Hydrogen Peroxide Increases [^3H]-2-Deoxyglucose uptake via MAPKs, cPLA $_2$, and NF- κB Signaling Pathways in Mouse Embryonic Stem Cells

Sun Im Na, Min Young Lee, Jung Sun Heo and Ho Jae Han

Department of Veterinary Physiology, Biotherapy Human Resources Center, College of Veterinary Medicine, Chonnam National University

Key Words

Embryonic stem cells • H_2O_2 • MAPKs • cPLA $_2$ • NF- κB

Abstract

Hydrogen peroxide (H_2O_2) has been shown to act as a signaling molecule that is involved in many cellular functions. This study investigated the effect of H_2O_2 on the [^3H]-2-deoxyglucose (2-DG) uptake and its related signaling pathways in mouse embryonic stem (ES) cells. H_2O_2 significantly increased the level of 2-DG uptake in a time- (> 4 hr) and concentration- (> 10^{-4} M) dependent manner due to an increase in V_{max} but not K_m . Indeed, H_2O_2 increased the mRNA and protein level of glucose transporter 1 (GLUT 1). PD 98059 (a p44/42 MAPKs inhibitor, 10^{-5} M), SB 203580 (a p38 MAPK inhibitor, 10^{-6} M), and SP 600125 (a SAPK/JNK inhibitor, 10^{-6} M) blocked the H_2O_2 -induced increase in 2-DG uptake. H_2O_2 also increased phosphorylation of p44/42 mitogen activated protein kinases (MAPKs), p38 MAPK, and stress-activated protein kinase/Jun-N-terminal kinase (SAPK/JNK). In addition, H_2O_2 stimulated the translocation of cytosolic phospholipase A $_2$ (cPLA $_2$) from the cytosolic fraction to the membrane fraction, the release of arachidonic

acid, and the activation of NF- κB . AACOCF $_3$ or mepacrine (cPLA $_2$ inhibitors, 10^{-6} M), SN 50 (NF- κB nucleus translocation inhibitor, 500 ng/ml) or Bay11-7082 (a I κB - α phosphorylation inhibitor, 2×10^{-5} M) blocked the H_2O_2 -induced increase in 2-DG uptake. H_2O_2 increased the protein level of glucose transporter 1 (GLUT 1), which was blocked by PD 98059, SB 203580, SP 600125, mepacrine, or Bay11-7082. In conclusion, H_2O_2 increases the 2-DG uptake via MAPKs, cPLA $_2$, and NF- κB signaling pathways in mouse ES cells.

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Introduction

Oxygen is an important component of the oviductal and uterine environments and may have important roles in regulating embryonic development, particularly through the regulation of metabolism [1-3]. Embryos from mice [4, 5] cultured *in vitro* under low (5%) O_2 concentrations have been reported to show higher developmental rates than those cultured in 20% O_2 [6, 7]. Studies in several species report that low (5%) O_2 concentrations enable greater numbers of embryo to develop to the blastocyst stage *in vitro* [8, 9]. In contrast, this suggests that a high O_2 concentration during the *in vitro* culture reduces

developmental ability, possibly by increasing the accumulation of reactive oxygen species (ROS) in the cytoplasm of the developing embryos [10, 11]. This oxygen gradient may have an important regulatory function in the programming of normal embryo development. Indeed, ROS now appear to play a role as key second messengers in several signaling pathways, eliciting a wide variety of biological responses ranging from transcriptional regulation, differentiation and proliferation to oncogenic transformation [12, 13]. Recent evidence suggests that ROS, such as superoxide anions and hydrogen peroxide, function as intracellular second messengers [14, 15]. The production of ROS is an inevitable consequence of the normal oxidative metabolism. ROS generation at low concentrations by cells may play roles as signaling molecules within the signal transduction cascades triggered by growth factors, cytokines, and hormones [16]. In addition, they may act as direct cellular stimuli themselves, mimicking in this manner the action of these receptor-directed stimuli. Therefore, oxidative stress of tissues and cells is widely recognized as a key factor in the development of embryos. However, high levels of ROS can be lethal [17, 18].

