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A Potential Mechanism for Short Time Exposure to Hypoxia-Induced DNA Synthesis in Primary Cultured Chicken Hepatocytes: Correlation Between Ca^{2+}/PKC/MAPKs and PI3K/Akt/mTOR

Sang Hun Lee,[1] Min Young Lee,[1] Jang Hern Lee,[2] and Ho Jae Han[1]*

[1]Department of Veterinary Physiology, Biotherapy Human Resources Center (BK 21), College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea
[2]Department of Veterinary Physiology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea

Abstract Less information is available concerning the molecular mechanisms of cell survival after hypoxia in hepatocytes. Therefore, this study examined the effect of hypoxia on DNA synthesis and its related signal cascades in primary cultured chicken hepatocytes. Hypoxia increased [^{3}H] thymidine incorporation, which was increased significantly after 0–24 h of hypoxic exposure. Indeed, the percentage of cell population in the S phase was increased in hypoxia condition. However, the release of LDH indicating cellular injury was not changed under hypoxic conditions. Hypoxia increased Ca^{2+} uptake and PKC translocation from the cytosol to the membrane fraction. Among the PKC isoforms, hypoxia stimulated the translocation of PKC α and ε. Hypoxia also phosphorylated the p38 and p44/42 mitogen-activated protein kinases (MAPKs), which were blocked by the inhibition of PKC. On the other hand, hypoxia increased Akt and mTOR phosphorylation, which was blocked in the absence of intra/extracellular Ca^{2+}. The inhibition of PKC/MAPKs or PI3K/Akt pathway blocked the hypoxia-induced [^{3}H] thymidine incorporation. However, hypoxia-induced Ca^{2+} uptake and PKC translocation was not influenced by LY 294002 or Akt inhibitor and hypoxia-induced MAPKs phosphorylation was not changed by rapamycin. In addition, LY 294002 or Akt inhibitor has no effect on the phosphorylation of MAPKs. It suggests that there is no direct interaction between the two pathways, which cooperatively mediated cell cycle progression to hypoxia in chicken hepatocytes. Hypoxia also increased the level of the cell cycle regulatory proteins [cyclin D_1, cyclin E, cyclin-dependent kinase (CDK) 2, and CDK 4] and p-RB protein but decreased the p21 and p27 expression levels, which were blocked by inhibitors of upstream signal molecules. In conclusion, short time exposure to hypoxia increases DNA synthesis in primary cultured chicken hepatocytes through cooperation of Ca^{2+}/PKC, p38 MAPK, p44/42 MAPKs, and PI3K/Akt pathways. J. Cell. Biochem. 104: 1598–1611, 2008.
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The liver has the potential for complete recovery from an acute loss of function. If the ischemic injury is not too severe, the surviving hepatocytes proliferate and the liver function improves. It has been suggested that the survival of a cell depends on the balance between the survival and death signals [Ebert, 2006; Kietzmann et al., 2006]. Although most studies have focused on the signals promoting cell death leading to the development of acute hepatic failure, there has been less emphasis on the cellular mechanisms involved in cell survival in the postischemic liver. Indeed, less information is available concerning the molecular mechanisms of cell survival after hypoxia in hepatocytes. Oxygen is a potent signaling molecule which has received increasing recognition for its ability to affect the fundamental

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*Correspondence to: Ho Jae Han, Department of Veterinary Physiology, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea.
E-mail: hjhan@chonnam.ac.kr

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characteristics of various types of stem cells [Csete, 2005]. Oxidative stress is also an important physiological and pathological regulator that might influence the whole life span, from embryogenesis and development to the maintenance of normal function, dysfunction, and aging [Agarwal et al., 2005; Martin and Grotewiel, 2006]. It is generally assumed that living organisms respond to reduced oxygen concentrations in a cell type- and tissue-specific manner. The production of reactive oxygen species (ROS) is inevitable consequence of the low oxygen culture. ROS generation at low concentrations in cells may play roles as signaling molecules within the signal transduction cascades [Prata et al., 2004]. However, high concentrations of ROS can significantly inhibit cell growth and induce apoptosis [Holbrook and Ikeyama, 2002; Juranek and Bezek, 2005].

The proliferation of mammalian cells is controlled largely during the G1 phase of their growth cycles. The decision to initiate a new round of DNA synthesis requires the integration of signals from the extracellular environment such as growth factors, cytokines, and extracellular matrix proteins mediated by different transduction cascades [Brennan et al., 1997; Gao et al., 2004; Zhu et al., 2005]. The two signaling cascades have emerged as major players in the mitogenic and antiapoptotic response in many cells: the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways. The MAPKs are serine/threonine kinases involved in regulating various cellular responses, such as cell proliferation, differentiation, and apoptosis [Tomaselli et al., 2005; Takahashi et al., 2006]. p38 MAPK is a member of the stress-activated kinases, or SAPK, which also includes JNK. Other MAPK so far identified-ERK1 and 2 (or p44/42) MAPK are known as "classic" MAPK. The SAPKs have been shown to be variably upregulated under conditions of cellular stress (such as altered pH, increased temperature, or hypoxia) [Scott et al., 1998] but, unlike JNK, p38 MAPK appears to be consistently involved in the hypoxia-mediated proliferation of pulmonary artery fibroblasts. In addition, serine/threonine kinase Akt, which is also known as protein kinase B, plays an important role in cell proliferation, differentiation, and survival [Katso et al., 2001; Gerasimovskaya et al., 2005]. It is activated by the PI3K-dependent signaling pathway in response to various stimuli including growth factors and certain cytokines. The activation of these kinases may be important for the survival of hepatocytes exposed to ischemia and thereby increasing the proliferative activity. However, there is little known regarding the signaling pathway involved in activating these kinases and their potential roles in a postischemic liver. Although it has been reported that MAPKs phosphorylation is enhanced after ischemia in vivo, it is unclear if MAPKs and Akt phosphorylation in hepatocytes are activated by hypoxia, which is an in vitro version of ischemia in vivo.

A primary culture of hepatocytes was used in 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 through the expression of liver-specific functions [Lee et al., 2006a,b; Suh et al., 2008]. Indeed, the primary chicken hepatocytes culture system used in this study was also recognized to retain the in vitro the differentiated phenotype typical of the liver, which included albumin expression [Goldberg et al., 1997], P450 1A induction [Hou et al., 2001], tyrosine aminotransferase expression [Sasaki et al., 2001], and ascorbate recycling [Sasaki et al., 2000]. In addition, the G0/G1 transition occurs after tissue disruption of the cell–cell contact during cell isolation [Etienne et al., 1988]. This mimics entry into the G1 phase of proliferating hepatocytes in vivo in a regenerating liver after a partial hepatectomy [Fausto et al., 1995; Michalopoulos and DeFrances, 1997]. In the primary culture, hepatocytes spontaneously progress in the G1 phase up to a restriction point located at the 2/3 of the G1 phase [Loyer et al., 1996]. Therefore, this study examined effects of short time exposure to hypoxia on DNA synthesis and its related signal pathways in primary cultured chicken hepatocytes.

