Branched-chain amino acids and arginine suppress MaFbx/atrogin-1 mRNA expression via mTOR pathway in C2C12 cell line

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【 学位論文題目 】

Branched-chain amino acids and arginine suppress MaFbx/atrogin-1 mRNA expression via mTOR pathway in C2C12 cell line(分岐鎖アミノ酸及びアルギニンは C2C12 細胞において mTOR パスウェイを介して MaFbx/atrogin-1 mRNA 発現を抑制する)

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Summary

The effect of amino acids on muscle protein degradation remains unclear. Recent studies have elucidated that proteolysis in catabolic conditions occurs through ubiquitin-proteasome proteolysis pathway and that muscle-specific ubiquitin ligases (atrogin-1 and MuRF1) play an important role in protein degradation. Atrogin-1 is a muscle-specific F-box type E3 ligase that is induced 8 to 40 fold in muscle atrophy during fasting, diabetes, cancer and renal failure, up to 3 fold in hind limb suspension, immobilization and denervation, and until 10 fold in cachectic or dexamethasone administration model. MuRF1 is a Ring Finger type muscle-specific E3 ligase that initially found in association with myofibril and suggested to play an important role in myofibrillar protein breakdown. Both muscle-specific E3 ligases are considered to play a pivotal role in muscle atrophy because knockout mice lacking these E3 ligases are prevented from muscle atrophy (56% sparing for atrogin-1/MaFbx$^{-/-}$ and 36% for MuRF1$^{-/-}$).

Amino acids are reported to suppress muscle protein breakdown. However, the underlying mechanism is known less than that in protein synthesis. Whereas previous studies have reported the effect of various amino acids on C2 and C8 proteasome subunits and proteasome activity, the effect of amino acids on the expression of E3 ligases have not been reported. In the present study, we examined the effect of amino acids on atrogin-1 and MuRF1 mRNA expressions. Furthermore, we studied which cell signaling system is involved in the effect of amino acids that regulate atrogin-1 and MuRF1 mRNA expressions using muscle cell line, C2C12 cells and a series of inhibitors for intracellular signal transductions.

Results

By using 5th days-differentiated C2C12 and 6 hours serum-free conditions as pre-treatment conditions, we found that 5mM of leucine, isoleucine, valine and arginine suppressed atrogin-1 mRNA expression (≈50%) at 6 hours stimulations, as well as MuRF1 expression but at 24 hours only MuRF1 mRNA were still suppressed. The suppressive effect of leucine, isoleucine or arginine was reversed by rapamycin. However, wortmannin did not reverse the inhibitory effects of these amino acids. PD98059 and HA89 did not influence the inhibitory effects, although they reduced basal atrogin-1 expression.

Discussion

In the present study, we revealed that leucine, valine, isoleucine and arginine suppressed atrogin-1 mRNA expression. It is well known that BCAA, especially leucine, stimulates phosphorylation of mTOR, which in turn phosphorylates S6K and 4E-BP1 and finally increases protein synthesis. On the other hand, although it has been reported that BCAAs attenuate protein degradations, the mechanism of attenuating protein degradation remains unclear. Since the physiological significance of atrogin-1 to protect muscle atrophy has been reported in the atrogin-1-knockout mice experiment, a decrease in atrogin-1 mRNA expression by BCAAs and arginine may lead to attenuating protein degradation.

Next, we revealed involvement of mTOR in the decrease in atrogin-1 mRNA by BCAAs and arginine. In addition, we found no involvement of PI3K in the inhibitory effect of amino acids on atrogin-1 mRNA levels. It is already reported that IGF-I attenuates the increased expression of atrogin-1 mRNA in denervation, hind limb suspension, fasting, diabetic condition, and sepsis or tumor-bearing model. The underlying mechanism to decrease atrogin-1 mRNA has been reported through the phosphorylation of PI3K. PI3K, in turn, stimulates Akt phosphorylation that increases the phosphorylation of Foxo3a. After the phosphorylation, Foxo3a translocates to cytosol from nucleus and as a result, atrogin-1 transcription activated by Foxo3a is decreased. However, there are some reports indicating the involvement of mTOR, which is located downstream of Akt, in the action of IGF-I. Whichever pathway is used in IGF-I signaling pathway, IGF-I activates PI3K and then stimulates divergent pathways. Our results showed that suppressive effects of BCAA and arginine were mediated through mTOR but not via PI3K. However, we cannot exclude the possibility that the amino acid signal may converge with IGF-1 signal at downstream of PI3K. Frost et al. showed that sepsis-induced increase in atrogin-1 mRNA was suppressed by IGF-I but not leucine in vivo study, which is consistent with our results in that different pathways are likely present. It is already known that BCAAs and arginine stimulate protein synthesis in muscles. The effect of BCAAs, at least in part, is via mTOR, although the underlying mechanism of arginine is still not clear. Interestingly, BCAAs and arginine showed a similar pattern in blocking experiments with various inhibitors although they have different structures, and

mTOR pathway was involved in the regulation of atrogin-1 expression by those amino acids. The regulatory pathway for atrogin-1 expression by BCAA and arginine may be related to the pathway that is involved in protein synthesis by the amino acids.

Inhibition of MEK and PKA pathways reduced atrogin-1 mRNA levels and no further reduction was observed after amino acids stimulations. This result may be explained as follows; atrogin-1 expression through MEK or PKA pathway was activated in basal condition and inhibition of these pathways might suppress atrogin-1 expression to the lowest expression level, therefore no further reduction by amino acids was observed. Alternatively, MEK and PKA pathways may be not involved in the effect of amino acid. Involvement of MAPK system in muscle atrophy and atrogin-1 expression has been reported, in particular, when NFκb is activated by inflammation or oxidation stress. Although p38 MAPK is a most likely kinase regulating muscle atrophy among MAPKs, ERK also appears to be involved in it, being consistent with our result. On the other hand, there are no reports concerning the effect of PKA on atrogin-1 expression.