Glucose is the main fuel for most cells, and its importance as an energy substrate has led to intense research on its cellular metabolites as well as on the mechanism controlling its uptake. A vast number of studies have been carried out on the glucose metabolism in mammalian preimplantation embryos. Most reports have shown that the presence of glucose in the early preimplantation embryos up to the 8-cell stage is detrimental to further embryonic development *in vitro* in several species such as mice [19] and humans [20]. In contrast, there are reports that glucose is not always an inhibitor of mouse preimplantation development *in vitro* [21, 22].

Glucose enters the cells as a result of its concentration gradient through a well-conserved group of transporter protein isoforms (GLUTs) present in the plasma membrane. The most abundant isoform, GLUT1, which was first identified as the erythrocyte glucose transporter, exhibits a broad distribution among the cells and provides low levels of glucose for the basal cellular metabolism [23]. Its expression is detectable throughout pre-implantation development from the oocytes through the blastocyst stage [24] and increases 11-fold in developing embryos from the two-cell stage to the blastocyst stage [25]. This may reflect in part the finding that GLUT1 is one of the major glucose transporters in the inner cell mass of the pre-implantation embryo. H_2O_2

has been reported to stimulate many cellular processes, particularly, glucose transport in various cell types [26]. These reports suggest that H_2O_2 is retained in mouse ES cells, causing a modification of the ES cells functions, which is caused, in part, by alterations affecting the glucose transporters of mouse ES cells. H_2O_2 can affect the function of ES cells through a similar spectrum of diverse signaling pathways. However, the mechanisms underlying the effects of H_2O_2 on the glucose transporter of ES cells have not been reported.

Embryonic stem cells are pluripotent cell lines derived from the blastocyst-stage of an early mammalian embryo. These unique cells are characterized by their capacity for prolonged undifferentiated proliferation in tissue culture, with the potential to differentiate into derivatives of all three germ layers [27]. Therefore, mouse ES cells may be regarded as a versatile biological system that has led to major advances in cellular and developmental biology [28, 29]. The growth of mouse ES cells in culture is likely to require the increased uptake of glucose and other substrates [30]. Therefore, mouse ES cells might be a powerful tool for examining the effect of H_2O_2 on glucose transport in the inner cell mass of an early embryo [31, 32]. In order to investigate the mechanism by which H_2O_2 modulates 2-DG uptake by mouse ES cells, this study examined the effect of H_2O_2 on the uptake of glucose as well as its related signal cascades in mouse ES cells.

Materials and Methods

Materials

The mouse ES cells were obtained from the American Type Culture Collection (ES-E14TG2a). Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, MD, USA). Hydrogen peroxide (H_2O_2), N-acetyl-L-cysteine (NAC), catalase, genistein, SB 203580, SP 600125, arachidonyl trifluoromethyl ketone (AACOCF₃), mepacrine, and monoclonal anti- β -actin were supplied by Sigma Chemical Company (St. Louis, MO, USA). Herbimycin A, PD 98059, and SN 50 were acquired from Calbiochem (La Jolla, CA). Bay11-7082 was purchased from Biomol (Butler Pike, PA). [³H]-2-deoxyglucose was obtained from Dupont/NEN (Boston, MA, USA). Rabbit anti-GLUT-1, cPLA₂, and NF- κ B p65 antibodies were supplied by Santa Cruz Biotechnology (Delaware, CA, USA). Phospho-p44/42, p44/42, phospho-p38, p38, phospho-SAPK/JNK, and SAPK/JNK antibodies were acquired from New England Biolabs (Herts, UK). Goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Liquiscint was obtained from National Diagnostics (Parsippany, NY, USA). All other reagents were of the highest purity commercially available.

ES cell culture

The mouse ES cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL, Gaithersburg, MD) 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, and 15% fetal bovine serum (FBS) without a feeder layer. This was followed by further culturing for five days in a standard medium plus leukemia inhibitory factor (LIF). The cells were grown on gelatinized 12-well plates or 60 mm culture dishes in an incubator maintained at 37°C in an atmosphere containing 5% CO₂ in air. The media were changed to serum-free DMEM (5 mM glucose) at 24 hr prior to the experiment.

