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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.

2.6. Statistical analysis

All data are expressed as mean ± SEM. Student's *t*-tests were used to evaluate statistical significance. *P*-values <0.05 were considered statistically significant.

3. Results and discussion

We compared the effects of amino acids on intracellular ROS generation and autophagy regulation in H4-II-E cells. ROS have been identified as signaling molecules in various pathways regulating cell survival and cell death [16]. Several studies have shown that ROS mediates starvation-induced autophagy [17,18].

3.1. Effects of starvation and amino acid treatment on ROS production and autophagy

We first examined the formation of ROS under starvation conditions using DCFDA as a fluorescent probe. This probe diffuses into the cell and reacts mainly with peroxides to form the highly fluorescent 2′,7′-dichlorofluorescein (DCF) [26]. Since H_2O_2 is the major peroxide in cells, it is generally accepted that the DCF level is proportional to the ROS level [27]. Measurements were conducted in H4-II-E cells under complete starvation and nutrient-rich conditions. As early as 15 min into starvation induction, increases in the DCF signal were evident (Fig. 1A). Over 90 min, the DCF signal sharply increased compared with nutrient-rich DMEM medium, clearly indicating that the stress induced by nutrient starvation caused the release of ROS generation from mitochondria. The presence of the oxidative signal suggested that ROS, mostly as H_2O_2, served as a good signaling molecule. The absence of abnormal increases or decreases in fluorescent signals indicated the stability of the reaction throughout the indicated time period. This method was therefore applied to amino acid mixtures.

Amino acids are end products of autophagic proteolysis and are well-known to be prime nutrient regulators of autophagy [4]. Specifically, RegAA were defined to be direct suppressors of autophagic proteolysis in the perfused liver [25]. As shown in Fig. 1B, the change in the cytosolic LC3 ratio, a marker of autophagy flux, ob-

