Possible Involvement of Calcium Signaling Pathways in L-Leucine-Stimulated Protein Synthesis in L6 Myotubes

Yutaka Miura,† Taro Nakazawa, and Kazumi Yagasaki

Department of Applied Biological Science, Tokyo Noko University, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

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1-L-Leucine is known to stimulate protein synthesis in L6 myotubes. In the present study, we examined the possible involvement of calcium signaling pathways in the stimulation of protein synthesis induced by l-leucine in L6 myotubes. After 16 h of treatment with l-leucine-depleted medium, the re-addition of l-leucine for 4 h augmented protein synthesis by about 50% as compared with an l-leucine-depleted control. Ryanodine receptor antagonists almost completely abolished the stimulatory effect of l-leucine, while IP3 receptor antagonists showed partial inhibition when added simultaneously with l-leucine. These results suggest the possibility that calcium signaling pathways are involved in l-leucine-stimulated protein synthesis.

Key words: l-leucine; protein synthesis; calcium; myotubes

Protein synthesis in mammalian cells is regulated by various factors, such as hormones, growth factors, amino acids, and carbohydrates. Among these regulatory factors, amino acids in particular the branched-chain amino acids (BCAAs: leucine, isoleucine, and valine), are known to exert powerful regulatory influences on protein turnover.1-3 We have reported that l-leucine stimulated protein synthesis in RLC-16 hepatocytes4) and L6 myotubes cultured in l-leucine-deficient media.5) Under l-leucine-deficient conditions, l-leucine did not exert such an effect.4)5) Likewise, l-histidine was found specifically to stimulate protein synthesis under basic amino acids (histidine, arginine, and lysine)-deficient conditions.6) Although many studies have been performed to elucidate the modes of action of BCAA, the precise intracellular process regulated by BCAA remains unknown. For example, the mechanism of BCAA recognition by cells and the molecular events following amino acid recognition remain largely unknown. But, several signaling molecules have been reported to be involved in the BCAA signaling pathway.7) Particularly, mTOR is believed to play the crucial role in amino acid-induced protein synthesis, while an mTOR-independent regulatory pathway (PKB/GSK-3/eIF2B axis) has also been reported.8) Upon amino acid stimulation, the PI-3K/PKB axis is activated, leading to the activation of mTOR. Activated mTOR then phosphorylates eIF2, which accelerates dissociation of eIF-2B from eIF-2B, or directly activates p70 S6 kinase and eEF2. We have reported the possible involvement of PKC in RLC-16 hepatocytes4) and a rapid activation of PKC in plasma membrane and cytosol in L6 myotubes,3) when l-leucine was added to either cell cultured in l-leucine-deficient media. PKC is known to be activated by diacylglycerol, which is produced by PLCβ-mediated cleavage of PIP2. IP3 is simultaneously produced by cleavage of PIP2 and is thought to induce calcium release from the endoplasmic reticulum (intracellular calcium store) by binding to its specific receptor, IP3 receptors. We have observed that IP3 receptor mRNA was induced in L6 myotubes cultured in l-leucine-depleted medium for 16 h (Nakazawa et al., unpublished observation). This result suggests an up-regulation of the IP3 receptor-mediated signaling pathway under l-leucine deficiency. In fact, calcium signaling is known to play an important regulatory role in protein synthesis.9,10) Moreover, a calcium-dependent transcriptional pathway (calcium/calcineurin/NF-AT pathway) has been reported to be involved in heart cardiac hypertrophy.11) There are, however, few reports that imply the possible role of calcium signaling in BCAA-stimulated protein synthesis. These facts prompted us to examine the possible involvement of the calcium signaling pathway in l-leucine-induced protein synthesis in L6 myotubes.

