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Journal of Cellular Biochemistry 105:1410–1419 (2008)

Journal of Cellular Biochemistry

L-Leucine Increases [^{3}H]-Thymidine Incorporation in Chicken Hepatocytes: Involvement of the PKC, PI3K/Akt, ERK1/2, and mTOR Signaling Pathways

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ABSTRACT

This study examined how L-leucine affected DNA synthesis and cell cycle regulatory protein expression in cultured primary chicken hepatocytes. L-Leucine promoted DNA synthesis in a dose- and time-dependent manner, with concomitant increases in cyclin D1 and cyclin E expression. Phospholipase C (PLC) and protein kinase C (PKC) mediated the L-leucine-induced increases in [^{3}H]-thymidine incorporation and cyclin D1/CDK4 and cyclin E/CDK2 expression, as U73122 (a PLC inhibitor) or bisindolylmaleimide I (a PKC blocker) inhibited these effects. L-Leucine also increased PKC phosphorylation and intracellular Ca^{2+} levels. L-Leucine-mediated increases in [^{3}H]-thymidine incorporation and cyclin/CDK expression were sensitive to LY 294002 (PI3K inhibitor), Akt inhibitor, PD 98059 (MEK inhibitor). It was also observed that L-leucine-induced increases of cyclin/CDK expression were inhibited by PI3K siRNA and ERK siRNA; L-leucine increased extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt phosphorylation levels. Bisindolylmaleimide I attenuated L-leucine-induced phosphorylation of ERK1/2 but did not influence Akt phosphorylation, and PI3K siRNA and LY 294002 inhibited L-leucine-induced ERK1/2 phosphorylation, suggesting some cross-talk between the PKC and ERK1/2 or PI3K/Akt and ERK1/2 pathways. L-Leucine also increased the levels of phosphorylated molecular target of rapamycin (mTOR) and two of its targets, ribosomal protein S6 kinase (p70S6K), and 4E binding protein 1 (4E-BP1); furthermore, rapamycin (an mTOR inhibitor) blocked all of the mitogenic effects of L-leucine. In addition, Akt inhibitor blocked L-leucine-induced mTOR phosphorylation. In conclusion, L-leucine stimulated DNA synthesis and promoted cell cycle progression in primary cultured chicken hepatocytes through PKC, ERK1/2, PI3K/Akt, and mTOR. J. Cell. Biochem. 105: 1410–1419, 2008.

KEY WORDS: L-LEUCINE; [^{3}H]-THYMIDINE INCORPORATION; CHICKEN HEPATOCYTES

The branched-chain amino acids (BCAAs; valine, leucine, and isoleucine) share unique biochemical properties that make them useful tools to modulate altered physiological states. They are abundant in body proteins and possess various nutritional [Watford, 2007], therapeutic [Khanna and Gopalan, 2007], and pathophysiological functions [Hörster and Hoffmann, 2004]. For example, these amino acids exert a mitogenic effect on both adult and fetal hepatocytes and hepatoma cells in the presence of insulin, insulin-like growth factor-I (IGF-I), or other growth factors [Patti et al., 1998; Dubbelhuis and Meijer, 2002; Krause et al., 2002]. BCAAs were also recently identified as important signaling agents in initiating and modulating hepatocyte proliferation.

One of the BCAAs, leucine, specifically activates signaling pathways that enhance cell growth and exerts mitogenic effects in various cell types [Lynch et al., 2000]. However, the liver metabolizes leucine poorly compared to other amino acids, since BCAA transaminase is nearly inactive in the liver. For these reasons, a better understanding of the molecular mechanisms behind the biological function of leucine will aid in developing more effective treatments for hepatic injury.

Presumably, L-leucine exerts its mitogenic effects on hepatocytes by modulating one or more components of the cell cycle, but the exact molecular mechanism behind this process remains a mystery. Cell cycle progression through the G1/S transition requires cyclin D–CDK4/CDK6 and cyclin E–CDK2 activation [Matsushime et al., 1994; Sherr and Roberts, 1995]. Although CDK activity is transcriptionally regulated, CDK inhibitors (CKIs), such as the members of the $p21^{WAF/Cip1}$ family, also regulate CDK activity throughout the cell cycle.

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Received 10 April 2008; Accepted 5 September 2008 • DOI 10.1002/jcb.21959 • 2008 Wiley-Liss, Inc.
Published online 3 November 2008 in Wiley InterScience (www.interscience.wiley.com).

Protein kinase C (PKC) is intimately involved in controlling progression through the cell cycle [Livneh and Fishman, 1997], but it is not clear whether PKC or any of the related pathways participate in L-leucine-induced proliferation of hepatocytes. Nishitani et al. [2002] reported that leucine acts through PKC to promote glucose uptake in skeletal muscle under insulin-free conditions. In addition, several studies implicated the mitogen-activated protein kinases (MAPKs) in leucine-induced hepatocyte mitogenesis [Kimura and Ogihara, 2005]. The serine/threonine kinase mammalian target of rapamycin (mTOR) is central to regulating cell growth and protein synthesis [Schmelzle and Hall, 2000], and other reports suggested that the activation of two of its downstream effectors, ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein (4E-BP1), mightplay a part in L-leucine-mediated hepatocyte proliferation [Anthony et al., 2001a; Fujita et al., 2007].

Despite various implications from these studies, the mechanisms of the mitotic regulation by leucine in chicken hepatocytes are not completely understood. In order to address this critical knowledge gap, this study examined the effect of L-leucine on DNA synthesis and its related signaling pathways. We chose cultured primary chicken hepatocytes as our experimental model system, because in culture they retained many liver-specific functions and hormone responses in previous biophysiological liver function studies. The primary chicken hepatocyte culture system utilized herein also retains many typical differentiated liver phenotypes, including albumin expression [Hou et al., 2001], p4501A induction [Hou et al., 2001], tyrosine aminotransferase expression [Sasaki et al., 2000], and ascorbate recycling [Sasaki et al., 2001], in an in vitro tissue culture setting. These properties make this system ideal for elucidating the cellular components of L-leucine-mediated hepatocyte proliferation.

MATERIALS AND METHODS

MATERIALS

Two-week-old White Leghorn male chickens were obtained from Dae Han Experimental Animal Co., Ltd. (Chungju, Korea). All animal management procedures 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 protocols. Experimental sample management and quality control of the laboratory facilities and equipment were appropriately maintained. Class IV collagenase and soybean trypsin inhibitor were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum was purchased from Biowhittaker (Walkersville, MD). L-Leucine, PD 98059, LY 294002, and rapamycin were obtained from Sigma Chemical Company (St. Louis, MO). Akt inhibitor (1L6-hydroxymethyl-chiro-inositol-2-(*R*)-2-*O*-methyl-3-*O*-octadecyl-*sn*-glycerocarbonate) was purchased from Calbiochem (La Jolla, CA). [^{3}H]-thymidine was purchased from NEN (Boston, MA). The phospho-ERK1/2, phospho-Akt (Thr^{308}, Ser^{473}), phospho-mTOR, phospho-p70S6K1, and phospho-4E-BP1 antibodies were acquired from New England Biolabs (Herts, UK). The CDK-2, CDK-4, cyclin D1, and cyclin E antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was obtained from Jackson Immunoresearch (West Grove, PA). 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 perfusing them 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 per 60-mm collagen-coated dish) in incubation medium (basal Eagle medium supplemented with essential amino acids) containing 75 U/ml penicillin, 75 U/ml streptomycin, and 5% fetal bovine serum, and incubated for 4 h at 37°C in 5% CO_2 in the gas phase. The medium was then replaced with fresh incubation medium, and the hepatocytes were incubated for another 20 h 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 the presence or absence of L-leucine for 24 h and subsequently pulsed with 1 μCi [methyl-^{3}H]-thymidine for 4 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 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 experiments were performed in triplicate, and the values were converted from absolute counts to a percentage of the control in order to compare the results between experiments.