2-DG uptake experiment

The 2-DG uptake experiments were carried out using a slight modification of the method described by Henriksen et al [33]. The uptake of 2-DG was examined by removing the culture medium by aspiration, and gently washing the monolayers twice with the uptake buffer (140 mM NaCl, 2 mM KCl, 1 mM KH₂PO₄, 10 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, 5 mM L-alanine, 5 μ M indomethacin, and 10 mM HEPES/Tris, pH 7.4). After washing, the cells were incubated at 37°C for 30 min in an uptake buffer containing 1 μ Ci/ml 2-DG. At the end of the incubation period, the cells were again washed three times with ice-cold uptake buffer, and dissolved in 1 ml 0.1% sodium dodecyl sulfate (SDS). The amount of 2-DG uptake incorporated intracellularly was determined by removing 900 μ l of each sample and counting the radioactivity using a liquid scintillation counter (LS 6500, Beckmann Coulter, Inc., Fullerton, CA, USA). The remainder of each sample was used for protein determination. The radioactivity counts in each sample were then normalized to the protein level and were corrected for zero-time uptake per mg protein. All the uptake measurements were made in triplicate.

AA release

AA release from the cultures was determined by a modification of the method described by Xing et al [34]. To summarize, confluent monolayers of ES cells were incubated for 24 hr in basal medium containing 0.5 μ Ci [³H]-AA/ml, as well as the three growth supplements. The cells were washed three times with culture medium, and incubated (at 37°C) for 1 hr in incubation medium containing specified agents. At the end of the 1 hr incubation period, the incubation medium was removed, and transferred to ice-cold tubes containing 100 μ l of 55 mM EGTA and EDTA (final concentration, 5 mM each). The incubation medium was then centrifuged at 12,000 g to eliminate cell debris. To determine radioactive levels, aliquots of the samples were placed in scintillation vials containing scintillation fluid, and the radioactivity was counted using a liquid scintillation counter. The cells which remained attached to the plates were scraped into 1 ml of 0.1% SDS, and 900 μ l of the resulting cell lysate was utilized for scintillation counting. The remaining 100 μ l of the cell lysate was utilized for protein determinations. The quantity of [³H]-AA released in each condition (determined as described above) was then standardized with respect to protein. Subsequently, this

standardized level of released [³H]-AA was compared percentage-wise to the level of total [³H]-AA which had been incorporated into the cells at the start of the 1 hr incubation period with the specified agents (equivalent to the quantity of total released radioactivity plus the cell-associated radioactivity at the end of the 1 hr incubation period).

Preparation of cytosolic and total membrane fractions

Preparation of cytosolic and total membrane fractions were prepared using a slight modification of the method reported by Mackman et al [35]. DMEM of mouse ES cells were exchanged 48 hr prior to the experiments. The medium was then removed and the cells were washed twice with ice-cold PBS, scraped, harvested by micro-centrifugation and re-suspended in buffer A [137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 μ g/ml leupeptin (pH 7.5)]. The re-suspended cells were then mechanically lysed on ice by trituration with a 21.1-gauge needle. The lysates were initially centrifuged at 1,000g for 10 min at 4°C. The supernatant was then centrifuged at 100,000g for 1 hr at 4°C to prepare the cytosolic and total particulate fractions. 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 resulting pellet was re-suspended in buffer A containing 1% (v/v) Triton X-100. The particulate fractions, which contained the membrane fraction, were washed twice and re-suspended in buffer A containing 1% (v/v) Triton X-100. The protein in each fraction was quantified using the Bradford procedure [36]. Twenty micrograms of protein was used for Western blot analysis.

RNA isolation and RT-PCR

The total RNA was extracted from the mouse ES cells using STAT-60, which is a monophasic solution of phenol and guanidine isothiocyanate from Tel-Test, Inc. (Friendwood, Tex., USA). Two micrograms of purified RNA was synthesized into cDNA using avian leukemia virus reverse transcriptase with oligodT(18)-primers. Subsequently, 5 μ l of RT products was amplified using Taq DNA polymerase followed by denaturation at 94°C for 5 min and 40 cycles of [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 used were 5'-AGC GTC ATC TTC TCC CAG -3' (sense), 5'-CCA CAA TGC TCA GGT AGG AC-3' (antisense) for GLUT-1 (544 bp) and 5'-AAC CGC GAG AAG ATGA CCC AGA TCA TGT TT-3' (sense), 5'-AGC AGC GTG GCC ATC TCT TGC TCG AAG TC-3' (antisense) for β -actin (350 bp). In addition, PCR of β -actin was performed as a control for the quantity of RNA.

