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Diversity of amino acid signaling pathways on autophagy regulation: A novel pathway for arginine

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ABSTRACT

Autophagy is the intracellular bulk degradation process to eliminate damaged cellular machinery and to recycle building blocks, and is crucial for cell survival and cell death. Amino acids modulate autophagy in response to nutrient starvation and oxidative stress. We investigated the relevance of reactive oxygen species (ROS) production on the regulation of autophagy using amino acids, both as a mixture and individually, in rat hepatoma H4-II-E cells. Nutrient starvation elevated ROS production and stimulated autophagy. Treatment with complete (CAA), regulatory (RegAA) and non-regulatory (NonRegAA) amino acid mixtures showed significant suppression of ROS production, whereas only CAA and RegAA exhibited significant suppression of autophagy, suggesting a dissociation of the two responses. The effects of individual amino acids were examined. Leucine from RegAA decreased ROS production and suppressed autophagy. However, methionine and proline from RegAA and arginine, cystine and glutamic acid from NonRegAA suppressed autophagy with an opposite increase in ROS production. Other amino acids from the NonRegAA group showed stimulating effects on ROS production without an autophagic response. Arginine's effect on autophagy suppression was not blocked by rapamycin, indicating an mTOR-independent pathway. Inhibitor studies on arginine-regulated autophagy may indicate the involvement of NO pathway, which is independent from ROS and mTOR pathways.

1. Introduction

Autophagy is the intracellular bulk degradation process to eliminate damaged cellular machinery and to recycle building blocks, and is crucial for cell survival and cell death [1]. It is controlled by major cellular stressors, such as nutrient and energy deprivation, endoplasmic reticulum stress, mitochondrial damage, and hypoxia. A rapid and efficient response of autophagy to disturbances in nutrient levels is crucial for cell survival. Therefore, the control of autophagy by nutrient intake and dietary factors is a topic of interest in aging and cancer treatment [2,3].

Amino acids are the most well-known nutrient regulators of autophagy [4], but the amino acids which are directly effective vary depending on cell type and physiological conditions. How amino acids are sensed by cells and how their signal is transduced in cells for autophagic control remains poorly understood. The first question was raised two decades ago and the possibility of an amino acid sensing system at the plasma membrane of hepatocytes was suggested [5,6]; this, however, is still under debate. An answer to the second question was proposed by Meijer's pioneer work [7]; a correlation between amino acid inhibition of autophagy and phosphorylation of S6K1 which leads to the involvement of the mTOR signaling pathway. Since then mechanisms for intracellular signaling of amino acids have been proposed, either being mTOR-dependent [8,9] or -independent pathways [10,11], possibly depending on cell type and amino acid type. Recently, Sabatini's group proposed a novel amino acid signaling pathway in which amino acids control the mTORC1 complex through Rag GTPases,

Abbreviations: AG, aminoguanidine; CAA, complete amino acids; DMEM, Dulbecco's Modified Eagle's Medium; EBSS, Earle's Balanced Salt Solution; LC3, microtubule-associated protein 1 light chain 3; LC3-IIs, cytosolic (soluble) form of LC3-II; L-NMMA, NG-monomethyl-L-arginine acetate salt; mTOR, mammalian target of rapamycin; NonRegAA, non-regulatory amino acids; RegAA, regulatory amino acids; SNAP, S-nitroso-N-acetyl-DL-penicillamine.

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and change subcellular distribution of mTORC1 from the cytosol to lysosomes in combination with the Ragulator complex [9,12,13].

Recently, there is growing evidence that oxidative stress can cause induction of autophagy [14,15]. Oxidative stress occurs in cells when an imbalance favoring production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), over antioxidant defenses exists. It is now well-recognized that, although H_2O_2 leads to cell damage at high doses, at low doses it has important roles as a signaling molecule in many biological processes [16]. Scherz-Shouval et al. reported that H_2O_2 is essential for autophagy in starvation through blocking the activity of Atg4 to keep LC3 lipidation [17]; while Chen et al. indicated superoxide as a major ROS for controlling autophagy [18]. There are also reports on relationships between ROS and mTOR signaling [19,20]. Therefore, in the present study, the mechanisms for autophagy control by amino acids were examined in relation to ROS production, since amino acids are the most effective nutrients for suppressing starvation-induced autophagy. In addition, there are reports that suggest that starvation also regulates autophagy by activating the c-Jun N-terminal kinase (JNK1)/Bcl-2/Beclin 1 pathway, which is independent from the mTOR pathway [21,22]. Nitric oxide (NO), a reactive nitrogen species, is one of the most widespread but ubiquitous signaling molecules that participates in many cellular functions, and is synthesized from L-arginine by a family of NO synthases (NOS). Recently, Sarker et al. [23] showed that *S*-nitrosylation of JNK1 and IKKβ by NO affected autophagy, suggesting another signaling pathway for amino acids.

