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Effect of EGF on [^{3}H]-Thymidine Incorporation and Cell Cycle Regulatory Proteins in Primary Cultured Chicken Hepatocytes: Involvement of Ca^{2+}/PKC and MAPKs

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Abstract The reported studies on the metabolism in chicken hepatocytes in comparison with those of mammals are quite different. Therefore, this study examined the effect of EGF on DNA synthesis along with its related signal cascades in primary cultured chicken hepatocytes. EGF stimulated DNA synthesis in a dose (≥10 ng/ml)-dependent manner, which correlated with the increase in CDK-2 and CDK-4 expression. The EGF-induced increase in [^{3}H]-thymidine incorporation was blocked by AG 1478 (an EGF receptor tyrosine kinase antagonist), genistein, and herbimycin A (tyrosine kinase inhibitors), suggesting a role in the activation and tyrosine phosphorylation of the EGF receptor. In addition, the EGF-induced stimulation of [^{3}H]-thymidine incorporation was prevented by staurosporine, H-7, or bisindolylmaleimide I (protein kinase C inhibitors), suggesting a role of PKC. In addition, PD 98059 (a MEK inhibitor), SB 203580 (a p38 MAPK inhibitor), and SP 600125 (a JNK inhibitor) blocked the EGF-induced stimulation of [^{3}H]-thymidine incorporation and CDK-2/4 expression. Indeed, EGF increased the translocation of PKC from the cytosol to the membrane fraction, and increased the activation of p44/42 MAPK, p38 MAPK, and JNK. Moreover, EGF increased the CDK-2, CDK-4, cyclin D1, and cyclin E expression levels but decreased the p21 and p27 expression levels. These EGF-induced increases were blocked by an EGF receptor antagonist, tyrosine kinase inhibitors, PKC inhibitors, and MAPKs inhibitors. In conclusion, EGF stimulates DNA synthesis of primary cultured chicken hepatocytes via Ca^{2+}/PKC and the MAPKs signaling pathways. J. Cell. Biochem. 99: 1677–1687, 2006.

Key words: EGF; PKC; MAPKs; cyclins/CDKs; chicken hepatocytes

Chickens have many differences in their lipid, glucose, and glycogen metabolism, and in the regulation of hormones, compared with mammals [Pearce, 1977]. The reported studies on the metabolism in chicken hepatocytes in comparison with those of mammals are quite interesting. The epidermal growth factor (EGF) is an essential regulatory polypeptide in the function of hepatocytes [Tsai et al., 2001] and in the regulation of cellular proliferation [Suhardja and Hoffman, 2003]. The mitogenic effects of EGF on chickens include a sudden increase in egg production of laying hens, an increase in chicken hatchability, along with increased weight gain. A further study on this subject is needed because some functions of hepatocytes in chickens are different from mammalian hepatocytes [Sasaki et al., 2000]. Therefore, this study was carried out to determine the effect of EGF on the proliferation of chicken hepatocytes.

Cell-cycle progression through the G_1/S transition requires the activation of cyclin D-CDK4/CDK6 and cyclin E-CDK2 [Matsushime et al., 1994; Sherr and Roberts, 1995]. The CDK activity is regulated by CDK inhibitors (CKIs) as well as by changes in the CDK expression levels. $p27^{kip1}$ and $p21^{WAF1/Cip1}$ are major

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kip/cip families of the CKIs. The involvement of PKC in proliferation of hepatocytes has not been clarified. PKC is an important component in controlling the cell cycle [Livneh and Fishman, 1997]. Marino et al. [2000] reported that EGF regulates amino acid transport in chick embryo hepatocytes via protein kinase C, suggesting a role of PKC in chicken hepatocytes. In addition, several studies suggest that the MAPKs are important for EGF-induced mitogenesis in hepatocytes [Kong et al., 2000]. However, the mechanisms of the mitotic regulation by EGF in chicken hepatocytes are not completely understood.

A primary culture of hepatocytes has been used for many biophysiological studies on the liver function because a primary culture of hepatocytes retains many of the liver-specific functions and responds to various hormones by the expression of liver-specific functions. The primary chicken hepatocytes culture system which was utilized in this study has also recognized to retain in vitro the differentiated phenotype typical of the liver, including albumin expression [Hou et al., 2001], P450 1A induction [Hou et al., 2001], tyrosine aminotransferase expression [Sasaki et al., 2000], and ascorbate recycling [Sasaki et al., 2001]. Therefore, this study examined the effect of EGF on DNA synthesis and its related signaling pathways in primary cultured chicken hepatocytes.

MATERIALS AND METHODS

Materials

Two weeks old White Leghorn male chickens were obtained from Dae Han Experimental Animal Co, Ltd. (Chungju, Korea). All the procedures for animal management followed the standard operation protocols of Seoul National University. The Institutional Review Board at Chonnam National University approved the research proposal and the relevant experimental procedures including animal care. The appropriate management of the experimental samples and quality control of the laboratory facilities and equipments were maintained. The class IV collagenase and a soybean trypsin inhibitor were purchased from Life Technologies (Grand Island, NY). The fetal bovine serum was purchased from Biowhittaker (Walkersville, MD). The EGF, AG 1478, herbimycin A, genistein, PD 98059, SB 203580, and SP 600125 were obtained from the Sigma Chemical Company (St. Louis, MO). The [^{3}H]-thymidine was purchased from NEN (Boston, MA). The phospho-EGFR, EGFR, phospho-p44/42 MAPKs, p44/42 MAPKs, phospho-p38 MAPK, and p38 MAPK antibodies were acquired from New England Biolabs (Herts, UK). The SAPK/JNK, CDK-2, CDK-4, cyclin D1, cyclin E, $p21^{WAF1/Cip1}$, and $p27^{kip1}$ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The goat anti-rabbit IgG was obtained from Jackson Immunoresearch (West Grove, PA). The liquiscint was obtained from National Diagnostics (Parsippany, NY). All other reagents were of the highest purity commercially available.