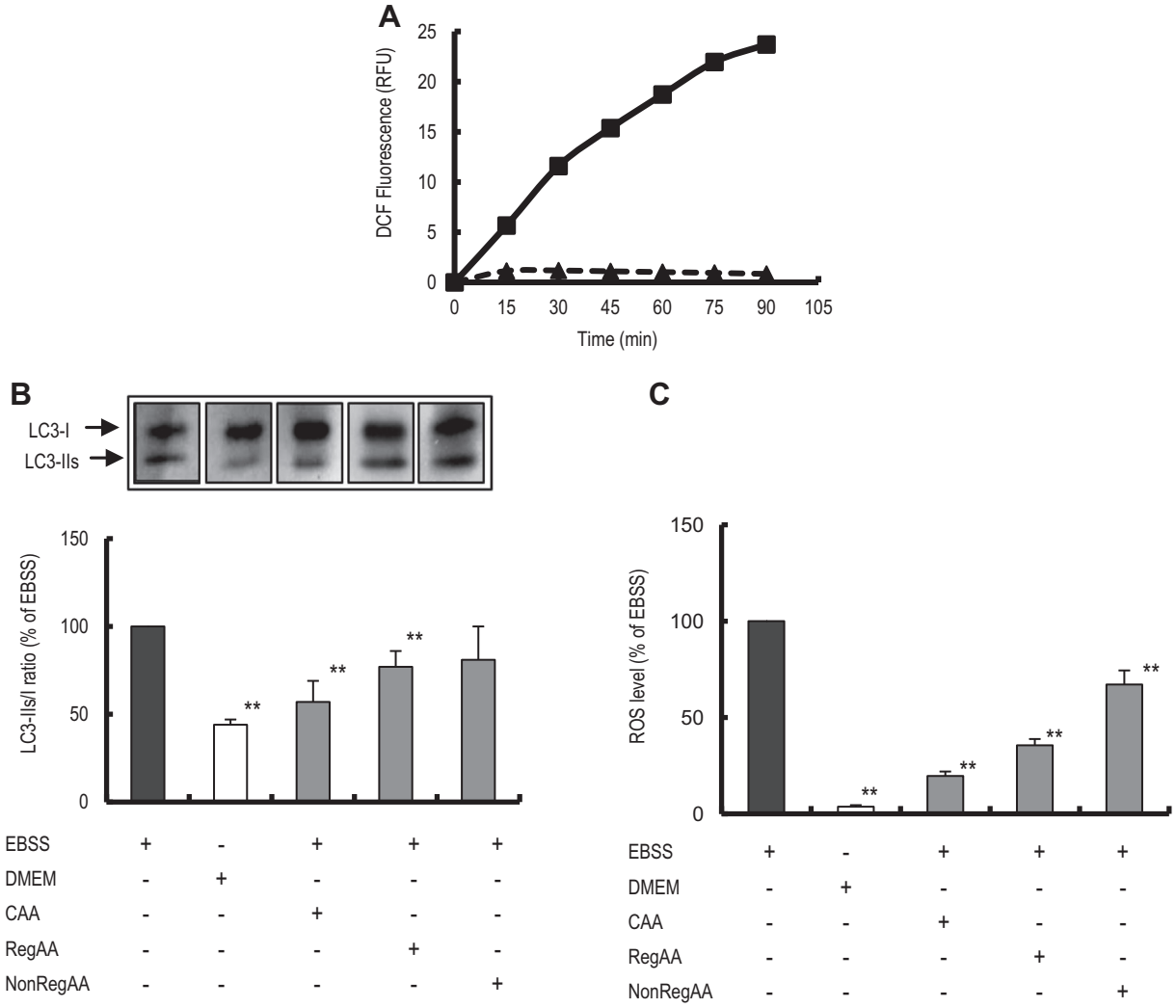

Fig. 1. Effects of starvation and amino acid mixtures on ROS production and autophagy. (A) H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates, and treated with 30 μM DCFDA at 37 °C for 15 min. Medium was changed to EBSS (completely starved; square and solid line) or DMEM (nutrient-rich; triangle and broken line). Cells were monitored by DCF fluorescence with a fluorometer for 90 min. (B) Cells were incubated in DMEM or starved in EBSS. Starved cells were treated with 4-fold CAA, 4-fold RegAA, and 4-fold NonRegAA mixtures for 1 h, then homogenized. LC3 bands in the 100,000*g* supernatant (cytosol) fraction were analyzed and visualized by SDS–PAGE and Western blotting. The cytosolic LC3 ratio (LC3-IIs/-I) was calculated by densitometry. (C) Cells were grown in control medium (DMEM), seeded in 6-well plates, treated with 30 μM DCFDA at 37 °C for 15 min, washed, and starved by changing to EBSS and treated with 4-fold CAA, 4-fold RegAA, and 4-fold NonRegAA mixtures. Cells were subsequently analyzed with a fluorometer for 1 h. Values are mean ± SEM ($n = 3$). $^{**}P < 0.01$ *vs.* EBSS.

Table 1
Effects of individual amino acids on ROS production and autophagy in H4-II-E cells.

Treatment	ROS level (% of EBSS)	LC3-IIs/-I (% of EBSS)
EBSS (control)	100	100
RegAA		
Alanine	58.5 ± 22.8*	80.0 ± 23.4
Glutamine	47.6 ± 11.8*	76.7 ± 21.1
Histidine	52.2 ± 10.4*	97.8 ± 15.5
Leucine	45.4 ± 9.5*	66.7 ± 7.1*
Methionine	127.9 ± 24.3*	60.0 ± 10.4*
Proline	162.8 ± 35.8*	62.2 ± 13.2*
Tryptophan	53.9 ± 2.1*	82.2 ± 23.2
Tyrosine	66.5 ± 8.6*	69.9 ± 25.6
NonRegAA		
Arginine	120.1 ± 0.3*	50.0 ± 6.2**
Asparagine	128.4 ± 19.6	84.4 ± 26.6
Aspartic acid	163.9 ± 21.2*	81.1 ± 19.2
Cystine	157.5 ± 26.7*	75.5 ± 6.3*
Glutamic acid	138.5 ± 10.0*	71.1 ± 3.6*
Glycine	117.4 ± 11.4	90.0 ± 11.1
Isoleucine	101.8 ± 18.3	60.0 ± 7.2*
Lysine	181.1 ± 11.8*	90.0 ± 11.2
Phenylalanine	99.7 ± 10.1	83.3 ± 10.6
Serine	115.1 ± 12.2	86.7 ± 6.2
Threonine	128.4 ± 20.2	88.9 ± 16.1
Valine	184.5 ± 11.7*	73.3 ± 11.4

