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AUTOCATALYTIC INACTIVATION OF LYSOSOMAL CATHEPSINS IS ASSOCIATED WITH INHIBITION OF PROTEIN BREAKDOWN BY INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) IN MYOTUBES

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SUMMARY: Protein breakdown was monitored in C2C12 myotubes as the rate of release of radioactivity after prelabeling cell protein with [^{3}H] tyrosine. IGF-1 (13 nM) and insulin (100 nM) prolonged the half-life of long-lived proteins. Enzymatic activities of cathepsins B and B+L were inhibited by the addition of IGF-1 or insulin. Immunoblotting of cathepsins B and L revealed extensive degradation of heavy chain forms by IGF-1. However, neither expression of cathepsins B and L genes nor expression of cystatin β, an intrinsic inhibitor for cathepsins, was influenced. The addition of E-64, L-3-carboxy-trans-2,3-epoxypropionyl-leucylamide-(4-guanidino) butane, a inhibitor of cathepsins B and L, increased protein contents of heavy chains of cathepsins B and L in the IGF-1 treated cells. Inhibition of protein breakdown by IGF-1 is mediated by autocatalytic inactivation of lysosomal cathepsins B and L.

IGF-1 has both anabolic effect on protein synthesis and anti-catabolic effect on protein degradation in skeletal muscle as does insulin (1-3). IGF-1 can simulate multiple parameters of insulin actions, such as lowering blood glucose, inhibiting gluconeogenesis, and inducing changes in leucine flux that are indicative of an inhibition of protein breakdown (4). These actions may be mediated by coupling to IGF-1 receptor. In cultured muscle cells, stimulation of glucose uptake in parallel with an increase in expression of glucose transporter is caused by insulin and IGF-1, which is mediated via IGF-1 receptor (5). Protein metabolism is more sensitive to IGF-1 infusion than to insulin infusion, and it is considered not to be mediated by insulin receptors (6).

Since the action of insulin on protein breakdown involves a reduction in the number and size of autophagic vacuoles (7, 8) and an increase of the latent form of cathepsin D (9, 10), there are enough reasons to speculate that IGF-1 may modulate lysosomal proteolytic system to reduce protein breakdown through the receptor mediated process. Lysosomal cathepsins B, H, and L are major endopeptidases that are important in intracellular protein degradation (11).

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Among them, cathepsin L is one of the most powerful lysosomal cysteine proteinases, and it is thus a strong candidate as the initiator of protein breakdown in lysosomes (12). Postreceptor events connecting IGF-1 binding with its anti-catabolic action are not fully elucidated. Investigations on gene expression, protein level and enzymatic activities of lysosomal cathepsins by IGF-1 and insulin gives a clue to the mechanism of inhibition of proteolysis.

C2C12 mouse skeletal muscle cell line isolated from dystrophic muscles (13) differentiates completely to form multinucleated myotubes, expresses a variety of muscle specific proteins (14), and can be used for research on muscle metabolism. This study was designed to examine a relationship between protein breakdown and lysosomal cathepsins, using IGF-1 treated C2C12 myotubes. The results of the present study demonstrated that enhanced autocatalysis may be responsible for inactivation of cathepsins, and implied a potential linkage between IGF-1 mediated postreceptor events and lysosomal proteolytic system.

MATERIALS AND METHODS

Materials. Insulin-like growth factor-1 (IGF-1) was kindly gifted from Fujisawa Pharmaceutical Co. (Osaka, Japan). L-3-carboxy-trans-2,3-epoxypropionyl-leucylamide-(4-guanidino) butane (E-64), Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, Arg-MCA, and 7-amido-4-methylcoumarin (AMC) were purchased from the Peptide Institute, Protein Research Foundation (Osaka, Japan). Other chemicals were purchased from Nakarai Tesque Co. (Kyoto, Japan).

Cell culture system. C2C12 mouse skeletal muscle cells were obtained from the American Type Culture Collection (ATCC). C2C12 myoblasts were seeded in 100-mm culture dishes (initial seeding density of 5.0×10^5 cells/dish) and were then grown in the growth medium, composed of Dulbecco's modified Eagle's medium with low glucose (DMEM) supplemented with 10% FBS, in a 5% CO_2 atmosphere at 37 °C. After 48 h, myoblasts became confluent. These cells were employed as myoblasts. When the medium was replaced with the fusion medium, composed of DMEM supplemented with 1% FBS, the cells started differentiation. The medium was then exchanged every other day. After a culture of 6 days, the cells fused completely to form myotubes.