Primary Culture of Chicken Hepatocytes

The chicken liver cells were prepared and maintained in a monolayer culture as described elsewhere [Hou et al., 2001]. Briefly, the chicken hepatocytes were isolated by perfusion with 0.05% collagenase from a liver that had been starved for 3 h. Hepatocytes with more than 90% viability, as verified by a Trypan blue exclusion test, were used for subsequent plating. The hepatocytes were plated (5.0×10^5 cells/60 mm collagen-coated dish) with an incubation medium (Basal Medium Eagle supplemented with essential amino acids) containing 75 U/ml penicillin and 75 U/ml streptomycin, 1 μg/ml insulin, 10^{-12} M dexamethasone, 5 μg/ml transferrin, 10^{-8} M T_3, and 5% calf serum, and incubated for 4 h at 37°C in 5% CO_2 in the gas phase. The medium was subsequently replaced with fresh incubation medium, and the hepatocytes were incubated for further 20 hr in order to achieve a monolayer culture.

[^{3}H]-Thymidine Incorporation

A final media change was carried out when the cells had reached 50–60% confluence. The thymidine incorporation experiments were conducted as described by Gabelman and Emerman [1992]. The cells were incubated in a medium in the presence or absence of EGF for 24 h and were pulsed with 1 μCi of [methyl-^{3}H]-thymidine for 24 h at 37°C. The cells were then washed twice with PBS, fixed in 10% trichloroacetic acid (TCA) at room temperature for 15 min, and then washed twice in 5% TCA. The acid-insoluble material was dissolved in 2 N NaOH at room temperature and the level of radioactivity was counted using a liquid scintillation counter (LS 6500, Beckman Instruments,

Inc, Fullerton, CA). All the experiments were performed in triplicate. The values were converted from absolute counts to a percentage of the control in order to compare the results between experiments.

Measurement of $[Ca^{2+}]_i$

The changes in $[Ca^{2+}]_i$ were monitored by using Fluo-3/AM that had initially been dissolved in dimethylsulfoxide and stored at $-20^{\circ}C$. Chicken hepatocytes 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)]. They were then incubated in Bath Solution containing 3 μM fluo-3/AM with 5% CO_2 to 95% O_2 at 37°C for 40 min, rinsed twice with the Bath Solution, mounted on a perfusion chamber, and scanned at 1 s intervals using confocal microscopy (400×) (fluoview 300, Olympus). The fluorescence was excited at 488 nm and the emitted light was observed at 515 nm. All analyses of $[Ca^{2+}]_i$ were processed in a single cell, and the results were expressed as the fluorescence intensity (F/F_0, arbitrary unit).

Membrane Preparation for Western Blotting

The medium was 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 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 resuspended cells were then mechanically lysed on ice by trituration using a 21.1-G needle. The lysates were first centrifuged at 1,000*g* for 10 min at 4°C. The supernatants were centrifuged at 100,000*g* for 1 h at 4°C to prepare the cytosolic and total particulate fractions. The particulate fractions, which contained the membrane fraction, were washed twice and resuspended in buffer A containing 1% Triton X-100. The protein level in each fraction was quantified using the Bradford [1976] procedure.

Western Blot Analysis

The cell homogenates (20 μg of protein) were separated on 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The blots were then washed with H_2O, blocked with 5% skim milk powder in TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20] for 1 h 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 (1: 5,000) conjugated to horseradish peroxidase. The bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK).

Statistical Analysis

The results are expressed as a mean ± standard error (SE). The difference between the two mean values was analyzed by ANOVA. A P-value <0.05 was considered significant.

RESULTS

Effect of EGF on DNA Synthesis and CDK Expression Levels

In order to examine the effect of EGF on DNA synthesis, the level of [^{3}H]-thymidine incorporation as a result of exposing the cells to various EGF doses (0–1,000 ng/ml) for 24 h was first examined. As shown in Figure 1A, EGF increased the level of [^{3}H]-thymidine incorporation in a dose (≥10 ng/ml)-dependent manner. The maximum increase in [^{3}H]-thymidine incorporation was observed between 100 and 1,000 ng/ml EGF after 24 h (Fig. 1A). Figure 1B shows that EGF significantly increased the CDK-2 and CDK-4 expression level at ≥10 ng/ml. In experiments designed at identifying the involvement of the EGF receptor as well as the tyrosine kinase phosphorylation of the EGF receptor in EGF-induced DNA synthesis, AG 1478 (EGF receptor tyrosine kinase antagonist), genistein, and herbimycin A (tyrosine kinase inhibitors) blocked EGF-induced DNA synthesis (Fig. 2).

