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HIGHLIGHTED TOPIC | *Regulation of Protein Metabolism in Exercise and Recovery*

Lipoic acid increases heat shock protein expression and inhibits stress kinase activation to improve insulin signaling in skeletal muscle from high-fat-fed rats

Anisha A. Gupte, Gregory L. Bomhoff, Jill K. Morris, Brittany K. Gorres, and Paige C. Geiger
Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas

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Gupte AA, Bomhoff GL, Morris JK, Gorres BK, Geiger PC. Lipoic acid increases heat shock protein expression and inhibits stress kinase activation to improve insulin signaling in skeletal muscle from high-fat-fed rats. *J Appl Physiol* 106: 1425–1434, 2009. First published January 29, 2009; doi:10.1152/japplphysiol.91210.2008.—The antioxidant α-lipoic acid (LA) has been shown to improve insulin action in high-fat (HF)-fed animal models, yet little is known about its underlying mechanisms of action. We hypothesize that LA acts by inducing heat shock proteins (HSPs), which then inhibit stress kinases known to interfere with insulin signaling intermediates. Male Wistar rats were fed a HF diet (60% calories from fat) for 6 wk, while controls received a chow diet (10% calories from fat). One-half of the rats in each group received daily LA injections (30 mg/kg body wt). In rats fed a HF diet, LA increased expression of HSP72 and activation of HSP25 in soleus muscle, but it had no effect on HSPs in muscle from chow-fed rats. LA treatment reduced phosphorylation of c-Jun NH_2-terminal kinase (JNK) and inhibitor of κB kinase-β (IKKβ) activity (IκBα protein levels) in rats fed a HF diet and effectively restored insulin responsiveness, as seen by insulin-stimulated phosphorylated Akt/Akt and 2-deoxyglucose uptake in soleus muscle. LA also induced activation of p38 MAPK and AMP-activated protein kinase, proteins previously implicated in insulin-independent glucose uptake. In addition, acute LA treatment induced HSPs in vitro in L6 muscle cells and prevented the activation of JNK and IKKβ with stimulants such as anisomycin and TNF-α, respectively. In conclusion, our results suggest chronic LA treatment results in stress kinase inhibition and improved insulin signaling through a HSP-mediated mechanism.

heat shock proteins; c-Jun NH_2-terminal kinase; inhibitor of κB kinase-β; glucose uptake; skeletal muscle

HIGH-FAT (HF)-DIET-INDUCED insulin resistance is associated with impaired insulin signal transduction and reduced glucose transport in skeletal muscle. Stress kinases, such as c-Jun NH_2-terminal kinase (JNK) and the inhibitor of κB kinase-β (IKKβ), are activated by oxidative stress, and activity levels of these kinases are high in skeletal muscles from insulin-resistant animals (14, 21, 40, 54). Chronically increased stress kinase activation is implicated in dysregulation of the insulin signaling pathway. JNK and IKKβ interfere with normal insulin signaling by phosphorylating the insulin receptor substrate (IRS-1) on serine 307, reducing its interaction with downstream effector phosphatidylinositol 3-kinase (PI3K) (55).

Specific inducible heat shock proteins (HSPs) have the potential to inhibit JNK and IKKβ (38, 39). HSPs are chaperone proteins, extensively studied in their role in mediating protein refolding, tissue protection, tissue repair, and cellular homeostasis (6). A study by Park et al. (38) showed that HSP72 can also function as a natural inhibitor of JNK by direct binding and subsequent prevention of its activation by upstream kinases. Similarly, the small HSP25 binds and inhibits the stress kinase IKKβ (2, 9, 39) and can regulate TNF-α-induced NF-κB activation (39). Our laboratory has recently demonstrated that HSP induction with heat treatment can improve whole body glucose tolerance and muscle-specific insulin sensitivity while reducing stress kinase activation in rats fed a HF diet (15). Another in vivo study by Chung et al. (8) demonstrated that increased HSP72 expression can protect against obesity-induced insulin resistance, and this was tightly associated with the prevention of JNK phosphorylation. Based on this evidence, a treatment that could induce HSPs in skeletal muscle has the potential to inhibit JNK and IKKβ and improve insulin action.

