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Involvement of protein kinase C activation in L-leucine-induced stimulation of protein synthesis in L6 myotubes

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Abstract

Effects of leucine and related compounds on protein synthesis were studied in L6 myotubes. The incorporation of [^{3}H]tyrosine into cellular protein was measured as an index of protein synthesis. In leucine-depleted L6 myotubes, leucine and its keto acid, α-ketoisocaproic acid (KIC), stimulated protein synthesis, while D-leucine did not. Mepacrine, an inhibitor of both phospholipases A_2 and C, canceled stimulatory actions of L-leucine and KIC on protein synthesis. Neither indomethacin, an inhibitor of cyclooxygenase, nor caffeic acid, an inhibitor of lipoxygenase, diminished their stimulatory actions, suggesting no involvement of arachidonic acid metabolism. Conversely, 1-*O*-hexadecyl-2-*O*-methylglycerol, an inhibitor of proteinkinase C, significantly canceled the stimulatory actions of L-leucine and KIC on protein synthesis, suggesting an involvement of phosphatidylinositol degradation and activation of protein kinase C. L-Leucine caused a rapid activation of protein kinase C in both cytosol and membrane fractions of the cells. These results strongly suggest that both L-leucine and KIC stimulate protein synthesis in L6 myotubes through activation of phospholipase C and protein kinase C.

Abbreviations: DMEM – Dulbecco's modified Eagle medium; EGTA – [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; FCS – fetal calf serum: HMG, 1-*O*-hexadecyl-2-*O*-methylglycerol; KIC – α-ketoisocaproic acid; Leu – Leucine; PBS(−) – Ca–Mg-free phosphate-buffered saline; P13K – phosphatidylinositol 3-kinase.

The proteins of living cells are constantly renewed by protein turnover. This involves the continuous process of degradation into, and subsequent re-synthesis from, free amino acids. L-Leucine (L-Leu), one of the branched-chain amino acids, reportedly inhibits protein degradation in the liver (Pösö et al. 1982; Mortimore et al. 1987), muscles (Tischler et al. 1982) and cultured L6 myotubes (Yagasaki et al. 1994), and stimulates protein synthesis in isolated muscles (Tischler et al. 1982) and cultured RLC-16 hepatocytes (Yagasaki et al. 2002a). A keto acid produced from leucine by transaminase catalysis, that is, α-ketoisocaproic acid (KIC), also inhibited protein degradation in the cultured muscle cells (Yagasaki et al. 1994) and stimulated protein synthesis in the cultured hepatocytes (Yagasaki et al. 2002a). The stimulatory action of leucine on protein synthesis is demonstrated to be mediated through phosphorylation of p70 S6 kinase and in part through protein

kinase mammalian target of rapamycin (mTOR) (Patti et al. 1998; Anthony et al. 2000; Anthony et al. 2001). Likewise, L-histidine, one of the basic amino acids, has been reported to affect protein synthesis in cultured cells (Ali and Evans 2001; Yagasaki et al. 2002b). Both leucine and histidine regulate global protein synthesis in L6 myoblasts through modulating the activity of eukaryotic initiation factor eIF2B (Kimball et al. 1998). These findings suggest that amino acids can act as direct initiators of signal transduction pathways, as well as precursors for protein synthesis. L6 myoblasts grow rapidly and fuse spontaneously into multinucleated myotubes, concomitantly with the cessation of DNA synthesis (Yaffe 1968). Thus, L6 myotubes provide a model of muscle tissues and are used for studies on muscle protein turnover. Protein anabolic actions of hormones such as insulin and nutrients such as amino acids are important to recover from muscle wasting after operation stress, for instance. In the present study, we investigated the effects of leucine and KIC on protein synthesis in L6 myotubes, comparing them with that of D-leucine (D-Leu). KIC as well as L-Leu stimulated protein synthesis in L6 myotubes, and their stimulatory actions are suggested to be mediated at least partly cellular phospholipid degradation process and protein kinase C activation.