Involvement of $[Ca^{2+}]_i$ and PKC in EGF-Induced Cell Proliferation

In experiments to examine if EGF-induced cell proliferation is involved in intracellular Ca^{2+}, EGF increased $[Ca^{2+}]_i$ (Fig. 3A), which was blocked by the pretreatment of neomycin (a PLC inhibitor, 10^{-4} M) (Fig. 3B) or EGTA (an extracellular calcium chelator, 4×10^{-3} M) and BAPTA-AM (an intracellular calcium chelator, 10^{-5} M) mixture (Fig. 3C). To further elucidate

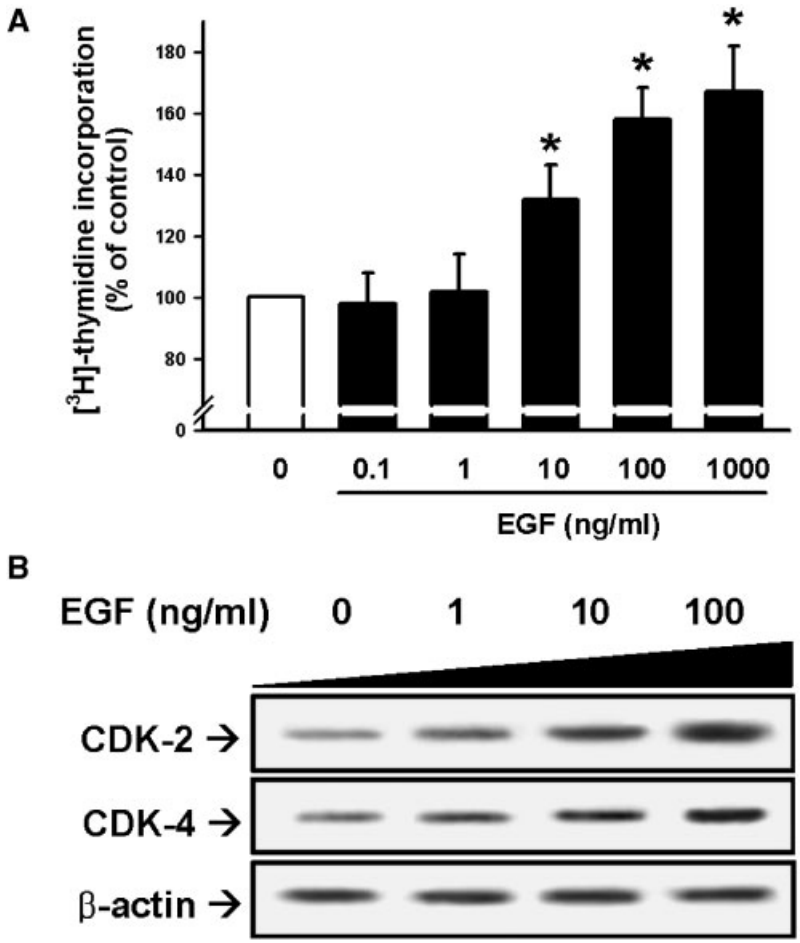

Fig. 1. Dose dependency of EGF on [^{3}H]-thymidine incorporation (**A**) and the expression of CDK-2 and CDK-4 (**B**). Chicken hepatocytes were treated with different EGF doses (0–1,000 ng/ml) for 24 h, and pulsed with 1 μCi of [^{3}H]-thymidine for 24 h. The whole cell extracts were probed by Western blotting using antibodies specific to CDK-2, CDK-4, and β-actin. The values are represented as a mean ± SE of three independent experiments with triplicate dishes. The lower panel of B depicting the bars denotes the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin. $*P < 0.05$ versus control.

the involvement of PKC in the EGF-induced cell proliferation, the translocation of PKC from the cytosolic to the membrane compartment was observed after treating the cells with 100 ng/ml EGF for 1 h (Fig. 4A). EGF also enhanced the translocation of PKC α, β_1, and δ isoforms to the membrane (Fig. 4B). Subsequently, the pretreatment of PKC blockers, staurosporine, H-7, or bisindolylmaleimide I (10^{-7} M) attenuated EGF-induced increase in [^{3}H] thymidine incorporation (Fig. 4C).

Involvement of MAPKs in EGF-Induced Cell Proliferation

The role of MAPKs in EGF-induced cell proliferation was next examined. As shown in Figure 5A, EGF increased each p44/42, p38 MAPK, or JNK in a time-dependent manner. In order to determine if adenylyl cyclase, PLC, and $[Ca^{2+}]_i$ are involved in the EGF-induced activation of MAPKs, the cells were pretreated with SQ 22536 (adenylyl cyclase inhibitor, 10^{-6} M),

Fig. 3. Effect of EGF on $[Ca^{2+}]_i$. **A**: Chicken hepatocytes were loaded with 2 μM fluo 3-AM in serum-free medium for 40 min and treated with EGF (100 ng/ml). Chicken hepatocytes were pretreated with (**B**) neomycin (10^{-4} M), (**C**) EGTA (4×10^{-3} M)/BAPTA-AM (10^{-5} M) mixture for 30 min prior to EGF treatment and then Ca^{2+} influx was measured. The changes in $[Ca^{2+}]_i$ were monitored by confocal microscopy, and the data is expressed as the fluorescence intensity (F/F_0, arbitrary unit). A 23187 (10^{-6} M) was used as the positive control. The example shown is a representative of three experiments.