α-Lipoic acid (LA) is a short-chain fatty acid that acts as a cofactor in the pyruvate dehydrogenase (PDH) complexes and is a potent biological antioxidant (32, 37). Administration of LA has demonstrated beneficial effects in several disease states associated with oxidative stress, such as diabetic neuropathy, heavy metal poisoning, and liver cirrhosis in humans (56). A recent study showed that HF diet-induced phosphorylated serine 307-IRS-1 in skeletal muscle was reduced by treatment with an antioxidant mixture containing LA (47). In addition, increased skeletal muscle glucose uptake following LA treatment in obese Zucker rats was associated with an increase in IRS-1 content and insulin-stimulated PI3K activation (43). Although improvements in IRS-1 function have been shown in previous studies with LA treatment, the mechanisms by which LA improves IRS-1 function are unknown. A recent study showed that LA treatment can increase production of HSPs in diabetic kidney and plasma (36, 45), yet its effect on skeletal muscle HSPs is unknown. Therefore, the purpose of this study is to determine whether chronic LA treatment increases the expression of HSPs in skeletal muscle and whether this induction can effectively inhibit stress kinases and improve insulin action.

Address for reprint requests and other correspondence: P. C. Geiger, Dept. of Molecular and Integrative Physiology, Univ. of Kansas Medical Center, MS 3043, 3901 Rainbow Blvd., Kansas City, KS 66160 (e-mail: pgeiger@kumc.edu).

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METHODS

Materials. (±)-α-LA was obtained from Sigma (St. Louis, MO) and dissolved in Tris·HCl (120 mM, pH 7.4). [^{14}C]mannitol and 2-deoxy-[1,2-^{3}H]glucose were purchased from American Radiolabeled Chemicals (St. Louis, MO). Dulbecco's modified Eagles's medium (DMEM; 4,500 mg/l glucose), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Sigma. Antibodies against phospho-SAPK/JNK (T183/Y185), total SAPK/JNK, phospho-Akt (S473), total Akt, IκBα, and phospho-PKC-θ (T538), phospho-p38 MAPK (T180/Y182), total p38 MAPK, phospho-AMP-activated protein kinase (AMPK) (T172), and total AMPK were purchased from Cell Signaling (Beverly, MA). Antibodies against HSP72, phospho-HSP25 (S82), and total HSP25 were obtained from Stressgen (Victoria, BC, Canada), an antibody against tubulin was obtained from Sigma, and the actin antibody was obtained from Abcam (Cambridge, MA). Total PKC-θ antibodies and goat-anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while goat anti-mouse HRP-conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA). KNK-437 was purchased from Calbiochem (San Diego, CA). Enhanced chemiluminescence reagents were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). All other reagents were obtained from Sigma.

L6 cell culture and treatment. L6 myoblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured (37°C, 5% CO_2) in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were differentiated into myotubes by changing culture media to DMEM + 2% FBS 4–6 days after plating (at 80% confluence). Myotubes were used 5–6 days later upon full differentiation. Cells were incubated with 300 μM LA for 18 h, washed, and stimulated with either 10 μg/μl anisomycin (30 min) or 20 ng/ml TNF-α (6 h) before harvesting. For the HSP inhibition experiments, an additional set of myotubes was incubated with 300 μM LA and KNK-437 (10 μM) for 18 h. Cells were washed and then incubated with either anisomycin or TNF-α, as described above.

Experimental animals and treatment. Male Wistar rats (100–120 g) were purchased from Charles River Laboratories (Wilmington, MA) and were housed in a temperature-controlled (22 ± 2°C) room with 12-h light and dark cycles. Animals were randomly divided into four groups (n = 8 rats/group): *1*) chow+Tris (chow); *2*) chow+LA; *3*) HF; and *4*) HF+LA. Chow rats were fed ad libitum (Harlan Teklad, Madison, WI, 10% calories from fat), whereas HF rats received a modified Kraegen diet (44) [60% calories from fat and 20% calories from carbohydrates (44) for 6 wk; Table 1].

Subgroups were injected with LA daily, intraperitoneally, at a dose of 30 mg/kg body wt, or with vehicle Tris (8.3 ml/kg body wt, pH 7.4). The rats were fasted 12 h before all experimental procedures to lower and normalize basal plasma glucose levels before insulin stimulation. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt) for the removal of the soleus muscle. All protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center.

