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Short communication

Possible involvement of phospholipase C and protein kinase C in stimulatory actions of L-leucine and its keto acid, α-ketoisocaproic acid, on protein synthesis in RLC-16 hepatocytes

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Abstract

Effects of leucine and related compounds on protein synthesis were studied in RLC-16 hepatocytes. The incorporation of [^{3}H]tyrosine into cellular protein was measured as an index of protein synthesis. In leucine-depleted RLC-16 cells, L-leucine and its keto acid, α-ketoisocaproic acid (KIC), stimulated protein synthesis, while D-leucine did not. Mepacrine, an inhibitor of both phospholipase A_2 and C canceled stimulatory actions of L-leucine and KIC on protein synthesis, suggesting a possible involvement of either arachidonic acid metabolism by phospholipase A_2, cyclooxygenase or lipoxygenase, or phosphatidylinositol degradation by phospholipase C in the stimulatory actions of L-leucine and KIC. 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 protein kinase 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. These results strongly suggest that both L-leucine and KIC stimulate protein synthesis in RLC-16 cells via activation of phospholipase C and production of diacylglycerol and inositol triphosphate from phosphatidylinositol, which in turn activate protein kinase C.

Abbreviations: HMG, 1-O-hexadecyl-2-O-methylglycerol; KIC, α-ketoisocaproic acid.

Introduction

L-Leucine, 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 myotubes (Yagasaki et al., 1994), and stimulates protein synthesis in isolated muscles (Tischler et al., 1982). Not only L-leucine but also α-ketoisocaproic acid (KIC), a keto acid produced from leucine by transaminase catalysis, inhibited protein degradation in the cultured muscle cells (Yagasaki et al., 1994). The regulation of leucine catabolism in the liver is different from that in the muscles; leucine is catabolized rapidly in the muscles (Tischler et al., 1982), while it is not oxidized rapidly in the liver because of low activity of transaminase (Pösö et al., 1982; Crabb and Harris, 1978). In this study, we investigated the effects of leucine and KIC on protein synthesis using an established cell line of RLC-16 rat hepatocytes, to know if leucine and KIC stimulate protein synthesis in hepatocytes like muscle cells despite the presence of the disparity in leucine catabolism between the two cells. KIC as well as L-leucine stimulated protein synthesis in RLC-16 hepatocytes, and their stimulatory actions are suggested to be mediated at least partly by cellular phospholipid degradation process and protein kinase C activation.

Materials and methods

Materials

Mepacrine, an inhibitor of phospholipases A_2 and C (Premecz et al., 1989; Fernandez et al., 1998), was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., indomethacin, an inhibitor of cyclooxygenase (Rodemann and Goldberg, 1982), from Sigma, caffeic acid, an inhibitor of 5-lipoxygenase (Koshihara et al., 1983), from Nacalai Tesque, Inc., Kyoto, Japan, and 1-O-hexadecyl-2-O-methylglycerol (HMG), an inhibitor of protein kinase C (Kramer et al., 1989), from Funakoshi Co., Ltd., Tokyo, Japan.

These 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 reagents were of the best grade commercially available.

Culture of RLC-16 hepatocytes

RLC-16 hepatocytes (Takaoka et al., 1975) were provided by the RIKEN Cell Bank (RCB069, Tsukuba, Japan). Stock cultures of RLC-16 cells were maintained in DM-160 medium (Kyokuto Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS, Hazleton, KS, U.S.A.), streptomycin (100 μg ml^{-1}, Meiji-Seika Kaisha, Tokyo, Japan) and penicillin (100 U ml^{-1}, Ban-yu Pharmaceutical Co., Tokyo, Japan) (10% FCS/DM-160). The cells (1.2×10^5 cells/well) were subcultured into Nunc 24-place multiwell plates and grown for 7 days in 10% FCS/DM-160, and then kept for 12 h in 0.2% FCS/leucine-free DM-160.

Measurement of protein synthesis

After the leucine depletion for 12 h, hepatocytes were exposed to serum-free, experimental media for 30 min. The cells then received 0.5 μCi of L-[ring-3,5-^{3}H]tyrosine (50 Ci mmol^{-1}, American Radiolabeled Chemicals Inc., St. Louis, MO, U.S.A.), and cultured for another 1 h. Then, plates were placed on ice and the medium was removed. After two washings with cold Ca-Mg-free phosphate-buffered saline (pH 7.4), cells were dissolved in 900 μl of 2 mM sodium dodecylsulfate, and 150 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 sec, 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). Portins 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^{-1} protein).