Materials and methods

Materials

L6 myoblasts (Yaffe 1968) were provided by Dr. T. Amano, Mitsubishi-Kasei Institute of Life Sciences, Tokyo, Japan. Dulbecco's modified Eagle medium (DMEM) was purchased from Nissui Pharmaceutical Co., Tokyo, fetal calf serum (FCS) from Hazleton, KS, USA, streptomycin from Meiji-Seika Kaisha, Tokyo, penicillin G from Ban-yu Pharmaceutical Co., Tokyo, and L-[ring-3,5-^{3}H]tyrosine (50 Ci/mmol) from American Radiolabeled Chemicals Inc., St. Louis, MO, USA. L-Cycloserine, an inhibitor of leucine transaminase (Tischler et al. 1982), mepacrine, an inhibitor of phospholipases A_2 and C (Premecz et al. 1989; Fernandez et al. 1998) and indomethacin, an inhibitor of cyclooxygenase (Rodemann and Goldberg 1982) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Caffeic acid, an inhibitor of 5-lipoxygenase (Koshihara et al. 1983) was obtained from Nacalai Tesque, Inc., Kyoto, Japan, and 1-*O*-hexadecyl-2-*O*-methylglycerol (HMG), an inhibitor of protein kinase C (PKC) (Kramer et al. 1989), from Funakoshi Co., Ltd., Tokyo. L-Cycloserine was dissolved in water and filtered before use. Other inhibitors were dissolved in ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan). They were added to the experimental media at the final ethanol concentration of 0.4%. The control medium contained 0.4% ethanol alone. All other chemicals were of the best grade commercially available.

Culture of L6 myotubes

Stock cultures of L6 myoblasts were maintained in DMEM supplemented with 10% (v/v) FCS, streptomycin (100 μg/ml), and penicillin G (100 U/ml) [10% FCS/DMEM] under an atmosphere of 5% CO_2/95% humidified air at 37 °C, as described previously (Yagasaki et al. 1991). The prefused cells (5×10^4 cells/well) were subcultured into Nunc 24-place multiwell plates and grown for 11 days to form myotubes in 0.4 ml of 10% FCS/DMEM. The medium was renewed every 3 days.

Measurement of protein synthesis

After the 11-day-old-myotubes were kept for 12 h in 0.2% FCS/DMEM minus L-leucine, L6 myotubes were washed twice with Ca-Mg-free phosphate-buffered saline [PBS(−)]. The cells were exposed to serum-free, experimental media for 2 h, then received 0.5 μCi/well of L-[ring-3,5-^{3}H]tyrosine (50 Ci/mmol), and cultured for another 1 h. Then, plates were placed on ice and the medium was removed. After two washings with cold PBS(−), cells were dissolved in 500 μl of 2 mM sodium dodecylsulfate, and 100 μl of the cell lysate was applied to Toyo cellulose nitrate membrane filter. After being dried at room temperature overnight, the filter was washed with 5% trichloroacetic acid at 90 °C for 10 s, dried and transferred to a mini-counting vial. Toluene–methyl cellosolve scintillator (4 ml) was added to the vial

and radioactivity was counted with a liquid scintillation spectrometer (LS5000 TD, Beckman) as described previously (Yagasaki et al. 1991). Portions of the cell lysate were taken for protein assay with bovine serum albumin as the standard (Lowry et al. 1951). The activity of protein synthesis was indicated by radioactivity per mg cellular protein (dpm/mg protein).

Cell fractionation and measurement of protein kinase C activity

The prefused cells (2.9×10^5 cells/dish) were subcultured into Nunc 60 mm dishes and grown for 11 days to form myotubes in 4 ml of 10% FCS/DMEM. After the 11-day-old-myotubes were kept for 12 h in 0.2% FCS/DMEM minus L-leucine, the cells were washed twice with PBS(−). The leucine-depleted L6 myotubes were exposed to serum-free, experimental media for 0, 1, 2, 5, 10 and 20 min.Then dishes were placed on ice and medium was removed. After two washings with cold PBS(−), cells were harvested by cell scraper (Becton Dickinson Labware, Franklin Lakes, NJ, USA), suspended in PBS(−), and centrifuged at 190 × *g* for 3 min. Cell pellets were resuspended in buffer A (20 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 1.2 mM [ethylenebis(oxyethylenenitrilo)] tetraacetic acid (EGTA), 2.5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride) and sonicated for 30 min in a sonicator (Tomy, UR-200P). Sonicates were centrifuged at 105,000 × *g* for 30 min in a Hitachi CP100H ultracentrifuge (RP100AT4-143 rotor). The supernatants were regarded as cytosol fractions (Cooper et al. 1987). The pellets were resuspended in buffer B (20 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 5 mM EGTA, 2 mM EDTA, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride) and incubated for 40 min at 4 °C. The solubilized membrane fractions were centrifuged at 105,000 × *g* for 30 min as above mentioned, and the supernatants were regarded as membrane fractions (Cooper et al. 1987). Both fractions were subsequently assayed for protein kinase C activity with a commercial kit (protein kinase C enzyme assay system with PB168 P-32, Amersham Japan, Tokyo, Japan). Protein was determined as described above. Protein kinase C activity was expressed as pmol/min/mg protein.