MATERIALS AND METHODS

Materials

Two-week old White Leghorn male chickens were obtained from the Dae Han Experimental Animal Co., Ltd. (Chungju, Korea). All the procedures for animal management were followed using 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 LY 294002 (PI3K inhibitor), rapamycin (mTOR inhibitor), PD 98059 (p44/42 inhibitor), SB 203580 (p38 inhibitor), BAPTA-AM (intracellular Ca^{2+} chelator), EGTA (extracellular Ca^{2+} chelator), anti-β-actin antibody were obtained from Sigma Chemical Company (St. Louis, MO). The bisindolylmaleimide I, staurosporine (PKC inhibitors), and Akt inhibitor (phosphatidylinositol ether analog) were supplied by Calbiochem (La Jolla, CA). The anti-p-Akt (Thr 308 and Ser 473) were obtained from Upstate Biotechnology (Charlottesville, VA). The anti-p-p44/42 (Thr202/Tyr204), p-p38 (Thr180/Tyr182), p-mTOR (Ser 2448), p-p70S6K1 (Thr 389), and p-4EBP1 (Thr37/46) were obtained from New England Biolabs (Herts, UK). The anti-p-pan PKC, PKC-α, ε, HIF-1α, cyclin D_1, cyclin E, CDK 2, CDK 4, $p21^{cip1}$, and $p27^{kip1}$ were purchased from Santa Cruz Biotechnology (Delaware, CA). The goat anti-rabbit IgG was purchased from Jackson Immunoresearch (West Grove, PA). The liquiscint was supplied by National Diagnostics (Parsippany, NY). All the other reagents were of the highest purity commercially available.

Primary Culture of Chicken Hepatocyte

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 >90% viability, as verified by the 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 streptomycin, 1 μg/ml insulin, 5 μg/ml transferrin, 10^{-8} M T_3, and 5% fetal bovine serum, and incubated for 4 h at 37°C in an atmosphere containing 5% CO_2. The medium was then replaced with a fresh incubation medium, and the hepatocytes were incubated for a further 20 h to achieve a monolayer culture.

Hypoxic Stimulation

The chicken hepatocytes were cultured in 35 or 60 mm culture dishes, and washed twice with phosphate-buffered saline (PBS). The media was then changed to fresh William's medium without any supplements. The experiments were performed in an incubator at 37°C under normoxic conditions by maintaining the cells in either 92.3% air and 5.5% CO_2 or under hypoxic conditions by incubating the cells in a modular incubator chamber containing 2.2% O_2, 5.5% CO_2, and 92.3% N_2 (Billups-Rotheberg Inc., CA) at a flow rate of 20 L/min for 30 min. After the chamber had been purged with gas, it was sealed and placed in a conventional incubator at 37°C.

[^{3}H] Thymidine Incorporation and Cell Counting

A final media change was carried out when the cells had reached 50–60% confluence. The ^{3}H thymidine incorporation experiments were conducted as described by Brett et al., (1993). The cells were incubated in a medium in the presence or absence of hypoxia 0–24 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 23°C for 15 min, and then washed twice with 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, Fullerton, CA). All the values are reported as the mean (±standard error, SE) of triplicate experiments. The values were converted from absolute counts to a percentage of the control to allow for a comparison between experiments.

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 bule solution and the number of living cells was determined using a hemocytometer.

Fluorescence-Activated Cell Sorter (FACS) Analysis

The cells were incubated under hypoxic conditions for 0–24 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 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, CA).

Immunofluorescence Staining with PKC

Cells were fixed and treated with monoclonal antibody against rabbit PKC (1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and then incubated for 30 min with fluorescein isothiocyanate-conjugated (FITC-conjugated) secondary antibody raised in goat against rabbit IgM (1:100). Fluorescence images were visualized with a fluorescence microscope (Fluoview 300, Olympus, Japan).

$^{45}Ca^{2+}$ Uptake Experiment

The $^{45}Ca^{2+}$ uptake experiments were carried out using a slight modification of the methodology reported by Chirayath et al., (1998). In order to measure the level of Ca^{2+} uptake, the culture medium was removed by aspiration, and the cells were gently washed twice with the uptake buffer (140 mM NaCl, 2 mM KCl, 1 mM KH_2PO_4, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 5 mM L-alanine, 5 μM indomethacin, and 10 mM HEPES/Tris, pH 7.4). After washing, the cells were incubated in an uptake buffer containing 1 μCi/ml $^{45}Ca^{2+}$ at 37°C for 30 min. At the end of the incubation period, the cells were washed three times with an ice-cold uptake buffer, and digested in 1 ml 0.1% SDS. In order to determine the level of $^{45}Ca^{2+}$ incorporated intracellularly, 900 μl of each sample was removed and the radioactivity was counted using a liquid scintillation counter (LS6500; Beckman Instruments, Fullerton, CA). The remainder of each sample was used to determine the protein level [Bradford, 1976]. The radioactivity in each sample was normalized to the protein level and corrected for the zero-time uptake per milligram protein. All the uptake measurements were carried out in triplicate.

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-gauge needle. The lysates were centrifuged at 1,000*g* for 10 min at 4°C. The supernatants were then centrifuged at 100,000*g* for 1 h at 4°C to prepare the cytosolic and 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 procedure [Bradford, 1976].

Western Blot Analysis

The cell homogenates (20 μg protein) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After the 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% skim milk for 1 h and incubated with the appropriate primary antibodies at the dilutions recommended by the supplier. The membrane was then washed, and the primary antibodies were detected using 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).

Statistical Analysis

The results are expressed as the mean ± SE. All the experiments were analyzed by ANOVA, which were followed in some experiments by a comparison of the treatment means with the control using a Bonnferroni–Dunn test. A P-value <0.05 was considered significant.

RESULTS

Effect of Hypoxia on DNA Synthesis

In order to ensure that the hypoxia used in this study induces typical cell responses to hypoxic stress, the alteration of the HIF-1α expression level at different times (0–120 min) was first observed. As shown in Figure 1A, the significant increase in HIF-1α appeared from 15 to 30 min of hypoxic exposure. The amount of LDH release was measured in order to determine if hypoxic conditions cause structural damage to the cell. As shown in Figure 1B,