MEASUREMENT OF INTRACELLULAR CA^{2+} CONCENTRATION ($[CA^{2+}]_i$)

Changes in $[Ca^{2+}]_i$ were monitored with Fluo-3/AM that had initially been dissolved in dimethylsulfoxide and stored at −20°C. Chicken hepatocytes in 35-mm culture dishes were rinsed twice with 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_2–95% O_2 at 37°C for 40 min, rinsed twice with bath solution, mounted on a perfusion chamber, and scanned at 1-s interval using confocal microscopy (Fluoview 300; Olympus, Orangeburg, NY), with fluorescence excitation set at 488 nm and emission at 515 nm. All analyses of $[Ca^{2+}]_i$ were processed in a single cell, and the results were expressed as fluorescence intensity (F/F_0, arbitrary units).

SMALL INTERFERING RIBONUCLEIC ACID TRANSFECTION

The cells were grown to 75% confluence in each dish and transfected for 48 h with either a ON-TARGET plus SMARTpool of small interfering RNAs specific to PI3KR1 (phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 p85 α) (target sequence: UCGGG-

AAUAUGAUAGAUUA, CGAUUACACUCUUACACUA, AAACAAAGCGGAGAACCUA, and GAACAACAAUAUGUCCUUG) or ERK1 [target sequence: GGAUACAGAUCUUAAAUUG, UGAGAGGGCUAAAGUAUAU, ACAAGAGGAUUGAAGUUGA, and UAUACCAAGUCCAUUGAUA] (200 pmol/L) or non-targeting small interfering RNA (as positive control; 200 pmol/L; Dharmacon, Inc., Lafayette, CO) using Dharmafect (Dharmacon, Inc.) according to the manufacturer's instructions.

WESTERN BLOT ANALYSIS

The cell homogenates (20 μl protein) were separated with 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blots were then washed with H_2O, blocked with 5% skimmed 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 in TBST, and the primary antibodies were detected with goat anti-rabbit-IgG (1:10,000) conjugated to HRP. The bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK).

STATISTICAL ANALYSIS

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

RESULTS

L-LEUCINE ENHANCED [^{3}H]-THYMIDINE INCORPORATION IN A TIME- AND DOSE-DEPENDENT MANNER

In order to examine the effect of L-leucine on DNA synthesis in cultured primary hepatocytes, we first measured changes in their [^{3}H]-thymidine incorporation as a result of exposing them to various L-leucine doses for 24 h. As shown in Figure 1A, L-leucine increased the level of [^{3}H]-thymidine incorporation in a dose-dependent manner. Addition of 10^{-5} M L-leucine yielded the maximum increase in [^{3}H]-thymidine incorporation. In addition, we determined [^{3}H]-thymidine incorporation at various time points (0–24 h) and found that L-leucine significantly increased [^{3}H]-thymidine incorporation in a time-dependent manner (Fig. 1B). We observed the maximum increase in [^{3}H]-thymidine incorporation using 10^{-5} M L-leucine for 8 h.

Next, we examined whether L-leucine affected cell cycle regulatory protein expression in a time-dependent manner. As shown in Figure 1C, L-leucine time-dependently increased the levels of cyclin D1 and cyclin E expression in these primary hepatocytes.

PLC/PKC WAS INVOLVED IN L-LEUCINE-INDUCED CELL CYCLE PROGRESSION

To determine whether PKC was involved in L-leucine-induced cell proliferation, we observed pan-PKC phosphorylation after treating

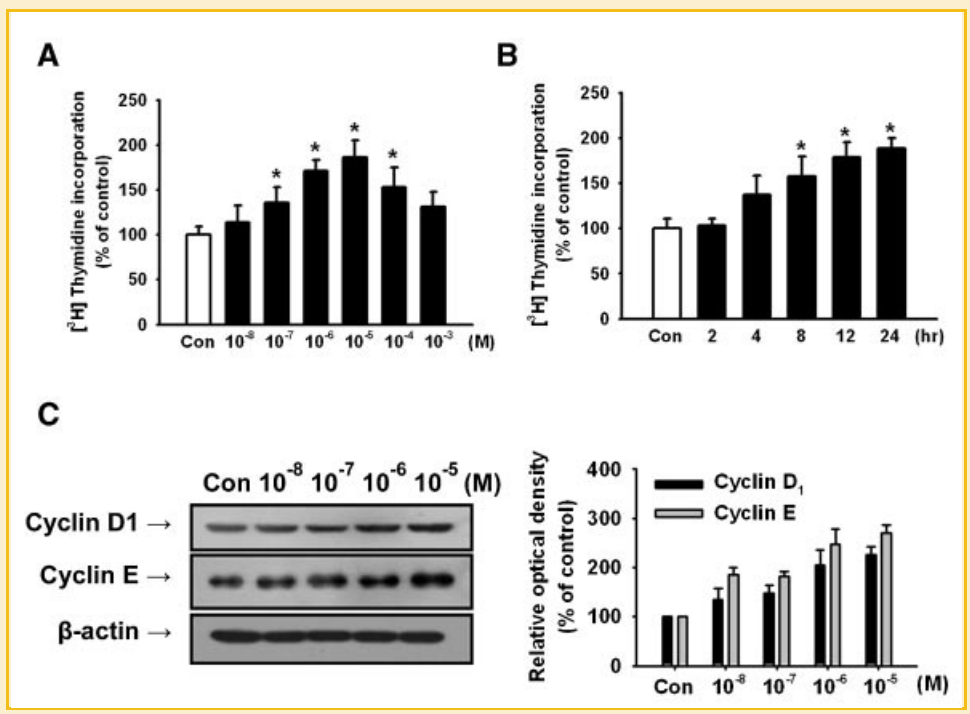

Fig. 1. L-Leucine affected [3H]-thymidine incorporation in a dose- and time-dependent manner. A: Chicken hepatocytes were incubated with various doses of L-leucine for 18 h and pulsed with 1 μCi [^{3}H]-thymidine for 1 h. B: Chicken hepatocytes were incubated in the presence of 10^{-5} M L-leucine for different time periods and pulsed with 1 μCi [^{3}H]-thymidine for 1 h. In (A) and (B), the values shown are means ± SE of four independent experiments from triplicate dishes. $^*P < 0.05$ versus control. C: L-Leucine dose-dependently upregulated cyclin D1 and cyclin E protein expression, as analyzed by Western blot. The cells were left untreated or given L-leucine (10^{-5} M) for 12 h. Each example shown is representative of three experiments. The graphs denote the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin.