Statistical analysis

Data are expressed as means ± SEM. Differences between group means were compared by Student's *t*-test, and $p < 0.05$ was considered statistically significant.

Results

Effects of L-leucine, L-leucine and KIC on protein synthesis

When L-Leu was added to serum free, leucine-free DMEM (control medium) at concentrations of 0.4, 0.8 and 1.6 mM, protein synthesis in L6 myotubes dose-dependently increased and reached plateau at 1.6 mM (data not shown). Thus, the effects of L-Leu, D-Leu and KIC on protein synthesis in myotubes were examined at the concentration of 1.6 mM (Table 1). Both L-Leu and KIC significantly stimulated protein synthesis in L6 myotubes and the stimulatory effect of L-Leu and KIC was identical, whereas D-Leu significantly suppressed the protein synthesis.

Effect of L-cycloserine on L-leucine- and KIC-induced stimulation of protein synthesis

Leucine transaminase converts L-Leu to KIC and *vice versa*. To learn whether change from L-Leu to KIC or *vice versa* would be required to stimulate protein synthesis, effect of L-cycloserine, an inhibitor of leucine transaminase, on L-Leu- or KIC-induced stimulation of protein synthesis in L6 myotubes was examined. As shown in Table 2,

Table 1. Effects of L-leucine, D-leucine and α-ketoisocaproic acid on protein synthesis in cultured L6 myotubes

Addition	Concentration (mM)	Protein synthesis (dpm × 10^{-4}/mg protein)
None (control)	–	2.65 ± 0.12
L-Leucine	1.6	2.95 ± 0.08*
D-Leucine	1.6	2.33 ± 0.07*
α-Ketoisocaproic acid	1.6	2.95 ± 0.08*

Each value represents the mean ± SEM of six incubations. Control medium was serum-free, leucine-free DMEM. To the control medium, L-leucine, D-leucine or α-ketoisocaproic acid was supplemented at a concentration of 1.6 mM. *Significantly different from the control group at $p < 0.05$.

Table 2. Effect of L-cycloserine on L-leucine- or α-ketoisocaproic acid-induced stimulation of protein synthesis in cultured L6 myotubes

Addition	Concentration (mM)	Protein synthesis (dpm $\times 10^{-4}$/mg protein)	
		L-Cycloserine condition	
		−	+
L-Leucine	–	2.96 ± 0.11	2.95 ± 0.06
	1.6	3.53 ± 0.13*	3.33 ± 0.16
α-Ketoisocaproic acid	–	2.97 ± 0.15	2.98 ± 0.07
	1.6	3.40 ± 0.12*	3.34 ± 0.14

Each value represents the means ± SEM of six incubations. Control medium was serum-free and leucine-free DMEM. To the control medium, 1.6 mM L-leucine or 1.6 mM α-ketoisocaproic acid was added in the absence or presence of 7.5 mM L-cycloserine. *Significantly different from the control (no leucine-no cycloserine or no α-ketoisocaproic acid-no cycloserine) groups at $p < 0.05$.

L-cycloserine did not exert any significant influence on both L-Leu- and KIC-induced stimulation of protein synthesis.

Effects of enzyme inhibitors on L-leucine- or KIC-induced stimulation of protein synthesis

To investigate whether or not molecules produced by some enzymes are involved as signal messengers in the stimulatory action of L-Leu on protein synthesis, influences of four enzyme inhibitors on protein synthesis were studied in L6 myotubes. As shown in Table 3, 10 μM mepacrine and 500 μM HMG significantly suppressed the L-Leu-induced increase in protein synthesis without affecting basal protein synthesis. However, 20 μM indomethacin and 100 μM caffeic acid did not cancel the stimulatory action of L-Leu on protein synthesis.

