Short communication

Possible involvement of phospholipase A₂ and cyclooxygenase in stimulatory action of L-histidine on protein synthesis in L6 myotubes

Kazumi Yagasaki*, Naoko Hatano, Motoki Fujii, Yutaka Miura & Ryuhei Funabiki

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

(* Author for correspondence; E-mail: yagasaki@cc.tuat.ac.jp; Fax: +81 42 367 5713)

Received 4 March 2002; accepted in revised form 25 July 2002

Key words: cyclooxygenase inhibitor, L-histidine, L6 myotube, phospholipase A₂ inhibitor, protein synthesis

Abstract

Effects of L-histidine and related compounds on protein synthesis were studied in cultured L6 myotubes. L-Histidine specifically stimulated protein synthesis, whereas D-histidine, histamine, L-arginine and L-lysine did not. Inhibitors of phospholipase A₂, phospholipase C and cyclooxygenase intercepted the stimulatory action of L-histidine on protein synthesis, while inhibitors of protein kinase C and 5-lipoxygenase did not. These results suggest an involvement of phospholipase A₂ and cyclooxygenase in the stimulatory action of L-histidine on protein synthesis in L6 myotubes.

Abbreviations: Arg, arginine; DMEM, Dulbecco's Modified Eagle Medium; FCS, fetal calf serum; His, histidine; HMG, 1-O-hexadecyl-2-O-methylglycerol; Lys, lysine; PKC, protein kinase C; PLA₂, phospholipase A₂; PLC, phospholipase C.

Introduction

The proteins of living cells are constantly renewed by protein turnover. This involves the continuous process of degradation into, and subsequent resynthesis from, free amino acids. Amino acids play important roles in regulating protein turnover. Leucine, one of the branched-chain amino acids, reportedly inhibits protein degradation and stimulates protein synthesis in isolated muscles (Tischler et al., 1982). The stimulatory effect 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., 2001). Likewise, histidine (His), one of the basic amino acids, has been reported to affect protein synthesis in cultured cells (Park et al., 1976; Ali and Evans, 2001). The amino acid stimulates global protein synthesis in L6 myoblasts through regulating the activity of eukaryotic initiation factor eIF2B (Kim-

ball 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 effect of His on protein synthesis in L6 myotubes, comparing it with those of other basic amino acids, arginine (Arg) and lysine (Lys). L-His specifically stimulated protein synthesis, and its stimulatory action was decreased by inhibitors of phospholipase (PL) A₂ and cyclooxygenase. Results obtained suggest an involvement of PLA₂ and cyclooxygenase in the stimulatory action of L-His on protein synthesis in L6 myotubes.

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, and penicillin G from Ban-yu Pharmaceutical Co., Tokyo. L-[ring-3,5-³H]tyrosine (50 Ci mmol⁻¹) from American Radiolabeled Chemicals Inc., St. Louis, MO, U.S.A. Mepacrine, an inhibitor of phospholipases A₂ and C (Premecz et al., 1989; Fernandez et al., 1998), manoalide, an inhibitor of phospholipase A₂ (Lombardo and Dennis, 1985), ibuprofen, an inhibitor of cyclooxygenase (Tompson et al, 1995), acetylsalicylic acid (aspirin), an inhibitor of cyclooxygenase (Gilroy et al., 1998), and indomethacin, an inhibitor of cyclooxygenase (Rodemann and Goldberg, 1982) were purchased from Sigma Chemical Co., St. Louis, MO. Caffeic acid, an inhibitor of 5-lipoxygenase (Koshihara et al., 1983) was from Nacalai Tesque, Inc., Kyoto, and 1-O-hexadecyl-2-O-methylglycerol (HMG), an inhibitor of protein kinase C (PKC) (Kramer et al., 1989), from Funakoshi Co., Ltd., Tokyo. 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 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⁻¹), and penicillin G (100 U ml⁻¹) (10% FCS/DMEM) under an atmosphere of 5% CO₂/95% humidified air at 37 °C, as described previously (Yagasaki et al., 1991). The prefused cells (5 × 10⁴ cells well⁻¹) 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 15 h in 0.2% FCS/DMEM, 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 0.4 µCi well⁻¹ of L-[ring-3,5-³H]tyrosine (50 Ci mmol⁻¹), and cultured for another 1 h. Then, plates were placed on ice and the medium was removed. After two washings with cold PBS(-), cells received 0.5 ml of 5% trichloroacetic acid (TCA) and stood on ice for 10 min. Cells were washed again with 0.5 ml of 10% TCA and 0.5 ml of water, and they were dissolved in 0.5 ml of 0.2 N NaOH solution containing 2 mM sodium dodecylsulfate, 0.1 ml of which being transferred to a mini-counting vial. Nonione-Toluene scintillator (4 ml) and 0.1 ml of 23.3% TCA solution was added to the vial and radioactivity was counted with a liquid scintillation spectrometer (LS5000 TD, Beckman) after mixing as described previously (Yagasaki et al., 1993). 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⁻¹ protein).

