml EGF. Cells were then incubated for 30 min with the indicated concentrations of 2-DG. 2-DG uptake was measured in a buffer containing [3 H]-2-DG in the presence of 0.5, 1, 2, or 4 mM unlabeled 2-deoxyglucose. Values are means $\pm$ S.E. of 4 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control.

Involvement of PLC/PKC in EGF-induced increase of 2-DG uptake

To evaluate the involvement of PLC/PKC in EGF-induced stimulation of 2-DG uptake, mouse ES cells were firstly treated with EGF after pretreatment of neomycin or U 73122, PLC inhibitors. Figure 6A showed that neomycin and U 73122 significantly prevented EGF-induced stimulation of 2-DG uptake. EGF also increased IPs formation by 79% compared to control at 90 sec and gradually decreased at 120 sec (Fig. 6B). Next, we investigated the involvement between EGF and PKC. As shown in Fig. 7A, PKC inhibitors, staurosporine or bisindolylmaleimide I, completely blocked EGF-induced increase of 2-DG uptake. Western blotting analysis was conducted to confirm the involvement of PKC in the EGF-induced increase of 2-DG uptake (Fig. 7B). The redistribution of PKC from the cytosolic to the membrane compartment is considered to be the first step in PKC activation and was used as a measure of PKC activation in response to EGF in mouse ES cells. PKC was found to be present primarily in the cytosolic fraction of untreated mouse ES cells. Following a 1 hr treatment with EGF, there was a loss of PKC from the cytosolic compartment, and a corresponding increase in the PKC level in the membrane compartment.

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

To investigate whether MAPKs are involved in EGF-induced increase of 2-DG uptake, SB 203580 (p38 MAPK inhibitor) or PD 98059 (p44/42 MAPKs inhibitor) was treated to the mouse ES cells for 30 min prior to the treatment of EGF. Figure 8A showed that SB 203580 and PD 98059 significantly prevented EGF-induced stimulation of 2-DG uptake ($P < 0.05$). Moreover, EGF enhanced phosphorylation of p38 MAPK and p44/42 MAPKs at 15 - 30 min after EGF treatment, respectively (Fig. 8B). Total expression of these MAPKs on Western blot showed no significant differences in the protein levels. These results suggest that p38 MAPK and p44/42 MAPKs are involved in the effect of EGF. We investigated the relationship between tyrosine kinase, PKC, and MAPKs in EGF-induced stimulation of 2-DG uptake. As shown in Fig. 9, genistein or bisindolylmaleimide I blocked EGF-induced phosphorylation of p38 MAPK and p44/42 MAPKs. On the other hand, genistein, bisindolylmaleimide I, SB 203580, or PD 98059 alone had no effect (data not shown). Furthermore, cycloheximide, herbimycin A, neomycin, staurosporine, SB 203580, or PD 98059 blocked the EGF-induced stimulation of GLUT1 protein expression level (Fig. 10).

Fig. 4. Effect of actinomycin D and cycloheximide on EGF-induced stimulation of 2-DG uptake and GLUT1 expression levels. (A) GLUT1, 2, 3, 4, and 5 mRNA expressions were determined by RT-PCR. Bands represent 544 bp GLUT1, 309bp GLUT2, 508bp GLUT3, 496bp GLUT4, 531bp GLUT5, and 350 bp β -actin. (B) Actinomycin D (10^{-7} M) or cycloheximide (4×10^{-6} M) was treated to the mouse ES cells for 30 min prior to the treatment of EGF. Values are means $\pm$ S.E. of 3 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. EGF alone. (C) GLUT1 mRNA expression level was determined by RT-PCR in mouse ES cells treated with EGF. (D) GLUT1 protein expression level was determined by Western blotting with plasma membrane protein as described in Materials and Methods in mouse ES cells which were treated with EGF (10 ng/ml) for 24 hr. Bands represent 50~60 kDa of GLUT1, and 41 kDa of β -actin, respectively.

Fig. 5. Effect of tyrosine kinase inhibitors on EGF-induced stimulation of 2-DG uptake. (A) ES cells were treated with AG1478 (10^{-5} M), herbimycin A, or genistein (10^{-6} M) for 30 min prior to treatment of EGF (10 ng/ml) for 24 hr. Values are means $\pm$ S.E. of 3 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. EGF alone. (B) Total lysates of mouse ES cells treated with EGF (10 ng/ml) for 5 min were subjected to SDS-PAGE and blotted with phospho-EGF receptor (EGFR) or total-EGFR antibody. The example shown is a representative of 5 experiments.

Fig. 6. Effect of PLC inhibitors on EGF-induced stimulation of 2-DG uptake and IP_s formation. (A) ES cells were treated with neomycin (10^{-4} M) or U 73122 (10^{-6} M) for 30 min prior to the treatment of EGF (10 ng/ml) for 24 hr. (B) Time course of EGF effect on IP_s formation. ES cells were treated with EGF for various time points (0 to 120 sec) before IP_s assay. Values are means $\pm$ S.E. of 3 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. EGF alone.