Measurement of degradation of intracellular long-lived proteins. Degradation rates of long-lived proteins in C2C12 myotubes were determined by the method of Gulve and Dice (15) with some modifications. Briefly, long-lived proteins were radiolabelled by incubating myotubes with 1 μCi/ml [^{3}H]tyrosine for 2 days in DMEM supplemented with 10% FBS. The cells were rinsed twice with 2 ml of ice-cold Hanks' balanced salt solution (HBSS) containing 2 mM non-radioactive tyrosine (HBSS-Tyr) and then incubated for 2 additional h in the chase medium, composed of DMEM containing 10% FBS and 2mM non-radioactive tyrosine, to degrade short-lived proteins. The myotubes were rinsed twice with ice-cold HBSS-Tyr and transferred to the degradation medium (3 ml), consisted of DMEM + 0.5 mg/ml BSA (or 1% FBS) + 2 mM tyrosine, supplemented with or without various amounts of IGF-1 or insulin. After various time-intervals, an aliquot (100 μl) was sampled to measure the radioactivity of [^{3}H]tyrosine in the medium released from the cells using a Wallac 1409 liquid scintillation counter in triplication. The remaining radioactivity in the attached cells was counted after harvesting them completely by trypsin (2.5%) treatment. Half-life of protein degradation was calculated by semi-log plotting of the percentage of remaining radioactivity in the cells at set time points.

Measurement of cathepsin activity. The cells were washed twice with the homogenization solution (250mM sucrose, 2mM EGTA, 2mM EDTA, 20mM Tris-HCl, pH7.4), homogenized in 1ml of the homogenization solution containing 0.2% Triton-X 100, and lysed by sonication. The homogenate was centrifuged at 18,000 g for 15 min. The supernatant was dialyzed against the same amount of glycerol and stored at -40°C until analysis. Activities of cathepsins B, B+L, and H were measured by the method of Barrett et al. (16) using fluorogenic peptides as respective substrates, namely Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, and Arg-MCA.

Cathepsin B activity was assayed with 10 μM Z-Arg-Arg-MCA at pH 6.0. To obtain a blank sample, the extract was preincubated with 1 μM of E-64 at 37°C for 5 min to inhibit cathepsin B activity. Cathepsin B+L activity was assayed by the same method of cathepsin B with Z-Phe-Arg-MCA. Since this synthetic substrate is not only hydrolyzed by cathepsin L but also cathepsin B, its hydrolysis is expressed as the activity of cathepsin B+L. The activity of cathepsin H was assayed with 10 μM Arg-MCA at pH 6.8.

RNA isolation and Northern blot analysis. Total RNA from C2C12 myotubes was extracted using guanidium thiocyanate as described (17) and quantified by absorbance at 260 nm. RNA samples (10 μg) were subjected to electrophoresis in 1.0% agarose gels and blotted overnight to Hybond-N^+ nylon membranes with 20 x standard saline citrate (SSC;0.15M NaCl and 15mM sodium citrate, pH7.0). The RNA in gels and filters was visualized with ethidium bromide and photographed by UV transillumination to ensure the integrity of RNA and the loading of equivalent amounts of RNA. The membrane was prehybridized for 1h and hybridized overnight with the Church buffer (18). The cDNAs encoding rat cathepsins B, L, and H (19, 20) and rat cystatin β (21) were used and radiolabelled probes were prepared by the random primer method. Filters were exposed to Kodak X-Omat AR films with an intensifying screen for 1-3 days at -80°C, and quantitation of the membranes was made by densitometry using a MCID system (Imaging Research Inc., Ontario, Canada).

