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Journal of Cellular Biochemistry 106:787–797 (2009)

Journal of Cellular Biochemistry

Arachidonic Acid Release by H_2O_2 Mediated Proliferation of Mouse Embryonic Stem Cells: Involvement of Ca^{2+}/PKC and MAPKs-Induced EGFR Transactivation

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ABSTRACT

Reactive oxygen species (ROS) generated by a variety of endogenous factors and roles in embryonic stem (ES) cells has yet to be identified. Thus, we examined role of arachidonic acid (AA) in H_2O_2-indued proliferation of mouse ES cells and its related signaling molecules. AA release was maximally increased in response to 10^{-4} M H_2O_2 for 1 h. In addition, H_2O_2 increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the phosphorylation of protein kinase C (PKC), p44/42, p38 mitogen-activated protein kinase (MAPK), and JNK/SAPK. Moreover, H_2O_2 induced an increase in the phosphorylation of epidermal growth factor receptor (EGFR), which was blocked by the inhibition of p44/42 or p38 MAPKs. The inhibition of each signal molecule with specific inhibitors blocked H_2O_2-induced cytosolic phospholipase A_2 ($cPLA_2$) activation and AA release. H_2O_2 increased NF-κB phosphorylation to induce an increase in the levels of cyclooxygenase (COX)-2 proteins. Subsequently, H_2O_2 stimulated PGE_2 synthesis, which was reduced by the inhibition of NF-κB activation. Moreover, each H_2O_2 or PGE_2 increased DNA synthesis and the number of cells. However, H_2O_2-induced increase in DNA synthesis was inhibited by the suppression of $cPLA_2$ pathway. In conclusion, H_2O_2 increased AA release and PGE_2 production by the upregulation of $cPLA_2$ and COX-2 via Ca^{2+}/PKC/MAPKs and EGFR transactivation, subsequently proliferation of mouse ES cells. J. Cell. Biochem. 106: 787–797, 2009.

KEY WORDS: H_2O_2; ROS; ARACHIDONIC ACID; PGE_2; EGFR; MOUSE ES CELL PROLIFERATION

Moderate levels of ROS have been shown to directly or indirectly modulate the functions of many enzymes and transcription factors via a multitude of signaling cascades, although excessive ROS results in oxidative damage and induces a cytotoxic effect in cells [Suzuki et al., 1997; Kunsch and Medford, 1999; Miura et al., 2003]. ROS play a role as a primary signal and/or second messenger in low concentration, eliciting a wide variety of biological responses ranging from transcriptional regulation, differentiation, and proliferation to oncogenic transformation [Seifried et al., 2007; Venkatesan et al., 2007]. Indeed, 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 [Prata et al., 2004]. Among the physiological roles, ROS was reported as one of factors inducing AA release through increased $cPLA_2$ activation [Chen et al., 1996]. The lipid-mediated metabolite of AA is emerging as key regulator of cell proliferation, chemotaxis, and signal transduction [Siegel et al., 1982; Axelrod et al., 1988; Peppelenbosch et al., 1995]. It has been suggested that PLA_2 is important for several stages of early embryonic development [Farber et al., 1999; Vitale et al., 2005]. The other coordinated AA–metabolizing enzyme is COX, which converts AA to eicosanoids including prostaglandins (PGs), leukotrienes and lipoxins. Moreover, the preimplantation embryos from zygote to the two-cell stage expressed COX-1, while the later stage embryos from eight-cell to blastocyst expressed COX-2 [Wang et al., 2002]. PGE_2 is known to be involved in a wide variety of physiological and pathological processes. The PGs generated by embryos have been implicated in a number of embryonic development processes [Lewis, 1989]. It has also been reported that PGE_2 was detected in the medium of cultured human embryos [Holmes et al., 1990] and modulated the expansion of hematopoietic stem cells [North et al., 2007]. Moreover, COX-2-induced PGE_2 protected against H_2O_2-mediated apoptosis in mouse ES cells [Liou et al., 2007]. However, signaling molecules contributed to the H_2O_2-

Grant sponsor: Korean Rural Development Administration (BioGreen 21 Program) and Stem Cell Research Center of the 21st Century Frontier Research Program (Ministry of Science and Technology); Grant number: 20070401034006-SC 2210.

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Received 9 September 2008; Accepted 7 November 2008 • DOI 10.1002/jcb.22013 • 2009 Wiley-Liss, Inc.
Published online 6 February 2009 in Wiley InterScience (www.interscience.wiley.com).

stimulated AA release and their roles remain to be clearly elucidated in ES cells.

ES cells hold a great deal of promise for the modeling of early development, and also may evidence therapeutic potential. These cells are defined as cells with self-renewal capacity and the ability to generate multiple differentiated cell types [Smith, 2001; Weissman et al., 2001]. Recently, approaches in the field of stem cell biology have emphasized the potential of signaling molecule phenotypic screens, not only for the identification of small molecules that can alter stem cell fate and define the relevant corresponding molecular pathways, but also in terms of their general biological implications in the field of stem cell research. Therefore, there is a growing appreciation of the need to replicate various aspects of the embryonic stem cells' in vivo environment in order to more effectively maintain their native characteristics. Many studies have used H_2O_2 as a model agent to study the mechanism of cell responses resulting from acute oxidative stress in various cell types. Thus, we examined role of AA in H_2O_2-indued proliferation of mouse ES cells and its related signaling molecules.

MATERIALS AND METHODS

MATERIALS

The mouse ES cells were acquired from the American Type Culture Collection (ES-E14TG2a). Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, MD). Hydrogen peroxide (H_2O_2), AG 1478, SB 203580, SP 600125, and monoclonal anti-β-actin were supplied by the Sigma Chemical Company (St. Louis, MO). PD 98059 and SN 50 were acquired from Calbiochem (La Jolla, CA). Bay11-7082 was purchased from Biomol (Butler Pike, PA). Rabbit anti-cPLA$_2$, and NF-κB p65 antibody were supplied by Santa Cruz Biotechnology (Delaware, CA). Goat anti-EGFR, and Phospho-EGFR (Try1068) antibody were supplied by Santa Cruz Biotechnology. Phospho-p44/42, p44/42, phospho-p38, p38, phospho-SAPK/JNK, and SAPK/JNK antibody were acquired from New England Biolabs (Herts, UK). Goat anti-rabbit IgG was purchased from Jackson Immunoresearch (West Grove, PA). Liquiscint was obtained from National Diagnostics (Parsippany, NY). All other reagents were of the highest commercially available purity.

ES CELL CULTURE

The mouse ES cells were cultured in 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 an additional 5 days of culturing in a standard medium plus leukemia inhibitory factor (LIF). The cells were then grown on gelatinized 12-well plates or 60 mm culture dishes in an incubator maintained at 37°C in an atmosphere containing 5% CO_2 in air. The media were changed to serum-free DMEM (5 mM glucose) 24 h prior to the experiment.

