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Insulin and amino acid availability regulate atrogin-1 in avian QT6 cells

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Abstract

New evidence has demonstrated that the expression of major genes, termed atrogenes, controls the ubiquitin–proteasome proteolytic pathway. The present work aimed to study the impact of insulin and amino acids on the expression of one of these atrogenes, the E3 ubiquitin ligase Muscle Atrophy F box (MAFbx, also called atrogin-1), in quail muscle (QT6) fibroblasts. First, we characterized atrogin-1 in QT6 cells and demonstrated the insulin sensitivity of these cells. Second, we showed that insulin reduced atrogin-1 mRNA via the phosphatidylinositol-3′kinase (PI3K)/protein kinase B (PKB or AKT)/target of rapamycin (TOR) pathway. Atrogin-1 expression also depended on the availability of an individual amino acid, i.e., methionine. Moreover, the amino acid-induced reduction of atrogin-1 was inhibited by rapamycin, indicating the involvement of the TOR pathway in such regulation. In conclusion, expression of the ubiquitin ligase atrogin-1 is regulated by both insulin and amino acids through the TOR pathway.

Keywords: Atrogin-1; Insulin; Methionine; AKT/TOR pathway; Avian fibroblasts

Signaling pathways that regulate muscle protein metabolism have been intensively studied in the last 10 years. One of the most explored is the mammalian target of rapamycin (mTOR) pathway, in which TOR integrates signals from nutrients such as amino acids, and mitogenic and growth factors, i.e., insulin/insulin-like growth factors (IGF) [1–7]. The signaling cascade initiated by growth factors is mediated by a complex pathway involving the tyrosine phosphorylation of insulin receptor substrate (IRS) proteins and activation of several intracellular kinases such as phosphatidylinositol-3′kinase (PI3K), protein kinase B (PKB or AKT) and TOR. The amino acid-induced signaling cascade also originates from TOR. The AKT/TOR pathway affects the phosphorylation of some major effectors involved in the regulation of translation initiation, e.g., p70 S6 kinase (S6K1) in mammals as in birds [1,5–9].

The PI3K pathway has been reported to be also involved in the insulin-induced inhibition of muscle proteolysis [10]. New evidence has demonstrated that AKT can regulate the expression of two important genes for controlling the ubiquitin–proteasome proteolytic pathway, i.e., E3 ubiquitin ligases Muscle Atrophy F box (MAFbx, also called atrogin-1) and Muscle Ring Finger-1 (MurRF-1) [11–14]. These two muscle specific E3 ligases have been shown to be overexpressed in atrophic conditions including diabetes, cancer, fasting but also following disuse [15–18]. They are regulated by growth factors such as IGF-1 via mechanisms involving the AKT/Forkhead box-O transcription factors (FOXO) and the AKT/TOR pathways [19–21]. Despite these interesting findings, the mechanisms by which AKT and TOR inhibit proteolysis are not completely understood. Moreover, it has not to date been established whether nutrient signal amino acids can be involved in the regulation of E3 ligases or not. The aim of the present study was therefore to clarify the signal transduction pathway involved in the ubiquitin–proteasome-dependent proteolysis and the potential role of

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amino acids by exploring the regulation of atrogin-1 in the avian QT6 fibroblasts. The QT6 cell line has been developed by Antin and Ordahl [22] from methylcholanthrene-induced *Pectoralis* fibrosarcomas of Japanese quail *Coturnix coturnix* japonica.

Materials and methods

Chemicals. Anti-phospho-S473 AKT/PKB, anti-phospho-S235/S263 S6, and anti-AKT antibodies, as LY294002 and rapamycin inhibitors were obtained from Cell Signaling Technology (Beverlt, MA, USA). Anti-vinculin and McCoy medium were from Sigma Chemical Company (St. Louis, MO, USA). All culture additives were obtained from Invitrogen (Carlsbad, CA, USA) and Eurobio (Les Ulis, Courtaboeuf, France). RPMI media were from Dutscher SA (Brumath, France). Human insulin was from LILLY France S.A.S. (Suresnes, France).

Cell culture and treatments. QT6 fibroblast cells were grown in McCoy medium supplemented with 10% fetal calf serum and 1% chicken serum to 90–100% confluence, fasted 16 h in serum-free medium, washed once with complete RPMI medium and incubated in the same medium for 2 h before treatments (0.1 μM insulin or amino acids). Complete RPMI medium or RPMI medium without methionine was used to test the effect of amino acid availability. In some studies, inhibitory treatments were performed using LY294002 at 20 μM and rapamycin at 25 nM (diluted in DMSO) for 30 min before stimulation by 0.1 μM insulin or amino acids for 5 h. All cultures were performed under a water-saturated atmosphere of 95% air/5% CO_2 at 37 °C.