the cells with 10^{-5} M L-leucine for 30 min. L-Leucine mediated a time-dependent increase in pan-PKC phosphorylation (Fig. 2A). To ascertain whether this apparent increase in PKC activity depended on Ca^{2+}, we monitored intracellular Ca^{2+} mobility in response to L-leucine. As shown in Figure 2B, L-leucine induced a transient increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), an increase that was sensitive to U73122 (PLC blocker, 10^{-7} M). Pretreating the cells with the PKC blocker bisindolylmaleimide I (10^{-7} M) attenuated the L-leucine-induced increase in the levels of the cell cycle regulatory proteins cyclin E, CDK2, cyclin D1, and CDK4, and it increased the levels of the CDKI $p21^{WAF1/Cip1}$ (Fig. 2C). In addition, treatment with U73122 or bisindolylmaleimide I blocked the L-leucine-induced increase in [^{3}H]-thymidine incorporation. Taken together, these results suggested that Ca^{2+}/PKC signaling participated in the mitogenic effects of L-leucine in primary hepatocytes.

PI3K/AKT WAS INVOLVED IN L-LEUCINE-INDUCED CELL CYCLE PROGRESSION

We next utilized Western blots to investigate whether phosphoinositide-3 kinase and protein kinase B (PI3K/Akt) were

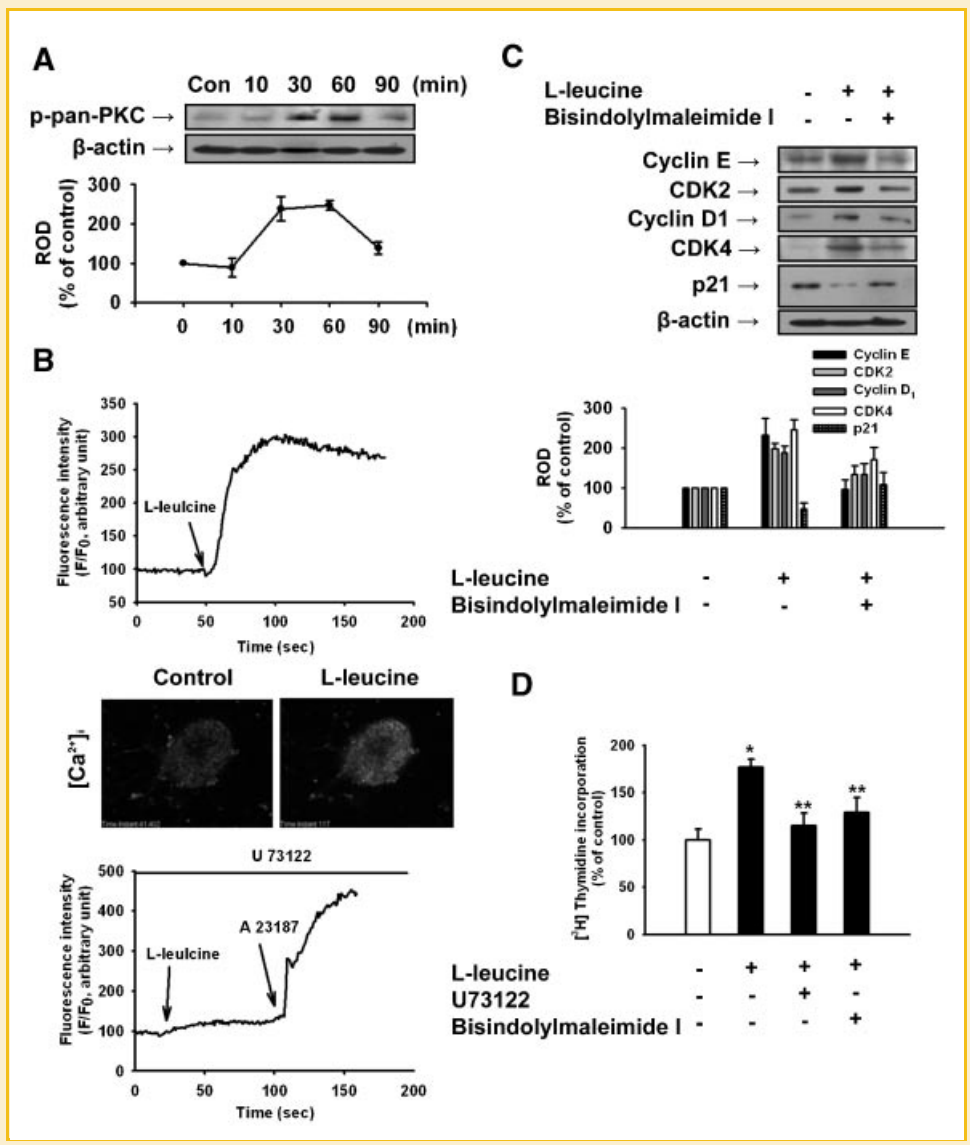

Fig. 2. Ca^{2+}/PKC was involved in L-leucine-induced [^{3}H]-thymidine incorporation. A: Chicken hepatocytes were treated with L-leucine (10^{-5} M) for various time points (0–90 min). The phosphorylation of pan-PKC was detected by Western blot analysis. Bands represent 80-kDa Pan-PKC and 41-kDa actin. Each example shown is representative of three experiments. The graphs denote the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin. B: Cells were loaded with 2 μM Fluo-3-AM in serum-free medium with or without U73122 (a PLC inhibitor, 10^{-7} M) for 40 min, treated with L-leucine as before, and changes in Ca^{2+} influx were monitored by confocal microscopy. The data are expressed as fluorescence intensity (F/F_0%, arbitrary units). C: Cells were treated with bisindolylmaleimide I (a PKC blocker, 10^{-6} M) for 30 min prior to L-leucine treatment for 8 h, and the levels of cyclin E, CDK2, cyclin D1, CDK4, and p21 were detected by Western blot. Each example shown is representative of three experiments. The graphs denote the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin. D: Cells were pretreated with U73122 or bisindolylmaleimide I for 30 min prior to L-leucine treatment for 12 h; cells were subsequently pulsed with 1 μCi [^{3}H]-thymidine for 1 h. The values are represented as means ± SE of four independent experiments from triplicate dishes. $^*P < 0.05$ versus control; $^{**}P < 0.05$ versus L-leucine alone. ROD, relative optical density.

also involved. L-Leucine induced Akt phosphorylation in a time-dependent manner (Fig. 3A). This activated PI3K/Akt pathway might affect cell cycle regulatory protein expression, because LY 294002 (a PI3K inhibitor, 10^{-6} M) inhibited both the L-leucine-induced modulation of cell cycle regulatory protein levels [increases in cyclin E, CDK2, cyclin D1, and CDK4, and decrease in p21 (Fig. 3B)] and the L-leucine-induced [^{3}H]-thymidine incorporation (Fig. 3C). In experiment to determine whether PI3K/Akt pathway is involved in PKC phosphorylation, PKC phosphorylation was not influenced by LY 294002 and Akt inhibitor. This result suggests that PI3K/Akt pathway and PKC are activated independently by L-leucine (Fig. 3D).

