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Effects of orally administered glycine on myofibrillar proteolysis and expression of proteolytic-related genes of skeletal muscle in chicks

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Summary. We examined the effects of orally administered glycine on myofibrillar proteolysis in food-deprived chicks. Food-deprived (24 h) chicks were orally administered 57, 113, and 225 mg glycine/100 g body weight and killed after 2 h. The plasma N^{τ}-methylhistidine concentration, used as myofibrillar proteolysis, was decreased by glycine. We also examined the expression of proteolytic-related genes by real-time PCR of cDNA from chick skeletal muscles. The mRNA expression of atrogin-1/MAFbx, proteasome C2 subunit, m-calpain large subunit, and cathepsin B was decreased by glycine in a dose-dependent manner. The plasma corticosterone concentration was also decreased by glycine, but the plasma insulin concentration was unaffected. These results indicate that orally administered glycine suppresses myofibrillar proteolysis and expression of proteolytic-related genes of skeletal muscle by decreasing the plasma corticosterone concentration in chicks.

Keywords: Glycine – Myofibrillar proteolysis – Atrogin-1/MAFbx – Corticosterone – Skeletal muscle – Chick

Introduction

Amino acids are potent modulators of protein synthesis and degradation in skeletal muscle. Muscle protein synthesis is increased by amino acids that are infused (Louard et al., 1990, 1995; Zanetti et al., 1999) or orally administered (Anthony et al., 1999, 2000), whereas degradation is decreased by infused (Louard et al., 1990, 1995) or dietary amino acids (Goodman and del Pilar Gomez, 1987). Oral leucine administration suppresses myofibrillar proteolysis in rats (Nagasawa et al., 2002). We previously reported that oral administration of leucine or isoleucine (Nakashima et al., 2005a), or glutaminate, glycine, alanine, serine, threonine, methionine, tryptophan, asparagine, glutamine, proline, lysine or arginine (Nakashima et al., 2006) also suppressed myofibrillar proteolysis in chicks. In particular, glycine strongly suppressed myofibrillar proteolysis. Despite these findings, the early response mechanism of muscle protein degradation to orally administered amino acids is largely unknown.

N^{τ}-methylhistidine is an amino acid formed by the post-translational methylation of specific histidine residues in myofibrillar proteins, actin and myosin. N^{τ}-methylhistidine does not charge tRNA; therefore, it is not to be recycled for protein synthesis. Further, N^{τ}-methylhistidine does not undergo catabolism. Thus, the output of N^{τ}-methylhistidine has been used as an index of myofibrillar proteolysis (Young et al., 1972). It has been reported that plasma N^{τ}-methylhistidine concentration, urinary N^{τ}-methylhistidine excretion, and N^{τ}-methylhistidine release from incubated muscle were increased by starvation in rats (Nagasawa et al., 1996). We also reported that plasma N^{τ}-methylhistidine concentration in chicks was increased by fasting and decreased by refeeding (Nakashima et al., 2005b). The recent findings that protein feeding (Nagasawa et al., 1998) and even feeding leucine alone (Nagasawa et al., 2002) rapidly reduced the plasma level of N^{τ}-methylhistidine, as a specific marker of myofibrillar proteolysis in vivo, together with its release from muscle incubation in vitro, strongly indicate that amino acids control myofibrillar proteolysis. It remains unclear whether this reduction in N^{τ}-methylhistidine is a result of myofibrillar proteolysis caused by amino acids, and the underlying mechanisms of individual amino acid effects on myofibrillar proteolysis in vivo has not yet been investigated.