Influences of enzyme inhibitors on protein synthesis were also examined in L6 myotubes cultured in KIC-supplemented medium (Table 3). Both 10 μM mepacrine and 500 μM HMG also canceled significantly the stimulatory action of KIC on protein synthesis in L6 myotubes without affecting basal protein synthesis. Neither 20 μM indomethacin nor 100 μM caffeic acid canceled the KIC-induced increase in protein synthesis.

Effect of L-leucine on protein kinase C activity

Time course of L-Leu effect on protein kinase C activity in L6 myotubes is shown in Figure 1. In 1 min of L-Leu addition, cytosolic protein kinase C activity increased, and peaked at 2 min. Cytosolic protein kinase C activity thereafter rapidly decreased to the control levels between 5–20 min (Figure 1a). In contrast to the change in cytosolic protein kinase C activity, membrane activity

Table 3. Effects of mepacrine (MEC), indomethacin (IND), caffeic acid (CAF) and 1-*O*-hexadecyl-2-*O*-methylglycerol (HMG) on L-leucine- or α-ketoisocaproic acid-induced stimulation of protein synthesis in cultured L6 myotubes

Inhibitor	Concentration (μM)	Protein synthesis (dpm $\times 10^{-4}$/mg protein)			
		L-Leucine condition		α-Ketoisocaproic acid condition	
		−	+	−	+
1.68 ± 0.04	1.90 ± 0.09*	1.83 ± 0.08	2.13 ± 0.09*		
MEC	10	1.53 ± 0.03	1.61 ± 0.04**	1.73 ± 0.06	1.87 ± 0.07**
None	–	1.79 ± 0.09	2.23 ± 0.08*	2.29 ± 0.15	2.83 ± 0.04*
IND	20	1.73 ± 0.09	2.08 ± 0.07	2.28 ± 0.06	2.93 ± 0.17
None	–	1.83 ± 0.08	2.23 ± 0.08*	2.21 ± 0.10	2.71 ± 0.10*
CAF	100	1.78 ± 0.02	2.27 ± 0.13	2.10 ± 0.08	3.03 ± 0.19
None	–	2.08 ± 0.06	2.42 ± 0.12*	1.93 ± 0.07	2.66 ± 0.07*
HMG	500	2.13 ± 0.06	2.14 ± 0.05**	2.06 ± 0.04	2.42 ± 0.03**

Each value represents the mean ± SEM of six incubations. Control medium was serum-free and leucine-free DMEM. To the control medium, 1.6 mM L-leucine or 1.6 mM α-ketoisocaproic acid was added in the absence or presence of various inhibitors. *Significantly different from the control (no leucine-no inhibitor, or no α-ketoisocaproic acid-no inhibitor) groups at $p < 0.05$. **Significantly different from the L-leucine- or α-ketoisocaproic acid-supplemented groups at $p < 0.05$.

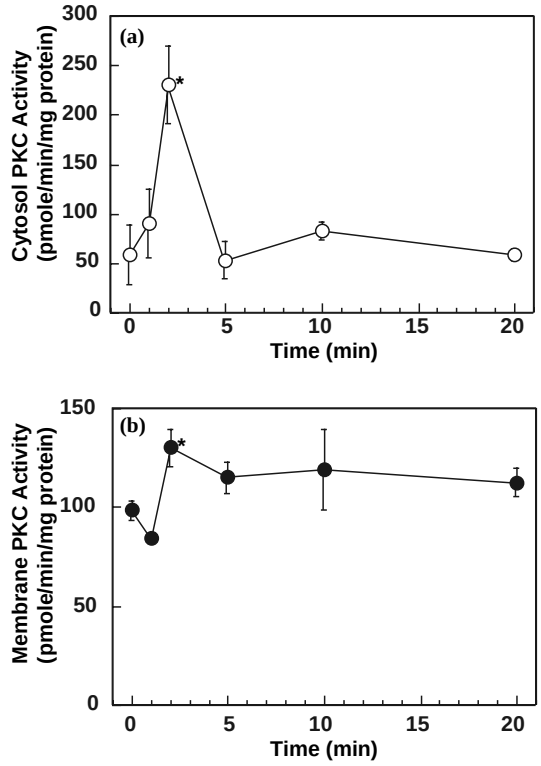

Figure 1. Time course of the effect of L-leucine treatment on protein kinase C activity in cytosol (a) and membrane (b) fractions. Each point and vertical bar represents the mean and SEM of triplicate assays. After 11-day-old-myotubes were kept for 12 h in a leucine-depleted medium, the myotubes were exposed to serum-free, L-leucine (1.6 mM)-supplemented DMEM for 0, 1, 2, 5, 10 and 20 min. At the end of the exposing periods, cells were collected, cytosol and membrane fractions were isolated, and protein kinase C activity in both fractions was measured as described in Materials and methods section of the text. *Significantly different from the corresponding initial (time zero) values at $p < 0.05$.

increased at 2 min and the high activity was maintained during L-Leu treatment (Figure 1b).