RNA isolation and RT-PCR. To our knowledge, no information was available on insulin signaling in QT6 fibroblasts. Consequently, we characterized insulin receptor (IR), AKT, and atrogin-1 in these cells. Total RNA was extracted using RNA Now (Biogentec, Seabrook, TX, USA) from QT6 fibroblasts according to the manufacturer's recommendations. 2.5 μg RNA was reverse transcribed using Super Script II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in the presence of Random Primers (Promega, Charbonnieres-les-Bains, France). The resulting cDNAs were submitted to PCR in the presence of UptiTherm DNA polymerase (Interchim, Montluçon, France) and primers designed to amplify parts of IR, AKT, and atrogin-1 as described in Table 1.

To quantify atrogin-1 mRNA, real-time RT-PCR was performed as previously described [23] using an ABI Prism 7000 apparatus (Applied Biosystems, Foster City, CA, USA). Atrogin-1 mRNA levels were estimated on the basis of PCR efficiency and threshold cycle (C_t) deviation of an unknown sample versus a control as previously described [23]. Runs were performed in duplicates. 18S ribosomal RNA was chosen as the reference gene.

Western blotting. Cell lysates were prepared as previously described [24]. QT6 lysates (40 μg of protein) were subjected to SDS–PAGE gel electrophoresis (10%) and Western blotting using the appropriate antibody. After washing, membranes were incubated with an Alexa Fluor secondary antibody (Molecular Probes, Interchim, Montluçon, France). Bands were visualized by Infrared Fluorescence by the Odyssey® Imaging System (LI-COR Inc. Biotechnology, Lincoln, NE, USA) and quantified by Odyssey infrared imaging system software (Application software, version 1.2).

Statistical analysis. Values are means ± SEM from at least 2–3 independent experiments. Statistical analysis was performed using analysis of variance (ANOVA, Statview Software program; SAS Institute, Cary, NC) to detect significant intergroup differences.

Results and discussion

Atrogin-1, IR and AKT characterization, and insulin response

Using the sequence of gallus gallus atrogin-1 available in the GenBank database at the NCBI (http://www.ncbi.nlm.nih.gov; NM_001030956), we characterized chicken atrogin-1 by RT-PCR and sequencing of the purified amplicons (data not shown). In addition, IR and AKT have been totally or partially sequenced in chickens [25–27]. Since no information was available on insulin signaling in QT6 fibroblasts, we investigated the presence of atrogin-1, IR, and AKT in these cells by RT-PCR analysis. Fig. 1A shows the amplification of cDNAs corresponding to fragments of atrogin-1 (152 bp), IR (235 bp), and AKT (440 bp), demonstrating atrogin-1, IR, and AKT gene expression in quail QT6 fibroblasts.

In mammals and birds, insulin induces AKT phosphorylation on S473 [28]. To further characterize insulin response in QT6 fibroblasts, cells were thus incubated with 0.1 μM insulin for 15 min or 5 h, and the phosphorylation of AKT on S473 was examined. Insulin treatment significantly increased the phosphorylation of AKT on S473 whatever the time of treatment (Fig. 1B). These findings demonstrate that QT6 fibroblasts are insulin sensitive, conversely to QM7 cells derived from the QT6 cell line which are lacking insulin receptors [8]. We next investigated the potential regulation of atrogin-1 by insulin in QT6 fibroblasts. Fig. 1C indicates that insulin treatment for 5 h clearly inhibited atrogin-1 expression ($P < 0.001$). The reduction of atrogin-1 mRNA levels by insulin is in good agreement with data obtained in mammalian C2C12 myotube cell cultures [20].

Table 1
Oligonucleotide primer sequences

Primer	Sequence	Accession No.	Product size (bp)
Atrogin-1			
Sense	5′-GACGCGCTTTCTCGATGAG-3′	NM_001030956	152
Antisense	5′-CCTTGTTATTCAGTAGGTCTTTTTTCCT-3′		
IR			
Sense	5′-TGCCACCACGTGGTTCGCCT-3′	XM_418250	235
Antisense	5′-GCCAGGTCTCTGTGAACAAA-3′		
AKT			
Sense	5′-CCGCGACATCAAGCTGGAGA-3′	AF181260	440
Antisense	5′-CCGCGACATCAAGCTGGAGA-3′		

The sequences of atrogin-1, insulin receptor (IR), and AKT are present in the GenBank database at the NCBI (http://www.ncbi.nlm.nih.gov).