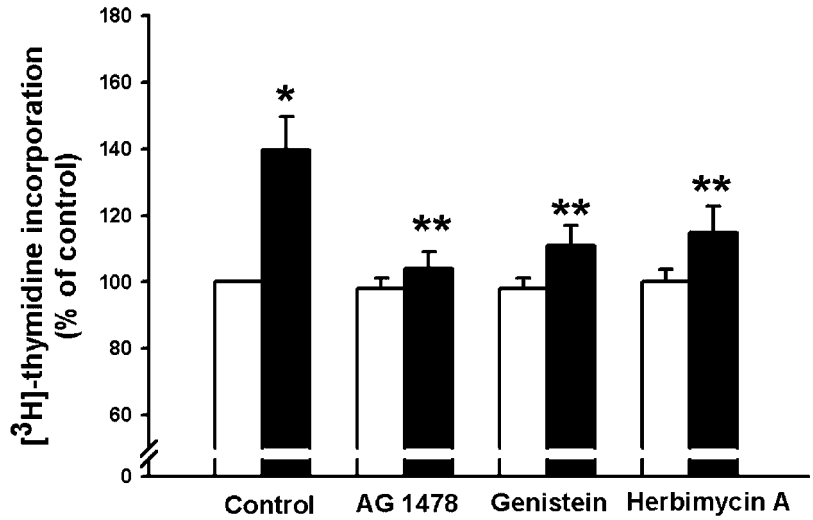

Fig. 2. Effect of the EGF receptor tyrosine kinase antagonist (AG 1478), tyrosine kinase inhibitors (genistein and herbimycin A) in EGF-induced [^{3}H]-thymidine incorporation. Chicken hepatocytes were treated with AG 1478 (10^{-5} M), genistein (10^{-6} M), or herbimycin A (10^{-6} M) for 30 min prior to the EGF treatment (100 ng/ml). The values are represented as a mean ± SE of three independent experiments with triplicate dishes. Symbols are: (□) control; (■) EGF. *$P < 0.05$ versus control. **$P < 0.05$ versus EGF alone.

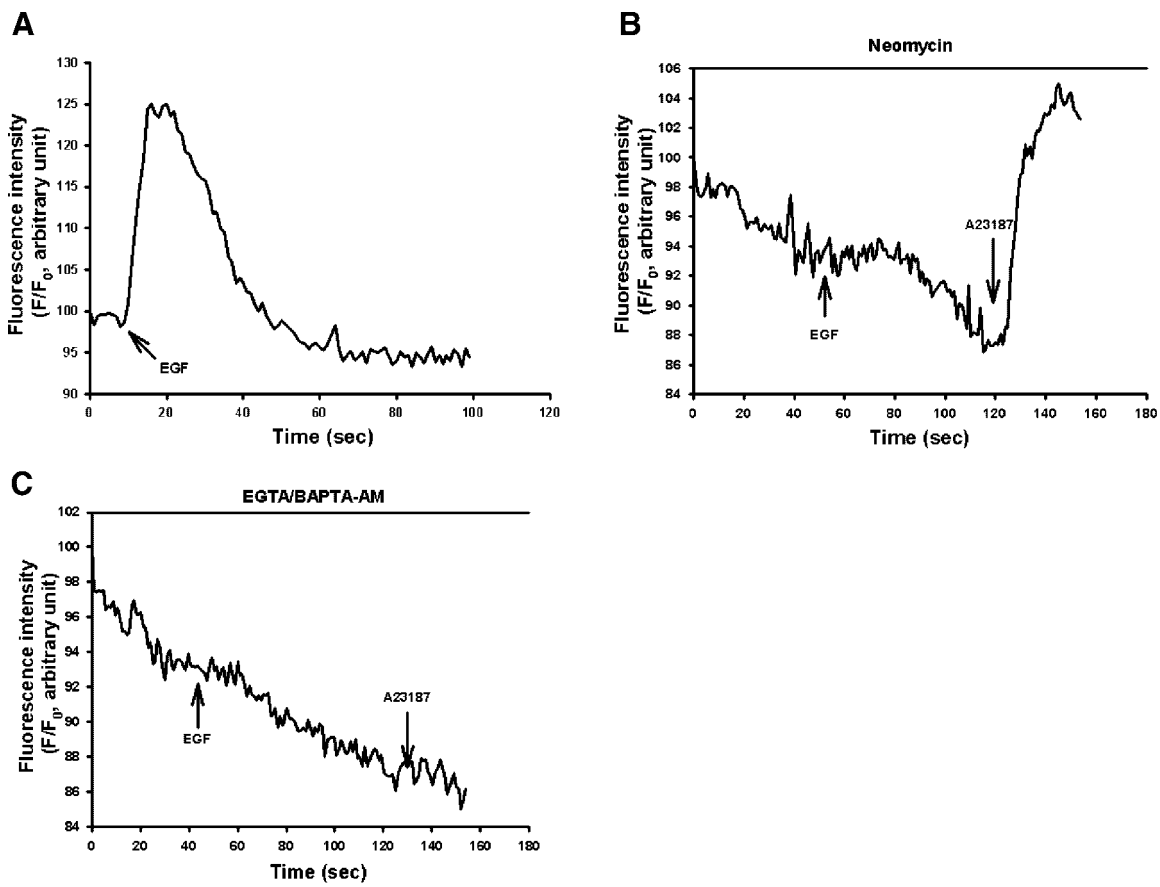

Fig. 3.