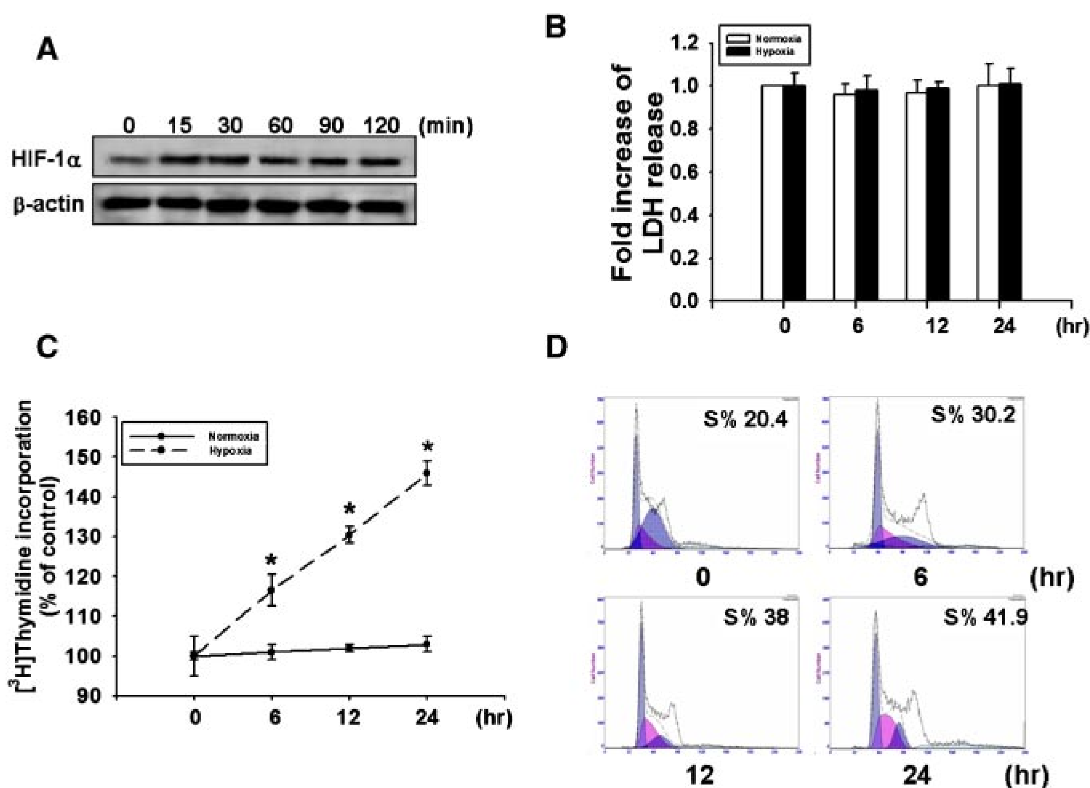

Fig. 1. Effect of hypoxia on HIF-1α expression and DNA synthesis. **A**: The cells were exposed to hypoxia for 0–120 min and HIF-1α was detected by Western blotting as described in Materials and Methods. **B**: The release of LDH was measured under hypoxia (0–24 h), as described in Materials and Methods. The values are reported as a mean ± SE of four independent experiments with triplicate dishes. **C**: The cells were exposed to hypoxia for 0–24 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. **D**: The cells were washed with PBS, fixed, stained with PI, and analyzed by flow-cytometer. The gates were configured manually to determine the percentage of cells at the G1, S, and G2/M phases based on the DNA content. The percentage of cells at the G0/G1, S, and G2/M phases was obtained from three independent experiments.

hypoxia did not affect the release of LDH up to 24 h. The effect of hypoxia on the level of DNA synthesis was examined by incubating the hepatocytes under hypoxic conditions for various times (0–24 h). As shown in Figure 1C, hypoxia increased the level of [^{3}H] thymidine incorporation in a time-dependent manner. In addition, the percentage of the cell population in the S phase was also increased after 0–24 h of hypoxic exposure (Fig. 1D), which is consistent with the observed increase in the level of [^{3}H] thymidine incorporation.

Fig. 2. Effect of hypoxia on Ca^{2+} and PKC activation. **A**: The cells were exposed to hypoxia for 0–24 h and the Ca^{2+} uptake was measured. The values are reported as the mean ± SE of three independent experiments with triplicate dishes (*$P < 0.05$ vs. Control). **B**: The cells were exposed to hypoxia for 30 min and immunofluorescence staining was carried out to detect PKC translocation. **C**: The PKC isoforms present in either the cytosolic or membrane compartments was detected by Western blotting, as described in Materials and Methods. The cells were exposed to hypoxia for 30 min and then determined phosphorylation of PKC isoforms. The bands represent the 80–90 kDa of PKC α and ε and 41 kDa band of β-actin. Each example shown is a representative of four experiments. **D**: The cells were pretreated with EGTA (4×10^{-3} M), BAPTA-AM (10^{-6} M), or bisindolylmaleimide I (10^{-7} M) for 30 min before being exposed to hypoxia for 30 min and then p-pan-PKC was detected with membrane fraction of cell lysates by Western blotting, as described in Materials and Methods. The bands represent the 80 kDa of Pan-PKC. The example shown is a representative of four experiments. The **lower panel** depicts the bars showing the mean ± SE of four experiments for each condition determined from densitometry relative to β-actin. *$P < 0.05$ versus Control, **$P < 0.05$ versus Hypoxia alone. **E**: The cells were pretreated with EGTA (4×10^{-3} M), BAPTA-AM (10^{-6} M), starusporine (10^{-7} M), or bisindolylmaleimide I (10^{-7} M) for 30 min before hypoxic exposure for 24 h and then pulsed with 1 μCi of [^{3}H] thymidine for 1 h before counting. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. *$P < 0.05$ versus Control, ** $P < 0.05$ versus Hypoxia alone.

Involvement of $[Ca^{2+}]_i$/PKC in Hypoxia-Induced DNA Synthesis

In order to determine if intracellular Ca^{2+} is involved in the hypoxia-induced DNA synthesis, the level of Ca^{2+} uptake was examined under hypoxic conditions. As shown in Figure 2A, hypoxia increased the level of Ca^{2+} uptake after 0–24 h of hypoxic exposure. Moreover, hypoxia induced PKC translocation from the cytosol to the membrane fraction (Fig. 2B). Hypoxia stimulated the translocation of PKC α