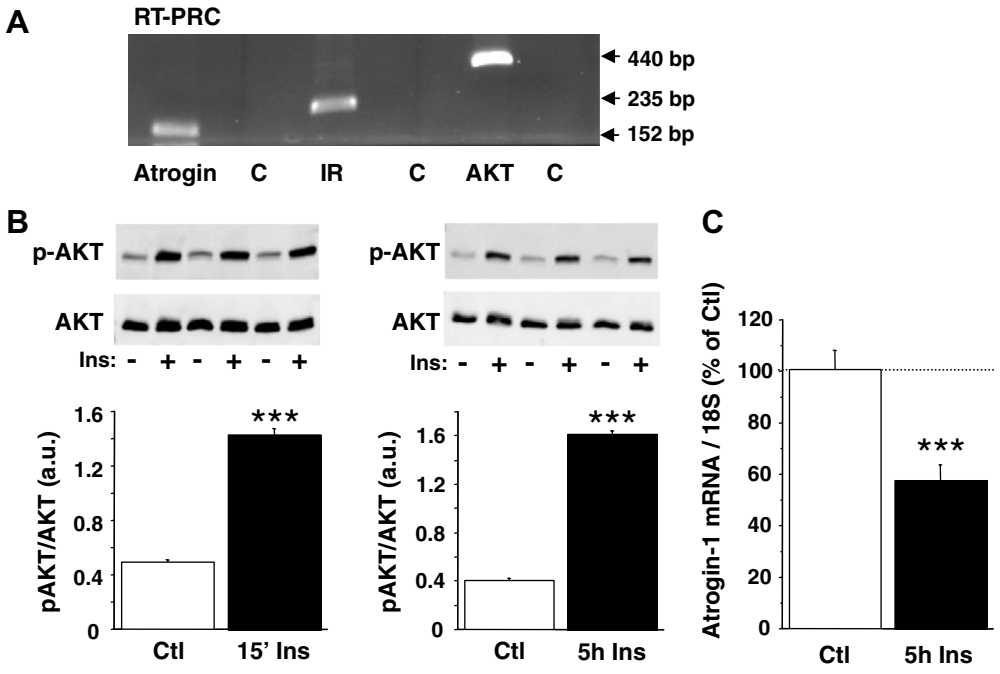

Fig. 1. Atrogin-1, IR and AKT characterization, and insulin response. (A) Atrogin-1, IR and AKT transcripts. RT-PCR analyses were performed using total RNA extracted from QT6 cells. C, negative controls (H_2O). (B) AKT phosphorylation. QT6 cells were stimulated or not by insulin for 15 min (Ins 15′) or 5 h (Ins 5 h). Representative Western blots. Blots were quantified and the ratio phospho-AKT/AKT was determined. (C) Atrogin-1 expression. RNA of QT6 cells stimulated or not by insulin for 5 h were prepared and subjected to real-time RT-PCR. The results were expressed as the atrogin-1 mRNA/18S RNA ratio ($n = 6$–9). $^{***}P < 0.001$ vs. Control (Ctl).