In addition to atrogin-1 mRNA, BCAAs and arginine reduced MuRF1 mRNA expression. However, a regulatory mechanism to reduce expressions appeared to be different, since rapamycin did not reverse the suppressive effect of amino acids. Atrogin-1 and MuRF1 are structurally different and have been reported to play a different role. Furthermore, there are some reports that indicate the different regulatory mechanism for atrogin-1 and MuRF1 expressions. IGF-I rapidly reduced atrogin-1 expression within 1 h by blocking mRNA synthesis, whereas IGF-I decreased MuRF1 mRNA slowly. Sepsis-induced increase in atrogin-1 expression was completely prevented by IGF-I, while the increased MuRF1 was not altered. Combined administration of TNF-α synthesis inhibitor and a β2-adrenergic agonist reduced increased expression of MuRF1 mRNA in cancer cachexia rat without modifying atrogin-1 mRNA levels.

In conclusion, we revealed that BCAAs and arginine decreased atrogin-1 mRNA levels in C2C12 cells and the involvement of mTOR-dependent pathway in the inhibitory effect of the amino acids. Although BCAAs and arginine also decreased MuRF1 mRNA levels, rapamycin did not reverse the inhibitory effect, suggesting different regulatory mechanism and different functions of two muscle-specific ubiquitin ligases in muscle atrophy.

論文審査の結果の要旨			
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（要旨は１，０００字～２，０００字程度）

Muscle atrophy, which is characterized by a decrease in protein synthesis and/or an increase in protein degradation, is a debilitating response found in a variety of catabolic conditions such as starvation, diabetes, and sepsis. Recent studies have shown that under such catabolic conditions, proteolysis occurs through the ubiquitin (Ub)-proteasome proteolysis pathway. The Ub-proteasome proteolysis pathway is a specific pathway for selective degradation of intracellular proteins. The degradation process of this pathway consists of two major steps; ubiquitination of protein and degradation of the ubiquitinated protein by 26S proteasome. In the first step, E1 Ub-activating enzyme, E2 Ub-carrier protein and E3 Ub-protein ligase mediate ubiquitination of the target proteins. Atrogin-1, a muscle-specific F-box type E3 ligase, is induced 8 to 40 fold in muscle atrophy found in fasting, diabetes, cancer and renal failure, 3 fold in hind limb suspension, immobilization, and denervation, and 10 fold in cachectic or dexamethasone administration model. On the other hand, MuRF1, a Ring Finger type muscle-specific E3 ligase, was initially found in association with myofibril and is suggested to play an important role in myofibrillar protein breakdown. Studies of knockout mice lacking these E3 ligases suggest that both Atrogin-1 and MuRF1 play a pivotal role in muscle atrophy.

Although amino acids have been reported to suppress breakdown of muscle proteins, the underlying mechanism is largely unknown.

The aim of this study is to elucidate the effect of amino acids on expressions of atrogin-1 and MuRF1 in muscles and the signaling pathways involved in these effects, using C2C12 cell line.

To examine the effect of amino acids on atrogin-1 and MuRF1 mRNA expressions, an in vitro model of differentiated C2C12 myotubes was used. A preliminary study of basal expression of atrogin-1 showed that 6h serum-free DMEM induced atrogin-1 mRNA expression to a level similar to that of 3 h PBS incubation. Dose-response experiment showed that leucine, at concentrations up to 5mM, suppressed both atrogin-1 and MuRF1. Leucine at 5mM was able to induce phosphorylation of mTOR signaling molecules such as mTOR and 4EBP1. Leucine, isoleucine, valine, or arginine suppressed atrogin-1 mRNA expression (≈50%) as well as MuRF1 expression.

To clarify the signaling pathways involved in the suppressive effect of amino acids, the effects of inhibitors for mTOR, PI3K, MAPK kinase, and PKA were examined. The suppressive effect of leucine, isoleucine or arginine was reversed by rapamycin. However, wortmannin did not reverse the inhibitory effects of these amino acids. These results suggest that the suppressive effect of branched-chain amino acids (BCAA) and arginine is mediated by mTOR but not PI3K. PD98059 and HA89 did not affect the inhibitory effects of amino acids, although they reduced the basal atrogin-1 mRNA expression. Inhibition of MEK and PKA pathways reduced atrogin-1 mRNA levels and no further reduction was found after amino acid stimulations. It has been shown that the MAPK system is involved in muscle atrophy and atrogin-1 expression, in particular, when NFkb is activated by inflammation or oxidation stress. Although p38 MAPK is a most likely kinase regulating muscle atrophy among MAPKs, ERK is also involved.

In summary, the present study shows that BCAAs and arginine decreased atrogin-1 mRNA levels in C2C12 cells and that the mTOR-dependent pathway is involved in the inhibitory effect of the amino acids. In addition, the present study also shows that although BCAAs and arginine decreased MuRF1 mRNA levels, rapamycin did not reverse the inhibitory effect. These results suggest distinct functions of two muscle-specific ubiquitin ligases in muscle atrophy.

The candidate, having completed studies on "Branched-chain amino acids and arginine suppress MaFbx/atrogin-1 mRNA expression via mTOR pathway in C2C12 cell line", with a specialty in muscle metabolism, and having advanced the field of knowledge in the area of the relationship between protein metabolism and muscle atrophy, is hereby recognized as having qualified for the degree of Ph.D.(Medicine).