ERK1/2 WAS INVOLVED IN L-LEUCINE-INDUCED CELL CYCLE PROGRESSION

Based on our results implicating Akt/PKB in L-leucine-induced cell proliferation, we next began to elucidate the effectors downstream of PKC signaling in L-leucine-induced cell proliferation by examining the role of extracellular signal-regulated kinases (ERK)1/2 in this process. As shown in Figure 4A, L-leucine increased

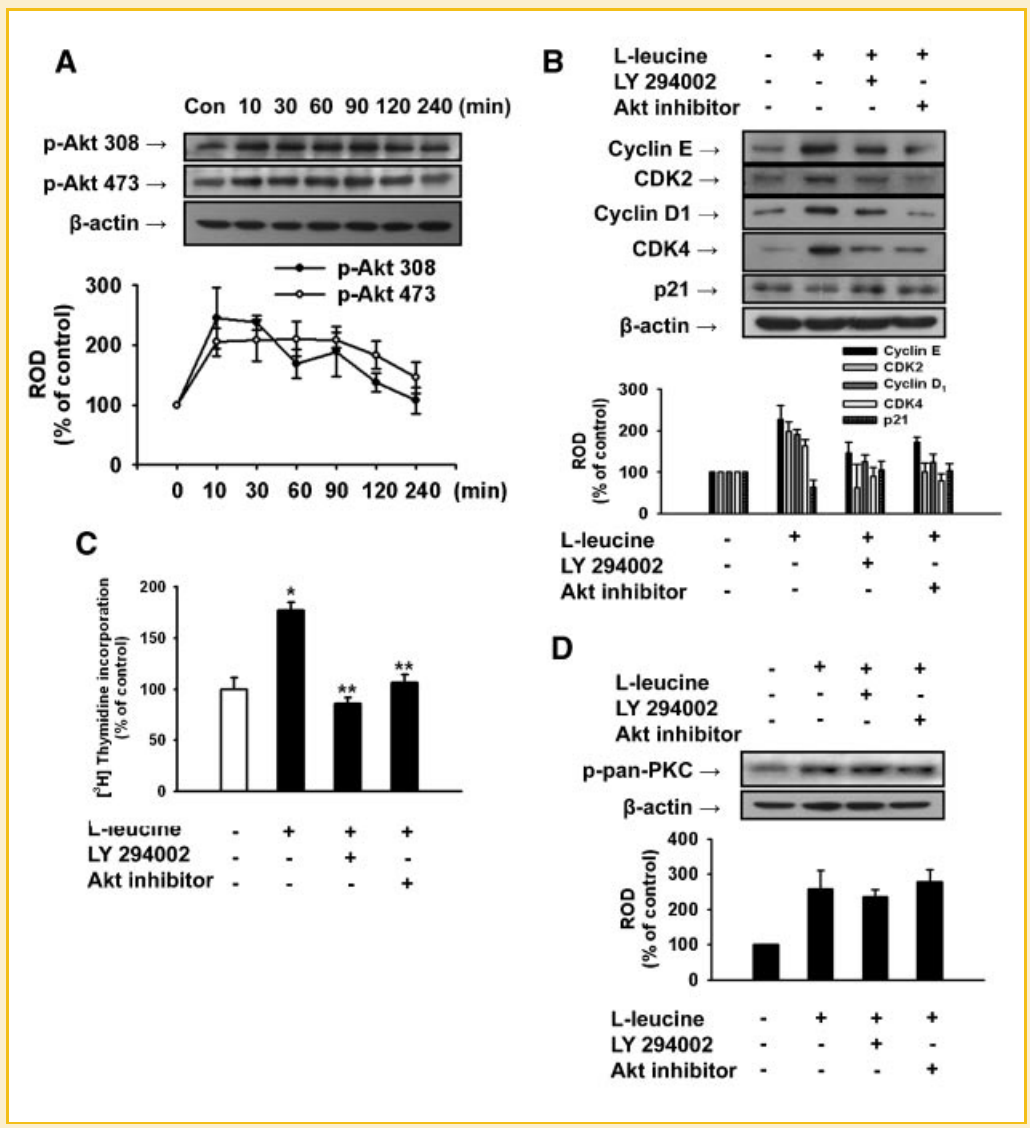

Fig. 3. PI3K/Akt was involved in L-leucine-induced [^{3}H]-thymidine incorporation. A: Chicken hepatocytes were treated with L-leucine (10^{-5} M) for various time points (0–240 min), and the levels of phosphorylated Akt 308 and Akt 473 were detected by Western blot. B: Cells were treated with LY 294002 (a PI3K inhibitor, 10^{-6} M) for 30 min prior to L-leucine treatment for 12 h; the levels of cyclin E, CDK2, cyclin D1, CDK4, and p21 were detected by Western blot. Each example shown is representative of three experiments. The graphs denote the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin. C: Cells were pretreated with LY 294002 for 30 min prior to L-leucine treatment for 12 h; cells were subsequently pulsed with 1 μCi [^{3}H]-thymidine for 1 h. The values represent means ± SE of four independent experiments from triplicate dishes. $^*P < 0.05$ versus control; $^{**}P < 0.05$ versus L-leucine alone. D: Cells were pretreated with LY 294002 (a PI3K inhibitor, 10^{-6} M) and Akt inhibitor (10^{-5} M) for 30 min prior to L-leucine treatment for 30 min. Phosphorylation of pan-PKC was detected by Western blot. Each example shown is representative of three experiments. The graphs denote the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin. ROD, relative optical density.