A major proteolytic pathway is the ubiquitin-proteasome system, which is also ubiquitous throughout the body, is dependent on ATP, and degrades ubiquitin-conjugated proteins via the 26S proteasome (Lecker et al., 1999). It is involved in many biologically important pro-

cesses, such as transcriptional regulation, cell cycle control, antigen processing, apoptosis, and DNA repair. Proteins degraded by this system turn over quite rapidly. In skeletal muscle, the proteasome is regulated to be the main proteolytic pathway for overall proteolysis (Lecker et al., 1999). Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which could play an important role in muscle atrophy. Intracellular proteolysis is carried out by lysosomal and non-lysosomal pathways in which intracellular proteases are directly responsible for the degradation of proteins. Calpains, cysteine proteases in the cytosol, are thought to be the main agents of non-lysosomal Ca^{2+}-dependent proteolysis that occurs within the myofibril, and have been shown to be capable of carrying out this initial step in myofibrillar proteolysis (Goll et al., 1991, 1992). Cathepsins, the main agents of lysosomal degradation, have been well established as contributors to muscle protein breakdown (Hall-Angeras et al., 1991). Lysosomal proteases degrade sarcoplasmic proteins and released myofibrillar proteins (Lowell et al., 1986). Amino acids have a regulatory effect on proteolysis in skeletal muscle tissue, but their mechanism of effect on the muscle proteolytic pathway has not been reported.

To clarify these issues, the present study investigated the effects of orally administered glycine on myofibrillar proteolysis and the expression of proteolytic-related genes in the skeletal muscle of chicks.

Materials and methods

Animal preparation and experimental protocol

One-day-old male layer chicks were supplied by a local commercial hatchery (Miyake-Furanjo, Chiba, Japan). They were housed in an electrically heated battery brooder and were provided with water and a commercial starter diet (Shimizukou-shiryou, Shizuoka, Japan) *ad libitum* for 7 days. On day 7, 24 birds of similar body weight (about 80 g) were selected and individually housed in wire-bottomed aluminum cages. The ambient temperature of the room was 25 °C, and relative humidity was maintained at 50–70% throughout the experiment. Chicks were allowed free access to a semi-purified corn soybean meal diet (Crude protein 20%, metabolizable energy 2900 kcal/kg diet) and water for the next 7 d. At the start of the experiment, 14-day-old chicks weighing 140 ± 10 g were divided into four groups and caged separately. Chicks were then deprived of all food for 24 h until glycine was administered. A solution of 38 g glycine/500 ml water was prepared, and chicks were administered calculated volumes of the solution corresponding to 57, 113, and 225 mg glycine/100 g body weight by oral gavage. Control chicks received an equal volume (5 ml) of saline by oral gavage. After the administration, chicks were returned to their cages where they were allowed free access to water. The dose of 225 mg glycine was equivalent to the amount of leucine consumed by chicks of this age and strain during 24 h of free access to a standard diet; the 113 mg dose was equivalent to the amount of glycine consumed by chicks of this age and strain during 24 h of free access to a standard diet; and the 57 mg dose of glycine was equivalent to the amount of glycine consumed by chicks of this age and strain during 2 h of free access to a standard diet after 24 h of diet deprivation. Two hours after glycine administration, the chicks were killed and gastrocnemius muscles and blood samples were obtained. The experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of the National Institute of Livestock and Grassland Science.

RNA isolation and real-time PCR

Gastrocnemius muscles were rapidly excised, frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from the muscles using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's directions. Complementary DNA was synthesized from 1 to 1000 ng of total RNA using random hexamer primers (TaKaRa, Tokyo, Japan) and ReverTra Ace (TOYOBO, Tokyo, Japan). Real-time PCR primers were designed with "Primer3" software available at http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi. Sequences of the forward (f) and reverse (r) primers were as follows: chicken atrogin-1/MAFbx, f-5′-CCA ACA ACC CAG AGA CCT GT-3′and r-5′-GGA GCT TCA CAC GAA CAT GA-3 (XM_418451); chicken proteasome C2 subunit, f-5′-AAC ACA CGC TGT TCT GGT TG-3′ and r-5′-CTG CGT TGG TAT CTG GGT TT-3 (AF027978); chicken m-calpain large subunit, f-5′-ACA TCA TCG TGC CCT CTA CC-3′ and r-5′-GAG ATC TCT GCA TCG CTT CC-3 (D38026); chicken cathepsin B, f-5′-CAA GCT CAA CAC CAC TGG AA-3′ and r-5′-TCA AAG GTA TCC GGC AAA TC-3 (U18083); and chicken glyceraldehyde -3- phosphate dehydrogenese (GAPDH), f-5′-CCT CTC TGG CAA AGT CCA AG-3′ and r-5′-CAT CTG CCC ATT TGA TGT TG-3 (K01458). Levels of mRNA expression were measured by real-time PCR analysis with LightCycler instrument (Roche Diagnostics, Mannheim, Germany) using the QuantiTect SYBR Geen PCR system (Qiagen KK, Tokyo, Japan) starting with 25 ng of reverse-transcribed total RNA. GAPDH expression was used as an internal control. For quantification of mRNA expression levels, primers of equivalent length and GC content (50–60%) were selected to yield PCR products roughly equivalent in size. PCR reactions were performed under the following conditions: 95 °C × 15 min, 45 × (94 °C × 15 sec, 55 °C × 20 sec, 72 °C × 12 sec).

