Published in final edited form as:

Shock. 2014 May ; 41(5): 420–428. doi:10.1097/SHK.0000000000000128.

Salutary effect of Aurintricarboxylic acid (ATA) on endotoxin- and sepsis-induced changes in muscle protein synthesis and inflammation

Lacey Laufenberg, MD, Abid A. Kazi, PhD, and Charles H. Lang, PhD

Departments of Cellular & Molecular Physiology, and Surgery, Pennsylvania State University
College of Medicine, Hershey, PA 17033

Abstract

Small molecule nonpeptidyl molecules are potentially attractive drug candidates as adjunct therapies in the treatment of sepsis-induced metabolic complications. As such, the current study investigates the use of aurintricarboxylic acid (ATA), which stimulates insulin-like growth factor (IGF)-I receptor and AKT signaling, for its ability to ameliorate the protein metabolic effects of endotoxin (LPS) + interferon (IFN) γ in C2C12 myotubes and sepsis in skeletal muscle. ATA dose- and time-dependently increases mTOR-dependent protein synthesis. Pretreatment with ATA prevents the LPS/IFN γ -induced decrease in protein synthesis at least in part by maintaining mTOR kinase activity, while post-treatment with ATA is able to increase protein synthesis when added up to 6 h after LPS/IFN γ . ATA also reverses the amino acid resistance which is detected in response to nutrient deprivation. Conversely, ATA decreases the basal rate of protein degradation and prevents the LPS/IFN γ -increase in proteolysis, and the latter change is associated reduced atrogen-1 and MuRF1 mRNA. The ability of ATA to antagonize LPS/IFN γ -induced changes in protein metabolism were associated with its ability to prevent the increases in IL-6 and NOS2 and decreases in IGF-I. In vivo studies indicate ATA acutely increases skeletal muscle, but not cardiac, protein synthesis, and attenuates the loss of lean body mass over 5 days. These data suggest ATA and other small molecule agonists of endogenous anabolic hormones may prove beneficial in treating sepsis by decreasing the inflammatory response and improving muscle protein balance.

Keywords

mTOR; 4E-BP1; S6K1; LPS; sepsis

INTRODUCTION

One hallmark of sustained catabolic illness is the erosion of lean body mass (LBM) and a reduction in muscle protein reserves which cannot be solely explained by a diminished

Address correspondence to: Charles H. Lang, PhD, Cell. & Molec. Physiology (H166), 500 University Drive, Penn State College Medicine, Hershey, PA 17033, 717.531.5538 (phone), 717.531.7667 (fax), clang@psu.edu.

DISCLOSURE

The authors have no conflict of interest to disclose.

caloric intake (1). Over the short-term, this catabolic response is potentially beneficial to the host by releasing amino acids used to support hepatic gluconeogenesis and acute phase protein secretion; however, prolonged cachexia produces skeletal muscle atrophy which negatively impacts muscle function and impairs recovery of this patient population (2). Muscle protein content represents an equilibrium between rates of synthesis and degradation, and gram-negative infection modulates both sides of this protein balance equation (3, 4). As the intake of food is intermittent and since individuals need to maintain circulating nutrients within a narrow physiological range, mechanisms have evolved to maintain whole-body and tissue protein homeostasis.

One such key metabolic regulator in eukaryotes is the mammalian or mechanistic target of rapamycin (mTOR). This protein is a serine (S)/threonine (T) kinase regulating protein translation and other metabolic processes (3–5). mTOR forms the catalytic core for two complexes which contain both common protein partners (i.e., GβL and Deptor) as well as unique accessory proteins. To date, the majority of studies have focused on mTOR complex (mTORC)-1 which regulates mRNA translation initiation by phosphorylation of the translational repressor protein eukaryotic initiation factor (eIF) 4E-binding protein (4E-BP1) as well as the AGC kinase family member S6 kinase (S6K)-1. Previous work has revealed sepsis decreases mTOR activity in skeletal muscle, as evidenced by a reduced phosphorylation of both 4E-BP1 and S6K1 (or its downstream substrate the ribosomal protein S6) (4, 6–8). A similar defect in the canonical pathway for muscle protein synthesis is also observed in related catabolic conditions produced by gram-negative endotoxin [lipopolysaccharide (LPS)] and excess proinflammatory cytokines (9–13). Moreover, this catabolic insult can be reproduced in vitro by culturing myocytes with a combination of LPS and interferon (IFN)- γ (14, 15).

In addition to decreasing basal muscle protein synthesis, sepsis also leads to the development of an “anabolic resistance” where various hormones and nutritional signals (e.g., growth hormone, insulin and leucine) fail to appropriately stimulate protein synthesis (7, 9, 16, 17). Hence, the provision of adequate nutritional support fails to completely resolve the loss of muscle and increased nitrogen excretion (1). In this regard, the negatively charged, polyaromatic carboxylic acid derivative aurintricarboxylic acid (ATA) has been reported to protect against viral infection, cardiac ischemia/reperfusion injury, and to increase cell survival in response to several stress conditions (18–21). Furthermore, other reports indicate that ATA stimulates protein synthesis under in vitro conditions in neural cells (18). The primary mechanism by which ATA exerts this biological function appears via activation of the insulin-like growth factor (IGF)-I receptor (IGF-R1) at the cell surface (19, 21), as this molecule is not believed to be cell membrane permeable (20). IGF-IR activation leads to downstream tyrosine phosphorylation of several different signaling pathways, including mitogen-activated protein kinase (MAPK) (20), Shc, phosphatidylinositol 3-kinase (PI-3K) and phospholipase C (PLC) γ (22), as well as inhibition of calcium-dependent nucleases (23, 24).

The purpose of this study was to investigate whether ATA-mediated activation of IGF-IR signaling regulates protein translation initiation via mTOR-mediated signal transduction mechanisms specifically in skeletal muscle. Additional studies were also performed to

determine whether ATA could prevent or ameliorate the reduction in muscle protein synthesis observed in vivo in response to peritonitis and/or in vitro in C2C12 myotubes incubated with a combination of LPS + IFN γ . Our studies address the hypothesis that ATA will reverse the sepsis- and LPS-induced decrease in protein synthesis by enhancing mTOR activity. These data will increase our understanding of mechanisms by which a prototypical non-peptide drug can modulate sepsis- and inflammation-induced changes in metabolism and in doing so may be useful in the design of other novel drugs to serve as adjunct therapeutic agents.