ARACHIDONIC ACID RELEASE

To measure the release of arachidonic acid (AA) via a modified version of the method described by Xing et al. [1997], the cells were incubated with 0.5 μCi/ml of [^{3}H] AA for 24 h. The cells were then washed and incubated for 1 h with the indicated agents. At the end of the incubation, the medium was transferred to ice-cold tubes containing 55 mM EGTA and 5 mM EDTA, centrifuged (12,000*g*), and the soluble material was counted in a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA). Both the [^{3}H] AA released and the cell-associated [^{3}H] AA were standardized with respect to protein. Then, the released [^{3}H] AA was compared by percentage to the cell-associated [^{3}H] AA (present at the beginning of the incubation).

MEASUREMENT OF $[Ca^{2+}]_I$

The changes in $[Ca^{2+}]_i$ were monitored using Fluo-3/AM dissolved in dimethylsulfoxide. The cells in 35 mm culture dishes were rinsed twice with a Bath Solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM HEPES, pH 7.4), incubated in Bath Solution containing 3 μM fluo-3/AM with 5% CO_2–95% O_2 at 37°C for 40 min, rinsed twice with the Bath Solution, mounted on a perfusion chamber, and scanned every second using confocal microscopy (400×) (fluoview 300, Olympus). The fluorescence was excited at 488 nm and the emitted light was read at 515 nm. All analyses of $[Ca^{2+}]_i$ were processed at a single cell level, and are expressed as the relative fluorescence intensity (RFI).

PROSTAGLANDIN E_2 ASSAY

Mouse ES cells plated on 60-mm culture plates were grown in a FBS-free medium for 24 h and divided into groups according to the experimental protocols. The PGE_2 concentration in the culture medium was measured with an enzyme-linked immunosorbent assay (ELISA) with a PGE_2 High Sensitivity Immunoassay kit (R&D Systems, Minneapolis, MN).

PREPARATION OF CYTOSOLIC AND TOTAL MEMBRANE FRACTIONS

The cytosolic and total membrane fractions were prepared via a slight modification of the method previously reported by Mackman et al. [1991]. The DMEM of mouse ES cells was exchanged 48 h prior to the experiments. The medium was then removed and the cells were washed twice in ice-cold PBS, scraped, harvested via micro-centrifugation and re-suspended in buffer A [137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 μg/ml leupeptin (pH 7.5)]. The re-suspended cells were then lysed mechanically on ice via trituration with a 21.1-gauge needle. The lysates were initially centrifuged at 1,000*g* for 10 min at 4°C. The supernatant was then centrifuged for 1 h at 100,000*g* 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 for 20 min at 20,000*g* at 4°C. The resultant pellet was then re-suspended in buffer A containing 1% (v/v) Triton X-100. The particulate fractions containing 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 via the Bradford procedure [1976]. Twenty micrograms of protein were used for Western blot analysis.

WESTERN BLOT ANALYSIS

The cell homogenates (20 μg protein) were separated via 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose. After the blots were washed in TBST [10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], the membranes were blocked for 1 h with 5% skimmed milk 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 IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. The bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK).

[^{3}H] THYMIDINE INCORPORATION

The [^{3}H] thymidine incorporation experiments were conducted using the methodology described by Chen et al. [2005]. Zhang et al. [2005] previously reported that most ES cells could be arrested in the G0/G1 phase using a serum deprivation culture. In addition, the synchronized ES cells were shown to successfully reenter a normal cell cycle after being resupplied with serum. In this study, the cells were cultured in a single well until reaching 50% confluence. They were then washed twice with PBS and maintained in serum-free DMEM including all the supplements. After 24 h of incubation, the cells were washed twice in PBS, and incubated with fresh serum-free DMEM including all the supplements and indicated agents. After the indicated incubation period, 1 μCi of [methyl-^{3}H] thymidine (specific activity: 74 GBq/mmol, 2.0 Ci/mmol; Amersham Biosciences, Buckinghamshire, UK) was added to the cultures. The cells were incubated for 1 h with [^{3}H] thymidine at 37°C. They were then washed twice in PBS, fixed for 15 min in 10% trichloroacetic acid (TCA) at 23°C, and washed twice with 5% TCA. The acid-insoluble material was dissolved in 0.2 N NaOH over a 12-h period at 23°C. Aliquots were removed and the level of radioactivity was determined using a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA). The control levels of [^{3}H] thymidine incorporation were determined under conditions in which the cells had been cultured in serum free DMEM without H_2O_2. The values were converted from absolute counts to a percentage of the control in order to allow for comparison between the experimental groups. In order 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 number of live cells was determined using a hemocytometer. Cells failing to exclude the dye were considered nonviable.

BROMODEOXYURIDINE INCORPORATION

The level of 5-bromo-2′-deoxyuridine (BrdU) (a thymidine analog) incorporation was measured to determine the level of DNA synthesis. The ES cells were serum-starved for 24 h prior to H_2O_2 stimulation. The ES cells were then treated with H_2O_2 for 24 h followed by the addition of 15 μM BrdU. Incubation was continued for an additional 1 h. After washing several times with PBS, the cells were fixed with methanol [10% (v/v) for 10 min at 4°C], followed by incubation in 1 N HCl for 30 min at room temperature. The cells were then washed and incubated with 0.1 M sodium tetraborate for 15 min. Alexa Fluor 488-conjugate Mouse anti-BrdU mAb (diluted 1:200, Molecular Probes, OR) in 2% BSA-PBS was incubated overnight at 4°C. After washing in PBS, coverslips were mounted onto glass slides with a Dako Fluorescent mounting medium using gelvatol and examined using optical microscopy (fluoview 300, Olympus). The mean ± SE number of BrdU-positive cells per field of vision was determined. At least 10 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, followed by BrdU staining. The fixed cells were incubated with the rabbit anti-Oct-4 antibody (1: 100, Santa Cruz Biotechnology) for 1 h at room temperature, and with the Alexa Fluor 555 anti-rabbit IgG (1:100, Molecular Probes, Eugene, OR) for 1 h at room temperature. This was followed by incubation in 1 N HCl, neutralization with 0.1 M sodium tetraborate, and incubation with Alexa Fluor 488-conjugate Mouse anti-BrdU mAb for 1 h at room temperature. After washing with PBS, the BrdU/Oct-4 stained cells were examined under confocal microscopy (fluoview 300, Olympus).