N^{τ}-methylhistidine analysis

Plasma N^{τ}-methylhistidine concentration was measured by HPLC after derivatization of fluorescamine with perchloric acid and heating (Wassner et al., 1980).

Glycine analysis

Plasma glycine concentration was measured with an automated amino acid analyzer (L-8500, HITACHI, Tokyo, Japan) after sulfosalicylic acid treatment (final concentration, 3%).

Insulin assay

Plasma insulin concentration was determined by enzyme immunoassay using an insulin assay kit (Rat Insulin ELISA Kit; Shibayagi), with chicken insulin as a standard.

Corticosterone assay

Plasma corticosterone concentration was determined by enzyme immunoassay using a corticosterone assay kit (Rat corticosterone EIA Kit; Diagnostic Systems Laboratories, Inc).

Statistical analysis

Data were analyzed with one-way analysis of variance and Tukey's multiple comparison test. A p value of <0.05 was considered statistically significant. Each result is expressed as the mean ± SD.

Results and discussion

In the present study, the effect of oral glycine administration on myofibrillar proteolysis in chicks was examined. Plasma glycine concentration increased in a dose-dependent manner upon oral administration of glycine (Fig. 1A), demonstrating that administration was an effective method and of sufficient concentration to elicit a change in the plasma levels.

The changes in plasma N^{τ}-methylhistidine concentration in response to glycine administration are shown in Fig. 1B. N^{τ}-methylhistidine concentration was clearly decreased by glycine in a dose-dependent manner, demonstrating an inhibitory effect on myofibrillar proteolysis by glycine in chicks. N^{τ}-methylhistidine is used as an index of myofibrillar proteolysis (Young et al., 1972; Thompson et al., 1996). The rate at which skeletal muscle protein is degraded can be determined by measuring the urinary excretion of N^{τ}-methylhistidine, a by-product of myofibrillar protein degradation that is not reused for protein synthesis (Young et al., 1972). Measuring the urinary excretion of N^{τ}-methylhistidine, however, will not detect acute changes in myofibrillar protein degradation, since the collection period for urine is usually 1 d. In contrast, measuring plasma levels of N^{τ}-methylhistidine concentration offers an effective method to measure acute changes in concentration and, consequently, early response myofibrillar protein degradation (Nagasawa et al., 1996, 1998). In a previous study, we found that oral administration of glycine, as well as leucine and isoleucine, reduced the plasma N^{τ}-methylhistidine concentration in chicks, providing clear evidence that glycine and other amino acids suppress myofibrillar proteolysis in chicks (Nakashima et al., 2005a). In the present study, we used the same system to examine the effects of three doses of glycine administration on myofibrillar proteolysis. Glycine at all concentrations decreased the plasma N^{τ}-methylhistidine concentration. Therefore, we conclude that glycine suppressed myofibrillar proteolysis in chicks.