Discussion

The present results demonstrated that EGF could help mouse ES cells meet the increased metabolic demands of the growth processes that initiated by concurrently stimulating glucose transport. Mao et al [36] reported that EGF (10 ng/ml) stimulated granulosa cell proliferation for developing a preantral follicular culture system in which the oocytes are capable of fertilization and embryonic development. Consistent with the report, in the present study, minimal effective dosage of EGF to

Fig. 7. Effect of PKC inhibitors on EGF-induced stimulation of 2-DG uptake and PKC activation. (A) ES cells were treated with staurosporine or bisindolylmaleimide I (10^{-7} M) for 30 min prior to the treatment of EGF (10 ng/ml) for 24 hr. Values are means $\pm$ S.E. of 3 independent experiments with triplicate dishes. (B) PKC protein expression levels which was presented in either the cytosolic and membrane fraction were detected by means of Western blotting, as described in Materials and Methods. The arrow indicates the 82 kDa band corresponding to PKC. The lower panel depicts the bars denote means $\pm$ S.E. of 3 experiments for each condition determined from densitometry relative to β -actin. * $P < 0.05$ vs. each control, ** $P < 0.05$ vs. EGF alone.

Fig. 8. Effect of MAPK inhibitors on EGF-induced stimulation of 2-DG uptake and MAPKs phosphorylation. (A) SB 203580 or PD 98059 (10^{-6} M) was treated to the ES cells for 30 min prior to the treatment of EGF (10 ng/ml) for 24 hr. Values are the means $\pm$ S.E. of 3 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. EGF alone. (B) Representative autoradiographs of time course of p38 and p44/42 MAPKs phosphorylation by EGF. Mouse ES cells were treated with EGF for different time point (0 to 90 min). Each example shown is a representative of 4 experiments.

Fig. 9. Effect of genistein, bisindolylmaleimide I, PD 98059, and SB 203580 on EGF-induced phosphorylation of p38 MAPK (A) and p44/42 MAPKs (B). ES cells were treated with genistein (10^{-6} M), bisindolylmaleimide I (10^{-7} M), SB 203580 (10^{-6} M), or PD 98059 (10^{-6} M) for 30 min prior to the treatment of EGF. The lower panel depicts the bars denote means $\pm$ S.E. of 4 experiments for each condition determined from densitometry relative to total p38 or p44/42 MAPKs, respectively. Each example shown is a representative of 4 experiments. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. EGF alone.

induce stimulation of 2-DG uptake is 10 ng/ml. This result suggested that the concentration of EGF used in this study have the physiological action of EGF on 2-DG uptake. By kinetic analysis, the incubation of EGF caused a significant increase of $V_{\max}$ for 2-DG uptake, not affecting K_m value. These results could suggest that the enhanced 2-DG uptake is induced by the increase of uptake sites

resulted from the elevation of GLUT1 gene and protein expression levels. Indeed, it seemed that the alteration of GLUT1 expression levels run parallel with those of 2-DG uptake. Studies in murine embryos have demonstrated that GLUT1 is the major glucose transporter in the inner cell mass that contains the ES cells [26, 37] and is known to be expressed throughout

Fig. 10. Effect of cycloheximide, herbimycin A, neomycin, staurosporine, SB 203580, and PD 98059 on EGF-induced stimulation of GLUT1 protein expression level. Mouse ES cells were treated with cycloheximide (4×10^{-6} M), herbimycin A (10^{-6} M), neomycin (10^{-4} M), staurosporine (10^{-7} M), SB 203580 (10^{-6} M), or PD 98059 (10^{-6} M) for 30 min prior to the treatment of EGF. The lower panel depicts the bars denote means $\pm$ S.E. of 3 experiments for each condition determined from densitometry relative to β -actin. The example shown is a representative of 3 experiments. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. EGF alone.

preimplantation embryonic development from the oocyte through the blastocyst stage [26]. In contrast, other embryonic glucose transporters first appear at later stage than GLUT1. This suggests GLUT1 plays an important nutritional role in embryonic development, and previous studies indicated GLUT1 may be important in supplying glucose for the glycolytic pathway [26]. Previous studies in GLUT1-deficient embryos demonstrated that apoptosis increased, suggesting GLUT1 was protecting them from programmed cell death [38]. Therefore, we hypothesized that GLUT1 would be one of efficient isoforms to supply glucose to mouse ES cells. On the other hand, inhibitory effect of cycloheximide on EGF-stimulated 2-DG uptake suggests that the effects of EGF on this process are partially dependent upon the synthesis of new protein. Indeed, growth hormone stimulates glucose transport in the blastocyst under conditions designed to identify the cumulative effects of recruitment and synthesis of transporter de novo [39]. Recently we also observed that angiotensin II stimulated 2-DG uptake by increasing GLUT1 protein expression level in mouse ES cells [23]. Inclusion of GLUT1 stimulation in the array of events activated by the EGF may be a consequence of the increased energy and biosynthetic requirements of actively dividing cells (for example, inner cell mass of the blastocyst

where proliferative effects of IGF are manifested) [39]. As such, it represents an example of coordination of proliferation and metabolism. Our previous and present results of EGF-induced increase of mouse ES cell proliferation may suggest EGF-induced cell proliferation is partially involved with increase of glucose transporter activity [24]. Thus, EGF is beneficial for improving the development of embryos indirectly by increasing glucose availability as well as by direct action as a growth factor. Consequently, the apparent synthesis of GLUT1 proteins might represent a part of the specific regulatory program elicited by EGF in mouse ES cells. However, the functional significance of these results cannot be determined without experiments using embryos, which we could not do in this study.