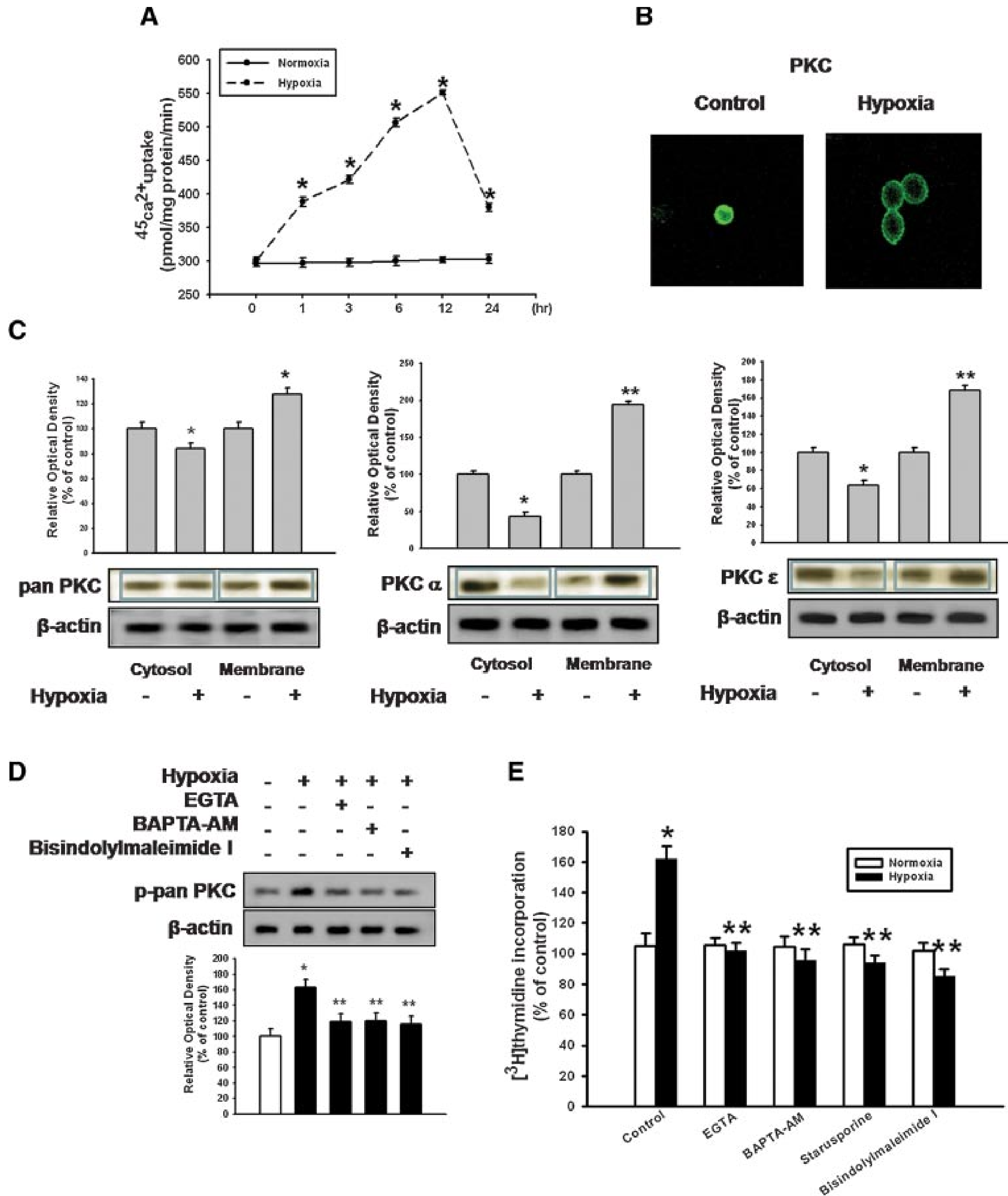

Fig. 2.

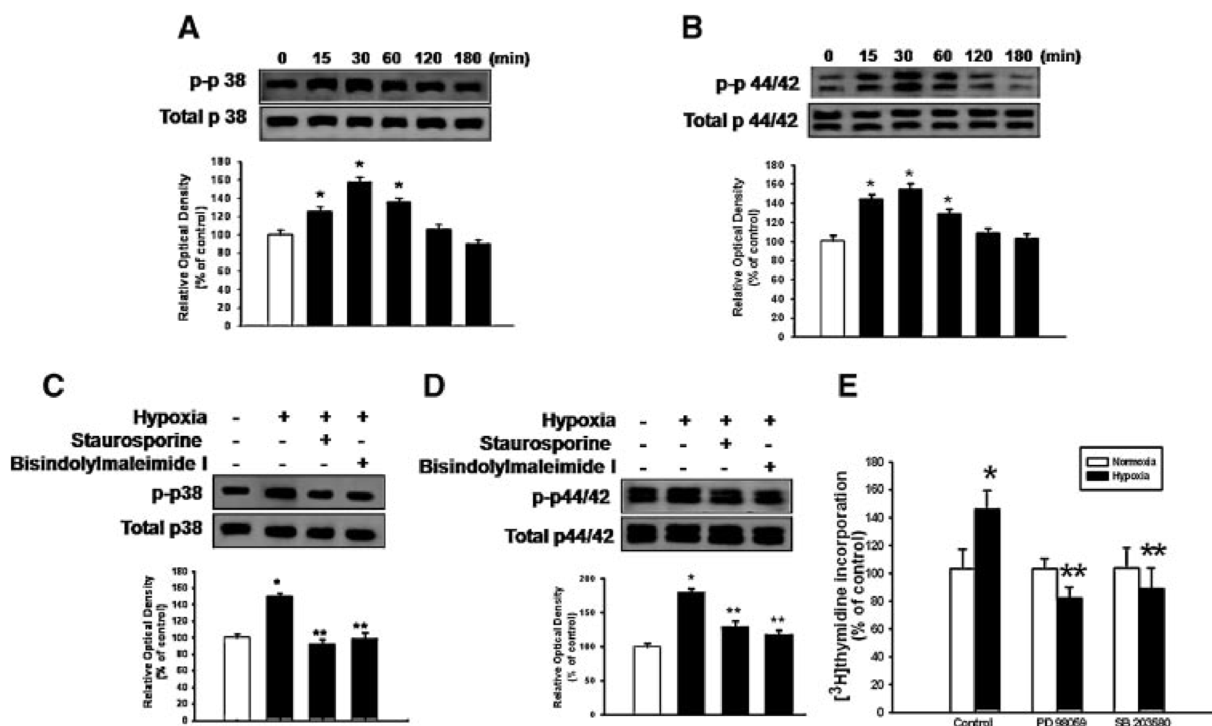

Fig. 3. Effect of hypoxia on MAPKs activation. The cells were exposed to hypoxia for 0–180 min and the phosphorylated p38 (**A**) and p44/42 MAPKs (**B**) were detected, as described in Materials and Methods. The cells were pretreated with staurosporine or bisindolylmaleimide I (10^{-7} M) for 30 min before being exposed to hypoxia for 30 min and examined p38 and p44/42 MAPKs phosphorylation by Western blotting (**C**, **D**). The **lower panels** of A, B, C, or D depict the bars showing the mean ± SE of four experiments for each condition determined from densitometry relative to β-actin. *$P < 0.05$ versus Control, **$P < 0.05$ versus Hypoxia alone. **E**: The cells were pretreated with PD 98059 (10^{-5} M) or SB 203580 (10^{-6} M) for 30 min prior to hypoxic exposure for 24 h and then pulsed with 1 μCi of [^{3}H] thymidine for 1 h prior to counting. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. *$P < 0.05$ versus Control, **$P < 0.05$ versus Hypoxia alone.

and ε from the cytosol to the membrane fraction (Fig. 2C). In the next step, pretreating the cells with EGTA (4×10^{-3} M, extracellular Ca^{2+} chelator), BAPTA-AM (10^{-6} M, intracellular Ca^{2+} chelator), or bisindolylmaleimide I (10^{-7} M, PKC inhibitor) inhibited the hypoxia-induced pan PKC phosphorytion (Fig. 2D). Each inhibitor alone did not affect PKC phosphorytion (date not shown). Consequently, the inhibition of the Ca^{2+} and PKC pathways blocked the hypoxia-induced DNA synthesis (Fig. 2E).

Involvement of MAPKs in Hypoxia-Induced DNA Synthesis

The phosphorylation of p38 and p44/42 MAPKs were examined to determine if the activation of MAPKs are involved in hypoxia-induced DNA synthesis. As shown in Figure 3A and B, hypoxia induced the phosphorylation of p38 and p44/42 MAPKs in a time-dependent manner (0–180 min). Moreover, pretreating the cells with either staurosporine or bisindolylmaleimide I (PKC inhibitors, 10^{-7} M) inhibited the hypoxia-induced phosphorytion of the MAPKs (Fig. 3C and D). Each inhibitor alone did not affect MAPKs phosphorytion (date not shown). Finally, the inhibition of the p38 and p44/42 MAPKs pathways blocked the hypoxia-induced DNA synthesis (Fig. 3E).