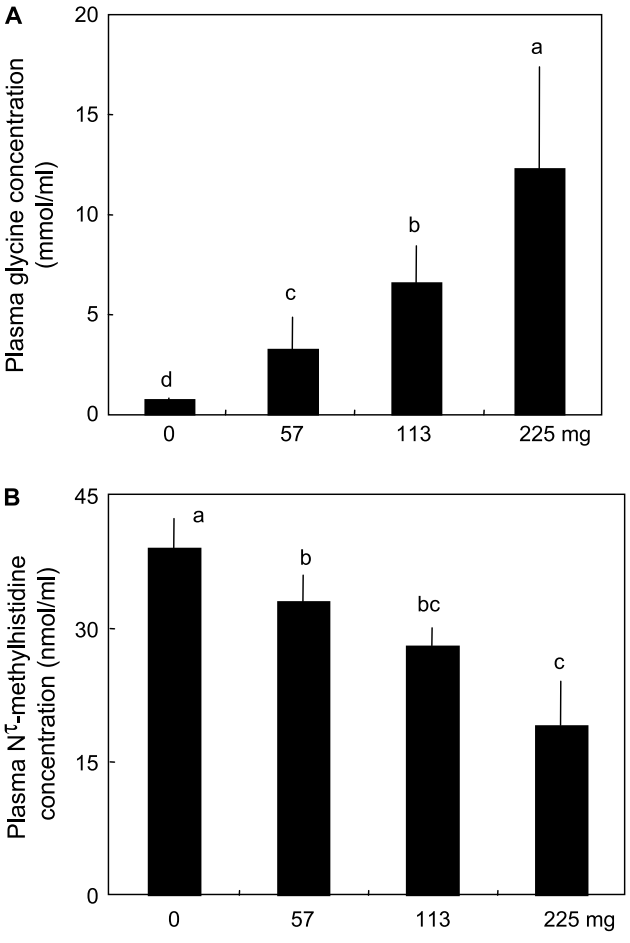

Fig. 1. Effect of orally administered glycine on plasma concentration of glycine (**A**) and N^{τ}-methylhistidine (**B**) in chicks. Food-deprived (24 h) chicks were orally administered 57, 113, and 225 mg glycine/100 g body weight and killed after 2 h. Data are expressed as means ± SD, $n = 6$

The main purpose of the present experiments was to determine the proteolytic mechanism(s) involved in the glycine-dependent suppression of myofibrillar proteolysis. The biochemical pathways for degradation of muscle protein and their mode of activation following administration of glycine have not yet been described. We measured mRNA expression of components in each of three protein degradation systems. The ubiquitin-proteasome system (non-lysosomal ATP-dependent proteolysis) was examined by measuring changes in atrogin-1/MAFbx and proteasome C2 subunit associated with glycine administration. The mRNA levels of atrogin-1/MAFbx and proteasome C2 subunit after glycine administration are shown in Fig. 2A and B. The mRNA expression decreased significantly in a dose-dependent manner for atrogin-1/MAFbx and proteasome C2 subunit ($p < 0.05$). Thus, orally administered glycine suppressed the expression of two genes involved in the ubiquitin-proteasome proteolytic pathway of skeletal muscle in chicks.

We next measured mRNA levels for calpain of the non-lysosomal Ca^{2+}-dependent proteolytic system and cathepsin B of the lysosomal-dependent proteolytic system. The mRNA expression decreased significantly in a dose-dependent manner for both m-calpain large subunit ($p < 0.05$, Fig. 2C) and cathepsin B ($p < 0.05$, Fig. 2D). These data demonstrate that glycine suppresses components of both the non-lysosomal Ca^{2+}-dependent and the lysosomal-dependent proteolytic systems.