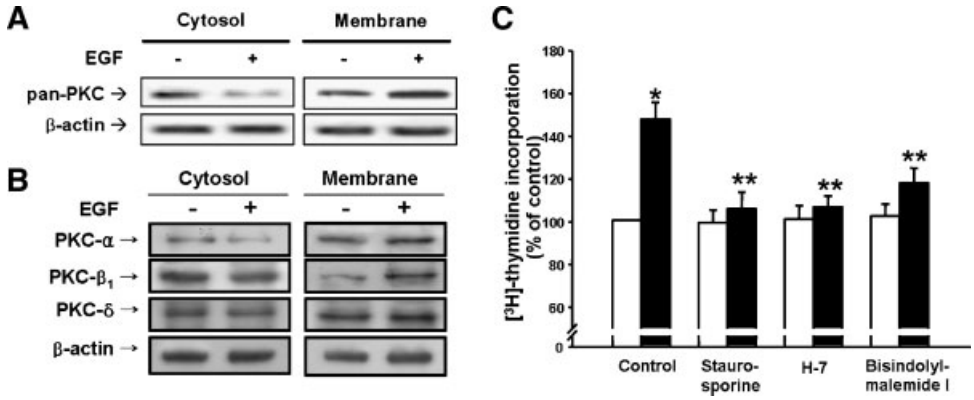

Fig. 4. Effect of EGF on PKC activation. **A**: The Pan-PKC protein and (**B**) PKC α, β_1, and δ isoforms which were present in either the cytosolic compartment or membrane compartment was detected by Western blotting, as described in Materials and Methods. Bands represent the 80 kDa of Pan-PKC, 80–90 kDa of PKC α, β_1, and δ and 41 kDa of β-actin. Each example shown is a representative of three experiments. **C**: Chicken hepatocytes were treated with staurosporine, H-7, or bisindolylmaleimide I (10^{-7} M) for 30 min prior to the EGF treatment (100 ng/ml) for 1 h. The values are represented as a mean ± SE of three independent experiments with triplicate dishes. $^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus EGF alone.

neomycin, and EGTA /BAPTA-AM mixture for 30 min prior to the EGF treatment. As shown in Figure 5B, each inhibitor attenuated the EGF-induced MAPKs activation. In addition, a pretreatment with AG 1478 (10^{-5} M), herbimycin A (tyrosine kinase inhibitor, 10^{-6} M), or bisindolylmaleimide I attenuated the EGF-induced MAPKs activation (Fig. 5C).

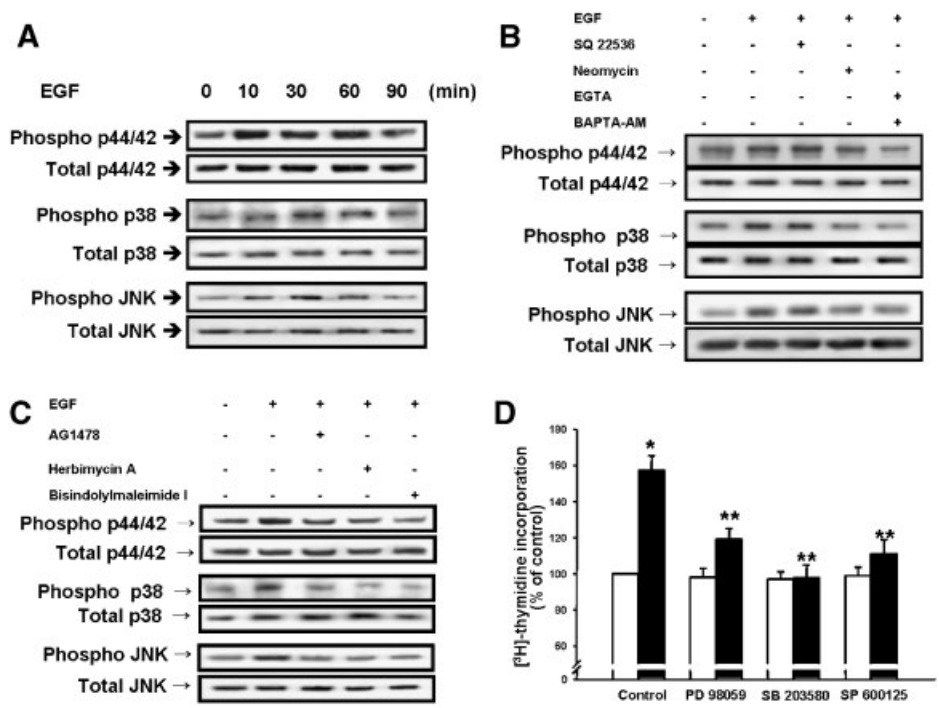

Fig. 5. Effect of EGF on MAPKs activation. **A**: Chicken hepatocytes were treated with EGF (100 ng/ml) for different time points (0–90 min). The phosphorylation of p44/42 MAPKs, p38 MAPK, and JNK was detected as described in the Materials and Methods, respectively. Chicken hepaotyctes were treated with (**B**) SQ 22536, Neomycin, EGTA/BAPTA-AM mixture, (**C**) AG1478 (10^{-5} M), herbimycin A (10^{-6} M), and bisindolylmaleimide I (10^{-7} M) for 30 min prior to the treatment of EGF. The phosphorylated p44/42 MAPK, p38 MAPK, and JNK were detected as described in the Materials and Methods. Each example shown is a representative of three experiments. **D**: The chicken hepatocytes were treated with PD 98059 (10^{-5} M), SB 203580, or SP 600125 (10^{-6} M) for 30 min prior to the EGF treatment. The values are represented as a mean ± SE of three independent experiments with triplicate dishes. Symbols are: (□) control; (■) EGF. $^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus EGF alone.

Consequently, the inhibition of each MAPK with PD 98059 (p44/42 MAPK inhibitor) (10^{-5} M), SB 203580 (p38 MAPK inhibitor), or SP 600125 (JNK inhibitor) (10^{-6} M) decreased EGF-induced increase in [^{3}H]-thymidine incorporation (Fig. 5D).