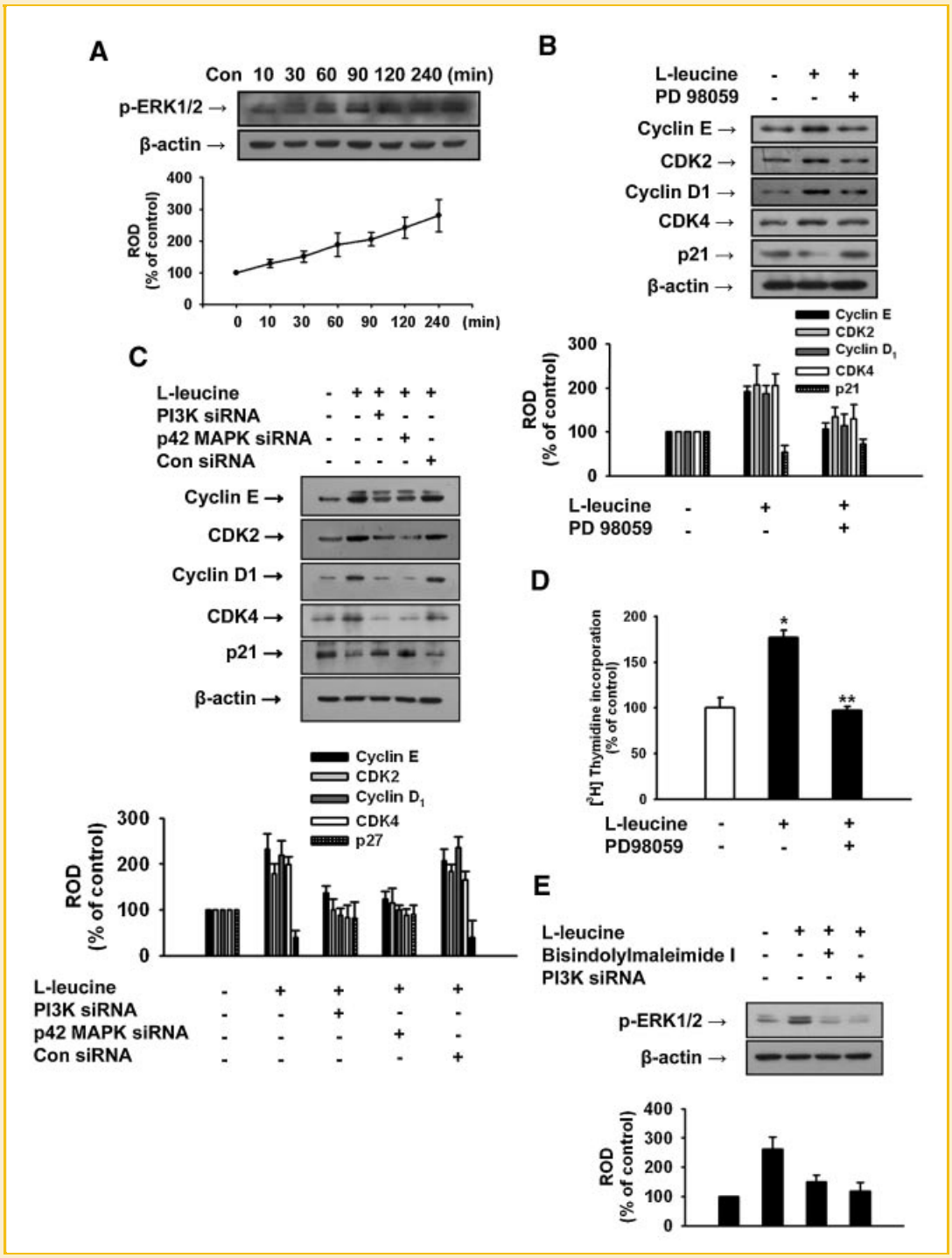

Fig. 4. ERK1/2 participated in L-leucine-induced [^{3}H]-thymidine incorporation. A: Chicken hepatocytes were treated with L-leucine (10^{-5} M) for various time points (0–120 min), and the levels of phosphorylated ERK1/2 were detected by Western blot. B: Cells were pretreated with PD 98059 (ERK1/2 blocker, 10^{-5} M) for 30 min prior to L-leucine treatment for 12 h, and the levels of cyclin E, CDK2, cyclin D1, CDK4, and p21 were detected by Western blot. C: Cells were transfected for 48 h with either SMART pool of PI3K, ERK siRNA, and a non-targeting control siRNA (200 pmol/L) using Dharmafect prior to L-leucine treatment for 12 h and the levels of cyclin E, CDK2, cyclin D1, CDK4, and p21 were detected by Western blot. Each example shown is representative of three experiments. The graphs denote the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin. D: Cells were pretreated with PD 98059 for 30 min prior to L-leucine treatment for 12 h, and they were subsequently pulsed with 1 μCi [^{3}H]-thymidine for 1 h. Values are means ± SE of four independent experiments from triplicate dishes. $^*P < 0.05$ versus control; $^{**}P < 0.05$ versus L-leucine alone. E: Cells were pretreated with bisindolylmaleimide I (a PKC blocker, 10^{-7} M) for 30 min or transfected for 48 h with SMART pool of PI3K siRNA prior to L-leucine treatment for 30 min. Phosphorylation of ERK1/2 was detected by Western blot. Each example shown is representative of three experiments. The graphs denote the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin. ROD, relative optical density.

ERK1/2 phosphorylation in a time-dependent manner. In addition, we investigated whether ERK1/2 was involved in L-leucine-induced cell cycle progression by treating chicken hepatocytes with PD 98059 [a MAP-ERK kinase (MEK) inhibitor, 10^{-5} M] for 30 min or transfection with PI3K siRNA and ERK siRNA for 48 h before treating them with L-leucine. As shown in Figure 4B,C, pretreatment with PD 98059, PI3K siRNA, or ERK siRNA blocked the L-leucine-induced modulation of cell cycle regulatory protein levels; namely, the increases in cyclin E, CDK2, cyclin D1, and CDK4, and the decrease in p21. In addition, PD 98059 also blocked this [^{3}H]-thymidine incorporation (Fig. 4D). These results suggest that L-leucine has a mitogenic effect, which is mediated by PI3K/Akt and ERK1/2 pathways. Furthermore, when we conducted experiments designed to determine whether PKC and PI3K/Akt were involved in L-leucine-induced activation of ERK1/2, the pretreatment of bisindolylmaleimide I (a PKC inhibitor, 10^{-7} M) or PI3K siRNA attenuated the L-leucine-induced activation of ERK1/2 (Fig. 4E). Taken together, these data support a model in which L-leucine-activated PKC and PI3K/Akt participate in cross-talk with ERK1/2 activation to mediate hepatocyte cell growth.

THE mTOR PATHWAY WAS INVOLVED IN L-LEUCINE-INDUCED CELL CYCLE PROGRESSION

We next examined whether L-leucine treatment affected the levels of mTOR/p70S6K/4E-BP1 phosphorylation, in order to determine if the mTOR pathway participated in L-leucine-induced DNA synthesis and cell cycle regulatory protein modulation. As shown in Figure 5A, L-leucine induced phosphorylation of mTOR, p70S6K, and 4E-BP1 in a time-dependent manner (0–240 min). In addition, pretreating cells with rapamycin (an mTOR inhibitor, 10^{-8} M) reversed the L-leucine-induced modulation of cyclin E, CDK2, cyclin D1, CDK4, and p21 expression levels (Fig. 5B) and blocked the increase of [^{3}H]-thymidine incorporation in response to L-leucine (Fig. 5C). In addition, as shown in Figure 5D, phosphorylation of mTOR was blocked by Akt inhibitor (10^{-5} M). Taken together, these results suggest a role for the mTOR pathway that is activated by PI3K/Akt pathway in L-leucine-induced cell cycle progression.

DISCUSSION

In this study, L-leucine stimulated cell cycle regulatory protein expression and DNA synthesis in cultured primary chicken hepatocytes. Understanding the mechanism behind this phenomenon, including the signaling pathways involved, is crucial to understanding the function of L-leucine in hepatocytes. L-Leucine increased [^{3}H]-thymidine incorporation at doses between 10^{-7} and 10^{-4} M, consistent with previous reports showing that L-leucine treatment increased the growth rate of several different cell types under various experimental conditions [Swenne, 1992; Kimura and Ogihara, 2005]. Several studies in a variety of cell types have reported L-leucine-induced mitogenic effects mediated by changes in expression of intracellular mitogens, such as TGF-α, HGF, or IGF-I [Sugiyama et al., 1998; Kimura and Ogihara, 2005; Tomiya et al., 2007], but the exact mechanism by which L-leucine alters cell cycle regulatory protein expression is unclear.