Western blot analysis. The cells were homogenized by sonication in 50 mM Tris-HCl buffer (pH 7.4), containing 250 mM sucrose, 5 mM EDTA, 1% SDS, 1 mM leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM N-ethylmaleimide, 1 mM pepstatin, and 0.1 U/ml trypsin inhibitor from soybean. The homogenate was centrifuged at 15,000 g for 20 min at 4°C, and the supernatant was used for analysis. Samples (20 μg of protein) separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in a 10-20 gradient gel (Daiichi Pure Chemicals Co., Tokyo, Japan) were transferred electrophoretically to an Immobilon-P membrane (Millipore Co., Bedford, MA, U.S.A.) with a semidry Trans-Blot (Bio-Rad Laboratories, Hercules, CA, U.S.A.). After blocking, the membrane was treated with various antibodies and anti-rabbit IgG conjugated with alkaline phosphatase using a ProtoBlot system (Promega Co., Madison, WI, U.S.A.) and stained by the alkaline phosphatase reaction. Rabbit polyclonal antibodies against rat liver cathepsins B (22) and L (23) were used. Protein concentration was determined by the method of Bradford (24) using a Bio-Rad reagent and bovine serum albumin as a standard.

Statistical analysis. Values were expressed as mean ± SD. Differences were tested for statistical significance with the analysis of varience with Scheffe's test. Significance was determined at the 95% confidence level.

RESULTS

As shown in Table 1, the addition of IGF-1 significantly prolonged the half-life of long-lived proteins in a dose-dependent manner and its effect was maximized at the concentration of 130 nM. The anti-proteolytic effect was also observed by the treatment of insulin and the maximum effect was observed at 1000 nM. The effect of IGF-1 was about ten times more potent than that of insulin. The addition of E-64 (10^{-4} M) prolonged the half-life of long-lived proteins indicating a potential participation of cathepsins in protein degradation.

As shown in Table 2, IGF-1 significantly decreased the activities of cathepsins B and B+L, dose-dependently. On the other hand, the activity of cathepsin H was not affected by IGF-1. Insulin had an inhibitory effect on the activities of cathepsins B and B+L, but its effect was much less potent than that of IGF-1. The results of Northern blotting are shown in Fig. 1. The levels of mRNA encoding cathepsins B and L were not influenced by the treatment of 13 nM IGF-1. The mRNA level of cathepsin H was undetectable not only in the control but also in the IGF-1 treated cells (data not shown). Likewise, the mRNA level of cystatin β, an endogenous inhibitor of cathepsins, was not influenced by IGF-1. Immunoblot analysis for cathepsins B and L was carried out as shown in Fig. 2. The antibody against cathepsin B

Table 1. Prolongation of half-life of long-lived proteins by IGF-1 and insulin in C2C12 myotubes

Treatments			Half-life of long-lived proteins (h)
In the presence of 1% fetal bovine serum(FBS)			
Control		(3)	28.9 ± 1.9
IGF-1	13 nM	(3)	43.7 ± 2.4 #
In the presence of 0.5 mg/ml bovine serum albumin(BSA)			
Control		(6)	18.5 ± 0.5
IGF-1	1.3 nM	(6)	19.6 ± 0.7
	13 nM	(6)	27.4 ± 1.3*
	130 nM	(6)	32.1 ± 0.6*
Insulin	1 nM	(6)	19.4 ± 1.5
	10 nM	(6)	23.8 ± 0.6*
	100 nM	(6)	30.8 ± 1.1*
	1000 nM	(6)	32.3 ± 1.1*
E-64	10^{-4} M	(6)	25.6 ± 3.0 *

Breakdown of long-lived proteins was measured in the presence of 1% FBS or 0.5 mg/ml BSA. Data are expressed as mean ± SD. ( ) indicates number of experiments. #P < 0.05, *P < 0.001 vs control.

reacts with two forms, composed of a single chain of high molecular weight (31 kDa), a mature form, and a heavy chain (29 kDa) (22). In myotubes, only a heavy chain form of cathepsin B was detectable, while myoblasts had the two forms. The treatment of IGF-1 decreased the protein level of a heavy chain by 40%, while the addition of E-64 in the IGF-1

Table 2. Inhibition of enzymatic activities of cathepsins B and B+L by IGF-1 and insulin in C2C12 myotubes

Treatments			Cathepsin activity (nmol AMC / h / mg protein)		
			Cathepsin B	B+L	H
Control		(9)	31.4 ± 14.7	422.3 ± 61.3	2.8 ± 0.2
IGF-1	13 nM	(9)	3.2 ± 3.9*	72.6 ± 20.5*	3.4 ± 0.7
	130 nM	(3)	8.2 ± 3.5*	68.1 ±13.9 *	N. D.
Control		(4)	38.3 ± 4.4	437.4 ± 50.8	N. D.
Insulin	100 nM	(4)	36.2 ± 5.3	269.6 ± 21.6¶	N. D.
	1000 nM	(4)	28.7 ± 3.0#	211.0 ± 15.6*	N. D.