The Effect of EGF on CDK-2, CDK-4, Cyclin D1, Cyclin E, p21, and p27 and its Related Signal Pathways

EGF increased CDK-2 and CDK-4 expression levels in the chicken hepatocytes (Fig. 1B). The EGF-induced increase in the levels of the cell-cycle regulator was blocked by AG 1478, herbymicin A, staurosporine, SB 203580, and PD 98059 (Fig. 6). Figure 7A shows that the EGF-induced increase in the cyclin D1 and cyclin E levels was blocked by AG 1478, herbimycin A, PD 98059, SB 203580, and SP 600125. In addition, AG 1478, herbimycin A, PD 98059, SB 203580, and SP 600125 blocked the EGF-induced decrease of $p21^{WAF1/Cip1}$ and $p27^{kip1}$ (Fig. 7B).

DISCUSSION

In this study, EGF stimulated DNA synthesis of the primary cultured chicken hepatocytes. Understanding the mechanism for this EGF induces cell proliferation is a key to understanding the EGF-induced signaling in chicken hepatocytes. In this study, a high dose of EGF (100 ng/ml) increased the rate of cell growth, even though the physiological levels of EGF are 3–10 ng/ml. This result is consistent with previous reports showing that a high dose of EGF (100 ng/ml) increased the rate of cell growth [Nakamura et al., 2001], where 100, 500, 1,000 ng/ml EGF were used under various experimental conditions and different cell types [Perry et al., 1998; D'Onofrio et al., 2003]. We suggest that the conditions used in the in vitro experimental systems and different cell types could have led to this result. The EGF-induced mitogenic effect on hepatocytes is mediated by the alteration of the mid/late G_1 phase, which are different from the other G protein-coupled receptors [Nilssen et al., 2002]. CDK-2, CDK-4, cyclin D1, and cyclin E play a key role in cell-cycle progression from the G_1 to S phase of hepatocytes, as in other cell types [Loyer et al., 1996; Kato et al., 1998]. In this study, an EGF treatment in chicken hepatocytes upregulated the protein levels of CDK-2, CDK-4, cyclin D1,

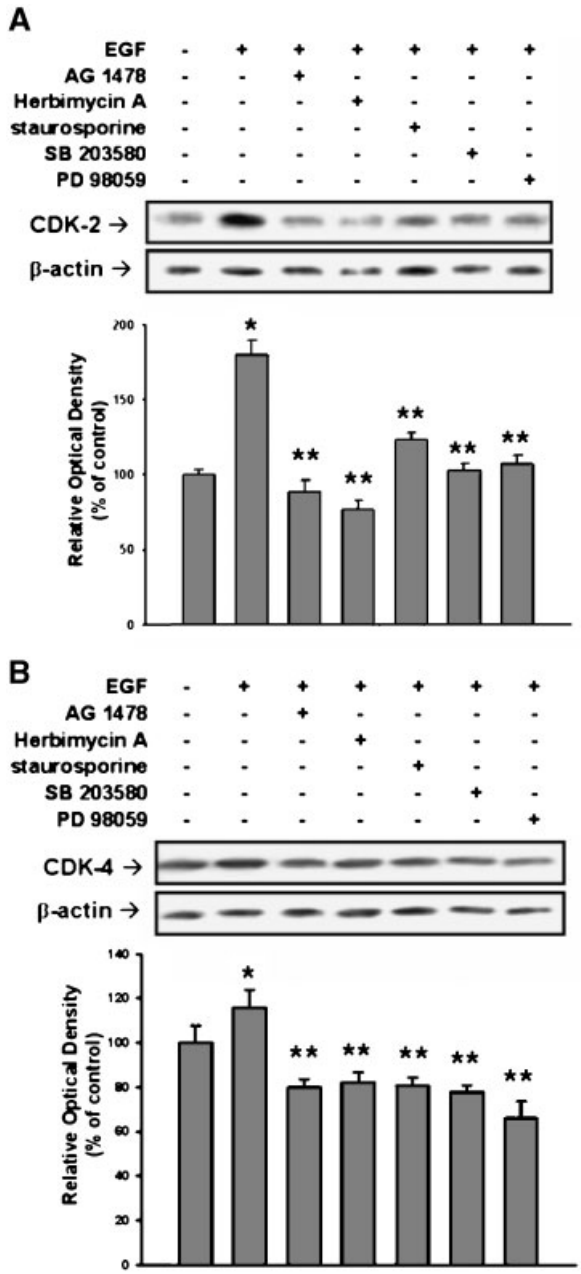

Fig. 6. Effect of EGF receptor tyrosine kinase, protein tyrosine kinase, PKC, and MAPKs inhibitors on EGF-induced expression of CDK-2 and CDK-4. Chicken hepatocytes were pretreated with AG 1478, herbimycin A, staurosporine, SB 203580, or PD 98059 for 30 min prior to the EGF treatment for 4 h. The total lysates were then subjected to SDS–PAGE and blotted with (**A**) CDK-2 or (**B**) CDK-4 antibody, respectively. Each example shown is a representative of three experiments. The lower panel of A and B depicting the bars denotes the means ± SE of three experiments for each condition determined from densitometry relative to β-actin. $^*P < 0.05$ versus Control, $^{**}P < 0.05$ versus EGF alone.