FLUORESCENCE ACTIVATED CELL SORTER (FACS) ANALYSIS

The cells were serum-starved for 24 h and pretreated with $AACOCF_3$, mepacrine or indometachin for 30 min before incubation with H_2O_2 for 24 h. They were then dissociated in trypsin/EDTA, pelleted by centrifugation, and resuspended at approximately 10^6 cells/ml in PBS containing 0.1% BSA. The cells were then fixed in 70% ice-cold ethanol, followed by the incubation in a freshly prepared nuclei staining buffer [250 μg/ml Propidium Iodide (PI) and 100 μg/ml RNase] for 30 min at 37°C. The cell cycle histograms were generated after analyzing the PI-stained cells by FACS (Beckman Coulter, CA). At least 10^4 events were recorded for each sample. The samples were analyzed using CXP software (Beckman Coulter).

STATISTICAL ANALYSIS

The results are expressed as the means ± standard errors (SE). 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

INVOLVEMENT OF Ca^{2+} AND PKC ACTIVATION IN H_2O_2-INDUCED ARACHIDONIC ACID RELEASE

The cells were treated with H_2O_2 ($0–10^{-2}$ M) for 1 h to confirm its effect on AA release. In this experiment, H_2O_2, at $\geq 10^{-4}$ M, significantly increased the level of AA (Fig. 1A). Moreover, in order to determine whether the Ca^{2+} and PKC pathways are involved in H_2O_2-induced AA release, Ca^{2+} influx and PKC translocation using confocal microscopy or Western blotting were assessed. As is shown in Figure 1B, H_2O_2 (10^{-4} M) treatment increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Moreover, H_2O_2 increased the translocation of PKC isoforms, including PKC α, δ, and ζ, from the cytosolic to membrane fraction (Fig. 1C). H_2O_2-induced phoshosphorylation of pan-PKC was inhibited by intra- and extracellular Ca^{2+} depletion using EGTA, BAPTA-AM, or EGTA plus BAPTA-AM (Fig. 1D). Consequently, in experiments conducted

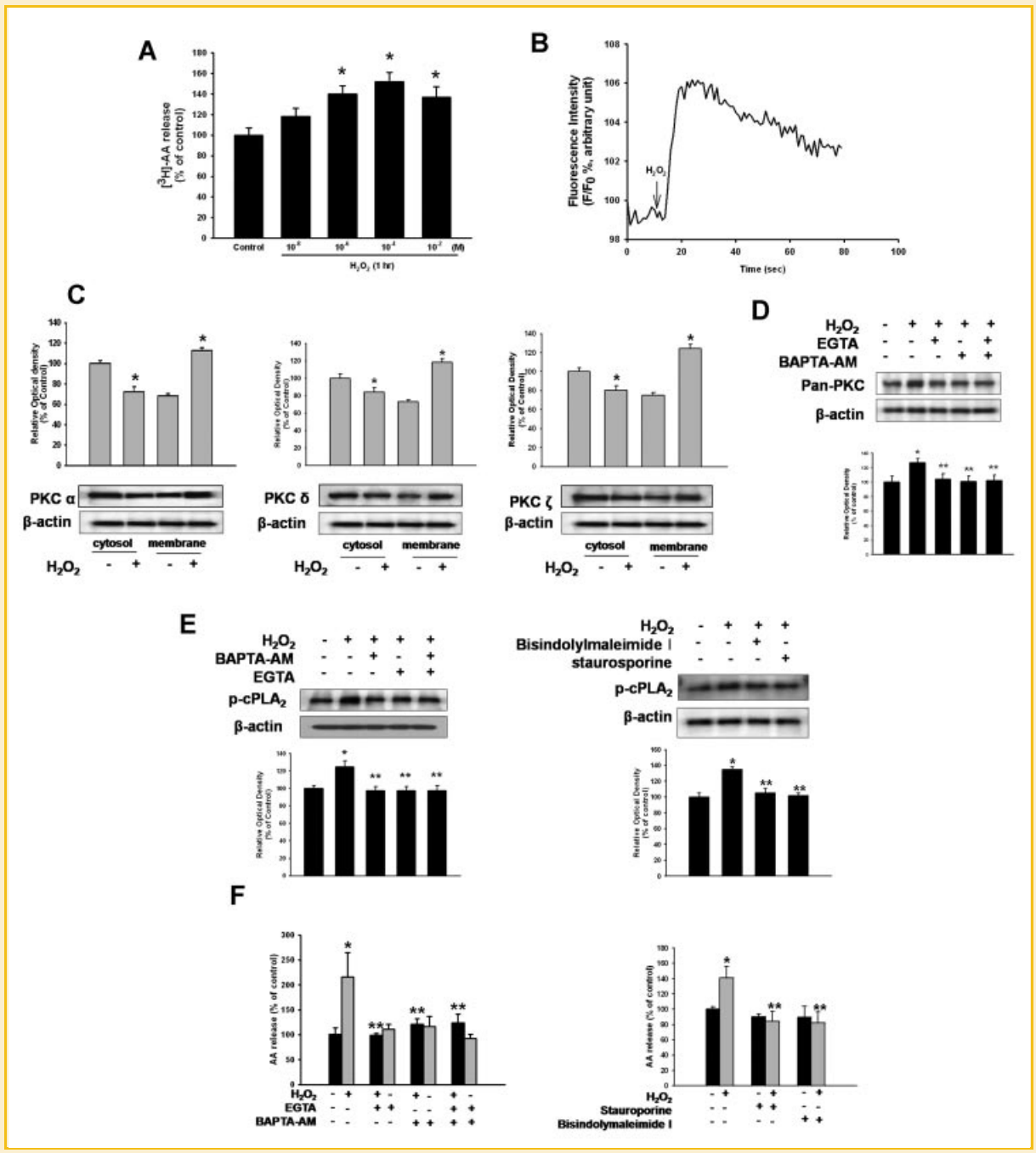

Fig. 1. Effects of Ca^{2+} and PKC on H_2O_2-induced [^{3}H]arachidonic acid (AA) release and cytoplasmic phospholipase A_2 ($cPLA_2$) phosphorylation. A: The ES cells were treated with different doses of H_2O_2 (0–10^{-2} M) for 1 h, after which AA release was assessed as described in the Materials and Methods Section. B: The ES cells were loaded with 2 μM fluo 3-AM in serum-free medium for 40 min and treated with H_2O_2 (10^{-4} M), and the H_2O_2-induced Ca^{2+} influx was then measured. The changes in $[Ca^{2+}]_i$ were monitored using confocal microscopy, and are expressed as the relative fluorescence intensity. C: The PKC α, δ, and ζ isoforms present in either the cytosolic or membrane compartments were detected via Western blotting, as described in the Materials and Methods Section. D: The ES cells were pretreated with EGTA, BAPTA-AM, or EGTA plus BAPTA-AM for 30 min before the 1 h H_2O_2 (10^{-4} M) and the Pan-PKC protein was detected in the membrane compartment. The bands represent 80–90 kDa for Pan-PKC, PKC α, δ, and ζ, and 41 kDa for β-actin. The ES cells were pretreated with EGTA, BAPTA-AM, EGTA plus BAPTA-AM, bisindolylmaleimide I, or staurosporine for 30 min before the 1 h H_2O_2 (10^{-4} M), then (E) phosphorylated $cPLA_2$ was detected by Western blotting and (F) effect of each inhibitor alone on AA release was assessed. Each of the examples shown is representative of four independent experiments. The lower panels (bars) of D and E denote the mean ± SE of four experiments for each condition determined from densitometry relative to the β-actin. The band represents 110 kDa for $cPLA_2$. The values are expressed as the means ± SE of four independent experiments with triplicate dishes. $^*P < 0.05$ versus Control, $^{**}P < 0.05$ versus H_2O_2 alone.