Western blot analysis

The cell homogenates (20 μ g protein) were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. After blots had been washed with TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], the membranes were blocked with 5% skimmed milk for 1 hr and incubated with the appropriate primary antibody at the dilutions recommended by the supplier. The membrane was then washed, and the primary antibodies were detected with goat anti-rabbit

Fig. 1. Time course (A) and dose response (B) of the effect of H_2O_2 on 2-DG uptake. The ES cells were treated with H_2O_2 (100 μM) for various time points (0 to 8 hr) or with different doses of H_2O_2 (0 to 1 mM) for 4 hr before the uptake experiment. The values are reported as the mean $\pm$ S.E. of 4 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control.

IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. The bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK).

Statistical analysis

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

Results

Effect of H_2O_2 on 2-DG uptake

In order to examine the effect of H_2O_2 on 2-DG uptake, the level of 2-DG uptake was first observed with 100 μM H_2O_2 at different times (0-8 hr), followed by various H_2O_2 concentrations (0-1 mM) for 4 hr. As shown in Fig. 1A and B, H_2O_2 increased the level of 2-DG uptake in both a time- and dose-dependent manner. The maximum increase in 2-DG uptake was observed after incubating the cells with 100 μM for 4 hr (57% increase vs. control; $P < 0.05$). Moreover, 100 μM H_2O_2 (38% increase vs. control; $P < 0.05$) significantly increased the level of ^3H -2-DG uptake after 4 hr incubation. The Lineweaver-Burk plot was used to determine if H_2O_2 affects either the affinity of 2-DG for the uptake process (K_m) or the maximal velocity ($V_{\max}$). As shown in Fig. 2, the kinetic analysis of the uptake data indicated a 41 % higher $V_{\max}$ for 2-DG uptake in the H_2O_2 -treated cells than the control, whereas there was no change in K_m .

Fig. 2. Effect of H_2O_2 on the kinetic parameters of 2-DG uptake. Mouse ES cells were incubated for 4 hr in the presence of 100 μM H_2O_2 . The cells were then incubated in different concentrations of 2-DG. The level of 2-DG uptake was measured in a buffer containing ^3H -2-DG in the presence of 0.5, 1, 2 or 4 mM unlabeled 2-deoxyglucose. To avoid curved plot Lineweaver-Burk plot was used so that data can be plotted to give a straight line. The slope and intercepts represent K_m and $V_{\max}$. The values are reported as the mean $\pm$ S.E. of 4 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control.

The expression of GLUT1 was detected by RT-PCR and Western blot to determine if the H_2O_2 -induced increase in 2-DG uptake is dependent on gene transcription and

Fig. 3. Effect of H_2O_2 on GLUT1 expression levels. (A) GLUT1 mRNA expression level was determined by RT-PCR in mouse ES cells treated with H_2O_2 . (B) The GLUT1 protein expression level in mouse ES cells treated with H_2O_2 (100 μ M) for 4 hr was determined by Western blotting with the plasma membrane protein, as described in Materials and Methods. The bands represent 50~60 kDa of GLUT1, and 41 kDa of β -actin, respectively. (C) Representative autoradiographs of the dose course of GLUT1 expression levels by H_2O_2 . The ES cells were treated with different doses of H_2O_2 (0 to 1 mM) for 4 hr. The bars in the lower panel 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 independent experiments. * P < 0.05 vs. Control.