Statistical analysis

Data are expressed as means ± SEM. Differences between group means were compared by Duncan's multiple-range test, and *P* < 0.05 was considered statistically significant.

Results

Effects of L-histidine, D-histidine, histamine, L-arginine and L-lysine on protein synthesis

As shown in Figure 1A, L-His significantly increased protein synthesis in L6 myotubes when compared with the control medium (His-free DMEM). The stimulatory effect of L-His on protein synthesis was maximum and significant at a concentration of 50 µM, although a possibility that lower concentrations might be most effective could not be ruled out. Selecting 50 µM, effects of D-His and histamine on protein synthesis were examined, but both compounds failed to increase it (data not shown). Furthermore, the effect of L-His (50 µM) on protein synthesis was estimated by adding the amino acid to either His-free DMEM or His-Arg-Lys-free DMEM (Figure 1B). L-His significantly increased protein synthesis even when it was added to His-Arg-Lys-free DMEM. Activity of

Figure 1. Effects of basic amino acids on protein synthesis in L6 myotubes. Dose-dependent effect of L-His on protein synthesis was evaluated by adding L-His to His-free DMEM (A). Stimulatory effect of L-His on protein synthesis was evaluated by adding L-His to either His-free DMEM (-H) or His-Arg-Lys-free DMEM (-HAL) (B). Values are the means $\pm$ SEM of six assays. Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

protein synthesis was identical between myotubes exposed to both the His-free DMEM and His-Arg-Lys-free DMEM, suggesting that neither L-Arg nor L-Lys could increase protein synthesis. In fact, both L-Arg and L-Lys failed to increase protein synthesis when they are added to His-Arg-Lys-free DMEM at concentrations of 0–400 μM (data not shown). Of three basic amino acids, L-His was therefore demonstrated to specifically increase protein synthesis in L6 myotubes under the present experimental conditions.

Effects of phospholipase inhibitors on L-histidine-induced protein synthesis

To learn mechanisms by which L-His stimulates protein synthesis in L6 myotubes, we selected L-His-free DMEM as control medium and 50 μM as standard L-His concentration, and examined the effects of inhibitors of enzymes affecting cellular signaling. Both 10 μM of mepacrine (an inhibitor of PLA_2 and PLC) and 5 μM of manoalide (an inhibitor of PLA_2) interrupted the stimulatory action of L-His on protein synthesis at the concentrations where inhibitors exerted no influence on basal protein synthesis by themselves (Figure 2).

Effects of protein kinase C and lipoxigenase inhibitors on L-histidine-induced protein synthesis

Figure 3 shows effects of HMG (an inhibitor of PKC) and caffeic acid (an inhibitor of 5-lipoxygenase) on L-His-induced protein synthesis in L6 myotubes. Neither 500 μM of HMG nor 100 μM of caffeic acid diminished the stimulatory action of L-His on protein synthesis, suggesting both PKC and 5-lipoxygenase might be scarcely involved in the L-His action.

Effects of cyclooxygenase inhibitors on L-histidine-induced protein synthesis

As depicted in Figure 4, cyclooxygenase inhibitors ibuprofen (100 μM) and acetylsalicylic acid (1 mM) diminished the stimulated protein synthesis without affecting basal protein synthesis by themselves. Likewise, another cyclooxygenase inhibitor indomethacin (20 μM) also canceled the stimulatory action of L-His on protein synthesis (data not shown).

Discussion

Of basic amino acids and their related compounds, L-His is demonstrated to specifically stimulate protein synthesis in L6 myotubes (Figure 1). This increase in protein synthesis in L6 myotubes was interrupted completely by mepacrine (an inhibitor of PLA_2 and

Figure 2. Effects of mepacrine (MEC, A) and manoalide (MLD, B) on the stimulatory effect of L-His on protein synthesis in L6 myotubes. After cultured for 15 h in 0.2% FCS/DMEM, L6 myotubes were cultured for another 3 h in serum-free experimental media, that is, L-His-free DMEM (None), None + inhibitor (+10 µM MEC or +5 µM MLD), None + L-His (+50 µM His) or None + L-His + inhibitor (+His + MEC or + His + MLD). The incorporation of [³H]tyrosine into cellular proteins for the final 1 h was measured as described in the Section 'Materials and methods'. Values are the means ± SEM of six assays. Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

Figure 3. Effects of 1-O-hexadecyl-2-O-methylglycerol (HMG, A) and caffeic acid (CAF, B) on the stimulatory effect of L-His on protein synthesis in L6 myotubes. After cultured for 15 h in 0.2% FCS/DMEM, L6 myotubes were cultured for another 3 h in serum-free experimental media, that is, L-His-free DMEM (None), None + inhibitor (+500 µM HMG or +100 µM CAF), None + L-His (+50 µM His) or None + L-His + inhibitor (+His + HMG or + His + CAF). The incorporation of [³H]tyrosine into cellular proteins for the final 1 h was measured as described in the Section 'Materials and methods'. Values are the means ± SEM of six assays. Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

PLC) and manoalide (an inhibitor of PLA₂) at concentrations where they had no influence on basal protein synthesis (Figure 2). These results strongly suggested an involvement of PLA₂ in L-His-stimulated protein synthesis in L6 myotubes.