to evaluate the effects of H_2O_2 on $cPLA_2$ activation and AA release, H_2O_2 increased both $cPLA_2$ activation and AA release, which were inhibited by pretreatment with EGTA, BAPTA-AM, EGTA plus BAPTA-AM, bisindolylmaleimide I, or staurosporine. Each inhibitor alone did not affect AA release (Fig. 1E,F).

INVOLVEMENT OF MAPKS AND EGFR ACTIVATION IN H_2O_2-INDUCED ARACHIDONIC ACID RELEASE

In experiments conducted to assess the involvement of MAPKs, H_2O_2 increased the phosphorylation of each p44/42, p38 MAPKs, or SAPK/JNK, but this effect was blocked by the inhibition of the Ca^{2+} and PKC pathways (Fig. 2A,B). Consequently, pretreatment with PD 98059 (a p44/42 MAPKs inhibitor), SB 203580 (a p38 MAPK inhibitor), or SP 600125 (a SAPK/JNK inhibitor) blocked H_2O_2 -induced $cPLA_2$ activation (Fig. 2C) and AA release (Fig. 2D). Moreover, H_2O_2 increased EGFR phosphorylation in a time-dependent manner (Fig. 3A). The activations of these EGFR were inhibited by AG 1478 (Fig. 3B). Also, H_2O_2-induced EGFR activation was attenuated by pretreatment with PD 98059 or SB 203580 (Fig. 3C). In addition, the inhibition of EGFR phosphorylation by AG 1478 blocked the H_2O_2-induced increase of AA release, which was consistent with the [^{3}H] thymidine incorporation results. Each inhibitor alone did not affect AA release and [^{3}H] thymidine incorporation (Fig. 3D,E).

INVOLVEMENT OF NF-κB ACTIVATION IN H_2O_2-INDUCED COX-2/PGE_2 PATHWAYS

H_2O_2 increased NF-κB phosphorylation (Fig. 4A), which was blocked via the inhibition of the Ca^{2+} and PKC pathways (Fig. 4B,C). In experiments conducted to assess the effects of H_2O_2 on COX activation, H_2O_2 increased the level of COX 2 protein, but not the level of COX 1, showing a maximum increase at 6 h (Fig. 4D). The H_2O_2-induced increase in COX 2 levels was blocked by the inhibition of NF-κB activation using each PDTC, SN 50 (NF-κB nucleus translocation inhibitor, 500 ng/ml), or Bay11-7082 (IκB-α phosphorylation inhibitor, 2×10^{-5} M) (Fig. 4E). Moreover, H_2O_2

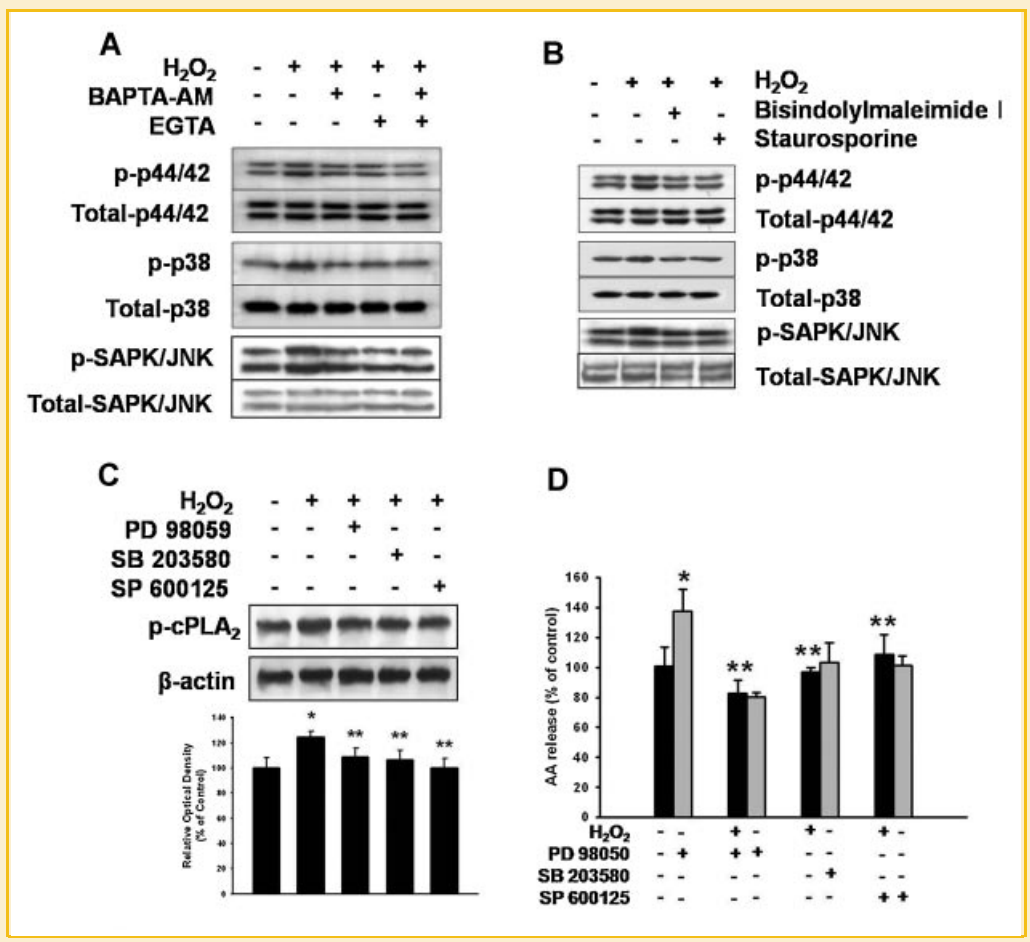

Fig. 2. Effect of MAPKs on H_2O_2-induced AA release and $cPLA_2$ phosphorylation. The ES cells were pretreated with (A) EGTA, BAPTA-AM, EGTA plus BAPTA-AM, (B) bisindolylmaleimide I, or staurosporine for 30 min before the 1 h H_2O_2 (10^{-4} M), then p44/42 MAPKs, p38 MAPK, and SAPK/JNK phosphorylation were detected. Each example shown is a representative of four experiments. The 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 (10^{-4} M) treatment for 1 h, then (C) phosphorylated $cPLA_2$ and (D) AA release were assessed in the presence or absence of each inhibitor. Each of the examples shown is representative of three independent experiments. The lower panels (bars) of C denote the mean ± SE of three experiments for each condition determined from densitometry relative to the β-actin. The values are expressed as the means ± SE of three independent experiments with triplicate dishes. $^{*}P < 0.05$ versus Control, $^{**}P < 0.05$ versus H_2O_2 alone.