MATERIALS and METHODS

Cell culture

The C2C12 mouse myoblast cell line was purchased from the American Type Culture Collection (Manassas, VA) and used for all in vitro studies. Cells were grown in 100 mm petri dishes and cultured in minimal essential medium (MEM) containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), and amphotericin B (25 μ g/ml) (Mediatech, Herndon, VA), as described (14, 15, 25). Cells were grown to near confluence and sub-cultured to six-well plates. In most experiments, cells were differentiated in 10% bovine calf serum (BCS) for 7 d or longer to obtain cultures composed of more than 90% myotubes. Experiments were performed with ultrapure *Escherichia coli* LPS 011:B4 (Invivogen, San Diego, CA) and mouse IFN γ (Biosource, Camarillo, TX). The tripalmitoylated peptide containing cysteine, serine, and lysine (PAM) was purchased from Calbiochem (La Jolla, CA). The concentrations of LPS, IFN γ , PAM and IGF-I used in these experiments were based on dose-response curves generated in these and previous studies (14, 15, 25), and each agent was dissolved in serum free (SF)-MEM prior to addition. ATA was dissolved in SFMEM and the pH adjusted to 7.3–7.4; control cells received the same volume of SFMEM. Unless otherwise noted, myotubes were incubated with 200 μ g/ml ATA. The Dulbecco's Phosphate Buffered Saline (DPBS), SFMEM, glucose, and 100 $\times$ MEM amino acids, and ATA were all obtained from Sigma Aldrich (St. Louis, MO). An additional experiment tested the efficacy of rapamycin (50 nM; Biomol, Plymouth Meeting, PA) to block mTOR signaling, and its concentration was based on dose-response curves from preliminary studies in C2C12 cells. Rapamycin was dissolved in ethanol and was diluted using serum-free MEM before addition to myotubes. The final ethanol concentration in media of cultured myotubes was 0.05% and did not alter protein synthesis (data not shown). In the rapamycin study, vehicle-treated myotubes were exposed to the same final concentration of ethanol. In some studies, C2C12 cells were switched to serum-free medium and transiently transfected with a pNF κ B-Luc reporter vector (BD Biosciences, Palo Alto, CA) or pSV- β -galactosidase control vector (Promega, Madison, WI) using electroporation and the cell line nucleofector kit V (Amaxa, Germany) following the manufacturer's protocol, as previously described (25, 26). In some studies, the interleukin (IL)-6 protein concentration was determined in culture media using a mouse-specific ELISA (BD Biosciences, San Diego, CA).

Western analysis

Cell extracts were electrophoresed on polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride, as previously described (6, 7, 9, 27). The resulting blots were blocked with 5% nonfat dry milk and incubated with the following antibodies from Cell Signaling Technology (Beverly, MA): phosphorylated (S240/244) and total ribosomal protein S6, phosphorylated (T389) and total S6K1, phosphorylated (S2448) and total mTOR, phosphorylated (T37/46) 4E-BP1, and phosphorylated (S473) and total Akt. In addition, atrogin-1 protein was determined by Western analysis (FBXO32, MyBioSource, LLC, San Diego, CA). Unbound primary antibody was removed by washing with Tris-buffered saline containing 0.05% Tween 20, and blots were incubated with anti-rabbit or anti-mouse immunoglobulin conjugated with horseradish peroxidase. Blots were briefly incubated with the components of an enhanced chemiluminescence detection system (Supersignal Pico; Pierce Chemical, Rockford, IL). Dried blots were used to expose x-ray film for 1–30 min to achieve a signal within the linear range. Each film was then scanned with a Microtek Scanmaker 4 scanner (Microtek, Cerritos, CA) to generate a digital image which was analyzed and quantified (Scion Image 3b, Scion Corp., Frederick, MD).

RNA extraction and real-time quantitative PCR

Total RNA was extracted using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) and RNeasy mini kit (Qiagen, Valencia, CA) following manufacturers' protocols, as previously described (28). Briefly, tissue was homogenized in Tri-reagent followed by phenol/chloroform extraction according to the manufacturer's instruction. An equal volume of 70% ethanol was added to the aqueous phase and the mixture was loaded on a Qiagen mini-spin column. The Qiagen mini-kit protocol was followed from this step onwards including the on-column DNase I treatment to remove residual DNA contamination. RNA was eluted from the column with RNase-free water and an aliquot was used for quantitation (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA). Quality of the RNA was analyzed on a 1% agarose gel. Total RNA (1 µg) was reversed transcribed to cDNA using superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) in a total reaction volume of 20 µl following manufacturer's instruction. Real-time quantitative PCR was performed on 1–2 µl of the reversed transcribed reaction mix in a StepOnePlus system using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for: Atrogin-1 (F-box protein 32; NM_026346.2), muscle RING-finger 1 (MuRF1; NM_001039048.2), interleukin (IL)-6 (NM_031168.1), nitric oxide synthase (NOS)-2 (NM_010927.3), and insulin-like growth factor-I (IGF, NM_010512.4), as well as the ribosomal protein L32 (NM_172086.2) which was used as an endogenous control for normalization. Two negative controls were run with each qRT-PCR assay; a) a minus-reverse transcriptase control to assess the amount of DNA contamination present in an RNA preparation (4 samples per gene of interest), and b) a no template control, which omits DNA or RNA template from a reaction, and is a general control for extraneous nucleic acid contamination. The comparative quantitation method 2-

Ct was used in presenting gene expression of target genes in reference to the endogenous control (i.e., L32).