This study demonstrated that L-leucine increased the expression of cyclin D1–CDK4 and cyclin E–CDK2. These kinase systems phosphorylate various substrates, including the product of the retinoblastoma susceptibility gene (pRB), in order to initiate DNA synthesis [Gad et al., 2004; White et al., 2005]. Cyclin D1–CDK4 and cyclin E–CDK2 play a key role in cell cycle progression from G1 to S phase in most cell types, including hepatocytes [Alisi et al., 2003; Koroxenidou et al., 2005]. Along with the increases in cyclins/CDKs, L-leucine treatment mediated a concomitant decrease in $p21^{WAF1/Cip1}$ expression. Since the cyclin/CDK pairs and their CKIs transduce various extracellular stimuli that control progression through the G1/S phase cell cycle restriction point, the results of this study suggested that L-leucine stimulates cell proliferation, reflected by increased [^{3}H]-thymidine incorporation, by facilitating cell cycle progression.

This study demonstrated that PKC phosphorylation was increased in response to L-leucine, as did the intracellular Ca^{2+} concentration. Taken together, these findings suggest that L-leucine activated PLC-associated second messenger cascades. Indeed, we observed that PLC influenced the L-leucine-induced increase in cell cycle regulatory proteins and DNA synthesis. PLC plays a pivotal role in transmembrane signaling, as follows: In order to transmit signals from activated extracellular membrane receptors in response to various extracellular stimuli (numerous hormones, growth factors, and neurotransmitters), PLC hydrolyzes a minor membrane phospholipid, phosphatidylinositol 4,5-bisphophate (PIP2), into the two second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3), which subsequently activate PKC and mediate intracellular Ca^{2+} release, respectively [Shin et al., 2002]. Lynch et al. [2000] reported that L-leucine executes its proliferative role through its functional receptor. In this study, we observed that L-leucine-induced increases in the intracellular Ca^{2+} concentration were inhibitable by neomycin pretreatment, suggesting that activation of the putative L-leucine receptor and subsequent PLC activation played a pivotal role in L-leucine-induced cell proliferation in chicken hepatocytes.

In addition to the PLC-associated Ca^{2+} influx, PKC activity increased in response to L-leucine. It is clear that PKC activation does not always produce the same pattern of CDK and cyclin modulation, because Bürger et al. [1994] reported that TPA (a PKC activator) decreased CDK-2 expression and upregulated cyclin D1. However, previous studies have consistently demonstrated that activation of the phospholipid-dependent PKC is associated with mitogenesis in various cell types [Rozengurt, 2007]. To our knowledge, the study herein is the first report to demonstrate that PKC activation is responsible for L-leucine-induced mitogenesis in chicken hepatocytes.

In the present study, L-leucine increased the levels of phosphorylated Akt. The serine/threonine protein kinase Akt is a well-characterized downstream target of PI3K during diverse processes, including glucose homeostasis, transcription, apoptosis, cell motility, angiogenesis, proliferation, and growth [Brazil and Hemmings, 2001]. Akt is a key component of growth control, and it acts as downstream of mitogens and nutrients to regulate protein translation and cell cycle progression in hepatocytes [Mullany et al., 2007]. Studies in a number of experimental models have demonstrated that Akt regulates cyclin–CDK activity and cell