Statistical analysis

Data are expressed as means $\pm$ 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, D-leucine and KIC on protein synthesis

In our preliminary experiment, protein synthesis in RLC-16 hepatocytes was found to be maximum when L-leucine concentration in the medium was 50% of original DM-160, that is, 1.5 mM (data not shown). Thus, the effects of L-leucine, D-leucine and KIC on protein synthesis were examined at the concentration of 1.5 mM (Figure 1). Both L-leucine and KIC significantly increased protein synthesis to the same extent. However, D-leucine failed to stimulate protein synthesis in RLC-16 cells.

Effects of enzyme inhibitors on stimulatory actions of L-leucine and KIC on protein synthesis

To investigate whether or not molecules produced by some enzymes are involved as signal messengers in the stimulatory action of L-leucine, influences of four enzyme inhibitors on protein synthesis were studied in RLC-16 cells. In our preliminary experiments, mepacrine below 20 μM did not affect basal protein synthesis, i.e., protein synthesis in the L-leucine-depleted RLC-16 cells. Likewise, indomethacin below 50 μM, caffeic acid below 200 μM, and HMG below 1000 μM did not exert any significant influence on basal protein synthesis in RLC-16 hepatocytes (data not shown). As shown in Table 1, 20 μM mepacrine and 1000 μM HMG significantly suppressed the L-leucine-induced increase in protein synthesis without affecting basal

Table 1. Influences of mepacrine (MEC), indomethacin (IND), caffeic acid (CAF) and 1-O-hexadecyl-2-O-methylglycerol (HMG) on [^{3}H]tyrosine incorporation into cellular protein in RLC-16 hepatocytes cultured in L-leucine- or α-ketoisacaproic acid (KIC)-supplemented medium

Inhibitor	Concentration	L-leucine condition		KIC condition	
		–	+	–	+
	(μM)	($\times 10^4$ dpm mg^{-1} protein)			
None	–	2.00±0.18	3.84±0.09[a]	2.60±0.07	5.30±0.24[a]
MEC	20	1.63±0.04	2.81±0.12[b]	2.52±0.16	4.13±0.38[b]
None	–	3.12±0.10	4.87±0.21[a]	3.11±0.09	4.36±0.29[a]
IND	50	2.97±0.05	4.70±0.22	2.94±0.13	4.34±0.09
None	–	2.57±0.05	4.13±0.30[a]	2.60±0.07	5.30±0.24[a]
CAF	200	2.77±0.13	4.33±0.15	2.72±0.08	4.92±0.20
None	–	2.89±0.10	4.06±0.13[a]	2.97±0.11	5.12±0.19[a]
HMG*	1000 or 500	2.69±0.25	3.56±0.08[b]	3.43±0.15	4.54±0.14[b]

Each value represents the mean ± SEM of five to six incubations.
* HMG was added at either 1000 μM (L-leucine experiment) or 500 μM (KIC experiment).
[a] Significantly different from the basal (no leucine-no inhibitor or no KIC-no inhibitor) groups at $P < 0.05$.
[b] Significantly different from the L-leucine- or KIC-supplemented groups at $P < 0.05$.

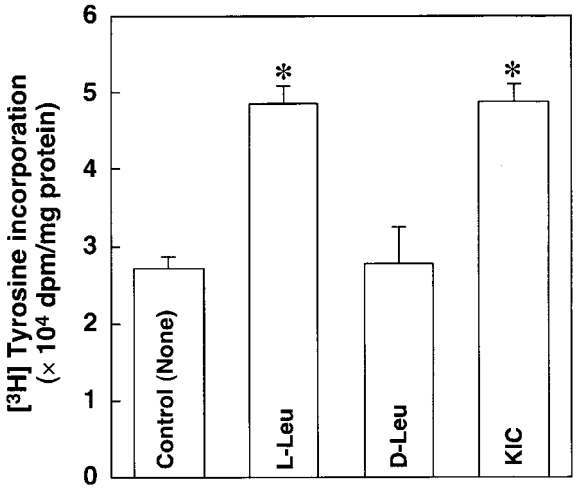

Figure 1. Effects of L-leucine, D-leucine and α-ketoisocaproic acid on protein synthesis in RLC-16 hepatocytes cultured in leucine-free medium. Results are expressed as the mean ± SEM of five to six incubations. Abbreviations: L-Leu, L-leucine; D-Leu, D-leucine; KIC, α-ketoisocaproic acid. Control cells were cultured in L-leucine-free medium. * Significantly different from the control (L-leucine-free) group at $P < 0.05$.

protein synthesis. However, 50 μM indomethacin and 200 μM caffeic acid did not cancel the L-leucine action.