protein synthesis. As shown in Fig. 3, H_2O_2 significantly increased the GLUT1 mRNA (52% increase vs. control; P < 0.05) and protein expression levels in the membrane fraction (64% increase vs. control; P < 0.05) (Fig. 3A, B). Moreover, the level of GLUT1 expression was H_2O_2 -dose dependent (Fig. 3C). Pretreating the cells with the antioxidants, NAC and catalase, blocked the H_2O_2 -induced stimulation of 2-DG uptake (Fig. 4). ES cells were preincubated with AG1478, EGF receptor tyrosine kinase inhibitor, or herbimycin A or genistein, tyrosine kinase inhibitors for 30 min prior to the H_2O_2 treatment to determine if intact tyrosine kinase activity is essential for the H_2O_2 -induced stimulation of 2-DG uptake. As shown in Figure 5A, H_2O_2 and EGF stimulated the phosphorylation of the EGF receptor. However, AG 1478 blocked the H_2O_2 -induced phosphorylation of the EGF receptor. Herbimycin A and genistein prevented H_2O_2 -induced stimulation of 2-DG uptake (Fig. 5B). The solvents itself were treated in control, respectively.

Fig. 4. Effect of antioxidants on the H_2O_2 -induced stimulation of 2-DG uptake. The mouse ES cells were pretreated with N-acetyl-L-cysteine (NAC, 1 μ M) or catalase (600 U/ml) for 30 min before the H_2O_2 treatment. The values are reported as the mean $\pm$ S.E. of 3 independent experiments with triplicate dishes. * P < 0.05 vs. Control, ** P < 0.05 vs. H_2O_2 alone.

Fig. 5. Effect of the tyrosine kinase inhibitors on the H_2O_2 -induced stimulation of 2-DG uptake. (A) The mouse ES cells were pretreated with AG1478 (EGF receptor tyrosine kinase inhibitor, 10^{-5} M) or herbimycin A (10^{-6} M) for 30 min prior to H_2O_2 treatment. The bars in the lower panel denote means $\pm$ S.E.

of 3 experiments for each condition determined from densitometry relative to β -actin. (B) The mouse ES cells were pretreated with herbimycin A or genistein (10^{-6} M) for 30 min before the H_2O_2 treatment. The values are reported as the mean $\pm$ S.E. of 3 independent experiments with triplicate dishes. * P < 0.05 vs. Control, ** P < 0.05 vs. H_2O_2 alone.

Fig. 6. Effect of H_2O_2 on MAPKs activation. (A) Representative autoradiographs of the time course of p44/42 MAPKs, p38 MAPK, and SAPK/JNK phosphorylation by H_2O_2 . The mouse ES cells were treated with H_2O_2 for different times (0 to 120 min). Each example shown is a representative of 4 experiments. (B) ES cells were treated with AG1478 (10^{-5} M) or herbimycin A (10^{-6} M) for 30 min prior to H_2O_2 (100 μ M) treatment of for 1 hr. Each example shown is a representative of 4 experiments. (C) ES cells were treated with PD 98059 (a p44/42 MAPK inhibitor, 10^{-5} M), SB 203580 (a p38 MAPK inhibitor, 10^{-6} M), and SP 600125 (a SAPK/JNK inhibitor, 10^{-6} M) for 30 min before the H_2O_2 (100 μ M) treatment of for 4 hr. The bars in the lower panel denote means $\pm$ S.E. of 3 experiments for each condition determined from densitometry relative to β -actin. (D) ES cells were treated with PD 98059, SB 203580, and SP 600125 for 30 min before the H_2O_2 (100 μ M) treatment for 4 hr. The values are reported as the mean $\pm$ S.E. of 3 independent experiments with triplicate dishes. * P < 0.05 vs. Control, ** P < 0.05 vs. H_2O_2 alone.

Fig. 7. Effect of H_2O_2 on [3H]arachidonic acid (AA) release and cytoplasmic phospholipase A_2 (cPLA $_2$) translocation and effect of cPLA $_2$ inhibitors on H_2O_2 -induced stimulation of 2-DG uptake. (A) The ES cells were treated with different doses of H_2O_2 (0 to 1 mM) for 60 min. The values are reported as the mean $\pm$ S.E. of 3 independent experiments with triplicate dishes. (B) cPLA $_2$ protein expression levels, which was presented in either the cytosolic and membrane fraction were detected by Western blotting, as described

in Materials and Methods. The band represent 110 kDa of cPLA $_2$. (C) ES cells were treated with AACOCF $_3$ or mepacrine (10^{-6} M) for 30 min before the H_2O_2 (100 μM) treatment for 4 hr. The values are reported as the mean $\pm$ S.E. of 3 independent experiments with triplicate dishes. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. H_2O_2 alone.