Involvement of PI3K/Akt/mTOR in Hypoxia-Induced DNA Synthesis

The level of Akt phosphorylation was examined to determine if the phosphorylation of PI3K/Akt/mTOR are involved in hypoxia-induced DNA synthesis. As shown in Figure 4A and B, hypoxia induced the phosphorylation of Akt 308 and Akt 473 in a time-dependent manner (0–24 h). Hypoxia also phosphorylated mTOR, p70S6K1, and 4EBP1, which are the downstream molecules of Akt, in a time-dependent manner (0–24 h) (Fig. 4C). In addition, pretreating the cells with EGTA and BAPTA-AM inhibited the hypoxia-induced phosphorylation of Akt 308 (Fig. 4D). Furthermore, hypoxia-induced phosphorylation

of mTOR was inhibited by pretreating the cells with LY 294002 and Akt inhibitor, but not by bisindolylmaleimide I treatment (Fig. 4E). Finally, the inhibition of the PI3K/Akt and mTOR pathways blocked the hypoxia-induced DNA synthesis (Fig. 4F).

The level of Ca^{2+} uptake was measured to observe the relationship between the hypoxia-induced phosphorylation of each signal pathway. The inhibition of the PI3K/Akt pathways had no effect on the hypoxia-induced increase in Ca^{2+} uptake (Fig. 5A). Moreover, it was observed that the hypoxia-induced PKC phosphorylation was unaffected by the inhibition of PI3K/Akt (Fig. 5B) and Akt phosphorylation was not influenced by the inhibition of the PKC pathway (Fig. 5C). Hypoxia-induced p44/42 MAPKs or p38 MAPK was not influenced by PI3K/Akt or mTOR inhibitor (Fig. 5D and E) and Akt or mTOR phosphorylation was not affected by the inhibition of p44/42 MAPKs or p38 MAPK (Fig. 5F).

Effect of Hypoxia on Cell Cycle Regulatory Proteins Expression Level

The protein levels of cyclin D_1, cyclin E, cyclin-dependent protein kinase (CDK) 2, and CDK 4 were measured using Western blotting to determine if hypoxia increases the levels of these cell cycle regulatory proteins. As shown in Figure 6A, hypoxia increased the level of these proteins in a time-dependent manner (0–24 h). Hypoxia also increased the pRB protein levels in a manner consistent with the cyclins and CDKs levels (Fig. 6B). On the other hand, the level of the CDK inhibitors, $p21^{cip1}$ and $p27^{Kip1}$ decreased after hypoxic exposure in a time-dependent manner (Fig. 6C). In the experiments examining the involvement of Ca^{2+}/PKC, MAPKs, and PI3K/Akt in the hypoxia-induced increases of cell cycle regulatory proteins, the inhibition of the Ca^{2+}/PKC, MAPKs, and PI3K/Akt/mTOR pathways blocked hypoxia-induced increases of cyclin E/D_1, CDK2/4, and pRB and decreases of p21 and p27. (Fig. 7A–D). In addition, this result was consistent with cell number counting experiment (Fig. 7E). Each inhibitor alone did not affect cell cycle regulatory protein expression (date not shown) and cell number.

DISCUSSION

In the present study, we demonstrated that short time exposure to hypoxia-induced increase in DNA synthesis was mediated through cooperation of Ca^{2+}/PKC/MAPKs and PI3K/Akt signal pathways in chicken hepatocytes. Several reports indicated that exposure of hepatocytes to 2% oxygen did not significantly induce cell death, while anoxic culture led to death via necrosis [Smith and Mooney, 2007]. Measurement of cell numbers over time demonstrated that culture in 0% oxygen killed a majority of hepatocytes within 2–3 days, and 2% oxygen slightly inhibited proliferation but did not cause cell death over same time period. In the present study, hepatocytes cultured at 2% oxygen were resistant to death and this degree of hypoxia did induce proliferation of the cells. In consistent with our result, there are reports that hypoxia obviously promoted the proliferation of pulmonary arterial smooth muscle cells or cardiomyocytes [Takahashi et al., 2006; Zhao et al., 2007]. However, we observed that long time hypoxic incubation generates excessive ROS and induced cellular damage in chicken hepatocyte (our unpublished work). Moreover, the results were different, even completely opposite in some investigations of this field [Dong et al., 2001; Guo et al., 2001]. It was possibly relevant to the time point of hypoxic exposure, different cell types, treatment and special measure method. Therefore, intriguing questions remain with regard to exactly how hypoxia initiates activation of individual signaling proteins in cells capable of proliferation under short time hypoxic condition.

Studies on hypoxic injuries have already shown a link between a lack of oxygen, an energy deficiency, and a disturbance in the Ca^{2+} homeostasis, leading to several biochemical tissue alterations [Piper, 1989]. Hypoxia increases the level of Ca^{2+} uptake in chicken hepatocyte. Several authors have suggested that an increase in Ca^{2+} uptake resulting from an energy deficiency is one of the causes of cell mortality and tissue necrosis during ischemia-reperfusion or hypoxia-reoxygenation [Piper, 1989; Arnould et al., 1992]. On the other hand, if the hypoxia and the incubation time are limited, as in the model examined here, the increase in $[Ca^{2+}]_i$ is not sufficient to induce cell death but is sufficient to activate cell proliferation [Piper, 1989]. In addition, involvement of PKC in DNA synthesis was clearly demonstrated, as shown by the lack of DNA synthesis in the presence of the PKC inhibitors as well as by the