Influences of enzyme inhibitors on protein synthesis were also studied in RLC-16 hepatocytes cultured in KIC-supplemented medium (Table 1). Both 20 μM mepacrine and 500 μM HMG also canceled significantly the stimulatory action of KIC on protein synthesis in RLC-16 cells without affecting basal protein synthesis. Neither 50 μM indomethacin nor 200 μM caffeic acid exerted any influence on the stimulatory action of KIC on protein synthesis.

Discussion

In this report, we studied the *in vitro* effects of L-leucine, D-leucine and KIC on protein synthesis in RLC-16 hepatocytes. KIC as well as L-leucine, but not D-leucine, stimulated the protein synthesis in the hepatocytes. These results are consistent with recent findings of Patti et al. who have reported that in cultured FAO cells L-leucine and KIC activate p70 S6 kinase, an intermediate which is important in the initiation of protein synthesis (Patti et al., 1998). Unlike in the muscles where leucine is catabolized rapidly (Tischler et al., 1982), the amino acid is not oxidized rapidly in the liver because of low activity of transaminase (Pösö et al., 1982; Crabb and Harris, 1978), indicating that leucine is not rapidly converted to KIC and KIC is not rapidly converted to leucine in the liver. If this is true of RLC-16 hepatocytes, L-leucine and

KIC are thought to independently stimulate protein synthesis without reciprocal conversion.

Since D-leucine failed to stimulate protein synthesis and three-dimensional structure of KIC seems more approximate to L-leucine than to D-leucine, we assumed that L-leucine and KIC might be recognized as extracellular signal molecules by RLC-16 hepatocytes and then their signaling might be propagated in the cells. To investigate this possibility, influences of inhibitors of several enzymes on protein synthesis were studied in RLC-16 cells (Table 1). Stimulatory actions of both L-leucine and KIC on protein synthesis were canceled by mepacrine, suggesting the involvement of either phospholipase A_2 or phospholipase C activity in stimulatory actions of L-leucine and KIC. Arachidonic acid is usually derived from the 2-position of phospholipids in the plasma membrane as a result of phospholipase A_2 activity, and is metabolized to prostagrandins and thromboxanes by the cyclooxygenase pathway or leukotrienes and lipoxins by the lipoxygenase pathway (Mayes, 2000). Neither indomethacin nor caffeic acid interrupted the stimulatory actions of L-leucine and KIC on protein synthesis, suggesting no involvement of cyclooxygenase, lipoxygenase and hence arachidonic acid metabolism in the stimulatory actions of L-leucine and KIC. This also suggests that phospholipase A_2 does not play any substantial role in the stimulation of protein synthesis by L-leucine and KIC. Conversely, HMG diminished stimulatory actions of L-leucine and KIC on protein synthesis, indicating an involvement of proteinkinase C in their actions. These results strongly suggest that both L-leucine and KIC activate phospholipase C and produce diacylglycerol and inositol triphosphate from phosphatidylinositol, which in turn activate protein kinase C. Measurements of phospholipase C and protein kinase C activities will clarify this aspect.

In this study, the blockage of the L-leucine effect by mepacrine and HMG is 56 and 43%, respectively, in RLC-16 hepatocytes. Likewise, the blockage of the KIC effect by mepacrine and HMG is 43 and 27%, respectively, in the cells. These results suggest that an alternative pathway, that is, phospholipase C-protein kinase C-independent pathway may also participate in the stimulatory actions of L-leucine and KIC on protein synthesis in RLC-16 hepatocytes.

In summary, L-leucine and its deaminated analogue KIC, but not D-leucine, stimulated protein synthesis in RLC-16 hepatocytes. Their stimulatory actions were partially canceled by inhibiting phospholipase C and protein kinase C. These results suggest some molecules produced by these enzymes are involved as signal messengers in the stimulatory actions of L-leucine and KIC on protein synthesis in RLC-16 cells.

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