Stem cell interacts with its environment through cell surface receptors that activate signaling pathways. In the present study, we suggest that EGF-stimulated 2-DG uptake in mouse ES cells is initiated by the binding of EGF to specific receptors because glucose transport in response to EGF was reduced by 97.5% in the presence of AG1478, EGF receptor tyrosine kinase inhibitor. Multiple signaling pathways in stem cell populations have been recognized and include those activated by cytokines [40]. Studies of the role of PLC in cell function are

Fig. 11. The hypothesized model for the signal pathways involved in EGF-induced increase of 2-DG uptake in mouse ES cells. EGF activates receptor tyrosine kinase (TK), which stimulates phospholipase C (PLC) to generate diacylglycerol (DAG). In turn, DAG activates protein kinase C (PKC), which stimulates mitogen-activated protein kinases (MAPKs) activation, continuously inducing the increase of 2-DG uptake. EGFR, EGF receptor; PIP₂, phosphatidylinositol-4,5-bisphosphate; GLUT1, glucose transporter 1. The solid line is the proposed pathway and the dashed line is suspected pathway.

hindered by the absence of a suitable specific cell permeable activator of PLC. In the absence of such an activator, the specific PLC inhibitor, U 73122 and neomycin were used in the present study. The fact that U 73122 and neomycin significantly blocked EGF-induced stimulation of 2-DG uptake indicates that PLC has a controlling role of glucose uptake in mouse ES cells. As ES cells are derived from the inner cell mass of the blastocyst, this work on the effect of PLC inhibition in ES cells is in agreement with the work of Stachecki and Armant [41] showing that the regulation of blastocyst formation in mice is mediated through a PLC-dependent pathway that can be inhibited by U 73122.

It is well known that PKC family, downstream of signaling by PLC, was activated by EGF or EGF receptor in various types of cells [42, 43]. The present results also demonstrated that EGF-induced stimulation of 2-DG uptake is involved in PKC pathway in mouse ES cells. Others have previously shown that, after exposure to phorbol esters, cultured fibroblasts exhibit increased expression of the mRNA encoding the HepG2/brain glucose transporter [44]. Our present results suggest that PKC activates GLUT1 expression, leading to a change of the kinetics of the transporter such as maximum velocity or turnover number. It was supported by that

PGF_{2α} enhance glucose transport in 3T3-L1 adipocytes by stimulating GLUT1 expression via a PKC-dependent mechanism [45]. The present results thus indicate that PKC may act in the regulation of GLUT1 gene expression. Indeed, several potential target sites for PKC are located in the promotor of GLUT1 gene [46, 47]. Furthermore, there is possibility that other signaling pathways must be involved in the regulation of glucose transport in the mouse ES cells under EGF treatment.

It has been known that PKC is responsible for the stimulation of MAPKs through the transactivation of EGF receptor [48] and PKC plays an important role in MAPKs-involved in keratinocyte cell proliferation through at least two PKC isoforms, PKCα and PKCμ [49]. In addition, PKC and p38 MAPK signaling pathways were involved in arsenite-stimulated GLUT1 expression and glucose uptake [50]. Thus, the activation of the PKC-MAPKs pathway is considered to be one of the important molecular mechanisms in the regulation of glucose transport. Recently, MAPK was reported as a regulator of GLUT2 and GLUT5 in intestinal brush-border membrane [51, 52]. In addition, Taha et al [53] reported that GLUT1 and GLUT3 were the downstream target induced by ERK1/2 and p38 MAPK. PKC is also known to activate MAPK through activation of the serine-threonine kinase Raf-1

[54]. In the present study, we observed that the inhibitors of p38 and p44/42 MAPKs blocked EGF-induced increase of 2-DG uptake. Therefore, it may be concluded that p38 and p44/42 MAPKs are involved in the activation of 2-DG uptake. Although cell type and experimental conditions were somewhat different, this link between p38 and p44/42 MAPKs and glucose uptake might be supported by our previous results [55]. The present results also showed that genistein or bisindolylmaleimide I blocked EGF-induced phosphorylation of p38 and p44/42 MAPKs. These results suggest the involvement of classic or novel PKCs in the signal transduction pathway following EGF receptor activation [24] and consequently EGF-induced PKC activation enhanced p38 and p44/42 MAPKs phosphorylation. To our knowledge, this is the first report to show EGF stimulated glucose uptake through activation

of PKC and p38 and p44/42 MAPKs in mouse ES cells (Fig. 11). According to the present and previous reported studies, EGF is in part suggested to play a role during ES cells self-renewal through interaction with glucose transporters. In conclusion, EGF increased the 2-DG uptake through PKC, p38 and p44/42 MAPKs pathways in the mouse ES cells.

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