Involvement of MAPKs in H_2O_2 -induced increase of 2-DG uptake

To determine if MAPKs are involved in the H_2O_2 -induced increase of 2-DG uptake, the ES cells were treated with 100 μM H_2O_2 for 120 min. H_2O_2 enhanced level of p44/42 MAPKs, p38 MAPK, and SAPK/JNK phosphorylation at 15-30 min after the H_2O_2 treatment (Fig. 6A). The total expression of these MAPKs on the Western blot was similar to the protein levels. As shown in Figure 6B, AG1478 or herbimycin A inhibited H_2O_2 -induced p44/42 MAPKs and p38 MAPK phosphorylation. Moreover, PD 98059 (p44/42 MAPKs inhibitor, 10^{-5} M), SB 203580 (p38 MAPK inhibitor, 10^{-6} M), or SP 600125 (SAPK/JNK inhibitor, 10^{-6} M) blocked the H_2O_2 -induced stimulation of the GLUT1 protein expression level (Fig. 6C) and prevented the H_2O_2 -induced stimulation of 2-DG uptake ($P < 0.05$) (Fig. 6D). This suggests that p44/42 MAPKs, p38 MAPK, and SAPK/JNK are involved in the H_2O_2 -induced stimulation of 2-DG uptake.

Involvement of cPLA $_2$ in H_2O_2 -induced increase of 2-DG uptake

The effect of H_2O_2 on the release of [3H]-AA was examined in order to determine the role of the cPLA $_2$

Fig. 8. Effect of MAPKs inhibitors on the H_2O_2 -induced stimulation of cPLA $_2$. ES cells were treated with PD 98059, SB 203580, and SP 600125 for 30 min before the H_2O_2 (100 μM) treatment for 4 hr. The bars in the lower panel denote means $\pm$ S.E. of 3 experiments for each condition determined from densitometry relative to β -actin. * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. H_2O_2 alone.

Fig. 9. Relationship between MAPKs, cPLA₂, and NF-κB on H₂O₂-induced stimulation of 2-DG uptake. (A) ES cells were treated with H₂O₂ for different time intervals (0-120 min). (B) ES cells were treated with PD 98059, SB 203580, SP 600125, and mepacrine for 30 min before the H₂O₂ treatment for 4 hr. The bars in the lower panel denote the means ± S.E. of 3 experiments for each condition determined from densitometry relative to β-actin. The example shown is a representative of 3 experiments. (C) ES cells were treated with SN 50 (NF-κB nucleus translocation inhibitor, 500ng/ml) or Bay 11-7082 (IκB-α phosphorylation inhibitor, 2x10⁻⁵ M) for 30 min before the H₂O₂ treatment for 4 hr. 2-DG uptake was assessed. (D) ES cells were treated with SN 50 (500 ng/ml) or Bay 11-7082 (2x10⁻⁵ M) for 30 min before the H₂O₂ treatment and then cPLA₂ was detected by Western blotting. The bars in the lower panel denote means ± S.E. of 3 experiments for each condition determined from densitometry relative to β-actin. The values are reported as the mean ± S.E. of 3 independent experiments with triplicate dishes. **P* < 0.05 vs. Control, ***P* < 0.05 vs. H₂O₂ alone.

signal pathway in H₂O₂-induced increase of 2-DG uptake. Figure 7A shows that 1 hr incubation with 100 μM H₂O₂ significantly increased the level of [³H]-AA released. The translocation of cPLA₂ from the cytosolic to the membrane compartment was observed after treating the cells with 100 μM H₂O₂ for 1 hr (Fig. 7B). Moreover, AACOCF₃ and mepacrine (cPLA₂ inhibitors, 10⁻⁶ M) blocked the H₂O₂-induced stimulation of 2-DG uptake (Fig. 7C). Next, the cells were pretreated with PD 98059, SB203580, and SP 600125 for 30 min prior to the H₂O₂ treatment to determine the relationship between MAPKs and cPLA₂. As shown in Fig. 8, each inhibitor attenuated the H₂O₂-induced stimulation of the cPLA₂ expression level.