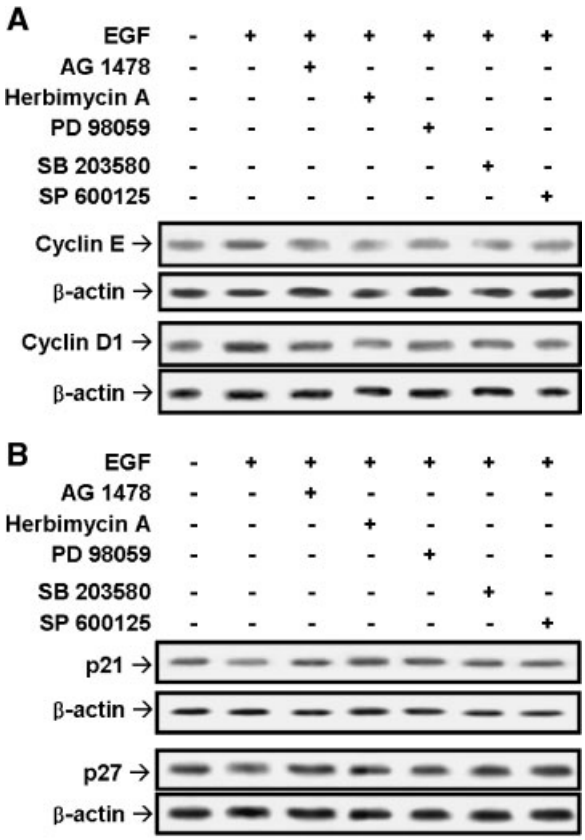

Fig. 7. Effect of EGF receptor tyrosine kinase, protein tyrosine kinase, PKC, and MAPKs inhibitors on EGF-induced expression of cyclin D1, cyclin E, p21, and p27. Chicken hepatocytes were pretreated with AG 1478, herbimycin A, PD 98059, SB 203580, or SP 600125 for 30 min prior to the EGF treatment for 4 h. The total lysates were then subjected to SDS–PAGE and blotted with (**A**) cyclin D/cyclin E or (**B**) p21/ p27 antibody. Each example shown is a representative of four experiments.

and cyclin E. Sheaff et al. [1997] reported that cyclin E/CDK-2 in murine fibroblasts causes the phosphorylation of the CKI $p27^{kip1}$ on T187, resulting in the elimination of p27 from the cell and allowing the cells to transit from the G_1 to S phase. This study demonstrated that the EGF treatment in primary cultured chicken hepatocytes induced the decrease in $p21^{WAF1/Cip1}$ and $p27^{kip1}$. This decrease correlated with the increase in cyclin D1/CDK-4 and cyclin E/ CDK-2, which are major translocators of various extracellular influences on the cell progression through the G_1 to S restriction point of the cell cycle, with promoting and inhibiting activities.

Although previous reports have shown that an alteration of CDK by EGF is required for EGF-induced mitogenesis, the mechanism by which CDK-2 and CDK-4 expression is altered is unclear. This study demonstrated that the selective EGF receptor blocker (AG 1478) and tyrosine kinase inhibitor (herbimycin A, genistein) completely inhibited the EGF-induced stimulation of cell proliferation and the alteration of the CDK complexes, which suggests that EGF stimulates the tyrosine kinase activity, resulting in the rapid phosphorylation of its own receptor [Krymskaya et al., 2000]. This result is accordance with the report showing that a treatment of human breast epithelial cells with an EGF receptor antagonist induced a rapid decrease in the cyclin D1 levels in Chou et al. [1999]. Therefore, upon activation, the EGF receptor is autophosphorylated at the specific tyrosine residues, which mediates the onset of the EGF signal. This study also showed that the increase of intracellular Ca^{2+} is involved in EGF-induced cell proliferation. Experiments were performed to determine if the activation of EGF receptor alters the intracellular Ca^{2+} concentration, which links EGF receptor to phospholipase C (PLC)-associated second messenger cascades, and to determine if the pattern of these alterations influences PKC and MAPKs activation. In this study, PLC was found to be involved in EGF-induced stimulation of Ca^{2+} influx. PLC plays a pivotal role in transmembrane signaling. In response to various extracellular stimuli, such as numerous hormones, growth factors, and neurotransmitters, PLC hydrolyzes a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), producing the two-second messengers diacylglycerol (DAG) and inositol 1, 4,5-triphosphate (IP_3), which mediate the activation of PKC and intracellular Ca^{2+} release, respectively [Berridge and Irvine, 1989; Shin et al., 2002]. In addition to PLC-associated Ca^{2+} influx, PKC activity increased in response to EGF. In this study, the involvement of the PKC pathway in the effect of EGF was evidenced by the following observations: (1) staurosporine or bisindolylmaleimide I (PKC inhibitors) prevented the EGF-induced stimulation of thymidine incorporation and CDK-2/4 expression, and (2) PKC was translocated to the membrane, accounting for PKC activation. These results are not in accordance with the report by Dajani et al. [1999], who suggested that EGF does not activate the DAG-dependent PKC pathway in rat hepatocytes. Although the reason for this discrepancy is unclear, and it might be due to

the different species used (chicken vs. rat). PKC activation does not always regulate the same pattern of CDK and cyclin because Burger et al. [1994] reported that TPA (PKC activator) induced a decrease in the expression of CDK-2 and the upregulation of cyclin D1. Previous studies have demonstrated that EGF-dependent mitogenesis is associated with the activation of phospholipid-dependent PKC in normal mammary epithelial cells [Birkenfeld et al., 1966], which might be consistent with the result of PKC translocation from the cytosolic to membrane fraction. To our knowledge, this is the first report showing that PKC activation is responsible for EGF-induced mitogenesis in chicken hepatocytes. This study demonstrated that an EGF treatment of chicken hepatocytes activates the phosphorylation of the EGF receptor, which translocates PKC from the cytosol to the membrane fraction. We also identified that PKC α, β_1, and δ are activated in response to EGF in chicken hepatocytes. However, Marino et al. [2000] reported that EGF translocated only PKC α and ε among PKC isoforms from cytosol to membrane fraction in chick embryo hepatocytes. This discrepancy may be due to the difference of developmental stage (postnatal vs. prenatal).