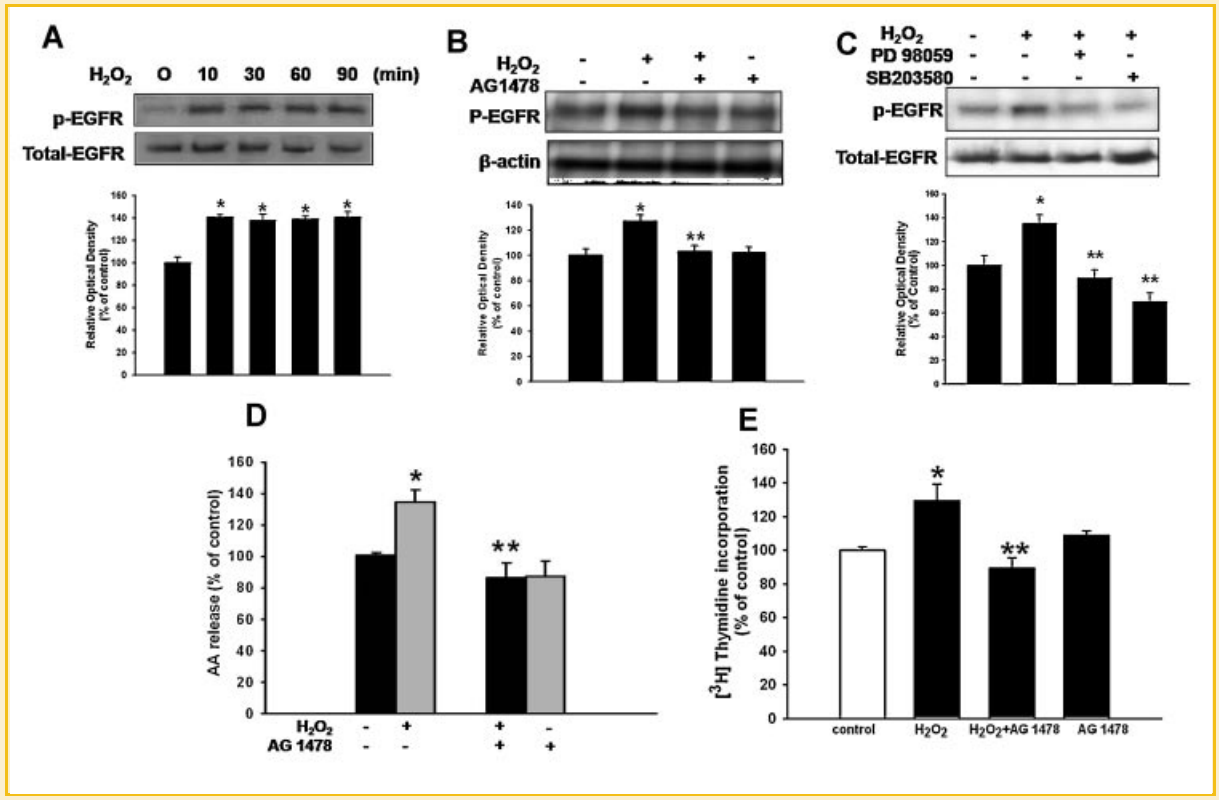

Fig. 3. Effect of EGFR activation on H_2O_2–induced AA release. A: The ES cells were treated with H_2O_2 for different times (0–90 min), then phosphorylated EGFR were detected. B: The ES cells were pretreated with AG 1478 (10^{-5} M) at 30 min prior to the H_2O_2 treatment for 1 h and the level of EGFR phosphorylation was assessed by Western blotting. C: The ES cells were treated with PD 98059 or SB 203580 for 30 min prior to the H_2O_2 treatment 1 h and the level of EGFR was assessed by Western blotting. Each of the examples shown is representative of three independent experiments. The lower panels (bars) of A, B, C denote the mean ± SE of three experiments for each condition determined from densitometry relative to the total-EGF receptor. The bands represent 175 kDa of the phospho-EGF receptor and EGF receptor. D,E: The ES cells were pretreated with AG 1478 for 30 min before the H_2O_2 treatment, then AA release and [^{3}H] thymidine incorporation was assessed. The values are expressed as the means ± SE of five independent experiments with triplicate dishes. $^*P < 0.05$ versus Control, $^{**}P < 0.05$ versus H_2O_2 alone.

treatment increased PGE_2 synthesis, which was blocked by the inhibition of NF-κB activation (Fig. 4F). The ES cells expressed the EP1, EP2, EP3, and EP4 receptors. Moreover, the results of RT-PCR showed that the treatment of H_2O_2 increased mRNA level of EP1 receptor, whereas those of other subtypes of EP receptors remained unchanged (Fig. 4G). In experiment to confirm the maintenance of pluripotency in the presence of H_2O_2 exposure, the protein expressions of Oct-4 and SSEA-1 (carbohydrate epitopes stage-specific embryonic antigen-1) were still maintained after treatment with H_2O_2 for 24 h (Fig. 5A). The number of BrdU-labeled cells was also increased in response to H_2O_2, which is consistent with the observed increase in the level of [^{3}H] thymidine incorporation (Fig. 5B–D). As shown in Figure 5E,F, treatment with H_2O_2 or PGE_2 increased thymidine incorporation (47% or 59% increase vs. control) and the number of cells. However, pretreatment with $AACOCF_3$, mepacrine ($cPLA_2$ inhibitors) or indometachin (COX-2 inhibitor) blocked the H_2O_2-induced increase in DNA synthesis (Fig. 5G). As shown in Figure 5H, H_2O_2 increased the percentage of the cell population in the S phase (control: 43.28 vs. H_2O_2: 61.07%). However, pretreatment of $AACOCF_3$, mepacrine, or indometachin decreased the accumulation in S phase by H_2O_2 (50.21%, 52.34%, or 54.21%, respectively).