Most studies on amino acid signaling and autophagy have used mixtures of amino acids, and the mechanisms whereby individual amino acids regulate autophagy remain obscure. We compared the effects of amino acids on intracellular ROS levels and autophagy to elucidate the underlying mechanisms of individual amino acids using the cytosolic LC3 ratio method [24], as a sensitive autophagy index, in rat hepatoma H4-II-E and human liver carcinoma Hep G2 cell lines. We aimed to clarify: (1) whether amino acids as a mixture control autophagy through ROS production or not; (2) when examined individually, which amino acids control autophagy through ROS; and (3) if there are amino acids that control autophagy through another mechanism. The results suggest that arginine, a new regulatory amino acid, is not mTOR-dependent but NO-dependent. This is a novel pathway for autophagy control.

2. Materials and methods

2.1. Reagents

All individual amino acids and aminoguanidine were obtained from Wako Pure Chemical Industries (Osaka, Japan). N^G-monomethyl-L-arginine acetate salt (L-NMMA), S-nitroso-N-acetyl-DL-penicillamine (SNAP), and rapamycin were purchased from Sigma Aldrich (St. Louis, MO, USA). Polyclonal rabbit anti-LC3 antibody was from ThermoFisher Scientific (Waltham, MA, USA) and peroxidase-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Trypsin–EDTA was from Invitrogen (Grand Island, NY, USA). The ECL Western blotting detection kit was from GE Healthcare Life Sciences (Pittsburgh, PA, USA). 2′-7′-dichlorofluorescein diacetate (DCFDA) was from Molecular Probes (Grand Island, NY, USA) and dissolved at 10 mg/188 μl in DMSO as a stock solution and stored at −20 °C. It was diluted with medium to the appropriate concentration before use.

2.2. Cell cultures

Rat hepatoma H4-II-E cells and human liver carcinoma Hep G2 cells were obtained from Riken Cell Bank (Tsukuba, Japan). Cells were grown in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM; Sigma–Aldrich) supplemented with 10% (v/v) fetal bovine serum (Gibco-Life Technologies), 1% antibiotic–antimycotic (Invitrogen) and 2.5 mM L-glutamine. Both cell lines were maintained in humidified conditions with 5% CO_2 at 37 °C. Prior to experiments, confluent cells were washed twice with phosphate buffered saline (PBS) and maintained in a nutrient-rich condition (DMEM). Autophagy was induced by replacing the medium with Earle's Balanced Salt Solution (EBSS; Sigma–Aldrich), and suppressed by treatment with complete amino acids (CAA), regulatory amino acids (RegAA; Ala, Gln, His, Leu, Met, Pro, Trp, Tyr), and non-regulatory amino acids (NonRegAA; Arg, Asn, Asp, Cys, Glu, Gly, Ile, Lys, Phe, Ser, Thr, Val), of which grouping was defined in the perfused liver study [25]. Amino acids were added as multiples of normal plasma concentrations. The normal concentration (1-fold) of each amino acid was (μM): L-His, 92; L-Leu, 204; L-Met, 60; L-Pro, 437; L-Trp, 93; L-Ala, 475; L-Gln, 716; L-Tyr, 98; L-Arg, 220; L-Asn, 101; L-Asp, 53; L-Cys(tine), 34; L-Glu, 158; L-Gly, 370; L-Ile, 114; L-Lys, 408; L-Phe, 96; L-Ser, 657; L-Thr, 329; L-Val, 250. A 4-fold concentration was regarded as the physiologically maximum level.

2.3. Western blotting

The cytosolic LC3 ratio was employed as it is an appropriate, sensitive autophagy index [24]. Briefly, cell samples were resuspended in a buffer of 0.25 M sucrose-1 mM EDTA (pH 7.4) and homogenized by 120 strokes using a tightly fitting Dounce homogenizer on ice. Homogenates were centrifuged at 100,000*g* for 1 h at 4 °C. LC3 in the soluble cytosolic fraction (supernatant) was separated and transferred onto PVDF membranes. The membrane was blocked with 8.1% (w/v) skim milk for 1 h. To detect LC3, the membrane was incubated with polyclonal rabbit anti-LC3 antibody for 1 h followed by incubation with peroxidase-conjugated goat anti-rabbit IgG diluted in PBS at 4 °C overnight. The LC3 bands were detected using a Western blot detection kit and quantified by densitometric analysis (Scion Image 1.63.1, NIH image), and calculated as the ratio of LC3-IIs/LC3-I. The accuracy of quantitativeness was certified without using loading control [24].

2.4. ROS measurements

ROS was measured according to the method of Cathcart et al. [26] as modified by Takanashi et al. [27]. Briefly, cells were seeded into 6- or 24-well plates and allowed to adhere overnight before being treated with 30 μM DCFDA for 15 min and maintained at 37 °C in an incubator. Cells were washed with PBS followed by starvation or amino acid treatment. Cells' DCF signals were measured using a SpectraMax Gemini spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) set to 485 nm excitation and 535 nm emission and kept at 37 °C for 90 min. The results from starved cell experiments were set at 100% and other treatments were normalized accordingly. Results presented for all fluorometric measurements are mean ± SEM of at least three experiments in duplicate.

2.5. Inhibitor study for cytosolic LC3 ratio

H4-II-E and HepG2 cells were maintained with DMEM, and autophagy was induced by replacing the medium with EBSS, and suppressed by adding CAA or arginine to EBSS. Individual inhibitors, aminoguanidine (1 mM), L-NMMA (100 μM), rapamycin (100 nM), and a NO donor, SNAP (100 and 500 μM) were added to the medium as indicated in the Figures.