Protein synthesis and degradation – in vitro

For in vitro studies, C2C12 myotubes were cultured as described above in the presence or absence of a combination of LPS and IFN γ for 12 h. Cells were then labeled with 2 μ Ci/well of [3 H]-phenylalanine (132 Ci/mmol; Amersham, Arlington Heights, IL) in the presence of excess unlabeled phenylalanine for 1 h, as previously described (14, 15, 26). Cells were washed with MEM and isolated, and cell protein was precipitated overnight at 4 °C in a final concentration of 10% trichloroacetic acid (TCA). TCA pellets were washed with 10% TCA, solubilized in 2 N NaOH and radioactivity determined (Scintsafe; Pittsburgh, PA). Protein synthesis measurements were linear with the amount of tracer added to the cells, the number of cells per well, and the incubation time (data not shown).

Protein degradation was determined as previously described (29). Briefly, myotubes were pulse labeled for 48 h with [3 H]-L-tyrosine (2 μ Ci/well). Some cells were collected at this time (pulse cells), whereas for other cells the radiolabeled medium was removed and replaced with fresh medium lacking radioactivity (chase). Cells were then chased for 24 h in the absence (control) or presence of either LPS/IFN γ $\pm$ ATA (200 μ g/ml). Cells were collected and precipitated in 10% TCA and the TCA-precipitable counts determined. Data were normalized to cell protein and expressed as a percent change from control values.

Animals and in vivo experimental protocol

The animal protocol was approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine and adhere to the National Institutes of Health (NIH) guidelines for the use of experimental animals. Mice were randomized between experimental groups and treatment ($\pm$ ATA) performed in a blinded manner.

Polymicrobial peritonitis was produced by cecal ligation and puncture (CLP), as described previously (27, 30). Specific pathogen-free male C57Bl/6 mice (8–10 wks of age, ~25 g, Charles River, Wilmington, MA) were anesthetized using isoflurane (3% induction +1.5–2% maintenance) (Abbott Laboratories, North Chicago, IL). The abdomen was shaved and the skin prepared with povidone-iodine; a midline incision (1.5 cm) was made below the diaphragm. The cecum was isolated, ligated, punctured once using a 25-gauge needle, and a small amount of cecal material extruded. The cecum was returned to the abdomen, the muscle incision closed with 4–0 surgical suture (Ethicon, Inc., Somerville, NJ), and metal wound clips were used to close the skin incision. Before suturing the skin, 2 to 3 drops of lidocaine (Abbott Laboratories) were administered to the wound for analgesia. All mice were housed individually after surgery and each received 1 mL of warmed (37 °C) 0.9% sterile saline containing 0.05 mg/kg of Buprenorphine (Reckitt Benckiser Pharmaceuticals Inc, Richmond, VA) administered subcutaneously every 12 h for the remainder of the experimental protocol. Sham control mice were subjected to the same surgical laparotomy and cecal isolation, but the cecum was neither ligated nor punctured; control mice were also administered saline/Buprenorphine every 12 h to match that given to septic mice. Control (nonseptic) and septic mice were injected with ATA (15 mg/kg; IP) twice daily starting 12 h after CLP, at a dosing regimen previously shown to be effective to antagonize enterotoxin-induced apoptosis in vivo in mice (31). For these studies, ATA was dissolved in 0.9% sterile saline and the pH adjusted to 7.3–7.4; control animals received an equal volume of saline.

Survival was assessed in all four experimental groups in a blinded manner by recording deaths every 12 h for 5 days; this study was performed two separate times. Although survival was an endpoint, any mouse determined to be moribund was anesthetized and killed instead of waiting for spontaneous death (32). Food (Teklad Global 2019; Harlan, Teklad, Boston, MA) and water were offered ad libitum. At the end of the 5-day observation period, whole-body lean mass and muscle protein synthesis were determined on all surviving mice. LBM was quantitated using ^1H -NMR (Bruker Minispec, LF90, Woodlands, TX), as previously reported (27, 28, 30). Next, a final injection of ATA or vehicle was administered and the in vivo rate of protein synthesis in the gastrocnemius-plantaris complex (hereafter referred to as skeletal muscle) and heart was determined 1 h later. Protein synthesis was determined using the flooding-dose technique (27, 28). Mice were injected intraperitoneal with [^3H]-L-phenylalanine (Phe; 150 mM, 30 $\mu\text{Ci}/\text{ml}$; 1 ml/100 g BW) and blood was collected 15 min later for determining the plasma Phe specific activity. Thereafter, skeletal muscle were rapidly excised, freeze-clamped and then stored at -70°C . The rate of protein synthesis was calculated by dividing the amount of radioactivity incorporated into protein by the plasma Phe specific radioactivity. The specific radioactivity of the plasma Phe was measured by high performance liquid chromatography analysis of supernatant from TCA extracts of plasma.

Statistics

Values are means $\pm$ SEM. For in vitro studies, each experimental condition was tested in at least triplicate and each experiment was repeated at least twice. Data were analyzed by 2-way ANOVA followed by Student-Newman-Keuls test. Differences in survival were analyzed by a Kaplan-Meier survival plot and the log-rank statistic. Statistical significance was set at $P < 0.05$.

RESULTS

ATA increases protein synthesis in C2C12 myotubes

Incubation of differentiated myotubes with ATA increased global protein synthesis in a dose-dependent manner, with doses of ATA $> 0.1 \mu\text{g}/\text{ml}$ producing a significant increase in protein synthesis; protein synthesis in response to $0.01 \mu\text{g}/\text{ml}$ ATA did not differ from time-matched vehicle control values (Figure 1A). A comparable ATA-induced increase in protein synthesis was also seen in undifferentiated *myoblasts* (data not shown). This increase in protein synthesis was associated with a time-dependent increase in T389-phosphorylation of the mTOR substrate S6K1 (Figure 1B). The ATA-induced increase in S6K1 phosphorylation was evident as early as 0.25 h and persisted at least up to 6 h post-treatment. The increased phosphorylation of S6K1 by ATA appeared as great as the stimulation produced by a maximal-stimulating concentration of IGF-I (Figure 1B). The ATA and IGF-I induced changes in S6K1 phosphorylation were independent of a change in total S6K1 protein in these conditions (data not shown). ATA also increased the phosphorylation of AKT (S473), 4E-BP1 (T46/37) as well as the auto-phosphorylation of mTOR at S2448 (Figure 1C). Finally, the increased protein synthesis in response to ATA was mTORC1-dependent, as rapamycin prevented the ATA-induced increase in protein synthesis in (Figure 1D). Rapamycin also prevented the ability of IGF-I to increase protein

synthesis. Collectively, these data suggest the ATA-induced increase in basal protein synthesis is primarily regulated by an mTORC1-mediated increase in translation initiation. Subsequent studies investigated whether ATA could prevent or reverse sepsis- or inflammation-induced changes in muscle protein synthesis.