H4-II-E cells were incubated in DMEM and then starved in EBSS. Starved cells were treated with individual amino acids from RegAA and NonRegAA. ROS production and LC3-I and LC3-IIs proteins as an autophagy index were analyzed by fluorometry and Western blotting, respectively, as described under "Section 2". Data are mean ± SEM (n = 3–6).
*P < 0.05, **P < 0.01 *vs.* EBSS.

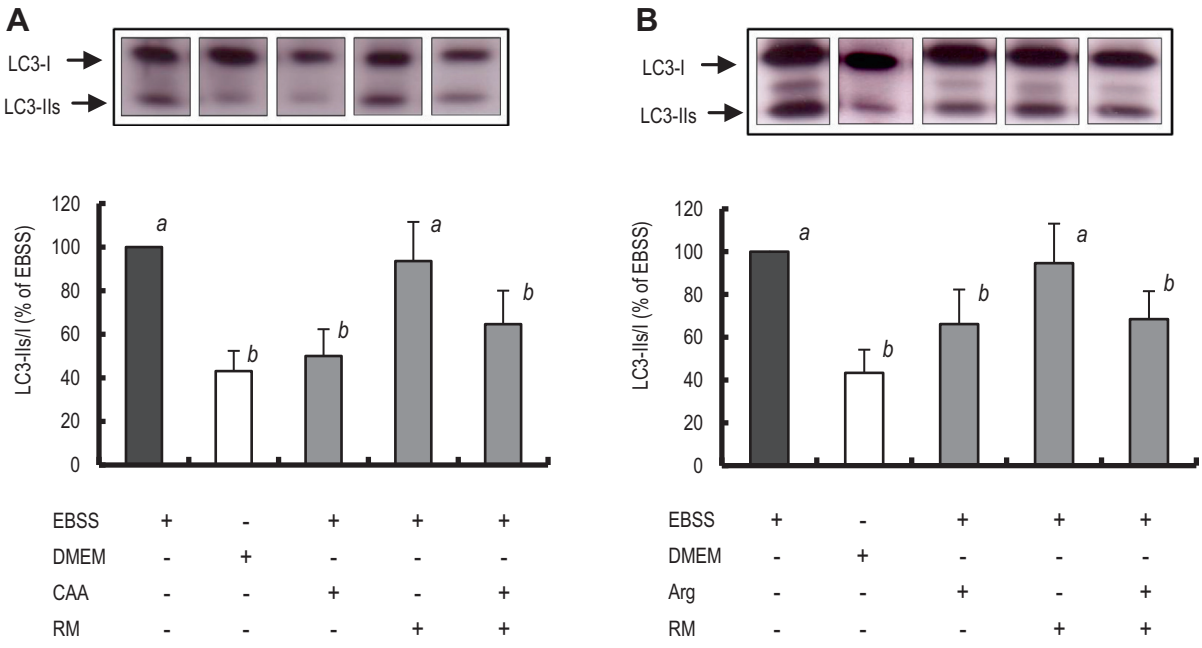

Fig. 2. Effects of CAA, Arg and rapamycin on autophagy. H4-II-E cells were incubated in DMEM or starved in EBSS (control). Starved cells were treated with (A) 4-fold CAA, 100 nM rapamycin, and 4-fold CAA with rapamycin or (B) 4-fold Arg, 100 nM rapamycin, and 4-fold Arg with rapamycin for 1 h and homogenized. LC3 bands in the cytosol fraction were analyzed and visualized by SDS–PAGE and Western blotting. The cytosolic LC3 ratio was calculated by densitometry. Data are mean ± SEM (n = 5–9); $^{a-b}$values without common letters were significantly different at P < 0.05.