DISCUSSION

The present study demonstrated that H_2O_2 increased AA release and PGE_2 production via Ca^{2+}/PKC/MAPKs activation and EGFR transactivation, consequently stimulating proliferation in mouse ES cells. H_2O_2 stimulates proliferation in mammalian cells in low concentrations and enhances survival in a wide variety of cell types [Stone and Collins, 2002]. Our previous study showed when ES cells were treated with 10^{-4} M H_2O_2, glucose transporter activity was significantly stimulated [Na et al., 2007], which in turn the increase of glucose uptake into ES cells led cell proliferation [Lee et al., 2007]. In the other research, the incubation of mesangial cells with 10^{-4} M H_2O_2 did not induce cytotoxicity [Johnson et al., 1994]. Therefore, there is possibility that 10^{-4} M H_2O_2 enhanced mouse ES functions including growth and survival through a wide variety of signaling molecules.

The present study suggests that Ca^{2+}, PKC, and MAPKs represent key signaling pathways in H_2O_2-induced $cPLA_2$ activation and AA release of ES cells. In previous study, certain PKC isoforms play pivotal, specific, or partly antagonistic roles in the regulation of AA production and cellular proliferation [Griger et al., 2007]. Our findings suggest that H_2O_2 regulates PKC α, δ, and ζ to provide

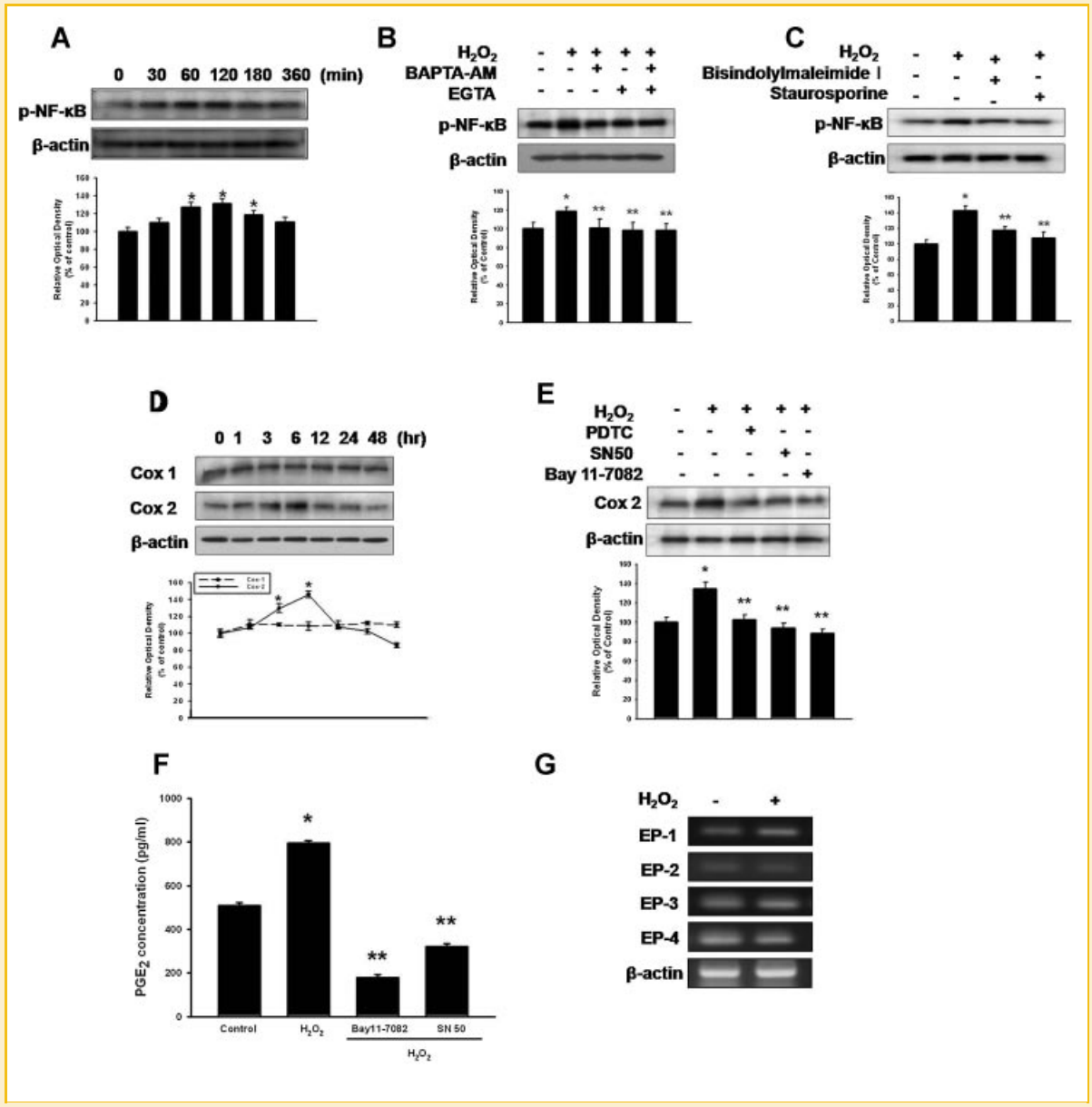

Fig. 4. Effects of NF-κB on H_2O_2-induced PGE_2 synthesis. A: The ES cells were treated with H_2O_2 for different times (0–360 min), then phosphorylated NF-κB were detected. The ES cells were pretreated with (B) EGTA, BAPTA-AM, EGTA plus BAPTA-AM, (C) bisindolylmaleimide I, or staurosporine for 30 min prior to the 2 h H_2O_2 (10^{-4} M), then NF-κB phosphorylation was assessed. The example shown is a representative of three experiments. D: The ES cells were treated with H_2O_2 for different times (0–48 h), then COX 1 and COX 2 protein expression levels were detected. D: The ES cells were treated with PDTC, SN 50 (NF-κB nucleus translocation inhibitors, 500 ng/ml) or Bay 11-7082 (IκB-α phosphorylation inhibitor, 2×10^{-5} M) for 30 min prior to H_2O_2 treatment for 6 h, after which COX 2 was detected via Western blotting. Each of the examples shown is representative of three independent experiments. The lower panels (bars) of A, B, D, and E denote the mean ± SE of three experiments for each condition determined from densitometry relative to the β-actin. The bands represent 70–72 kDa of the COX 1 and COX 2. F: The ES cells were treated with SN 50 or Bay 11-7082 for 30 min prior to H_2O_2 treatment and then PGE_2 synthesis was analyzed as described in the Materials and Methods Section. The values are expressed as the means ± SE of three independent experiments with triplicate dishes. $^{*}P < 0.05$ versus Control, $^{**}P < 0.05$ versus H_2O_2 alone. G: The ES cells were incubated with H_2O_2 (10^{-4} M) for 24 h, and the expression levels of EP1, 2, 3, 4 receptors, and β-actin (350 bp) mRNA were assessed by RT-PCR. Each example shown is representative of five independent experiments.