ATA inhibits LPS-induced changes in protein synthesis

We have previously reported that incubation of myotubes with LPS + IFN γ decreases protein synthesis via an mTORC1-dependent suppression of translation initiation (19, 20). Figure 2A illustrates that protein synthesis was decreased 60% in myotubes cultured with LPS + IFN γ for 12 h and then label with [3 H]-phenylalanine for an additional 1 h. Protein synthesis in control (e.g., no LPS/IFN γ) cells incubated with ATA for 12 h did not differ from untreated control cells. This lack of response, which differs from that reported in Figure 1, presumably occurs because the ATA-induced increase in protein synthesis was transient and had returned to basal levels by the 12 h time point. However, pretreatment of myotubes with ATA prior to addition of LPS + IFN γ prevented the decrease in protein synthesis. The ability of ATA to prevent the LPS-induced decrease in protein synthesis was associated with a reversal of the LPS-induced decrease in the phosphorylation of 4E-BP1 and ribosomal protein S6 (a downstream substrate for S6K1) as well as the increased NOS2 protein content in myotubes (Figure 2B).

The preceding studies were performed under conditions where ATA was added 30 min prior to LPS/IFN γ . We next examined whether ATA could reverse the LPS/IFN γ -induced decrease in protein synthesis when added at various times *after* LPS. ATA completely prevented the decrease in protein synthesis observed 12 h after LPS when ATA was added at either 1 h or 4 h after LPS/IFN γ treatment (Figure 3A and 3B). Even when added 12 h after LPS/IFN γ , ATA partially reversed the normally observed reduction in myotube protein synthesis (Figure 3C).

Nutrient deprivation is a well-characterized model in which mTOR activity and translation initiation are severely depressed (20). Essentially no 4E-BP1 phosphorylation was detected in myotubes cultured under nutrient deprived conditions (e.g., DPBS) either in the absence or presence of LPS/IFN γ (Figure 4A). Addition of 1 $\times$ total amino acids (AAs) in combination with glucose markedly increased 4E-BP1 phosphorylation after 30 min in control cells, but not in cells treated with LPS/IFN γ . These data demonstrate the ability of LPS/IFN γ to produce a nutrient-resistant condition *in vitro*. In this long-term nutrient deprivation model, restoration of mTOR activity requires the addition of a carbon source (e.g., glucose) and amino acids (20). Addition of 1 $\times$ AAs alone to nutrient deprived cells did not increase S6 or 4E-BP1 phosphorylation (data not shown). In contrast, incubation of myotubes with ATA during the period of nutrient deprivation restored the ability of 1 $\times$ AA + glucose to stimulate 4E-BP1 phosphorylation in LPS/IFN γ treated cells (Figure 4B). Comparable results were obtained for S6K1 phosphorylation (data not shown).

ATA prevents LPS-induced increases in protein degradation

The other side of the protein balance equation involves protein degradation which is regulated by a diverse number of mechanisms (33). One such mechanism involves activation

of the ubiquitin-proteasome pathway and is tightly associated with an increase in the muscle-specific E3 ligases atrogin-1 and MuRF1. Atrogin-1 protein and mRNA content of myotubes was elevated 3- to 4-fold in response to LPS/IFN γ and ATA dose-dependently suppressed this increase (Figure 5A and 5B). Atrogin-1 mRNA did not differ in SFMEM-treated control (no added LPS/IFN γ) myotubes in the absence (1.00 ± 0.11 AU/L32) or presence (0.96 ± 0.13 AU/L32) of ATA (200 μ g/ml); the effect of ATA on atrogin-1 protein content under basal conditions was not assessed. Comparable changes in the mRNA content for the other E3 ligase MuRF1 were observed with LPS and ATA (data not shown). ATA also completely prevented the dexamethasone-induced increase in atrogin-1 (data not shown). Finally, in separate study, Figure 5C illustrates that ATA decreased the basal rate of protein degradation and also prevented the LPS/IFN γ -induced increase in proteolysis.

ATA inhibits LPS-induced changes in inflammatory markers

Many of the sepsis- and LPS-induced disturbances in muscle protein balance can be attributed at least in part to the over production of inflammatory mediators (3). LPS/IFN γ increased IL-6 and NOS2 mRNA in myotubes (Figure 6A and 6B, respectively). Moreover, this increase was associated with increased NF- κ B reporter activity in transiently transfected C2C12 cells (Figure 6C). Finally, LPS/IFN γ decreased the mRNA content for the anabolic hormone IGF-I and this drop was prevented by ATA (Figure 6D). Pretreatment of myocytes with ATA completely prevented the LPS/IFN γ -induced increase in these inflammatory endpoints and increased IGF-I mRNA. A comparable effect of ATA was also observed in TNF α -treated myotubes (data not shown). To verify the changes in mRNA content were coordinate with changes in protein, we also determined IL-6 secreted into the media (Figure 7). Incubation of cells with LPS/IFN γ increased the IL-6 protein concentration and pretreatment with ATA completely prevented this increase.

LPS was used in the previous studies to activate Toll-like receptor (TLR)-4. However, myotubes also contain other TLRs which might be of importance (18), for example TLR-2. Therefore, we also examined the ability of ATA to block the inflammatory response produced by a TLR-2 ligand (i.e., tripalmitoylated peptide containing cysteine, serine and lysine; PAM). Pretreatment of C2C12 cells with ATA completely prevented the increase in IL-6 and NOS2 mRNA content and the reduction in IGF-I mRNA produced by PAM (data not shown).