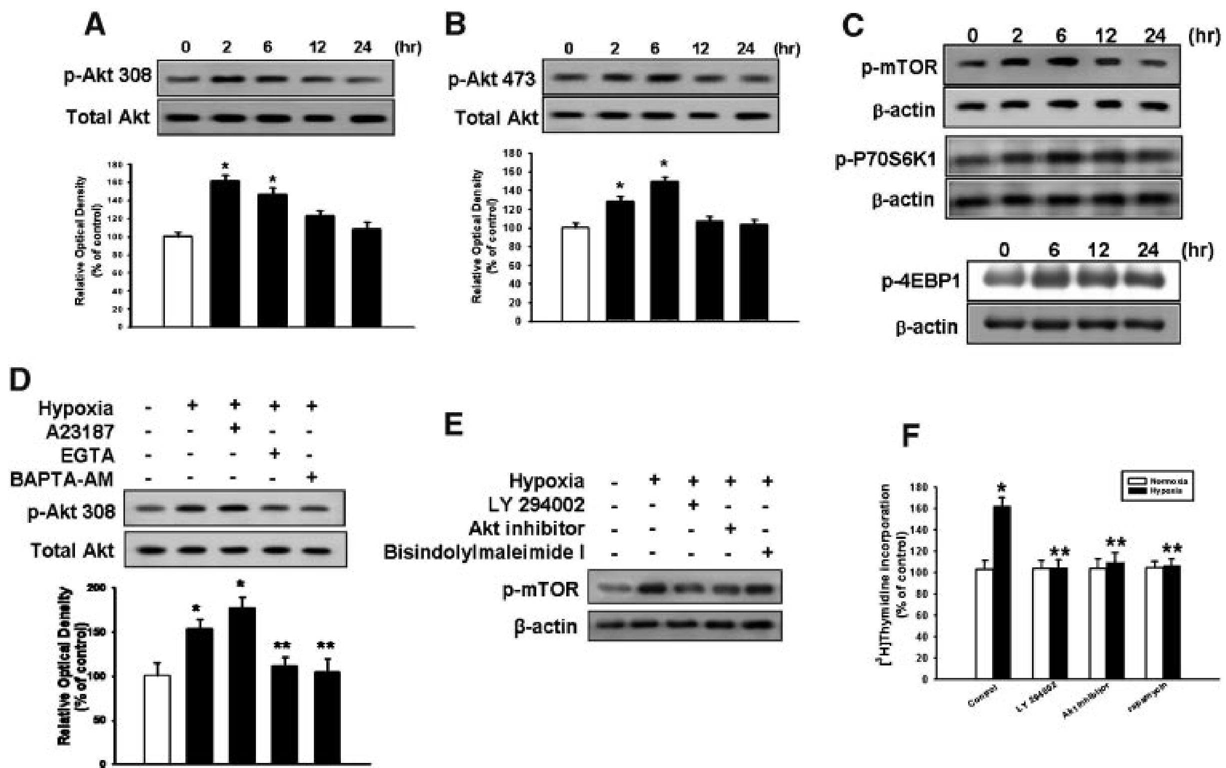

Fig. 4. Effect of hypoxia on the expression of PI3K/Akt and mTOR. The cells were exposed to hypoxia for 0–24 h and the phosphorylated (**A**) Akt 308 and (**B**) Akt 473 were detected, as described in Materials and Methods. **C**: The cells were exposed to hypoxia for 0–24 h and then phosphorylated mTOR, p70S6K1, and 4EBP1 were detected by Western blotting. **D**: The cells were pretreated with A 23187 (calcium inophore, 10^{-6} M), EGTA (4×10^{-3} M), and BAPTA-AM (10^{-6} M) for 30 min before being exposed to hypoxia for 2 h and examined Akt 308 phosphorylation by Western blotting. Each example shown is a representative of four independent experiments. The lower panels of A, B, or D depict the bars showing the mean ± SE of four experiments for each condition determined from densitometry relative to β-actin. *$P < 0.05$ versus Control, **$P < 0.05$ versus Hypoxia alone. **E**: The cells were pretreated with LY 294002, Akt inhibitor, and bisindolylmaleimide I pretreated for 30 min before being incubated in hypoxic condition for 2 h and then phosphorylated mTOR were detected by Western blotting. **F**: The cells were pretreated with LY 294002 (10^{-7} M) or Akt inhibitor (10^{-7} M) for 30 min prior to hypoxic exposure for 24 h and then pulsed with 1μCi of [^{3}H] thymidine for 1 h prior to counting. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. *$P < 0.05$ versus Control, **$P < 0.05$ versus Hypoxia alone.

translocation of the isoform from the cytosol to the membrane fraction of the tissue lysate (a decrease in the cytosolic levels of the protein and corresponding increase in the membrane levels). In agreement with these results, the stimulation of PKC by various phorbol esters was reported to increase the level of DNA synthesis [Sahai et al., 1997]. PKCs are involved in the increase in DNA synthesis after exposure to hypoxia in chicken hepatocytes. It is unclear if the differences in dependency on the different PKC isoforms reflect the variances in the cell model and/or the injuries used in these experiments. In addition to activation of PKC, several reports have shown that extracellular stimuli elicit a broad spectrum of biological response through activation of MAPK cascades [Lacchini et al., 2006]. In the present study to examine link between PKC and the p44/42 MAPKs, p44/42 MAPKs phosphorylation appeared to occur downstream of PKC. This is because the PKC inhibitors block the phosphorylation of the p44/42 MAPKs during hypoxia. It is interesting that PKC phosphorylates the p44/42 MAPKs, even if this pathway is not the only pathway capable of converging to this subfamily of MAPKs.

In addition, various calcium-dependent signaling cascades with kinases and phosphatases directly or indirectly influence cellular signaling, including PI3K/Akt signaling pathway [Yano et al., 1998]. Recent reports suggested that CaMKII can activate PI3K in monocytes [Ma et al., 2007] and that hypoxia activates CaMKII [Yuan et al., 2005]. Therefore, as shown in present study, there is possibility