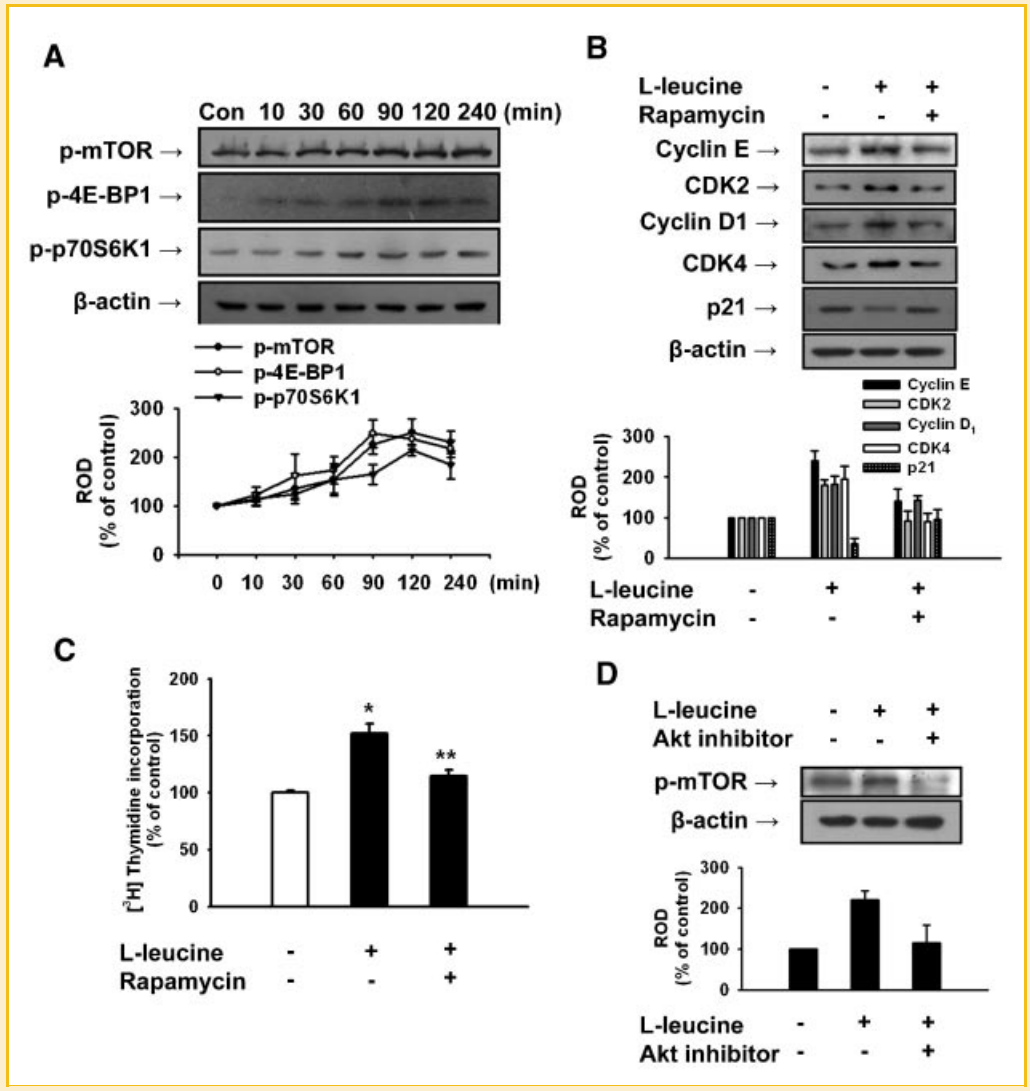

Fig. 5. The mTOR pathway is involved in L-leucine-induced [^{3}H]-thymidine incorporation. A: Chicken hepatocytes were treated with L-leucine (10^{-5} M) for various time points (0–240 min), and the levels of phosphorylated mTOR, p70S6K, and 4E-BP1 were detected by Western blot. B: Cells were treated with rapamycin (an mTOR inhibitor, 10^{-8} M) for 30 min prior to L-leucine treatment for 12 h, and the levels of cyclin E, CDK2, cyclin D1, CDK4, and p21 were detected by Western blot. Each example shown is representative of three experiments. The graphs denote the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin. C: Cells were pretreated with rapamycin for 30 min prior to L-leucine treatment for 12 h; cells were subsequently pulsed with 1 μCi [^{3}H]-thymidine for 1 h. The values represent means ± SE of four independent experiments from triplicate dishes. $^*P < 0.05$ versus control; $^{**}P < 0.05$ versus L-leucine alone. D: Cells were pretreated with Akt inhibitor (10^{-5} M) for 30 min prior to L-leucine treatment for 2 h. Phosphorylation of mTOR was detected by Western blot. Each example shown is representative of three experiments. The graphs denote the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin. ROD, relative optical density.

cycle progression through several mechanisms. For example, in one study, Akt induced expression of cyclin D1, a key G1-phase regulatory protein [Muise-Helmericks et al., 1998]. Akt also inactivated CKI proteins (p21 and p27), thereby promoting CDK activity and cell cycle progression [Liang and Slingerland, 2003]. Consistent with these precedents, the Akt pathway was clearly involved in the L-leucine mitogenic effect, because L-leucine-induced stimulation of [^{3}H] thymidine incorporation and cyclin D1–CDK4, cyclin E–CDK2 expression, and its inhibition of $p21^{WAF/Cip1}$ protein expression was blocked by the inhibition of PI3K/Akt by LY 294002, PI3K siRNA, or Akt inhibitor.

This study also implicated ERK1/2 in L-leucine-induced modulation of cell cycle regulatory proteins and DNA synthesis in primary cultured chicken hepatocytes. The ERK1/2 pathway is an important growth-related signaling pathway for cell proliferation, and various mitogens can activate it [Deng et al., 2007]. In this study, L-leucine triggered ERK1/2 activation, and a MEK inhibitor, PD 98059 or ERK siRNA blocked L-leucine-induced increases in expression of cyclin D1, CDK4, cyclin E, and CDK2. In addition, L-leucine-induced DNA synthesis was also blocked by PD 98059. Therefore, ERK1/2 activation plays an important role in cell cycle progression and cell proliferation by L-leucine.

In cell culture systems, Akt promoted growth by regulating several components of the translational apparatus, including the mTOR pathway [Gingras et al., 1998; Bjornsti and Houghton, 2004]. In this study, we showed that L-leucine promoted phosphorylation of mTOR and two of its most well-described targets, p70S6K and the translational repressor 4E-BP1. These results are consistent with a number of studies showing that a PI3K inhibitor blocked the leucine-induced activation of p70S6K or phosphorylation of 4E-BP1 [Xu et al., 1998; Wang et al., 2005]. Both these proteins are critical for translation of specific mRNAs involved in cell cycle progression, including cyclin D1 [Mamane et al., 2006]. A number of studies reported that L-leucine stimulated protein synthesis by increasing mRNA expression of several effectors [Anthony et al., 2001b; Yoshizawa, 2004; Stipanuk, 2007]. Our observations that L-leucine induced increases in cyclin/CDK protein expression levels and decreases in $p21^{WAF/Cip1}$ protein expression, and that rapamycin blocked this effect were consistent with these previous studies and provided another piece of the mechanism behind the results of our [^{3}H]-thymidine incorporation experiment.

In conclusion, we propose a model in which L-leucine stimulates DNA synthesis and expression of cell cycle regulatory proteins through the Ca^{2+}/PKC/ERK1/2, PI3K/Akt, and mTOR signaling pathways in primary cultured chicken hepatocytes (Fig. 6). Further research will elucidate the exact nature of these intracellular signal transduction pathways, which should add to our understanding of how L-leucine modulates hepatocyte proliferation and clarify its role in regulating physiological processes like liver regeneration.

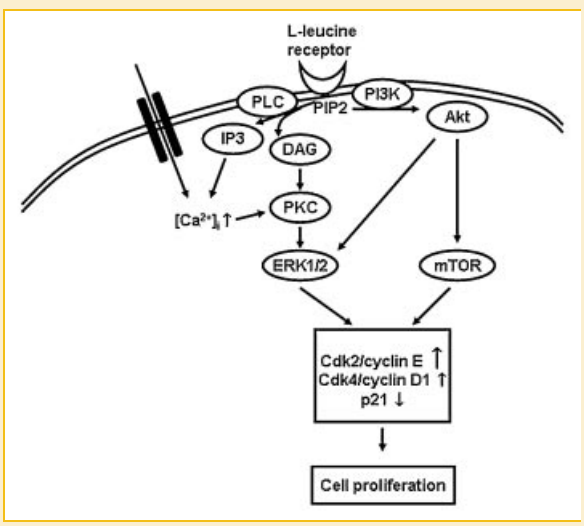

Fig. 6. The hypothesized model for the signal pathways involved in L-leucine-induced cell proliferation. L-leucine activates PLC, which increases intracellular Ca^{2+} concentration and activates PKC. PKC subsequently induces ERK1/2 activation. In addition, L-leucine concomitantly activates Akt, which activates ERK1/2 and mTOR activation. ERK1/2 and mTOR increase cell cycle regulatory protein expression, which induces cell proliferation. PLC, phospholipase C; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; ERK1/2, extracellular signal regulated kinase1/2; mTOR, mammalian target of rapamycin; Cdk, cyclin-dependent protein kinase. Arrows represent the proposed signaling pathways.

REFERENCES

Alisi A, Spagnuolo S, Leoni S. 2003. Treatment with EGF increases the length of S-phase after partial hepatectomy in rat, changing the activities of cdks. Cell Physiol Biochem 13(4): 239–248.

Anthony TG, Anthony JC, Yoshizawa F, Kimball SR, Jefferson LS. 2001a. Oral administration of leucine stimulates ribosomal protein mRNA translation but not global rates of protein synthesis in the liver of rats. J Nutr 131:1171–1176.

Anthony JC, Anthony TG, Kimball SR, Jefferson LS. 2001b. Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. J Nutr 131(3): 856S–860S.

Bjornsti MA, Houghton PJ. 2004. The TOR pathway: A target for cancer therapy. Nat Rev Cancer 4(5): 335–348.

Brazil DP, Hemmings BA. 2001. Ten years of protein kinase B signalling: A hard Akt to follow. Trends Biochem Sci 26:657–664.

Bürger C, Wick M, Müller R. 1994. Lineage-specific regulation of cell cycle gene expression in differentiating myeloid cells. J Cell Sci 107:2047–2054.

Deng Y, Xu H, Riedel H. 2007. PSM/SH2-B distributes selected mitogenic receptor signals to distinct components in the PI3-kinase and MAP kinase signaling pathways. J Cell Biochem 100(3): 557–573.