that H_2O_2 activates conventional, novel, or atypical PKCs with affecting their translocation, and then causes $cPLA_2$ phosphorylation and release of AA in ES cells. It was also shown that the PKC isoforms very often play central roles in the proliferation and other functional processes in ES cells [Heo and Han, 2006; Heo et al., 2006, 2007]. Among the PKC capacity, the present study showed that PKC mediates the activation of MAPK pathways including p44/42, p38 MAPKs, and JNK/SAPK to promote activation of $cPLA_2$ /AA pathway. Other researchers demonstrated that $cPLA_{2\alpha}$ activity in cells is regulated by the intracellular translocation and phosphorylation of the enzyme by MAPKs such as ERK1/2 [Das et al., 2003; Hirabayashi et al., 2004]. Moreover, phosphorylation of $cPLA_2$ at

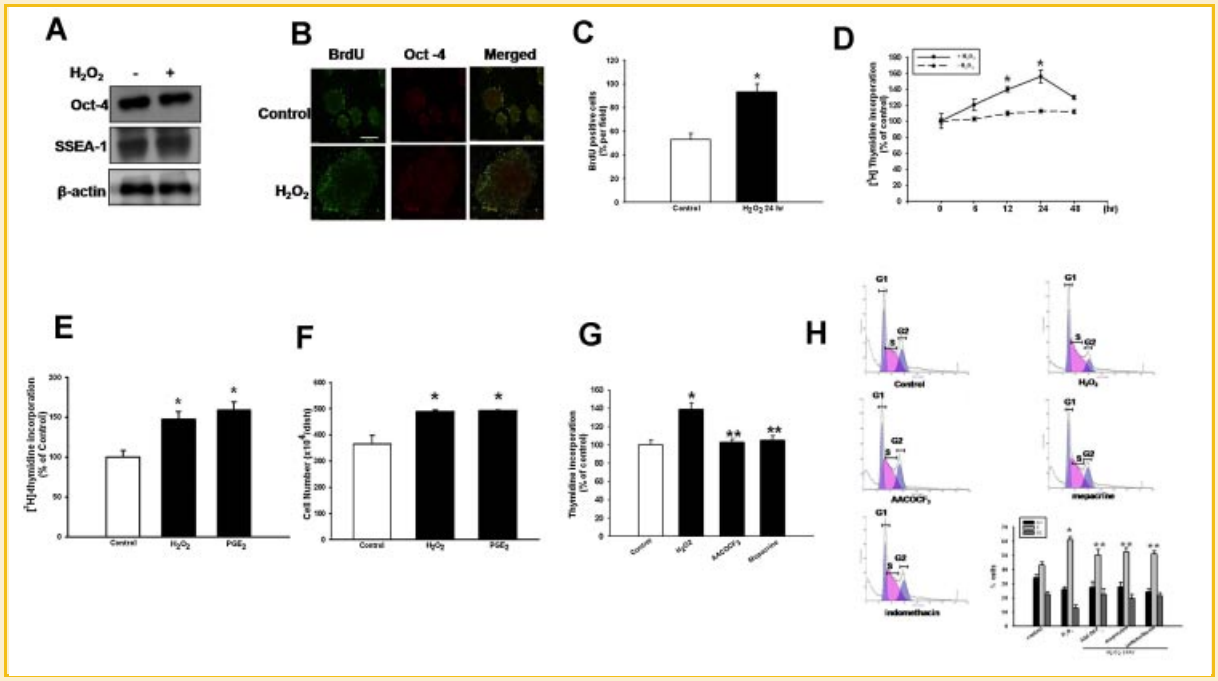

Fig. 5. Relationship between H_2O_2-induced PGE_2 synthesis and cell proliferation. A: The ES cells were treated with H_2O_2 (10^{-4} M) for 24 h and then Oct-4 and SSEA-1 were detected via Western blotting. Each of the examples shown is representative of three independent experiments. B: The ES cells were treated with H_2O_2 for 24 h and double-labeled with BrdU and Oct-4. The scale bars represent 20 μm. C: The percentage of BrdU-positive cells was determined by counting the number of BrdU-positive cells per field of vision. At least 10 fields of vision per coverslip were counted. $^*P < 0.05$ versus Control. D: The ES cells were exposed to H_2O_2 for 0–48 h and pulsed with 1 μCi of [^{3}H] thymidine for 1 h prior to counting ($^*P < 0.05$ vs. Control). The values are reported as a mean ± SE of three independent experiments with triplicate dishes. The ES cells were treated with H_2O_2 (10^{-4} M) or PGE_2 (10^{-8} M) for 24 h, then (E) [^{3}H] thymidine incorporation and (F) cell counting were evaluated as described in the Materials and Methods Section. G: After pretreatment with $AACOCF_3$ or mepacrine (10^{-6} M) prior to H_2O_2 treatment, [^{3}H] thymidine incorporation was determined. D: The ES cells were pretreated with $AACOCF_3$, mepacrine (10^{-6} M), indometachin (10^{-7} M) for 30 min prior to before the H_2O_2 treatment for 24 h, and the cells were washed with PBS, fixed, stained, and analyzed by flow cytometry. The gates were configured manually to determine the percentage of cells at the S phases based on the DNA content. The percentage of cells at the S phases was obtained from three independent experiments. The values are expressed as the means ± SE of four independent experiments with triplicate dishes. $^*P < 0.05$ versus Control, $^{**}P < 0.05$ versus H_2O_2 alone. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Ser^{505} by ERK1/2 or p38 MAPK has been shown to cause an increase in enzyme activity [Lin et al. 1993; Kramer et al. 1996; Gijon et al. 2000]. Distinct from other cell types, JNK/SAPK has been shown to involve in H_2O_2-induced $cPLA_2$ activation and AA release indicating that activation of JNK also results in H_2O_2-induced responses in ES cells. These phenomenon can be agreement with that multiple phosphorylation sites are present in $cPLA_2$ [de Carvalho et al. 1996; Gijon et al. 1999] and that various protein kinases may regulate its activity [Geijsen et al., 2000; Gijon et al., 2000]. Thus, these findings suggest that H_2O_2, which are regulators of not only Ca^{2+} but also various protein kinases, had significant effects on the process of AA release in ES cells.