In vivo protein synthesis, body composition and survival in CLP sepsis

Finally, we determined whether in vivo administration of ATA could modulate sepsis-induced changes in protein synthesis and body composition. In nonseptic control mice, ATA did not alter body weight, LBM or gastrocnemius weight in control mice, but did increase skeletal muscle protein synthesis 20% (Figure 8). At the end of the 5-day septic period, body weight and LBM were reduced 14%, gastrocnemius mass reduced 38% and skeletal muscle protein synthesis reduced 32%, compared to time-matched nonseptic mice. Twice daily treatment of septic mice with ATA ameliorated these sepsis-induced changes and returned skeletal muscle protein synthesis back to basal nonseptic values. There was no significant difference ($P = \text{NS}$) for myocardial protein synthesis in nonseptic (2.21 ± 0.14 nmol/h/mg protein) and septic (2.13 ± 0.11 nmol/h/mg protein) mice under control (no ATA) conditions,

or in response to ATA (nonseptic = 2.28 ± 0.32 nmol/h/mg protein; septic = 2.07 ± 0.31 nmol/h/mg protein).

Figure 8E indicates that muscle atrogin-1 mRNA was still elevated 5 days after induction of sepsis in vehicle-treated mice, but this increase was not seen in septic mice treated with ATA. In contrast, there was no sepsis-induced change in the mRNA content for the other E3-ligase, MuRF1, in gastrocnemius after 5 days, and there was no ATA effect on either nonseptic or septic mice (data not shown). Likewise, ATA antagonized the sepsis-induced increase in muscle IL-6 mRNA (Figure 8F).

Despite the general anabolic nature of ATA in septic mice, 5-day survival did not differ between the septic + vehicle (27% survival; 22 dead/30 total) and septic + ATA (33% survival; 20 dead/30 total) groups. Survival at 5 days was 100% for nonseptic controls + vehicle as well as nonseptic controls + ATA.

DISCUSSION

This study investigates the potential of a small molecular weight (473 Da) nonpeptidyl molecule capable of mimicking, both in vitro and in vivo, the metabolic and cell protective effects of the endogenous hormone IGF-I. Such drugs can potentially be given orally and thereby circumvent one of the major limitations associated with exogenous administration of native peptide hormones. The primary cellular mechanism by which ATA functions appears to be via binding and activation of IGF-IR, where ATA functions as a competitive agonist (19, 21). In vitro studies in neuronal cells have reported that ATA induces phosphorylation of the IGF-IR and subsequent downstream activation of the MAPK and PI3K-AKT signal transduction networks (19, 22, 34). Moreover, there appears to be a more rapid dephosphorylation of the IGF-I-activated IGF-IR, as opposed to a slower dephosphorylation by ATA (19). In addition or alternatively, ATA may also inhibit protein tyrosine phosphatase activity (20), a potential mechanism which was not the focus of the current study. Collectively, these data suggest ATA might prove beneficial as a therapeutic adjunct in conditions where the synthesis or bioavailability of the endogenous ligand is reduced, such as sepsis.

Our results extend previous findings by demonstrating that ATA activates the AKT-mTOR signaling pathway in muscle cells, both under in vitro and in vivo conditions. Our studies in differentiated myotubes show ATA stimulation of AKT and mTOR activity is more prolonged than that induced by IGF-I, which is consistent with the presumption that ATA-induced change in IGF-IR configuration and activation of downstream signaling pathways differs from that of the natural ligand. Importantly, we have also shown mTOR activation leads to a pronounced stimulation in protein synthesis, a physiologically relevant endpoint in skeletal muscle. Importantly, we have shown that ATA is also capable of stimulating mTOR-dependent protein synthesis in LPS/IFN γ -treated myotubes at a time when protein synthesis is depressed (current data) and their ability to respond to anabolic stimuli is reduced (15). The ability of ATA to prevent the LPS/IFN γ -induced decrease in protein synthesis was associated with its ability to prevent the increase in IL-6 and NOS2 which is likely related to the suppression of NF- κ B activation. These data are consistent with

previous observations that ATA prevents the LPS-induced increase in NOS2 protein expression in RAW 264.7 cells (35) and inhibits NF- κ B activation in macrophages (35). Additionally, our data show ATA also prevents the LPS/IFN γ -induced reduction in IGF-I mRNA content. As elevated endogenous production of IL-6 and NO as well as decreased IGF-I can impair muscle protein synthesis (26), this might be one mechanism by which ATA preserves protein balance in myocytes. Although not extensively investigated in the present study, we demonstrated that pretreatment with ATA also prevented the inflammatory response produced by TLR2 activation suggesting a broad efficacy for this small molecule. ATA also appeared to effectively sensitize myotubes to the anabolic action of amino acids, which might prove important in stimulating muscle protein synthesis after meal feeding in conditions associated with the presence of elevated inflammatory cytokines and/or reduced IGF-I concentrations. Finally, our in vitro data indicate that ATA need not be given preemptively to overcome the catabolic effects of LPS/IFN γ . That is, ATA treatment of myotubes for up to at least 6 h (but not 12 h) after LPS/IFN γ was capable of stimulating protein synthesis.

The sepsis-induced loss of muscle mass can also in part be mediated via activation of the ubiquitin-proteasome pathway (33) which may be overcome by activation of the IGF-IR. Our data show ATA prevents the increased proteolysis observed in response to LPS/IFN γ and this inhibitory effect was associated with a reduction in both atrogen-1 and MuRF1, which are muscle-specific E3 ligases central for ubiquitin-proteasome-mediated protein degradation. These data are complementary to previous reports that ATA is a calpain inhibitor (24).