served after changing the media from DMEM to EBSS, indicated sensitivity of H4-II-E cells to nutrient change. In starvation conditions, maximum activation of autophagy was observed. Furthermore, the effectiveness of amino acids as an autophagy regulator was confirmed. Treatment with 4-fold CAA and RegAA mixtures significantly suppressed autophagy by 43% and 23%, respectively, in H4-II-E cells, although these values were equal in the perfused liver [25], which may respond differently depending on cell specificity. A NonRegAA mixture showed a non-significant suppression, but similar to RegAA.

We next examined the response of ROS production to the amino acid mixtures. Treatment resulted in decreases in ROS production (Fig. 1C). Complete suppression of ROS production was obtained in the DMEM medium, and as much as 80% and 64% decreases were observed with CAA and RegAA, respectively. The results supported the hypothesis that autophagy regulation by these mixtures is

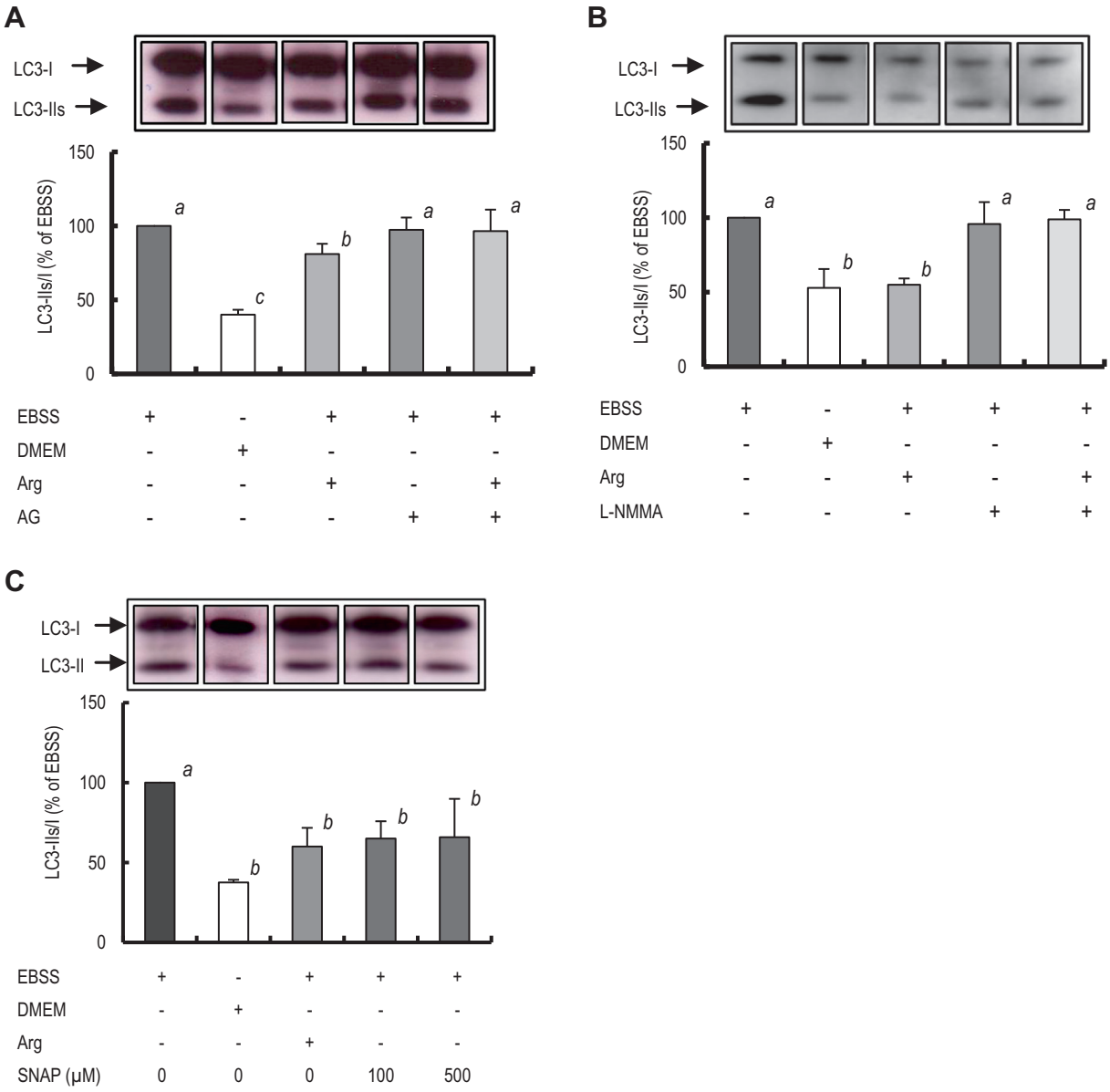

Fig. 3. Effects of NOS inhibitors and an NO donor on Arg-regulated autophagy. H4-II-E and Hep G2 cells were incubated in DMEM or starved in EBSS (control). (A) Starved H4-II-E cells treated with 4-fold Arg, 1 mM aminoguanidine (AG), and Arg with AG; and (B) Starved Hep G2 cells with 4-fold Arg, 100 μM L-NMMA, and Arg with L-NMMA. (C) Starved H4-II-E cells treated with 4-fold Arg and SNAP, an NO donor (100 and 500 μM) for 1 h and homogenized. LC3 bands were analyzed and visualized by SDS–PAGE and Western blotting. The cytosolic LC3 ratio was calculated by densitometry. Data are mean ± SEM (n = 4–5); $^{a-c}$values without common letters were significantly different at $P < 0.05$.

mediated by suppression of ROS production. Indeed, autophagy that increased with H_2O_2 was suppressed by α-lipoic acid, a typical antioxidant in a previous study [28]. Even NonRegAA exhibited a less but significant suppression of ROS production, which exhibited antioxidant properties Therefore, we deduced that ROS suppressed by CAA and RegAA plays as a signaling role in autophagy regulation. The results of NonRegAA treatment, however, suggest that the antioxidant properties of this group may not be tightly linked to autophagy regulation.