Arachidonic acid and other C₂₀ fatty acids with methylene-interrupted bonds give rise to eicosanoids, physiologically and pharmacologically active compounds known as prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), and lipoxins (LX).

Arachidonic acid is usually derived from the 2-position of phospholipids in the plasma membrane as a result of PLA₂ activity, and is metabolized to PG and TX by the cyclooxygenase pathway or LT and LX by the lipoxygenase pathway (Mayes, 2000). The effects of known inhibitors of these enzymes were examined in the presence and absence of L-His. Caffeic acid (an inhibitor of 5-lipoxygenase), whose ID₅₀ has been reported to be 3.7 µM (Koshihara et al., 1983), did not show any effect at 100 µM on L-

Figure 4. Effects of ibuprofen (IBP, A) and acetylsalicylic acid (ASA, B) on the stimulatory effect of L-His on protein synthesis in L6 myotubes. After cultured for 15 h in 0.2% FCS/DMEM, L6 myotubes were cultured for another 3 h in serum-free experimental media, that is, L-His-free DMEM (None), None + inhibitor (+100 μ M IBP or +1 mM ASA), None + L-His (+50 μ M His) or None + L-His + inhibitor (+His + IBP or + His + ASA). The incorporation of [³H] tyrosine into cellular proteins for the final 1 h was measured as described in the Section 'Materials and methods'. Values are the means $\pm$ SEM of six assays. Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

His-stimulated protein synthesis in L6 myotubes (Figure 3B). In contrast, the stimulatory action of L-His on protein synthesis was significantly canceled by ibuprofen (Figure 4A) and indomethacin (data not shown) that inhibit cyclooxygenases 1 and 2 by competing with arachidonic acid and by acetylsalicylic acid (Figure 4B) that inhibits cyclooxygenases by acetylation (Mayes, 2000). These data are similar to the previous observations in L6 myoblasts (Palmer and Bain, 1989; Southorn and Palmer, 1990; Thompson et al., 1995) and L6 myotubes (Yagasaki et al., 1991) that inhibitors of PLA₂ and cyclooxygenase block the stimulation of protein synthesis by insulin in the muscle cells cultured in serum-free DMEM. Thus, our present results strongly suggest that the stimulatory action of L-His on protein synthesis is mediated by arachidonic acid metabolite(s) produced via cyclooxygenase pathway in L6 myotubes, as seen in insulin action. Since PGF_{2 α} reportedly increased protein synthesis in skeletal muscle (Rodemann and Goldberg, 1982; Reeds and Palmer, 1983) and stimulated both S6 phosphorylation and protein synthesis in Swiss/3T3 cells (Thomas et al., 1982), it is likely that L-His also stimulates protein synthesis in L6 myotubes via PG including PGF_{2 α} . Further studies are required to identify PG concerned.

PLC results in the generation of inositol triphosphate, which liberates stored intracellular Ca²⁺, and diacylglycerol, which activate PKC. The activated PKC phosphorylates specific substrates, which then

alter physiologic processes (Granner, 2000). Mepacrine inhibits PLC (Fernandez et al., 1998) as well as PLA₂ (Premecz et al., 1989). In the present study, mepacrine interrupted the stimulatory action of L-His on protein synthesis in L6 myotubes, suggesting a possible involvement of PLC in L-His action. HMG, an inhibitor of PKC, failed to eliminate the stimulation of protein synthesis by L-His (Figure 3A). This result suggests that PLC plays no or little, if any, role in the stimulation of protein synthesis by L-histidine, although PKC activity is not always equivalent to PLC activity and no evidence directly excluding the possibility of PLC involvement has been obtained yet. Use of specific inhibitor of PLC will clarify this aspect.

In summary, of three basic amino acids and related compounds, L-His was demonstrated to specifically stimulate global protein synthesis in L6 myotubes, and the stimulatory action of L-His was suggested to be mediated by a product(s) of PLA₂-cyclooxygenase pathway. This phenomenon is similar to that observed in the stimulatory action of insulin on protein synthesis in L6 muscle cells in culture. Further studies are needed to clarify precise mechanisms for L-His action.

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