C2C12 myotubes maintained in the fusion medium (DMEM + 1%FBS) were incubated for 48 h in the presence of the indicated amounts of IGF-1 or insulin. Activities of cathepsins B, B+L, and H were measued in duplication, using respective fluorogenic peptides as substrates, namely Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, and Arg-MCA. Data are means ± SD. ( ) indicates number of experiments. #P < 0.05, ¶ P < 0.01, *P < 0.001 vs control. N.D., not determined.

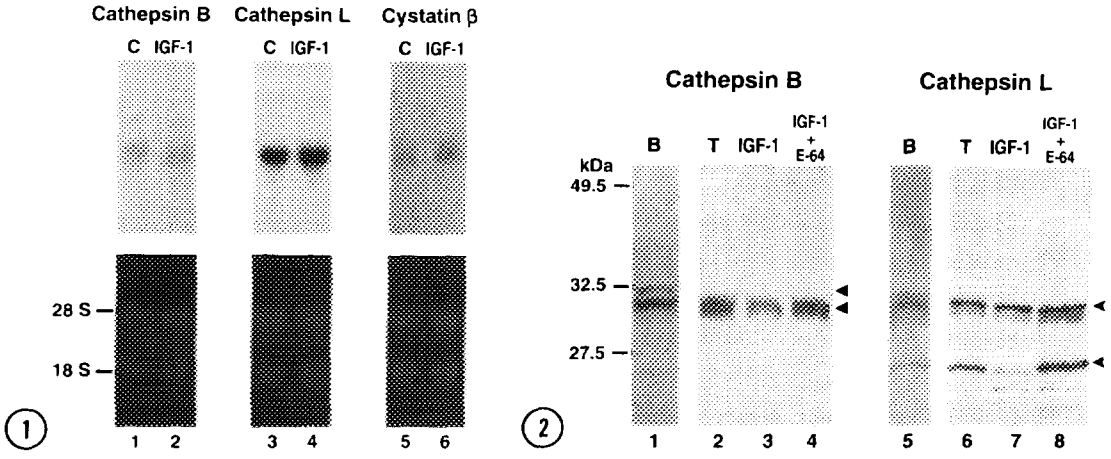

Fig.1. Expression of cathepsins (B,L) and cystatin β genes and effects of IGF-1 C2C12 myotubes were cultured in the absence (C) or the presence of IGF-1 (13 nM=100 ng/ml) (IGF-1) for 24 h. Ten μg of total RNA extracted from harvested cells was examined by Northern blot analysis. Lanes 1-2, Northern blotting with cDNA for rat cathepsin B; 3-4, with cDNA for rat cathepsin L; 5-6, with cDNA for rat cystatin β. The bottom panels show the pattern of total RNA stained with ethidium bromide.

Fig.2. Western blot analysis of cathepsins B and L and effects of IGF-1 and E-64 C2C12 myotubes cultured in the absence (T) or the presence of IGF-1 (13 nM) without (IGF-1) or with E-64 (10^{-4} M) (IGF-1 + E-64) for 24 h were harvested and their crude extracts (20 μg of protein) were used for immunoblot analysis of cathepsins B and L. Extract from C2C12 myoblasts (B) was also analyzed. Lanes 1-4, immunoblotting with the rabbit anti-cathepsin B (rat liver) polyclonal antibody; 5-8, with the rabbit anti-cathepsin L (rat liver) polyclonal antibody. There were two forms of cathepsin B, composed of a single chain of high molecular weight (31 kDa) and a heavy chain (29 kDa), and two forms of cathepsin L, composed of a single chain (30 kDa) and a heavy chain (25 kDa), as indicated by arrows.

treated cells restored its protein content. The antibody against cathepsin L reacts with two forms, composed of a single chain (30 kDa), a mature form, and a heavy chain (25 kDa) (23). IGF-1 decreased the protein content of a heavy chain by 50% and its effect was blocked by the addition of E-64, while the content of a single chain form was not affected by either IGF-1 or E-64.