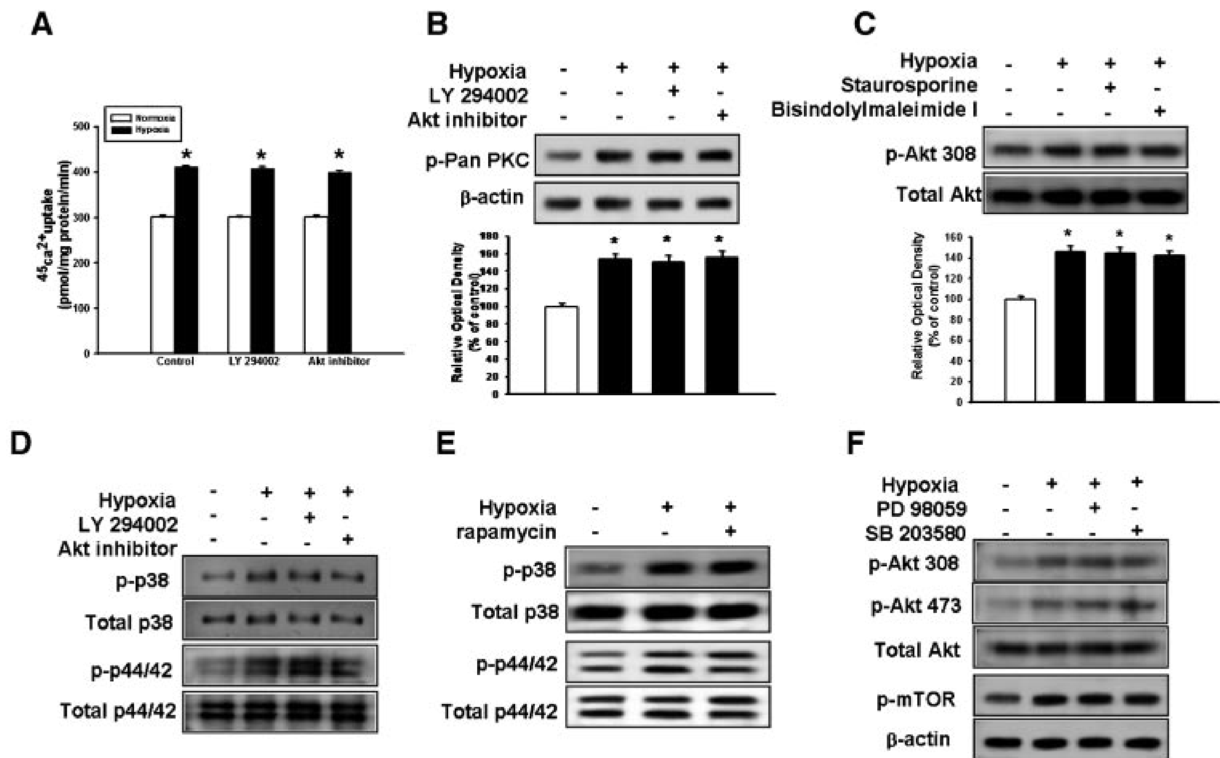

Fig. 5. Relation of PI3K/Akt, mTOR, and MAPKs in hypoxia-induced signal cascades. **A**: The cells were pretreated with LY 294002 or the Akt inhibitor for 30 min before being exposed to hypoxia for 12 h. The Ca^{2+} uptake was then measured. The values are reported as the mean ± SE of three independent experiments with triplicate dishes (*$P < 0.05$ vs. Control). **B**: The cells were pretreated with LY 294002 or Akt inhibitor (10^{-7} M) for 30 min before being exposed to hypoxia for 30 min. p-pan-PKC was detected by Western blotting, as described in Materials and Methods. The bands represent the 80 kDa of Pan-PKC. **C**: The cells were pretreated with staurosporine (10^{-7} M) or bisindolylmaleimide I (10^{-7} M) for 30 min before being exposed to hypoxia for 2 h. Phosphorylated Akt 308 was then detected by Western blotting. The **lower panels** of B, C depict the bars showing the mean ± SE of four experiments for each condition determined from densitometry relative to β-actin. *$P < 0.05$ versus Control. **D**, **E**: The cells were pretreated with LY 294002 (10^{-7} M), Akt inhibitor (10^{-7} M), or rapamycin (10^{-9} M) for 30 min before being exposed to hypoxia for 30 min. Phosphorylated p38 and p44/42 MAPKs were detected by Western blotting. **F**: The cells were pretreated with PD 98059 and SB 203580 for 30 min before being exposed to hypoxia for 2 h. Phosphorylated Akt 308, Akt 473, and mTOR was detected by Western blotting. Each example shown is a representative of four independent experiments.

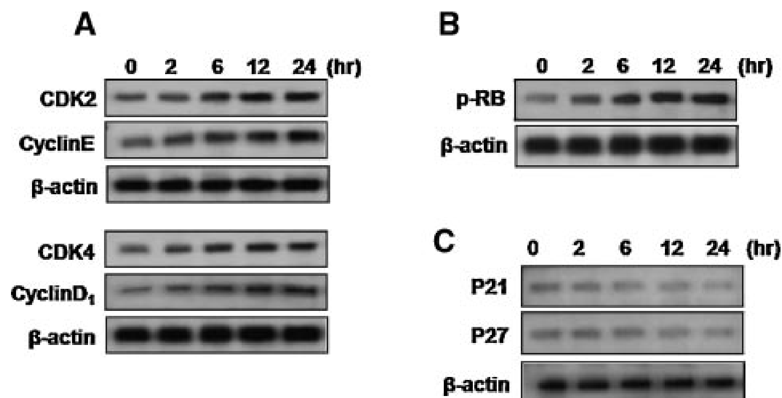

Fig. 6. Effect of hypoxia on the expression of the cell cycle regulatory proteins. Cells were exposed to hypoxia for various times (0–24 h) and the total proteins were then subjected to SDS-PAGE and blotted with the CDK 2, CDK 4, cyclin D1, cyclin E, pRB, p21, or p27 antibody (**A**, **B**, **C**). Each example shown is a representative of four independent experiments.