3.2. Effects of individual amino acids on ROS production and autophagy

The unexpected result, that NonRegAA suppressed ROS, led us to consider whether some amino acids with antioxidant activity cannot control autophagy. Therefore, we extended our study to examine the effects of individual amino acids comprehensively using the same method. We expected that each amino acid mimicked responses of autophagy and ROS generation in their mixture groups. Physiologically maximum levels of amino acids (4-times their normal plasma levels) were employed [25]. Most individual RegAA caused a considerable decrease in ROS production, except for Met and Pro (Table 1). From these results, it was concluded that ROS suppression exhibited with the mixture of RegAA reflected an accumulating effect of individual RegAA. However, amino acids Met and Pro showed an opposite increase of ROS; 28% and 63%, respectively. Furthermore, Leu, Met, and Pro exhibited a significant suppression of autophagy individually, as demonstrated by decreases in their LC3 ratios. Other amino acids in RegAA also tended to suppress individually, but not significantly. His had no effect at all. The slight difference in the RegAA group in H4-II-E cells may be because of the difference of cell type; rat hepatocytes.

Individual NonRegAA exhibited mostly an increase or no change in ROS production. This was surprising, as we had observed a sig-

nificant decrease in ROS production in the mixture form (Fig. 1C). Arg gave a 20% increase in ROS production and 50% autophagy suppression. Cys and Glu exhibited 58% and 39% increases in ROS production and 25% and 29% autophagy suppression, respectively. Ile showed 40% suppression of autophagy without any change in ROS production. The lack of correlation between ROS production and autophagy suppression was evident. From the above experiments, these six amino acids, Met, Pro, Arg, Cys, Glu, and Ile, can be defined as regulatory amino acids in H4-II-E cells through signaling pathways other than ROS.