Dubbelhuis PF, Meijer AJ. 2002. Hepatic amino acid-dependent signaling is under the control of AMP-dependent protein kinase. FEBS Lett 521(1–3): 39–42.

Fujita S, Dreyer HC, Drummond MJ, Glynn EL, Cadenas JG, Yoshizawa F, Volpi E, Rasmussen BB. 2007. Nutrient signalling in the regulation of human muscle protein synthesis. J Physiol 582:813–823.

Gabelman BM, Emerman JT. 1992. Effects of estrogen, epidermal growth factor, and transforming growth factor-α on the growth of human breast epithelial cells in primary culture. Exp Cell Res 201:113–118.

Gad A, Thullberg M, Dannenberg JH, te Riele H, Stromblad S. 2004. Retinoblastoma susceptibility gene product (pRb) and p107 functionally separate the requirements for serum and anchorage in the cell cycle G1-phase. J Biol chem 279:13640–13644.

Gingras AC, Kennedy SG, O'Leary MA, Sonenberg N, Hay N. 1998. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. Genes Dev 12(4): 502–513.

Hörster F, Hoffmann GF. 2004. Pathophysiology, diagnosis, and treatment of methylmalonic aciduria—Recent advances and new challenges. Pediatr Nephrol 19(10): 1071–1074.

Hou DX, Kunitake T, Kusuda J, Fujii M. 2001. Primary culture of chicken hepatocytes as an in vitro model for determining the influence of dioxin. Biosci Biotechnol Biochem 65(1): 218–221.

Khanna S, Gopalan S. 2007. Role of branched-chain amino acids in liver disease: The evidence for and against. Curr Opin Clin Nutr Metab Care 10(3): 297–303.

Kimura M, Ogihara M. 2005. Effects of branched-chain amino acids on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. Eur J Pharmacol 510(3): 167–180.

Koroxenidou L, Ohlson LC, Porsch Hällström I. 2005. Long-term 17α-ethinyl estradiol treatment decreases cyclin E and cdk2 expression, reduces cdk2 kinase activity and inhibits S phase entry in regenerating rat liver. J Hepatol 43(3): 478–484.

Krause U, Bertrand L, Maisin L, Rosa M, Hue L. 2002. Signalling pathways and combinatory effects of insulin and amino acids in isolated rat hepatocytes. Eur J Biochem 269(15): 3742–3750.

Liang J, Slingerland JM. 2003. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle 2(4): 339–345.

Livneh E, Fishman DD. 1997. Linking protein kinase C to cell-cycle control. Eur J Biochem 248(1): 1–9.

Lynch CJ, Fox HL, Vary TC, Jefferson LS, Kimball SR. 2000. Regulation of amino acid-sensitive TOR signaling by leucine analogues in adipocytes. J Cell Biochem 77(2): 234–251.

Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N. 2006. mTOR, translation initiation and cancer. Oncogene 25(48): 6416–6422.

Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ, Kato JY. 1994. D-type cyclin-dependent kinase activity in mammalian cells. Mol Cell Biol 14(3): 2066–2076.

Muise-Helmericks RC, Grimes HL, Bellacosa A, Malstrom SE, Tsichlis PN, Rosen N. 1998. Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. J Biol Chem 273(45): 29864–29872.

Mullany LK, Nelsen CJ, Hanse EA, Goggin MM, Anttila CK, Peterson M, Bitterman PB, Raghavan A, Crary GS, Albrecht JH. 2007. Akt-mediated liver growth promotes induction of cyclin E through a novel translational mechanism and a p21-mediated cell cycle arrest. J Biol Chem 282(29): 21244–21252.

Nishitani S, Matsumura T, Fujitani S, Sonaka I, Miura Y, Yagasaki K. 2002. Leucine promotes glucose uptake in skeletal muscles of rats. Biochem Biophys Res Commun 299(5): 693–696.

Patti ME, Brambilla E, Luzi L, Landaker EJ, Kahn CR. 1998. Bidirectional modulation of insulin action by amino acids. J Clin Invest 101(7): 1519–1529.

Rozengurt E. 2007. Mitogenic signaling pathways induced by G protein-coupled receptors. J Cell Physiol 213(3): 589–602.

Sasaki K, Kitaguchi Y, Fukuda T, Aoyagi Y. 2000. Ascorbic acid supplementation to primary culture of chicken hepatocytes with non-serum medium. Int J Biochem Cell Biol 32:967–973.

Sasaki K, Kitaguchi Y, Koga K, Narita R, Fukuda T, Aoyagi Y. 2001. Dehydroascorbic acid reduction in several tissues and cultured hepatocytes of the chicken. Biosci Biotechnol Biochem 65(10): 2288–2290.

Schmelzle T, Hall MN. 2000. TOR, a central controller of cell growth. Cell 103(2): 253–262.

Sherr CJ, Roberts JM. 1995. Inhibitor of mammalian G1 cyclin-dependent kinases. Genes Dev 9(10): 1149–1163.

Shin SY, Yoon SC, Kim YH, Kim YS, Lee YH. 2002. Phosphorylation of glycogen synthase kinase-3β at serine-9 by phospholipase Cγ1 through protein kinase C in rat 3Y1 fibroblasts. Exp Mol Med 34:444–450.

Stipanuk MH. 2007. Leucine and protein synthesis: mTOR and beyond. Nutr Rev 65(3): 122–129.

Sugiyama K, Yu L, Nagasue N. 1998. Direct effect of branched-chain amino acids on the growth and metabolism of cultured human hepatocellular carcinoma cells. Nutr Cancer 31(1): 62–68.

Swenne I. 1992. Pancreatic beta-cell growth and diabetes mellitus. Diabetologia 35(3): 193–201.

Tomiya T, Nishikawa T, Inoue Y, Ohtomo N, Ikeda H, Tejima K, Watanabe N, Tanoue Y, Omata M, Fujiwara K. 2007. Leucine stimulates HGF production by hepatic stellate cells through mTOR pathway. Biochem Biophys Res Commun 358(1): 176–180.

Wang X, Beugnet A, Murakami M, Yamanaka S, Proud CG. 2005. Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins. Mol Cell Biol 25(7): 2558–2572.

Watford M. 2007. Lowered concentrations of branched-chain amino acids result in impaired growth and neurological problems: Insights from a branched-chain α-keto acid dehydrogenase complex kinase-deficient mouse model. Nutr Rev 65(4): 167–172.

White J, Stead E, Faast R, Conn S, Cartwright P, Dalton S. 2005. Developmental activation of the Rb-E2F pathway and establishment of cell cycle-regulated cyclin-dependent kinase activity during embryonic stem cell differentiation. Mol Biol Cell 16(4): 2018–2027.

Xu G, Kwon G, Marshall CA, Lin TA, Lawrence JC, Jr., McDaniel ML. 1998. Branched-chain amino acids are essential in the regulation of PHAS-I and p70 S6 kinase by pancreatic β-cells. A possible role in protein translation and mitogenic signaling. J Biol Chem 273(43): 28178–28184.

Yoshizawa F. 2004. Regulation of protein synthesis by branched-chain amino acids in vivo. Biochem Biophys Res Commun 313(2): 417–422.