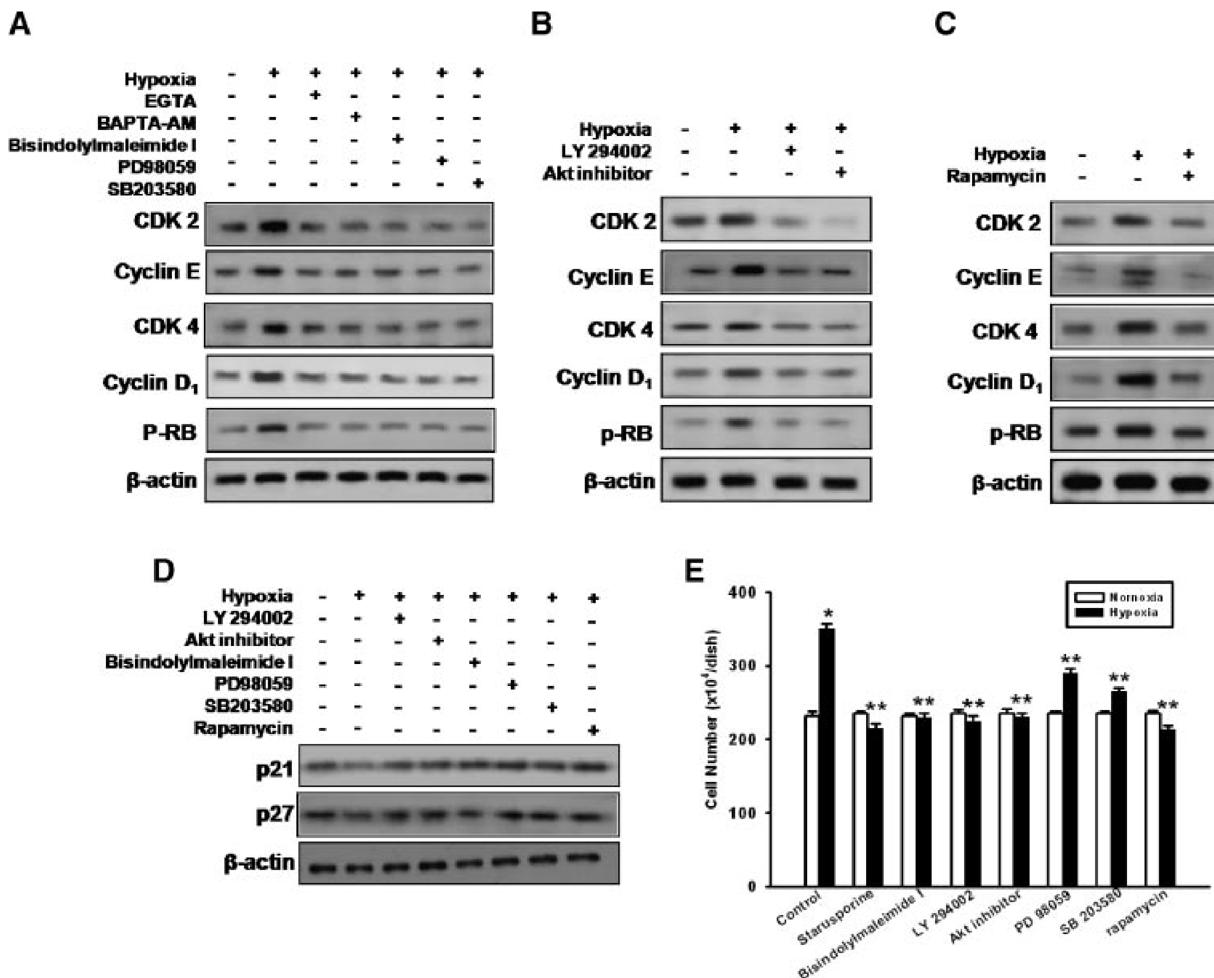

Fig. 7. Effect of Ca^{2+}/PKC, MAPKs, PI3K/Akt, and mTOR on cell cycle regulatory protein expression levels. **A**: The cells were pretreated with EGTA (4×10^{-3} M), BAPTA-AM (10^{-6} M), bisindolymaleimide I (10^{-7} M), PD 98059, and SB 203580 (10^{-6} M) for 30 min before being exposed to hypoxia for 12 h and examined CDK 2, CDK 4, cyclin D_1, cyclin E, and p-RB protein expression level by Western blotting. **B**, **C**: The cells were pretreated with LY 294002, Akt inhibitor (10^{-7} M), or rapamycin (10^{-9} M) for 30 min before being exposed to hypoxia and examined CDK 2, CDK 4, cyclin D_1, cyclin E, and p-RB protein expression level by Western blotting. **D**: The cells were pretreated with LY 294002, Akt inhibitor, bisindolylmaleimide I, PD 98059, SB 203580, and rapamycin for 30 min before hypoxic incubation and p21 and p27 protein expression level was measured by Western blotting. Each example shown is a representative of four independent experiments. **E**: The cells were pretreated with each inhibitors for 30 min prior to hypoxic exposure for 24 h and then counted using a hemocytometer. The values are reported as a mean ± SE of 3 independent experiments. *$P < 0.05$ versus Control, **$P < 0.05$ versus Hypoxia alone.

that CaMKII underlies the PKC-independent but Ca^{2+}-dependent PI3K/Akt signaling. The present result shows that PI3K is involved in cell proliferation and differentiation. The addition of the PI3K inhibitor, LY 294002, induced G1 cell cycle arrest and increased the expression of the cyclin-dependent kinase inhibitor, p27, in melanoma and osteosarcoma cell lines [Thomas et al., 1997; Casagrande et al., 1998]. It was suggested that the activities of PI3K and Akt are also important for the G1 cell cycle progression in chicken hepatocytes as well as for the expression of the proteins associated with G1 progression. The role of PI3K in the expression of the known regulators associated with the G1 cell cycle in these cells were examined in order to further understand the mechanism by which PI3K regulates the G1 cell cycle progression. During the G1-to-S progression of the cell cycle in response to a mitogen, the levels of D-type cyclin increase, bind to, and activate CDK4 and CDK6 [Matsushime et al., 1994]. These results suggest that the inhibition of PI3K by LY 294002 greatly decreases the

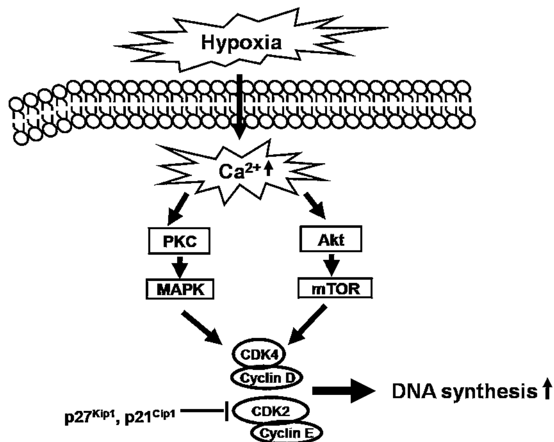

Fig. 8. A hypothesized model for the signal pathways involved in the hypoxia-induced hepatocyte DNA synthesis. Hypoxia increases the intracellular Ca^{2+} concentration, which activates PKC or Akt to activate MAPKs or mTOR. Therefore, PKC/MAPKs and the PI3K/Akt/mTOR signaling pathways independently stimulate hypoxia-induced hepatocyte DNA synthesis. PKC, protine kinase C; MAPK, mitogen-activated protein kinase; Akt, AGC family of protein kinase; mTOR, mammalian target of rapamycin; CDK, cyclin-dependent kinase.

expression of cyclin D1 and CDK4 and the phosphorylation of Rb. This indicates that PI3K is essential for cyclin D1/CDK4 interactions, which indicates Rb phosphorylation, E2F release, and the G1 cell cycle progression [Johnson and Schneider-Broussard, 1998]. These results are consistent with previous studies in other cell lines, indicating that PI3K/Akt is essential for inducing cyclin D1 [Muise-Helmericks et al., 1998] and E2F expression [Brennan et al., 1997; Takahashi et al., 2006]. In addition, mTOR is a major downstream target of Akt that regulates p70S6K1 [Romanelli et al., 2002; Torii et al., 2006]. Therefore, it is predicted that the inhibition of mTOR will have a similar effect on the inhibition of PI3K and Akt in chicken hepatocytes. This was further confirmed by the result showing that the indicated treatment of the cells with rapamycin markedly inhibited cyclin D1, CDK4, cyclin E, and CDK2. These results are similar to those obtained after treating the cells with LY 294002.

It has been shown that PI3K/Akt and MAPKs pathways can interact in multiple ways. Signaling through the ERK cascade is relatively well understood and significant progress in the inhibition of this signaling pathway has been achieved [Martin and Blenis, 2002]. However, as more is learned about this pathway, it becomes increasingly complex with many sites interacting with different pathways and substrates. The results obtained from this study demonstrated that treating the cells with LY 294002 markedly inhibited the phosphorylation of Akt, whereas it has no effect on the phosphorylation of ERK1/2. This suggests that the phosphorylation of Akt, but not ERK1/2 is directly involved in PI3K-dependent cell cycle regulation. Nevertheless, the requirement of PI3K for MAPK/ERK activity has been documented in other cell systems and with other growth factors [Duckworth and Cantley, 1997; Takami et al., 2002]. In this study, we demonstrate the failure to see involvement of PI3K in hypoxia-induced MAPK phosphorylation, indicating the cell-specific regulation of hypoxia-signaling. The difference between the results presented here and the previous studies in epithelial cells are likely to be explained by cell type difference in cross-talk between regulatory pathways downstream of hypoxia [Berra et al., 1998; Rommel et al., 1999]. The results presented here emphasize the importance of PKC/MAPKs and PI3K/mTOR in DNA replication. Even though both pathways can be regulated by hypoxia, this indicates that there is no direct interaction between the two pathways, which cooperatively mediated cell cycle progression to hypoxia in chicken hepatocytes (Fig. 8). It is encouraging that hepatocytes seem able to survive relatively in low oxygen levels, suggesting that even a slight improvement of oxygen transport at the ischemic/transplant site may have a positive effect. These results provide important insights into the intracellular signaling activation in the G1 phase, which is of prime importance understanding the hepatocyte growth and survival controls that regulate homeostasis during the regeneration process after a liver injury and disease. Furthermore, future understanding of signal transducing proteins assembly into signaling networks should have significant implications for cell proliferation under hypoxic conditions. In conclusion, short time exposure to hypoxia increases DNA synthesis via cooperation of Ca^{2+}/PKC, p38 MAPK, p44/42 MAPKs, and PI3K/Akt pathways in primary cultured chicken hepatocytes.

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