Involvement of NF-κB in H₂O₂-induced increase of 2-DG uptake

The ES cells were treated with H₂O₂ to determine if NF-κB is involved in the H₂O₂-induced stimulation of 2-DG uptake. H₂O₂ increased the level of p65 NF-κB phosphorylation in a time dependent manner (Fig. 9A). In addition, PD 98059, SB203580, SP 600125, and mepacrine inhibited the H₂O₂-induced stimulation of NF-κB expression (Fig. 9B). Moreover, the mouse ES cells were treated with SN 50 (NF-κB nucleus translocation inhibitor, 500 ng/ml) or Bay 11-7082 (IκB-α phosphorylation inhibitor, 2x10⁻⁵ M) for 30 min prior to the H₂O₂ treatment. Figure 9C shows that SN 50 and Bay 11-7082 prevented the H₂O₂-induced stimulation of

Fig. 10. Effect of mepacrine and Bay 11-7082 on the H₂O₂-induced stimulation of MAPKs activation (A) and GLUT1 expression (B). ES cells were treated with mepacrine and Bay 11-7082 for 30 min before the H₂O₂ treatment. The bars in the lower panel denote the 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. H₂O₂ alone.

2-DG uptake ($P < 0.05$), but not that of cPLA₂ expression (Fig. 9D). Furthermore, mepacrine and Bay11-7082 blocked the H₂O₂-induced stimulation of and GLUT1 protein expression (Fig. 10B) and 2-DG uptake (Fig. 7C, 9C), but not that of p44/42 MAPKs and p38 MAPK phosphorylation (Fig. 10A).

Discussion

These results show that 1) H₂O₂ stimulates the uptake of 2-DG by mouse ES cells; and 2) the stimulatory effect of H₂O₂ on 2-DG uptake is mediated via MAPKs, cPLA₂, and NF- κ B signal pathways. In particular, the activity of the glucose transporter system in the ES cells is notably affected under conditions of oxidative stress [37]. Previously, the effects of oxidative stress on the transport system have been examined in a number of other tissues, including the intestine and the placenta, which also have high expression levels of the glucose transporters [38]. These results show that H₂O₂ can help mouse ES cells meet the increased metabolic demands of the growth processes that are initiated by concurrently stimulating glucose transport. However, high concentration of H₂O₂ has negative effect on cell growth and functions.

Kinetic analysis revealed that the incubation of H₂O₂ caused a significant increase in $V_{\max}$ for 2-DG uptake,

not affecting the K_m . This suggests that the enhanced 2-DG uptake is induced by the increase in the number of uptake sites resulting from the increase in the GLUT1 gene and protein expression levels. Indeed, it appears that the alteration of the GLUT1 expression levels occurs in conjunction with the 2-DG uptake. Studies in murine embryos have demonstrated GLUT1 to be the major glucose transporter in the inner cell mass that contains ES cells [30, 39] and is expressed throughout preimplantation embryonic development from the oocytes through to the blastocyst stage. In contrast, other embryonic glucose transporters first appear at a stage later than GLUT1. This suggests GLUT1 plays an important nutritional role in embryonic development, and previous studies have indicated that GLUT1 may be important for supplying glucose for the glycolytic pathway [30]. Previous studies in GLUT1-deficient embryos demonstrated an increase in apoptosis, suggesting that GLUT1 offers protection against programmed cell death [40]. Moreover, previous study showed that GLUT1 expression has been described for mouse (D3) and human (H1) ES cells [41] and the GLUT1^{-/-} cells were non-viable [30], indicating that GLUT1 is the main glucose transporter isoform in ES cells. Recently, it was observed that hypoxia stimulated the uptake of 2-DG by increasing the GLUT1 protein expression level in mouse ES cells [42]. Therefore, H₂O₂ is beneficial for improving the

development of embryos indirectly by increasing the glucose availability. Consequently, the apparent synthesis of the GLUT1 proteins might represent a part of the specific regulatory program elicited by H_2O_2 in mouse ES cells. However, the functional significance of these results cannot be determined without experiments using embryos, which was not done in this study.