L6 myoblasts,12) kindly provided by Dr. T. Amano of the Mitsubishi-Kasei Institute of Life Sciences, Tokyo, were maintained in DMEM (Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal bovine serum (FBS, JRH, Lenexa, KS), streptomycin (100 μg/ml, Wako Pure Chemicals, Osaka), and penicillin G (100 U/
ml, Wako) (10% FBS/DMEM) under an atmosphere of 5% CO₂/95% humidified air at 37°C, as described previously. L6 myoblasts were seeded at a density of 5 x 10⁴ cells/0.6 ml/well of 24-well culture plates (NUNC, Roskilde, Denmark) in 10% FBS/DMEM, and cultured for 9 d to form a confluent state. The medium was renewed every 3 d. After culturing for 9 d, cytosine β-d-arabinofuranoside (Sigma-Aldrich Japan, Tokyo) was added at a concentration of 1 mM and cultured for another 3 d to form myotubes. Sufficient formation of myotubes was confirmed under phase contrast microscope in every experiment.

The protein synthesis activity of the L6 myotubes was determined as described previously. Briefly, the L6 myotubes precultured with L-leucine-depleted medium for 16 h were cultured in the presence or absence of L-leucine (0.8 mM, the concentration in the complete DMEM) and/or antagonists at the concentrations indicated in the figure for another 4 h. Protein synthesis was measured as described in the text. Data are expressed as means ± SEM for six wells. Statistical differences were compared by one-way analysis of variance followed by the Tukey-Kramer multiple comparisons test. Values not sharing a common letter are significantly different at P < 0.05.

Fig. 1. Effects of IP₃ Receptor Antagonists on l-Leucine-Induced Protein Synthesis in L6 Myotubes. Heparin was dissolved directly in DMEM. 2-APB was dissolved in ethanol and added to the medium at a final ethanol concentration of 0.1%. L6 myotubes precultured with l-leucine-depleted medium for 16 h were cultured in the presence or absence of l-leucine (0.8 mM, the concentration in the complete DMEM) and/or antagonists at the concentrations indicated in the figure for another 4 h. Protein synthesis was measured as described in the text. Data are expressed as means ± SEM for six wells. Statistical differences were compared by one-way analysis of variance followed by the Tukey-Kramer multiple comparisons test. Values not sharing a common letter are significantly different at P < 0.05.

The re-addition of L-leucine to L-leucine-depleted media augmented protein synthesis by about 50% (Figs. 1 and 2). To investigate the involvement of the calcium signaling pathway in L-leucine-induced protein synthesis, we examined the effects of antagonists for calcium release from intracellular calcium store on L-leucine-induced protein synthesis. Intracellular calcium is known to be released via two kinds of calcium releasing pathways in skeletal muscle cells, viz., IP₃-induced calcium release (ICIR) and calcium-induced calcium release (CICR). ICIR is mediated by the IP₃ receptor and CICR is mediated by the dihydropyridine receptor (DHPR)-induced depolarization of cell membrane coupled with ryanodine receptors. The effects of IP₃ receptor antagonists, 2-APB and heparin (sodium salt from bovine lung) (both obtained from Merck Japan, Tokyo) on L-leucine-induced protein synthesis were assessed. As shown in Fig. 1, 25 µM and 40 µM of 2-APB vials. Radioactivity was counted with a liquid scintillation spectrometer (LS5000 TD, Beckman-Coulter, Fullerton, CA). Portions of the lysate were taken, and protein concentration was assessed by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. The activity of protein synthesis was indicated by radioactivity/mg cellular protein (dpm/mg protein).

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Effects of Ryanodine Receptor Antagonists on L-Leucine-Induced Protein Synthesis in L6 Myotubes.

Ruthenium red was dissolved directly in DMEM. Ryanodine was dissolved in ethanol and added to the medium at a final ethanol concentration of 0.1%. L6 myotubes precultured with L-leucine-depleted medium for 16 h were cultured in the presence or absence of L-leucine (0.8 μM, the concentration in the complete DMEM) and/or antagonists at the concentrations indicated in the figure for another 4 h. Protein synthesis was measured as described in the text. Data are expressed as means ± SEM for six wells. Statistical differences were compared by one-way analysis of variance followed by the Tukey-Kramer multiple comparisons test. Values not sharing a common letter are significantly different at *P < 0.05.*
physiological significance of the phenomenon observed in this study, this is the first report that describes the possible involvement of calcium signaling pathways in L-leucine-induced protein synthesis.

References