Finally, given the positive anabolic response and anti-inflammatory effects of ATA in LPS/IFN γ -stimulated myotubes, an in vivo study was conducted to demonstrate efficacy and proof-of-principle. When assessed 1 h after IP injection, ATA increased protein synthesis in gastrocnemius of both control and septic mice, and this increase appeared to be of comparable magnitude. This comparable increase is noteworthy as sepsis produces anabolic resistance in skeletal muscle to growth factors and nutrient stimulants (3, 7, 8). Hence, ATA may potentially circumvent one or more of the sepsis-induced blocks in skeletal muscle protein synthesis. Conversely, ATA did not increase protein synthesis in heart. The reason why cardiac muscle lacked an anabolic response to ATA was not pursued but may be related to heart being less sensitive than gastrocnemius to anabolic stimulation and/or tissue-specific differences in the temporal activation of the synthetic response. ATA also appeared to down-regulate the sepsis-induced increase in skeletal muscle protein degradation, as evidenced by the reduction in the sepsis-induced increase in atrogen-1 mRNA, but a more in-depth analysis of this side of the protein balance equation is needed in future studies. Collective, the ATA-induced changes in skeletal muscle protein balance had an effect at the whole body level as ATA partially antagonized the sepsis-induced decrease in body weight and LBM. While the mechanisms for these ATA effects of muscle protein homeostasis were not investigated, we did note ATA was capable of preventing the sepsis-induced increase in muscle IL-6 mRNA. However, despite the positive anabolic effects, ATA treatment did not alter 5-day survival in after CLP. As these studies were designed as proof-of-principle, we cannot exclude the possibility that changing the dosing regimen (dose, time, route of administration, etc) might improve efficacy. Alternatively, it is also possible that sepsis-

induced death in the CLP model is primarily driven by mechanisms unrelated to the catabolic response. Collectively our data suggest ATA and potentially other small molecule agonists of endogenous anabolic hormones may prove beneficial in the treatment of sepsis by decreasing the inflammatory response and improving muscle protein balance.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Danuta Huber, Jay Nystrom, Anne Pruznak and Gina Deiter. We also thank Dr. Robert Frost who performed some of the initial studies using ATA in our laboratory.

GRANTS

This work was supported in part by National Institutes of Health Grant GM-38032.

REFERENCES

1. Kinney JM. Metabolic responses of the critically ill patient. *Crit Care Clin.* 1995; 11(3):569–585. [PubMed: 7552971]
2. Casaer MP, Mesotten D, Hermans G, Wouters PJ, Schetz M, Meyfroidt G, Van Cromphaut S, Ingels C, Meersseman P, et al. Early versus late parenteral nutrition in critically ill adults. *N Engl J Med.* 2011; 365(6):506–517. [PubMed: 21714640]
3. Frost RA, Lang CH. mTor signaling in skeletal muscle during sepsis and inflammation: where does it all go wrong? *Physiology (Bethesda).* 2011; 26(2):83–96. [PubMed: 21487027]
4. Cooney RN, Kimball SR, Vary TC. Regulation of skeletal muscle protein turnover during sepsis: mechanisms and mediators. *Shock.* 1997; 7(1):1–16. [PubMed: 8989831]
5. Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell.* 2007; 12(1):9–22. [PubMed: 17613433]
6. Kazi AA, Pruznak AM, Frost RA, Lang CH. Sepsis-induced alterations in protein-protein interactions within mTOR complex 1 and the modulating effect of leucine on muscle protein synthesis. *Shock.* 2011; 35(2):117–125. [PubMed: 20577146]
7. Lang CH, Frost RA. Glucocorticoids and TNFalpha interact cooperatively to mediate sepsis-induced leucine resistance in skeletal muscle. *Mol Med.* 2006; 12(11–12):291–299. [PubMed: 17380194]
8. Nystrom G, Pruznak A, Huber D, Frost RA, Lang CH. Local insulin-like growth factor I prevents sepsis-induced muscle atrophy. *Metabolism.* 2009; 58(6):787–797. [PubMed: 19375133]
9. Lang CH, Frost RA. Endotoxin disrupts the leucine-signaling pathway involving phosphorylation of mTOR, 4E-BP1, and S6K1 in skeletal muscle. *J Cell Physiol.* 2005; 203(1):144–155. [PubMed: 15389631]
10. Lang CH, Frost RA, Nairn AC, MacLean DA, Vary TC. TNF-alpha impairs heart and skeletal muscle protein synthesis by altering translation initiation. *Am J Physiol Endocrinol Metab.* 2002; 282(2):E336–E347. [PubMed: 11788365]
11. Vary TC, Deiter G, Lang CH. Cytokine-triggered decreases in levels of phosphorylated eukaryotic initiation factor 4G in skeletal muscle during sepsis. *Shock.* 2006; 26(6):631–636. [PubMed: 17117141]
12. Cooney RN, Maish GO 3rd, Gilpin T, Shumate ML, Lang CH, Vary TC. Mechanism of IL-1 induced inhibition of protein synthesis in skeletal muscle. *Shock.* 1999; 11(4):235–241. [PubMed: 10220298]
13. Vary TC, Owens EL, Beers JK, Verner K, Cooney RN. Sepsis inhibits synthesis of myofibrillar and sarcoplasmic proteins: modulation by interleukin-1 receptor antagonist. *Shock.* 1996; 6(1):13–18. [PubMed: 8828078]
14. Frost RA, Nystrom GJ, Lang CH. Endotoxin and interferon-gamma inhibit translation in skeletal muscle cells by stimulating nitric oxide synthase activity. *Shock.* 2009; 32(4):416–426. [PubMed: 19295495]