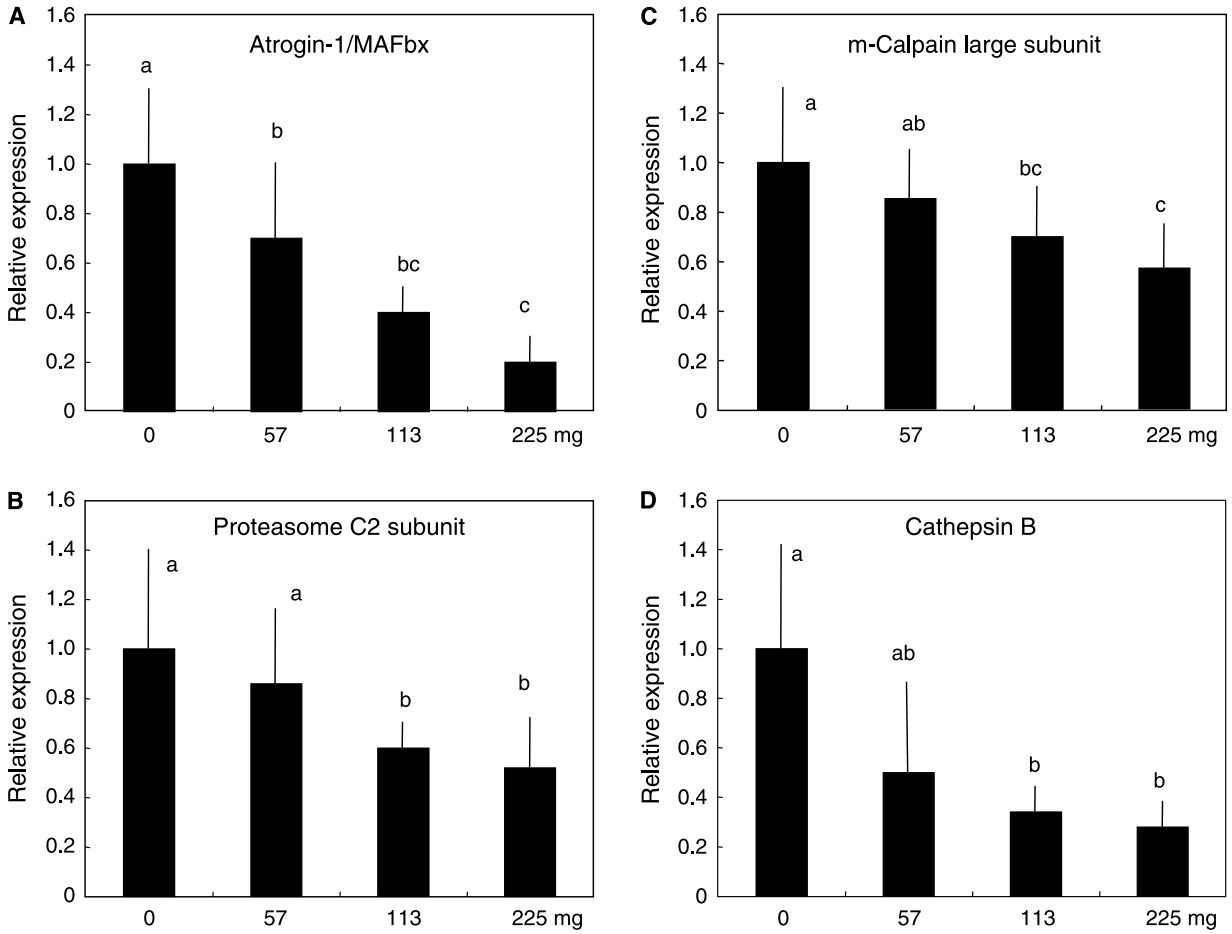

Fig. 2. Effect of orally administered glycine on the mRNA expression of atrogin-1/MAFbx (**A**), proteasome C2 subunit (**B**), m-calpain large subunit (**C**) and cathepsin B (**D**) in skeletal muscle of chicks. Food-deprived (24 h) chicks were orally administered 57, 113, and 225 mg glycine/100 g body weight and killed after 2 h. Data are expressed as means ± SD, $n = 6$

Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which might play an important role in muscle atrophy. Calpains (non-lysosomal Ca^{2+}-dependent proteolysis), ubiquitin-proteasome (non-lysosomal ATP-dependent proteolysis), and cathepsins (the main agents of lysosomal degradation) have been well established as contributing to muscle protein breakdown. However, prior to this report the effect of glycine on their expression in skeletal muscles in vivo had not been reported. In the experiments presented, we measured the expression of calpain, ubiquitin-proteasome, and cathepsin, and showed that glycine suppresses all three protein degradation pathways in chick skeletal muscle in vivo.