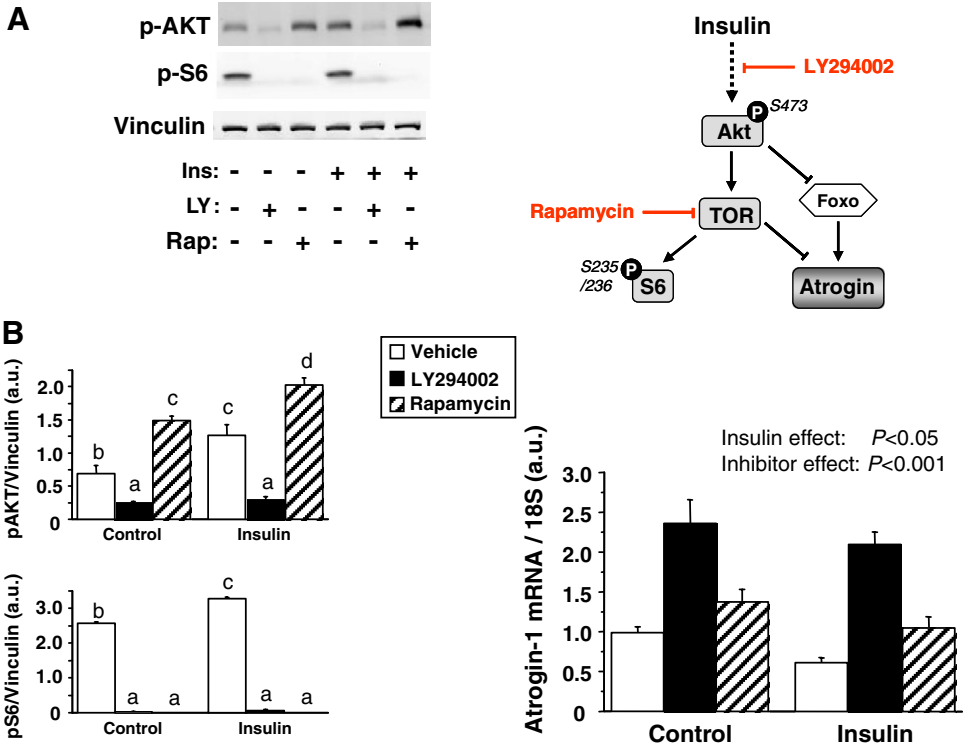

Fig. 2. Effect of insulin in QT6 cells incubated with or without LY294002 or rapamycin. QT6 cells were stimulated or not by insulin for 5 h. (A) Representative Western blots of phospho-AKT, phospho-S6, and vinculin as a loading control. A representation of the AKT/TOR/atrogin pathway is shown on the right; the inhibitors of PI3K/AKT and TOR signaling (LY294002 and rapamycin, respectively) are indicated. Controls with the diluent of the inhibitors were performed for these experiments. (B) On the left, AKT and S6 phosphorylation normalized with vinculin ($n = 3$). Different letters indicate significant differences between treatments at $P < 0.05$. On the right, atrogin-1 expression presented as the atrogin-1 mRNA/18S RNA ratio ($n = 10$). Results of two-way ANOVA are shown as the P-values associated with main effects (no significant interaction observed).

Signaling pathways involved in the insulin-related inhibition of atrogin-1 expression

To explore the signaling pathways involved in insulin-induced atrogin-1 inhibition, QT6 cells were incubated with or without 20 μM LY294002 or 25 nM rapamycin. LY294002 is an inhibitor of the PI3 K/AKT pathway as indicated by dephosphorylation of AKT in cells incubated with or without insulin (Fig. 2A and B). As a consequence, LY294002 drastically increased atrogin-1 mRNA levels by approximately twofold compared to control cells ($P < 0.001$). Higher levels of atrogin-1 mRNA were previously found with LY294002 treatments by Sacheck et al. [20]. These findings can be explained by lower AKT phosphorylation in LY294002-treated cells, suggesting a key role of the PI3 K/AKT pathway in the inhibition of atrogin-1 expression in avian as in mammalian models.

Rapamycin is a specific inhibitor of the TOR pathway as indicated by the dephosphorylation of protein ribosomal S6, a downstream target of TOR, in cells incubated with or without insulin (Fig. 2A and B). Rapamycin, which acts downstream of AKT, did not block the phosphorylation of AKT, but in contrast increased it. This feedback regulation of AKT signaling has been previously observed in various mammal cell models [29–31] and generated increasing interest in the last few years (see [32–34] for reviews). However, the precise mechanisms and consequences of this feedback regulation are so far poorly defined. In this regard, we showed here that rapamycin increased atrogin-1 expression ($P < 0.05$) in both insulin treated and untreated cells although to a lesser extent than LY294002 (Fig. 2B). The concomitant increase of AKT phosphorylation and atrogin-1 gene expression suggests that a mechanism independent of the inhibition of FOXO transcription factors is required in such regulation. Our results therefore demonstrate that TOR pathway is involved in the insulin control of the E3 ubiquitin ligase atrogin-1 in QT6 fibroblasts, as found in C2C12 myotubes [21].