15. Frost RA, Pereyra E, Lang CH. Ethyl pyruvate preserves IGF-I sensitivity toward mTOR substrates and protein synthesis in C2C12 myotubes. *Endocrinology*. 2011; 152(1):151–163. [PubMed: 21106878]
16. Lang CH, Deshpande N, Frost RA. Leucine acutely reverses burn-induced alterations in translation initiation in heart. *Shock*. 2004; 22(4):326–332. [PubMed: 15377887]
17. Carlson GL. Hunterian Lecture, Insulin resistance in human sepsis: implications for the nutritional and metabolic care of the critically ill surgical patient. *Ann R Coll Surg Engl*. 2004; 86(2):75–81. [PubMed: 15005922]
18. Heiduschka P, Thanos S. Aurintricarboxylic acid promotes survival and regeneration of axotomized retinal ganglion cells in vivo. *Neuropharmacology*. 2000; 39(5):889–902. [PubMed: 10699455]
19. Haimsohn M, Beery R, Karasik A, Kanety H, Geier A. Aurintricarboxylic acid induces a distinct activation of the IGF-I receptor signaling within MDA-231 cells. *Endocrinology*. 2002; 143(3):837–845. [PubMed: 11861505]
20. Cho H, Lee DY, Shrestha S, Shim YS, Kim KC, Kim MK, Lee KH, Won J, Kang JS. Aurintricarboxylic acid translocates across the plasma membrane, inhibits protein tyrosine phosphatase and prevents apoptosis in PC12 cells. *Mol Cells*. 2004; 18(1):46–52. [PubMed: 15359123]
21. Beery R, Haimsohn M, Wertheim N, Hemi R, Nir U, Karasik A, Kanety H, Geier A. Activation of the insulin-like growth factor 1 signaling pathway by the antiapoptotic agents aurintricarboxylic acid and Evans blue. *Endocrinology*. 2001; 142(7):3098–3107. [PubMed: 11416032]
22. Okada N, Koizumi S. A neuroprotective compound, aurin tricarboxylic acid, stimulates the tyrosine phosphorylation cascade in PC12 cells. *J Biol Chem*. 1995; 270(27):16464–16469. [PubMed: 7608219]
23. Lu H, Wei G, Wang D, Yung P, Ying W. Posttreatment with the Ca(2+)-Mg(2+)-dependent endonuclease inhibitor aurintricarboxylic acid abolishes genotoxic agent-induced nuclear condensation and DNA fragmentation and decreases death of astrocytes. *J Neurosci Res*. 2008; 86(13):2925–2931. [PubMed: 18506851]
24. Posner A, Raser KJ, Hajimohammadreza I, Yuen PW, Wang KK. Aurintricarboxylic acid is an inhibitor of μ - and m -calpain. *Biochem Mol Biol Int*. 1995; 36(2):291–299. [PubMed: 7663433]
25. Frost RA, Nystrom GJ, Lang CH. Multiple Toll-like receptor ligands induce an IL-6 transcriptional response in skeletal myocytes. *Am J Physiol Regul Integr Comp Physiol*. 2006; 290(3):R773–R784. [PubMed: 16254126]
26. Frost RA, Nystrom GJ, Lang CH. Lipopolysaccharide stimulates nitric oxide synthase-2 expression in murine skeletal muscle and C2C12 myoblasts via Toll-like receptor-4 and c-Jun NH(2)-terminal kinase pathways. *Am J Physiol Cell Physiol*. 2004; 287(6):C1605–C1615. [PubMed: 15282190]
27. Lang CH, Frost RA, Bronson SK, Lynch CJ, Vary TC. Skeletal muscle protein balance in mTOR heterozygous mice in response to inflammation and leucine. *Am J Physiol Endocrinol Metab*. 2010; 298(6):E1283–E1294. [PubMed: 20388826]
28. Lang SM, Kazi AA, Hong-Brown L, Lang CH. Delayed recovery of skeletal muscle mass following hindlimb immobilization in mTOR heterozygous mice. *PLoS One*. 2012; 7(6):e38910. [PubMed: 22745686]
29. Hong-Brown LQ, Brown CR, Navaratnarajah M, Huber DS, Lang CH. Alcohol-induced modulation of rictor and mTORC2 activity in C2C12 myoblasts. *Alcohol Clin Exp Res*. 2011; 35(8):1445–1453. [PubMed: 21438886]
30. Lang CH, Lynch CJ, Vary TC. BCATm deficiency ameliorates endotoxin-induced decrease in muscle protein synthesis and improves survival in septic mice. *Am J Physiol Regul Integr Comp Physiol*. 2010; 299(3):R935–R944. [PubMed: 20554928]
31. Liang D, Yang M, Guo B, Cao J, Yang L, Guo X, Li Y, Gao Z. Zinc inhibits H₂O₂-induced MC3T3-E1 cells apoptosis via MAPK and PI3K/AKT pathways. *Biol Trace Elem Res*. 2012; 148(3):420–429. [PubMed: 22434380]

32. Demers G, Griffin G, De Vroey G, Haywood JR, Zurlo J, Bedard M. Animal research. Harmonization of animal care and use guidance. *Science*. 2006; 312(5774):700–701. [PubMed: 16675685]
33. Smith IJ, Lecker SH, Hasselgren PO. Calpain activity and muscle wasting in sepsis. *Am J Physiol Endocrinol Metab*. 2008; 295(4):E762–E771. [PubMed: 18492780]
34. Okada N, Koizumi S. Tyrosine phosphorylation of ErbB4 is stimulated by aurintricarboxylic acid in human neuroblastoma SH-SY5Y cells. *Biochem Biophys Res Commun*. 1997; 230(2):266–269. [PubMed: 9016763]
35. Tsi CJ, Chao Y, Chen CW, Lin WW. Aurintricarboxylic acid protects against cell death caused by lipopolysaccharide in macrophages by decreasing inducible nitric-oxide synthase induction via IkappaB kinase, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase inhibition. *Mol Pharmacol*. 2002; 62(1):90–101. [PubMed: 12065759]

Figure 1.

Effect of aurointricarboxylic acid (ATA) on protein synthesis and mTORC1 substrate phosphorylation. *Panel A:* ATA dose response for protein synthesis. Myotubes were treated with increasing concentrations of ATA or vehicle (SFMEM) and protein synthesis measured using ^3H -phenylalanine incorporation over a period of 1 h. Data are expressed as a percent of vehicle-treated control value; * $P < 0.05$ compared to time-matched vehicle-treated control cells. *Panel B:* representative Western blots for T389-phosphorylated S6K1 in myotubes in the presence of a maximally- stimulating concentration of ATA (200 μg/ml) or

IGF-I (200 ng/ml) or vehicle (SFMEM) for the times indicated. At each time, the relatively amount of total S6K1 did not differ between SFMEM, ATA and IGF-I treated cells (data not shown). No significant stimulation of S6K1 phosphorylation was detected in either group at the 24 h time point (data not shown). *Panel C*: representative Western blots for total and phosphorylated 4E-PB1 and mTOR in control and ATA (200 µg/ml)- or SFMEM-treated myotubes at 1 h. *Panel D*; Protein synthesis in myotubes pretreated (-30 min) with the mTORC1 inhibitor rapamycin (50 nM) or vehicle (ethanol, final concentration 0.05%) prior to addition of IGF-I or ATA. Values are means $\pm$ SEM, with $n = 3-4$ wells of cells run in triplicate. Protein synthesis was determined over a 1 h period after addition of IGF-I or ATA. * $P < 0.05$ compared to time-matched control cells treated with vehicle. § $P < 0.05$, compared to control value from same treatment group. Where absent, error bars are too small to visualize.