Since p44/42 MAPKs are activated by the phosphorylation of threonine and tyrosine residues, the tyrosine phosphorylation of the EGF receptor mediates the activation of ERK. This result is consistent with a report showing that EGF stimulates p44/42 MAPKs in rat hepatocytes [Karihaloo et al., 2001; Nilssen et al., 2002]. Therefore, p44/42 MAPKs activation decreases the expression of the cyclin-dependent protein kinase inhibitor, $p21^{WAF1/Cip1}$, leading to cell-cycle (G_1 phase) progression and cell proliferation. It was reported that PD 98059 prevented the EGF-induced stimulation of cyclin D1 in human thyroid tumor cells, which is an important intermediate in inducing the cell-cycle cascade [Manole et al., 2001]. In addition, several reports support the suggestion that p38 MAPK and JNK also drive specific cell-cycle responses to extracellular stimuli, which can positively or negatively influence DNA synthesis [Wang et al., 2001; Simmons et al., 2004]. In this study, both JNK and p38 MAPK are involved in the EGF-induced stimulation of cyclin D1, cyclin E, CDK-2, and CDK-4 as well as in the decrease in $p21^{WAF1/Cip1}$ and $p27^{kip1}$ expression in chicken hepatocytes. This result is consistent with a report by Schwabe et al. [2003] showing that SP 600125 decreased the EGF-induced transcription of a cyclin D1 mRNA in rat hepatocytes. Interestingly, all three MAPKs are involved in the EGF-induced cell proliferation and alteration of CDKs. Although the signal pathways between the three MAPKs are different, there might be a cross-talk between the three MAPKs. A recent report also demonstrated cross-talk between the MAPKs [Shama et al., 2003].

Cross-talk between PKC and the MAPKs by agonists has been extensively studied in various cell systems [Rivedal and Opsahl, 2001; Fan et al., 2004]. Toyoda et al. [1998] reported that EGF-induced p21 expression in A431 cells was mediated by the MAP kinase-independent protein kinase C signaling pathway. Xu et al. [2002] also reported that EGF-induced ERK phosphorylation in human corneal epithelial cells is independent of the PKC isozymes.

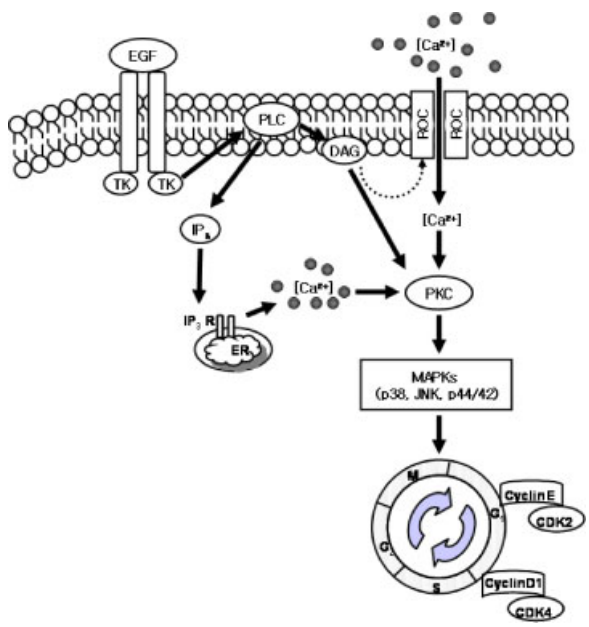

Fig. 8. The hypothesized model for the signal pathways involved in EGF-induced chicken hepatocyte proliferation. EGF activates receptor, which stimulate PLC to generate IP_3 and DAG. In turn, DAG activates PKC, which induces p44/42 MAPKs activation, continuously inducing cell-cycle progression. In another pathway, DAG activates ROC-mediated Ca^{2+} influx and IP_3 stimulates the release of Ca^{2+} from an intracellular Ca^{2+} pool. In turn, Ca^{2+} activates PKC and MAPKs leading to the cell-cycle progression. PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; ROC, receptor-operated channel; IP_3, 1,4,5-inositol-triphosphate; IP_3R, IP_3 receptor; ER, endoplasmic reticulum; CDK, cyclin-dependent kinase. The solid line is the proposed pathway and the dashed line is suspected pathway.

Unlike these reports, the present study revealed that both PKC and MAPKs activation by EGF receptor phosphorylation are involved in the regulation of cell progression in chicken hepatocytes. Furthermore, it was demonstrated that PKC is responsible for the activation of three MAPKs. This discrepancy might be due to the difference in species (chicken vs. human) or the cell specificity (hepatocytes vs. squamous cell carcinoma or corneal epithelial cells). Based on all of the results of this study, we would suggest that various downstream signal molecules of EGF receptor tyrosine kinase are required for cell-cycle control and finally these cascades lead to chicken hepatocyte proliferation (Fig. 8). In conclusion, EGF induces DNA synthesis in chicken hepatocytes through a change in CDK-2/4, cyclin D/E, and $p21^{WAF1/Cip1}$, $p27^{kip1}$ expression via the Ca^{2+}/PKC and MAPKs signal cascades.

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