Discussion

In the present study, we investigated the *in vitro* effect of L-Leu, D-Leu and KIC on protein synthesis in L6 myotubes. KIC as well as L-Leu, but not D-Leu, stimulated the protein synthesis in the myotubes (Table 1). Similar results have also been observed in RLC-16 hepatocytes (Yagasaki et al. 2002a). These results are consistent with the findings of Patti et al. who have reported that in cultured FAO cells L-Leu and KIC activate p70 S6 kinase, an intermediate which is important in the initiation of protein synthesis (Patti et a1. 1998).

Leucine transaminase converts L-Leu to KIC and *vice versa*. L-Cycloserine, an inhibitor of leucine transaminase, did not cancel the stimulatory actions of both L-Leu and KIC on protein synthesis in L6 myotubes (Table 2), suggesting that L-Leu and KIC can independently stimulate protein synthesis without reciprocal changes between L-Leu and KIC. Taking the failure of D-Leu to stimulate protein synthesis into consideration, we assumed that L-Leu and KIC might be recognized as extracellular signal molecules by L6 myotubes and then their signaling might be propagated in the cells. To verify this possibility, we examined influences of inhibitors of several enzymes on protein synthesis in L6 myotubes (Table 3). Stimulatory actions of L-Leu and KIC were canceled by mepacrine, suggesting the involvement of either phospholipase A_2 or phospholipase C activity in the stimulatory actions of L-Leu and KIC. Neither indomethacin nor caffeic acid interrupted the stimulation of protein synthesis by L-Leu and KIC, suggesting no involvement of cyclooxygenase, lipoxygenase and hence arachidonic acid metabolism in the stimulatory actions of L-Leu and KIC, unlike in that of L-histidine in L6 myotubes (Yagasaki et al. 2002b). This also suggests that phospholipase A_2 does not play any substantial role in the stimulatory actions of L-Leu and KIC. Conversely, HMG canceled the stimulatory actions of L-Leu and KIC on protein synthesis, strongly suggesting that L-Leu might activate phospholipase C and hence protein kinase C. We, therefore, measured protein kinase C activity when L-Leu was used as an extracellular stimulus (Figure 1). The results obtained clearly demonstrated that L-Leu activated protein kinase C in both cytosol and membrane fractions of L6 myotubes. The effect of KIC on protein kinase C activity should also be studied in the near future. In the cascade of events leading to insulin-induced glucose transport, protein kinase Cζ, an isoform of the kinase, is important in primary culture of rat skeletal muscle (Braiman et al. 2001). Dehydroepiandrosterone has been reported to stimulate glucose uptake, phosphatidylinositol 3-kinase (PI3K) activity and translocation of protein kinase Cζ from the cytosol to the membrane in rat adipocytes (Ishizuka et al. 1999). In L6 myotubes,

L-Leu (2 mM) caused a rapid but transient stimulation of PI3K (Peyrollier et al. 2000). The present result is the first evidence that L-Leu activates protein kinase C, but its isomer activated by L-Leu is unknown at present. Further intensive studies concerning correlation among protein synthesis, PI3K, protein kinase C and other molecules are required to learn exact signaling pathway(s) of L-Leu action on protein synthesis.

In this study, the blockage of the KIC effect by HMG is partial (33%) in L6 myotubes. This result suggests that an alternative pathway, that is, phospholiphase C–protien kinase C-independent pathway may also participate in the stimulatory action of KIC on protein synthesis in L6 myotubes.

In summary, L-Leu and its deaminated analogue KIC, but not D-Leu, stimulated protein synthesis in L6 myotubes. Their stimulatory actions were canceled by inhibiting phospholipase C and protein kinase C. L-Leu did cause a rapid elevation in protein kinase C activity. These results suggest that some molecules produced by these enzymes are involved as signal messengers in the stimulatory actions of L-Leu and KIC on protein synthesis in L6 myotubes.

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