Figure 2.

ATA prevents the LPS/IFN γ -induced decrease in protein synthesis and mTOR kinase activity. *Panel A:* Myotubes were cultured in the presence of ATA (200 μ g/ml) or SFMEM and/or LPS/IFN γ (1 μ g/ml + 3 ng/ml) for 12 h and protein synthesis determined over the subsequent 1 h period by the incorporation of 3 H-phenylalanine into TCA-precipitable protein. Bar graphs, values are means $\pm$ SEM ($n = 3-4$ wells of cells run in triplicate) with different superscripts (a, b, and c) being significantly different, $P < 0.05$. *Panel B:*

representative Western blots for phosphorylated 4E-BP1 and the ribosomal protein S6 as well as for NOS2 protein; n = 6 per treatment group.

A ATA Tx - 1 h post-LPS/IFN γ **B ATA Tx - 4 h post-LPS/IFN γ** **C ATA Tx - 12 h post-LPS/IFN γ** **Figure 3.**

ATA reverses LPS/IFN γ -induced decrease in protein synthesis. C2C12 myotubes were grown and treated with SFMEM (vehicle) or LPS/IFN γ . ATA (200 μ g/ml) or SFMEM was then added to myotubes at 1 h, 4 h or 12 h after the addition of LPS/IFN γ , and protein synthesis determined for a 1 h period after addition of ATA. Bar graphs, values are means $\pm$ SEM (n = 3–4 wells of cells run in triplicate) with different superscripts (a, b, and c) being significantly different, $P < 0.05$.

Figure 4.

LPS/IFN γ -induces resistance to amino acid mTOR signaling which is prevented by ATA. Western blots for the experimental conditions described below; blots are representative of 3 independent experiments. *Panel A:* C2C12 myotubes were cultured as described previously with the exception that they were treated with LPS/IFN γ for 6 h followed by a switch to DPBS for an additional 12 h. Some of the nutrient-deprived cells were restimulated with 1 $\times$ total amino acids (AA) + 1 mM glucose, and cell extracts were isolated after 30 min. Control cells were provided an equal molar amount of alanine + glucose. Extracts were run and transferred as described in METHODS and probed for 4E-BP1 phosphorylation (T37/46). *Panel B:* Myotubes were treated as above, except ATA was present during the period of nutrient deprivation. However, ATA was not present during the 30 min restimulation period following the addition of 1 $\times$ AA and glucose. Qualitatively similar results were obtained for S6K1 phosphorylation (data not shown).

Figure 5.

ATA alters rates of protein degradation and atrogin expression. *Panel A*: Representative Western blot for atrogin-1 protein in myotubes under control conditions (SFMEM) and in the absence or presence of LPS (1 μ g/ml) + IFN γ (3 ng/ml) and/or ATA ($\sim$ 200 μ g/ml) or SFMEM (vehicle). Tubulin was used as a loading control. Western blot is representative of three independent dose-response studies. *Panel B*: Quantitation of atrogin-1 mRNA content under conditions comparable to those described for panel A. Data were normalized to L32 and there was no difference in the L32 content between groups (data not shown). *Panel C*.

rates of in vitro-determined protein degradation, expressed as percentage of control values, was determined in myotubes under control conditions (SFMEM) and in the absence or presence of LPS (1 $\mu\text{g/ml}$) + IFN γ (3 ng/ml) and/or ATA (200 $\mu\text{g/ml}$) or SFMEM (vehicle). For both bar graphs, values are means $\pm$ SEM (n = 3–4 wells of cells run in triplicate) with different superscripts (a, b, and c) being significantly different, $P < 0.05$.

Figure 6.

ATA inhibits LPS + IFN γ -induced changes in inflammatory and metabolic markers.

Myotubes were treated in the presence or absence of LPS (1 μ g/ml) + IFN γ (3 ng/ml) and/or ATA (200 μ g/ml) or SFMEM (vehicle). Changes in the mRNA content for interleukin (IL)-6 (*panel A*) and nitric oxide synthase (NOS)-2 (*panel B*) were determined 6 h after addition of LPS/IFN γ and/or ATA. *Panel C*, NF- κ B reporter activity, where transfection efficiency was corrected by cotransfection with a pSV- β -Gal plasmid and normalization to β -galactosidase activity. *Panel D*, changes in insulin-like growth factor (IGF)-I mRNA content. All mRNA data were normalized to L32 and expressed as a percentage of the vehicle-treated control cells. Bar graphs, values are means $\pm$ SEM ($n = 3$ –4 wells of cells run in triplicate) with different superscripts (a, b, and c) being significantly different, $P < 0.05$.

Figure 7.

ATA inhibits LPS + IFN γ -induced changes in media IL-6 protein concentration in myotubes treated in the presence or absence of LPS (1 μ g/ml) + IFN γ (3 ng/ml) and/or ATA (200 μ g/ml) or SFMEM (vehicle). Values are means $\pm$ SEM (n = conditioned media from 3–4 wells of cells run in triplicate) with different superscripts (a, b, and c) being significantly different, $P < 0.05$.

Figure 8.

ATA ameliorates sepsis-induced decreases in body weight, lean body mass, muscle mass and muscle protein synthesis under in vivo conditions. ATA was injected twice daily (15 mg/kg, IP) starting 12 h after CLP. On day 5, body composition was determined and injected with the final injection of ATA. One hour thereafter in vivo protein synthesis was determined by IP injection of ^3H -phenylalanine and muscle collected 15 min later as in

METHODS. Values are means $\pm$ SEM; n = 8–10 mice per group. Means with different superscripts (a, b, and c) are significantly different, $P < 0.05$.