Table 1. *High-fat diet recipe*

Ingredient	g/kg
Casein	254
Sucrose	85
Cornstarch	169
Vitamin mix	11.7
Choline chloride	1.3
Mineral mix	67
Bran	51
Methionine	3
Gelatin	19
Corn oil	121
Lard	218

Muscle incubations. Following dissection, the soleus muscles were carefully trimmed and split longitudinally to allow adequate diffusion of substrates, as described previously (14, 19). Two muscle strips per rat were assessed for glucose transport, and two strips for Western blot analysis. Muscles strips, designated for Western blot analysis, recovered for 60 min in flasks containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol (recovery medium), and a gas phase of 95% O_2-5% CO_2. The flasks were placed in a shaking incubator maintained at 35°C. Following recovery, one muscle strip was treated with 1 mU/ml insulin, and the other muscle strip was left untreated in recovery medium (basal) for exactly 10 min and then clamp frozen in liquid nitrogen.

Measurement of glucose transport activity. The muscles recovered after dissection in recovery medium for 1 h at 35°C and then rinsed for 30 min at 29°C in 2 ml of oxygenated KHB containing 40 mM mannitol, with or without insulin (1 mU/ml). After the rinse step, muscles were incubated for 20 min at 29°C in flasks containing 2 ml KHB with 4 mM 2-[1,2-^{3}H]deoxyglucose (2-DG) (1.5 μCi/ml) and 36 mM [^{14}C]mannitol (0.2 μCi/ml), with or without insulin (1 mU/ml), with a gas phase of 95% O_2-5% CO_2, in a shaking incubator. The muscles were then blotted, clamp frozen, and processed, as described previously (12, 52), for determination of intracellular 2-DG accumulation (^{3}H dpm) and extracellular space (^{14}C dpm) on a scintillation counter.

Western blotting. Clamp-frozen soleus muscles were homogenized in a 12:1 (volume-to-weight) ratio of ice-cold buffer from Biosource (Invitrogen, Camarillo, CA) containing 10 mM Tris·HCl (pH 7.4); 100 mM NaCl; 1 mM each of EDTA, EGTA, NaF, and phenylmethylsulfonyl fluoride; 2 mM Na_3VO_4; 20 mM $Na_4P_2O_7$; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; and 250 μl/5 ml protease inhibitor cocktail. Homogenized samples were rotated for 30 min and then centrifuged for 30 min at 3,000 rpm at 4°C. The protein concentration of the supernatant was determined by the Bradford method (Bio-Rad). Samples were prepared in 2× Laemmli buffer containing 100 mM dithiothreitol and boiled in a water bath for 5 min.

Protein (30–100 μg) was separated on a SDS-PAGE (6.25–10% gel) followed by a wet transfer to a nitrocellulose membrane for 90 min (200 mA). To verify transfer of proteins and equal loading of lanes, the membranes were stained with Ponceau S (data not shown). Membranes were blocked for 1 h at room temperature in Tris-buffered saline, 0.1% Tween 20 (TBST) and 5% nonfat dry milk, followed by an overnight incubation with the appropriate primary antibodies at a concentration of 1:1,000. Antibodies were diluted in TBST, 5% BSA. Blots were incubated in TBST 1% nonfat dry milk, supplemented with an HRP-conjugated goat anti-rabbit secondary antibody at a concentration of 1:10,000 for 1 h at room temperature. Bands were visualized by ECL and quantified using Image J densitometry.

Statistical analysis. Two-way ANOVA was used when differences between both diet and LA treatments were studied. This was followed by a post hoc comparison using the Student-Newman-Keuls test when necessary. Statistical significance was set at $P < 0.05$.

RESULTS

Animal characteristics. Six weeks of the HF diet induced a small increase in body weight, with a trend for a decrease in body weight with LA treatment, although neither change was significant (chow 369 ± 35.4 g, chow+LA 360.5 ± 36.8 g, HF 397.25 ± 27.45 g, HF+LA 391 ± 30.07 g, P = 0.09). An intraperitoneal glucose tolerance test was performed to assess whole body glucose utilization in response to a glucose challenge. The HF-fed rats showed decreased glucose tolerance compared with both chow groups and did not effectively clear

the glucose bolus at the end of the 2-h period (Fig. 1*A*). LA treatment administered in parallel with the HF diet effectively improved glucose tolerance compared with nontreated HF-fed rats, in agreement with previous work (41). This is further reflected by a decrease in the glucose area under the curve (Fig. 1*B*).