In addition, H_2O_2 leads to an EGFR phosphorylation, which induces an increase of $cPLA_2$-dependent AA release. These processes can be classified into two categories—intracellular signaling molecules including Ca^{2+}, PKC, and Src [Prenzel et al., 2001], and matrix metalloprotease-dependent EGF ligand cleavage [Frank et al., 2003]. The finding that the blockage of H_2O_2-induced phosphorylation of p44/42 MAPKs and p38 MAPK inhibited H_2O_2-induced EGFR phosphorylation demonstrates that these two MAPKs are involved in the rapid kinetics of EGFR transactivation. Consistent with the results of our study, previous studies have shown that EGFR transactivation is required for the activation of p44/42 MAPKs in insulin-like growth factor-treated cells [Roudabush et al., 2000] and p38 MAPK mediated EGFR activation after oxidant injury in renal epithelial cells [Zhuang et al., 2005]. The present study showed that inhibition of EGFR tyrosine kinase led to attenuate H_2O_2-induced AA release. It has been known that stimulation of AA release is found in many cells expressing EGFR [Bonventre et al., 1990; Hack et al., 1991]. Moreover, EGFR tyrosine kinase has been implicated as a modulator of $cPLA_2$ activity to liberate AA [Goldberg et al., 1990; Clark and Dunlop, 1991]. Therefore, our findings suggest that H_2O_2 can indirectly regulate ES cell biology via transactivation with EGFR, raising the possibility that AA metabolism can be involved in maintenance of self-renewal.

The present study also showed that H_2O_2 regulated the production of COX-2 and PGE_2 via an increase in NF-κB activity. Therefore, NF-κB is a transcription factor that is required for the induction of COX-2 and PGE_2 by H_2O_2 in ES cells. The presence of $cPLA_2$ activity in the embryonic rat brain [Waldman et al., 1984; Yoshihara et al., 1992] and the embryonic expression of COX, an enzyme which utilizes the AA produced by PLA_2 activity for PG synthesis [Kawasaki et al., 1993; Piddington et al., 1996], suggest that these

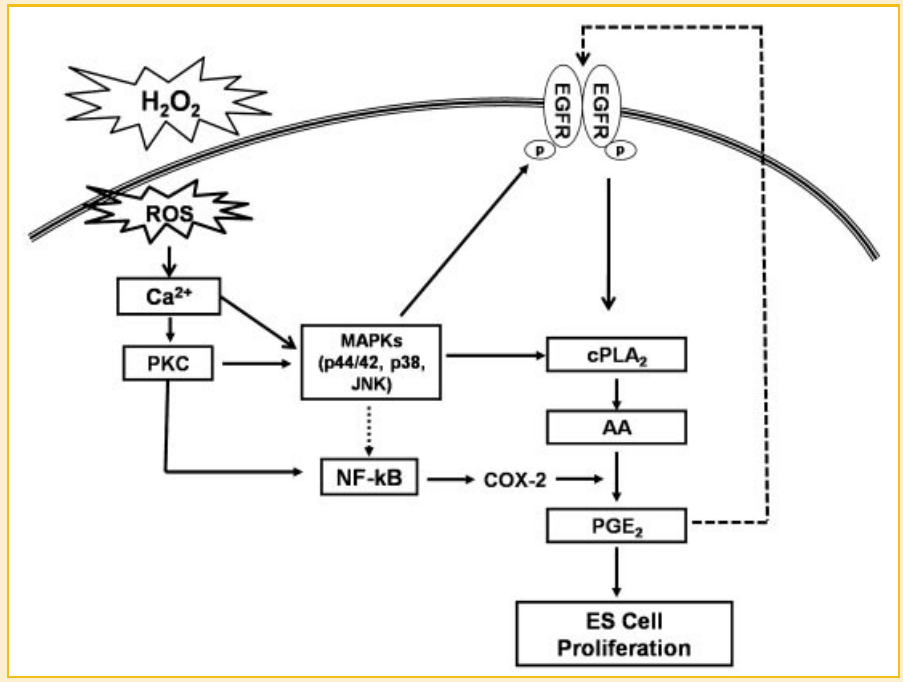

Fig. 6. The hypothesized model for the signal pathways involved in H_2O_2-induced AA release and cell proliferation. H_2O_2 increased intracellular Ca^{2+} level and PKC activation to induce MAPKs activation. Subsequently, $cPLA_2$ is activated to stimulate AA release. MAPKs also activate EGFR, which induces $cPLA_2$ phosphorylation and AA release. In another way, Ca^{2+}/PKC-induced NF-kB stimulates COX-2 activity to increase PGE_2 production, which finally influences ES cell proliferation. EGFR, EGF receptor; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; $cPLA_2$, cytosolic phospholipase A_2; COX-2, cyclooxygenase; PGE_2, prostaglandin E_2. The solid lines are the proposed pathways and the dashed lines are suspected pathways.

two enzymes may perform a developmental function in embryogenesis. Moreover, in hematopoietic stem cells, both COX-1 and -2 participate in the regulation of the stem cell niche and homeostasis [North et al., 2007]. The present study also demonstrated that H_2O_2 increased COX-2 expression, but COX-1 was constitutively expressed. Therefore, under the current experimental conditions, COX-2 is responsible for the increase in PG synthesis, especially PGE_2, which plays a critical role in many cellular functions. Recently, we showed that ES cell growth enhanced by high glucose is mediated through the induction of COX-2 expression and PGE_2 production [Kim and Han, 2008]. Moreover, E-type prostaglandin (EP) receptor subtypes (EP 1–4), which is specific G-protein coupled transmembrane receptors to induce biological actions of PGE_2, were expressed in mouse ES cells. In the present study, treatment with H_2O_2 increased EP1 mRNA expression, suggesting a pivotal role of the EP1 receptor in H_2O_2-induced PGE_2 metabolism of ES cells. Therefore, our findings indicate that the H_2O_2-mediated activation of signaling molecules contributes to both AA release and PGE_2 synthesis, and is subsequently linked to ES cell proliferation (Fig. 6). On the basis of these results, we think that the discovery of the role played by H_2O_2 in stimulating ES cells self-renewal, together with the other results shown in this study, represent a significant advance in our knowledge of how ES cells pluripotency is maintained by extracellular factors and has application in the development of ES cells culture systems. In conclusion, the results of the current study indicate that AA release via Ca^{2+}/PKC and MAPKs-induced EGFR transactivation by H_2O_2 mediated proliferation of mouse embryonic stem cells.

ACKNOWLEDGMENTS

This work was supported by Korean Rural Development Administration (BioGreen 21 Program) and Stem Cell Research Center of the 21st Century Frontier Research Program (code # 20070401034006 - SC 2210), Ministry of Education, Science, and Technology Korea. The authors acknowledge a graduate fellowship provided by the Ministry of Education and Human Resources Development through the Brain Korea 21 project, Republic of Korea.

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