Glucose transporter can be regulated by various signaling molecules. In previous report, insulin-activated glucose transport in ES cells is PKC dependent involving the activation of nonreceptor proline-rich tyrosine protein kinase-2, which in turn activates ERK pathway [43]. Our previous study also showed that epidermal growth factor increased glucose transporter activity through PKC, p38 and p44/42 MAPKs pathways in the mouse ES cells [32]. The present results suggest that the tyrosine kinase cascade is involved in stimulating the uptake 2-DG induced by H_2O_2 . It should be noted that although oxidative stress apparently activates protein tyrosine kinases, there is no evidence showing that oxidants stimulate these activities directly. Although there are many potential targets for the actions of H_2O_2 , oxidants can now be regarded as selective modulators of the signal transduction pathways [44]. Included among these targets are known substrates for MAPKs, including other protein kinases, PLA_2 , and transcription factors. However, the precise role of the MAPKs in the process by which H_2O_2 increases the activity of the glucose transporter system in mouse ES cells is not completely understood. The results of this study suggest that p44/42 MAPKs, P38 MAPK, and JNK are involved in mediating the stimulatory effects of H_2O_2 on 2-DG uptake. Not only was MAPKs phosphorylated after the H_2O_2 treatment but the MAPKs inhibitors blocked the H_2O_2 -induced increase in 2-DG uptake. This suggests that MAPKs itself may be an intracellular target of oxidative stress in the mouse ES cells that mediates, at least in part, the cell response to H_2O_2 . Previously, H_2O_2 was found to induce the release of arachidonic acid (AA) in other cell types, which could be attributed to the activation of p44/42 MAPKs, and the consequent phosphorylation and activation of cytosolic phospholipase A_2 (cPLA $_2$) [45]. These results showed that H_2O_2 individually stimulated the phosphorylation of p44/42 MAPKs, and the release of AA by mouse ES cells. These observations might be explained by the simple hypothesis that activation of the p44/42 MAPKs pathway by H_2O_2 is responsible for the phosphorylation and stimulation of cPLA $_2$, along with the consequent increase in AA release [46]. The activation of cPLA $_2$ has been associated with processes that lead to the uptake of 2-DG. Indeed, p44/

42 MAPKs has been shown to phosphorylate cPLA $_2$ at Ser505, causing a shift in the electrophoretic mobility of cPLA $_2$ as well as an increase in enzyme activity [47]. This type of cPLA $_2$ phosphorylation and activation by MAPKs is in all probability not the only route for the activation of cPLA $_2$. There are many possible phosphorylation sites on cPLA $_2$, and multiple protein kinases may regulate its activity [48].

Another signaling pathway that engages in unique cross-talk with ROS is the pathway mediating the activation of the nuclear factor-kappa B (NF- κ B) family transcription factors [49, 50]. New evidence has unveiled the existence of cross-talk between the ROS/MAPKs and NF- κ B pathways. This study provides evidence showing that NF- κ Bs are activated for more than 15 min after a H_2O_2 treatment and that the activation of NF- κ Bs is inhibited by pretreating the cells with MAPKs inhibitors. These results suggest that NF- κ B is activated in mouse ES cells possibly through the activation of MAPKs and oxidative stress. These observations of the activation of NF- κ B in response to H_2O_2 are in agreement with Gloire et al [51] and Li et al [52]. However, in these reports, NF- κ B activation by H_2O_2 resulted in the inhibition of glucose uptake, which is unlike the case with our mouse ES cells. The difference in response may be explained by cell type differences, as well as differences in the signaling pathways involved. The results in this study suggest that cPLA $_2$ activation is involved NF- κ B. Although the physiological effect of the H_2O_2 -induced up-regulation of the glucose transporters is not known, it has been suggested that the up-regulation of GLUT1 under H_2O_2 might be a mechanism for embryonic growth from a hypoxic status at the pre-implantation stage of development. However, further studies will be needed to determine the relevance of these findings to the onset of embryonic development or the functional regulation of embryonic stem cells. In conclusion, H_2O_2 increases, in part, the uptake of 2-DG in mouse ES cells via MAPKs, cPLA $_2$, and NF- κ B signal cascades.

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