Effect of amino acid availability

Since amino acids act on TOR signaling [1,5–7] that is involved in atrogin-1 control in avian QT6 fibroblasts (Fig. 2), we next studied the regulation of atrogin-1 by amino acids. We have previously demonstrated in avian myoblasts that the availability of individual amino acids such as methionine regulates S6K1, a downstream target of TOR [8]. Consequently, QT6 cells were incubated in RPMI media with or without methionine for two

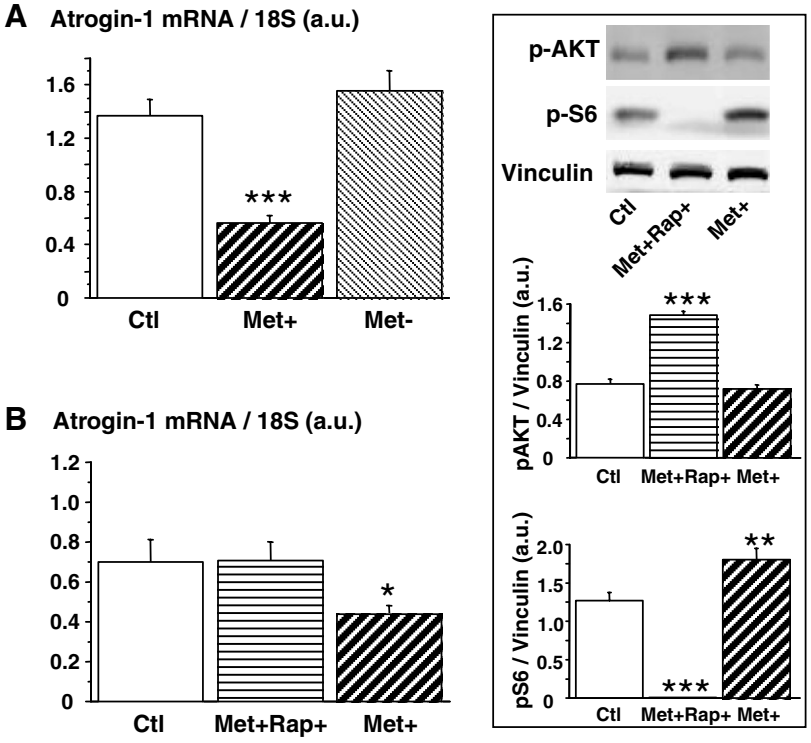

Fig. 3. Effect of amino acid and rapamycin. QT6 cells were incubated in RPMI media with or without methionine (Met) for two periods of 5 h. (A) Effect of amino acid availability on atrogin-1 expression presented as the atrogin-1 mRNA/18S RNA ratio ($n = 12$). Ctl, control cells maintained in complete RPMI medium; Met+, cells deprived of Met for 5 h to which the deprived Met was returned for 5 h; Met−, cells maintained in complete RPMI medium then deprived of Met for 5 h. (B) Effect of amino acid and rapamycin on atrogin-1 expression presented as the atrogin-1 mRNA/18S RNA ratio ($n = 8$). Ctl, control cells maintained in complete RPMI medium; Met+Rap+, cells deprived of Met for 5 h to which the deprived Met was returned for 5 h in the presence of rapamycin; Met+, cells deprived of Met for 5 h to which the deprived Met was returned for 5 h. Results of AKT and S6 phosphorylation are shown in the insert ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. Control (Ctl).

successive periods of 5 h. Atrogin-1 expression was not significantly modified by methionine deprivation for 5 h as compared to cells cultured in the complete medium containing amino acids (Fig. 3A). Conversely, re-supplying methionine after a period of deprivation clearly reduced the levels of atrogin-1 mRNA ($P < 0.001$). The present study is the first to provide evidence that methionine is able to modulate atrogin-1 expression. To our knowledge, the only two studies in which the potential effects of amino acids have been tested failed to support the hypothesis of an atrogin-1 control by nutrients [35,36]. These studies were performed using the branched-chain amino acid leucine clearly recognized as a nutrient signal regulator of mRNA translation and proteolysis [37–39]. However, under the experimental conditions used, muscle atrogin-1 was unresponsive to leucine. To determine how methionine influenced atrogin-1 expression, QT6 cells were then incubated with or without treatment with 25 nM rapamycin. Inhibiting TOR by rapamycin for 5 h as indicated by the results of AKT and S6 phosphorylation (Fig. 3B) abolished the amino acid-related decrease in atrogin-1 expression. Thus, our results indicate that atrogin-1 is regulated by growth factors but also amino acids in QT6 fibroblasts via mechanisms that imply TOR.

In conclusion, atrogin-1 is expressed in the quail muscle (QT6) cell line. This E3 ubiquitin ligase is regulated by insulin through signal transduction pathways involving AKT and TOR. In this study, we demonstrate for the first time that atrogin-1 is also regulated by amino acid availability through the TOR signaling pathway. Additional studies are needed to better understand the potential effects of amino acids in controlling the ubiquitin–proteasome-dependent proteolysis.

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