DISCUSSION

The results of the present study clearly demonstrated that both IGF-1 and insulin inhibited protein degradation in myotubes with decreasing lysosomal cathepsins (B and L) activities. Considering their concentrations causing maximum responses, IGF-1 was 10 times more potent than insulin. The affinity of insulin to the heterogeneous IGF-1 receptor is 40-60 nM, whereas that of IGF-1 to the homologous receptor is 0.5 nM in the gastrocunemius muscle (25). In ovine primary cultured muscle cells and L6 cells, 1 mM insulin causes the similar anti-catabolic effect with 1.3 nM IGF-1 (1). These facts indicated that inhibition of protein breakdown and lysosomal cathepsin activity may be mediated via the receptor for IGF-1, as

reported by Douglas et al. (6) that the effects of IGF-1 on muscle protein metabolism are not mediated by insulin receptors. It has been reported that IGF-1 is present at 2 x 10^{-8} M in normal human serum (26) and physiological concentrations of insulin and IGF-1 are 100 ng/ml (3). Therefore, IGF-1 can be effective on protein degradation and lysosomal cathepsins in muscles in an in vivo situation.

The similar observation with the present study was done by Ballard et al. (27). Protein degradation in rat L6 myoblasts is inhibited by a high concentration of insulin as well as by fetal bovine serum and bovine colostrum, mixtures rich in growth factors as IGF-1. Inhibition of protein breakdown is a consistent response to growth factors in cultured cells (28), any common mechanism for which is probably dependent on their binding to their surface receptors. Postreceptor events leading to inhibition of protein degradation are not clearly known, though lysosomes and the formation of autophagic vacuoles are supposed to be involved as reported on insulin mediated inhibition of protein degradation (7, 8). The formation of autophagic vacuoles comprises two stages: the pre-lysosomal autophagosome and, after fusion with a pre-existing lysosome, the autolysosome is formed to represent the place for proteolysis, and insulin acts at the pre-lysosomal level (8). On the other hand, postreceptor mechanisms leading to inactivation of lysosomal cathepsins has been entirely unknown. Possible mechanisms are as followings: suppression of cathepsin gene expression, decrease of cathepsin gene translation, decrease of processing from preprocathepsin to procathepsin or matured cathepsin (a single chain cathepsin), accelerated degradation of matured form or their processed two chain forms (a heavy chain and a light chain) (29, 30) and up-regulation of endogenous inhibitors, cystatins. Metal endopeptidases or an aspartic protease such as cathepsin D (31) are involved in the propeptide processing of cathepsins B, H and L to produce mature forms, and proteolytic cleavages of the mature single chain cathepsins are accomplished by cysteine proteinases in lysosomes (32), mainly by cathepsin L. The results of the present study demonstrated that cathepsin gene expression was not inhibited by IGF-1, and the amounts of heavy chains of cathepsins B and L were reduced by IGF-1, while a single chain of cathepsin L was not influenced. These findings indicate that once the mature forms of cathepsins B and L are cleaved, extensive autocatalytic degradation takes place by the treatment of IGF-1. When autocatalysis was blocked by E-64, both heavy chains levels returned to the control levels.

This paper reports for the first time the marked effect of IGF-1 on metabolic instability of proteolytic machinery, cathepsins. Mechanism for accelerated degradation of cathepsins B and L on the treatment of the cells with IGF-1 remains uncler, but following possibilities can be drawn: 1) IGF-1 may mediate elevation of cellular pH, which increases lysosomal pH and causes metabolic instability of cathepsins. 2) IGF-1 may promote the association of specific protein (s) to cathepsins and results in autocatalytic degradation of cathepsins as demonstrated in antizyme-induced degradation of ornithine decarboxylase (33). 3) Autophagy is stimulated in the cell condition employed in this study and substrares are provided from autophagosomes continuously. The presence of substrates in autolysosomes may prevent autocatatalytic degradation of cathepsins. IGF-1, as does insulin, blocks the formation of prelysosomal

autophagosomes and reduces the provision of substrates to lysosomes, which may result in autocatalytic degradation of cathepsins. These possibilities should be evaluated for the future study.

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