3.3. Lack of involvement of mTOR in the autophagy regulatory pathway by amino acids

Signaling pathways for autophagy regulation by amino acids are still not completely understood. Are they uniform or diverse? The mTOR pathway together with Rag GTPases upstream and the Ragulator complex and ULK1/2 downstream is the most popular idea [9,12,13]. Interaction between Bcl-2 and Beclin 1 with JNK1 is also a possible pathway [21,22]. In our study, we observed that treatment with arginine, which had been considered a NonRegAA in the perfused liver, resulted in inhibition of LC3 modification, implying suppression of autophagy. Therefore, we examined the possibility of a mTOR contribution using the mTOR inhibitor rapamycin.

A 50% suppression of autophagy was observed upon treatment with CAA (Fig. 2A). Addition of rapamycin (100 nM) alone, at a dose effective to block mTOR activity [11], showed no effect on autophagy. In addition, a significant inhibitory effect, compared with EBSS, was obtained from 4-fold CAA combined with rapamycin. The slight difference in LC3 modification between CAA and CAA combined with rapamycin may suggest a partial involvement of the mTOR pathway in autophagy regulation by CAA. However, under starvation conditions with EBSS, the LC3 ratio was inhibited by 35% and 33% with 4-fold Arg and 4-fold Arg combined with rapamycin, respectively (Fig. 2B). The absence of a significant difference in autophagy suppression between both conditions suggested independence of Arg-regulated autophagy from the mTOR pathway. Therefore, we searched for possible involvement of other signaling pathways that regulate autophagy. It has been reported that using NO-releasing chemicals, NO showed inhibition of autophagosome formation through S-nitrosylation inhibition of JNK1 and IKKβ, which in turn resulted in the disruption of the class III PI3K/hVps34/Beclin1 complex and mTORC1 activation, respectively [23].

3.4. Involvement of NO on arginine-regulated autophagy

On the basis of the results presented in Table 1, we postulated that the signaling mechanisms of individual amino acids are diverse. Because of significant autophagy suppression but an increase in ROS generation observed with arginine, we searched for the possible signaling pathway of arginine-regulated autophagy. The presence of NO and its metabolites are important in arginine-related physiological phenomenon. In the current study, we considered the possibility that NO acts as a signaling molecule in the Arg-regulated autophagy mechanism. To test this hypothesis an inhibitor study was performed. We used aminoguanidine and L-NMMA, as both are known as NOS inhibitors, to examine the effects of NO generation by NOS. Aminoguanidine is a partial arginine analog that shows irreversible inhibition in the substrate and mRNA expression levels of NOS in rat hepatocytes [29]. A significant inhibition of LC3 modification was displayed by 4-fold Arg administration, implying suppression of autophagy (Fig. 3A). The addition of 1 mM aminoguanidine had no effect on autophagy. A combination of Arg and aminoguanidine resulted in the disappearance of the suppressive effect of Arg on autophagy. This result suggests the possibility of Arg-derived NO modulating autophagy in H4-II-E cells.

To further elucidate the possible involvement of NO in Arg-regulated autophagy, another cell line, HepG2, was used. The pattern of LC3 was somewhat different, but the ratio responded to DMEM similarly and Arg suppressed the ratio more sensitively (Fig. 3B). L-NMMA inhibits NOS activity by competitive inhibition [30]. L-NMMA showed no effect on autophagy. The suppressive effect of Arg on autophagy disappeared with the addition of L-NMMA. We then further investigated the existence of an NO pathway in autophagy regulation in H4-II-E cells by introducing SNAP, an NO donor [31], to our system, and expected it to show a direct effect on autophagy. Treatment with different doses of SNAP resulted in inhibition of LC3 modification in parallel with the Arg effect (Fig. 3C). Taken together, these inhibitor studies present evidence that autophagy regulation by Arg may be NO pathway dependent.

In conclusion, the results show that individual amino acids have diverse stimulatory and inhibitory effects on ROS production, and suggest the possibility that individual amino acids may have their own signaling pathways to control autophagy, unique from an amino acid mixture. We propose that arginine-regulated autophagy may be NO pathway dependent. These findings provide novel insights on autophagy signaling mechanisms by amino acids.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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