We next measured plasma concentrations of insulin and corticosterone in glycine-administered chicks. Plasma insulin and IGF-1 are widely believed to be major regulators of the anabolic drive of nutrients. Indeed, insulin reduced muscle protein degradation, through a decrease in the expression of atrogin-1/MAFbx in an ATP-ubiquitin-dependent system (Rome et al., 2003). Plasma insulin concentration in glycine-administered chicks is shown in Fig. 3A. The insulin concentration was not affected by glycine, indicating that insulin is not involved in the suppression of myofibrillar proteolysis by the oral administration of glycine in chicks. In contrast to insulin, glucocorticoids stimulate muscle proteolysis and wasting (Kayali et al., 1987), and physiological levels of these steroids are required for the activation of proteolysis, accumulation of ubiquitin conjugates, and expression of ubiquitin mRNA during fasting, diabetes, sepsis, and uremia (Wing and Goldberg, 1993; Price et al., 1996). Glucocorticoids stimulate muscle protein degradation through an increase in the expression of atrogin-1/MAFbx, in an ATP-ubiquitin-dependent proteolytic system (Sandri et al., 2004; Stitt et al., 2004). The synthetic glucocorticoid, dexamethasone, has

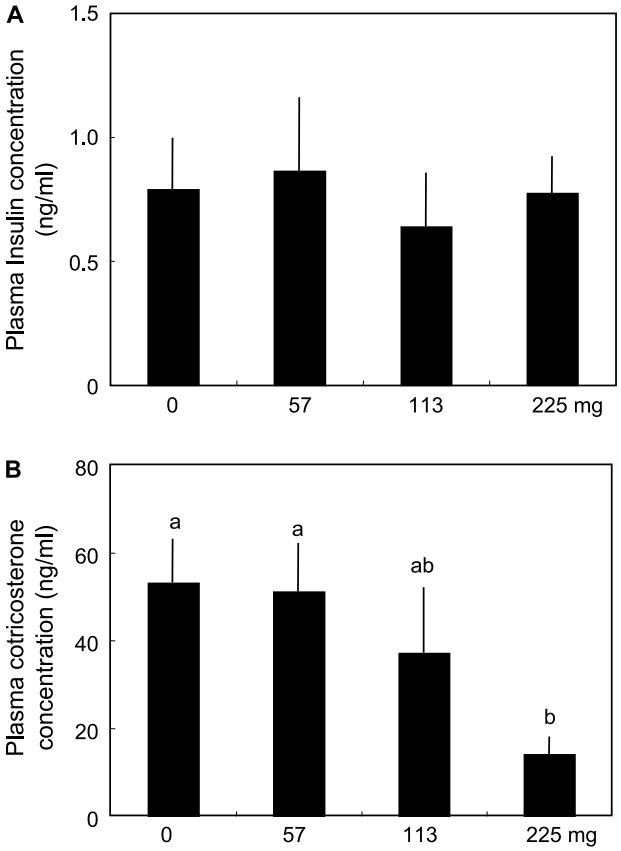

Fig. 3. Effect of orally administered glycine on plasma concentration of insulin (**A**) and corticosterone (**B**) in chicks. Food-deprived (24 h) chicks were orally administered 57, 113, and 225 mg glycine/100 g body weight and killed after 2 h. Data are expressed as means ± SD, $n = 6$

also been reported to promote proteolysis and to induce atrogin-1/MAFbx expression in myotube cultures (Wang et al., 1998; Bodine et al., 2001), as well as in skeletal muscles (Bodine et al., 2001). We measured the plasma concentration of corticosterone, in glycine-administered chicks. The concentration of plasma corticosterone was significantly decreased by glycine administration (225 mg/100 g body weight: $p < 0.05$, Fig. 3B) in chicks. We also showed that myofibrillar proteolysis and atrogin-1/MAFbx expression in the skeletal muscle were decreased by glycine administration (Fig. 2A). These results indicate that the oral glycine administration suppresses the plasma corticosterone concentration, resulting in a decrease of atrogin-1/MAFbx expression and subsequent decrease in myofibrillar proteolysis in the skeletal muscle of chicks.

In conclusion, the present study shows that orally administered glycine suppresses myofibrillar proteolysis and expression of proteolytic-related genes in the skeletal muscle by decreasing the plasma corticosterone concentration in chicks.

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