Effects of LA treatment on insulin action in the soleus muscle. Skeletal muscle accounts for the largest amount of insulin-stimulated glucose transport, and, consequently, insulin resistance in skeletal muscle can reflect whole body changes in glucose homeostasis (5). To investigate the effects of a HF diet and LA on skeletal muscle glucose uptake, we performed 2-DG uptake assays on rat soleus muscles. The HF diet resulted in reduced insulin-stimulated glucose transport compared with the chow-fed rats (54.7% reduction in HF compared with chow, Fig. 2*A*). LA had no effect on glucose uptake in chow-fed rats. However, LA treatment significantly improved 2-DG uptake in the HF-fed rats (55.7% increase in HF+LA over HF). Akt is a serine threonine kinase in the insulin/IRS-1/PI3K signaling cascade, activation of which is crucial for insulin-stimulated glucose transport. Activation of Akt in response to insulin stimulation, as measured by phosphorylation of Akt on serine 473, was reduced in the muscles from rats fed a HF diet (47.8% reduction in HF compared with chow, Fig. 2*B*). LA treatment did not induce further Akt activation in chow-fed rats; however, it partially restored Akt phosphorylation in the HF diet rats (74% increase in HF+LA over HF). No significant changes were seen between groups for basal (non-insulin stimulated) glucose uptake or Akt activation.

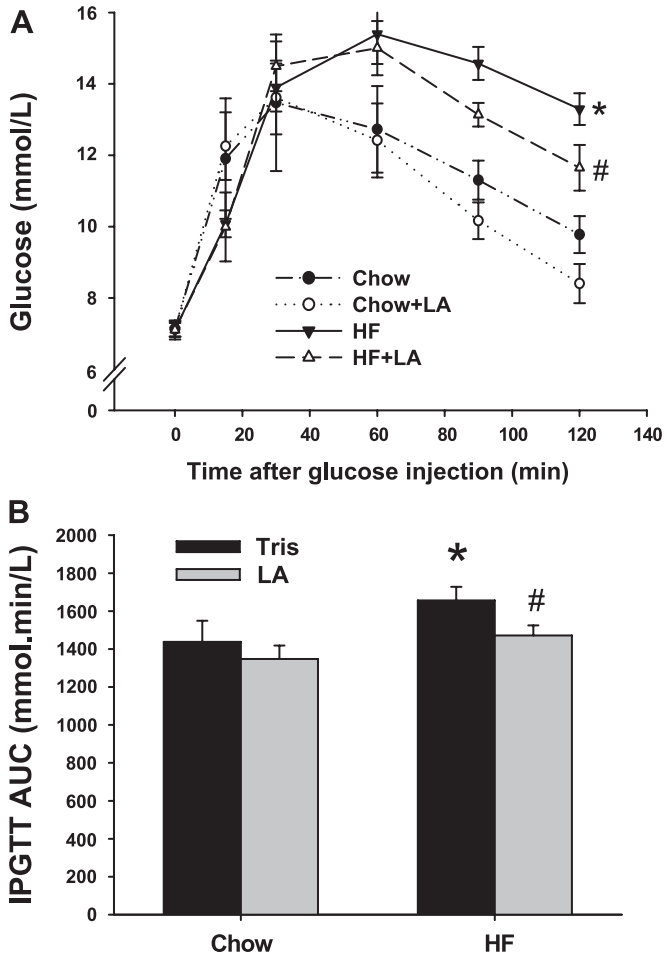

Fig. 1. Lipoic acid (LA) reverses high-fat (HF) diet-induced whole body insulin resistance. *A*: intraperitoneal glucose tolerance test (IPGTT). Overnight-fasted rats were injected with a glucose load of 2 g/kg body wt ip. Blood glucose was measured at 0, 15, 30, 60, 90, and 120 min postinjection using a glucometer. *B*: IPGTT is expressed as glucose area under the curve (AUC). Values are means ± SE for 6–8 rats/group. *$P < 0.05$, HF vs. chow. #$P < 0.05$, HF+LA vs. HF.

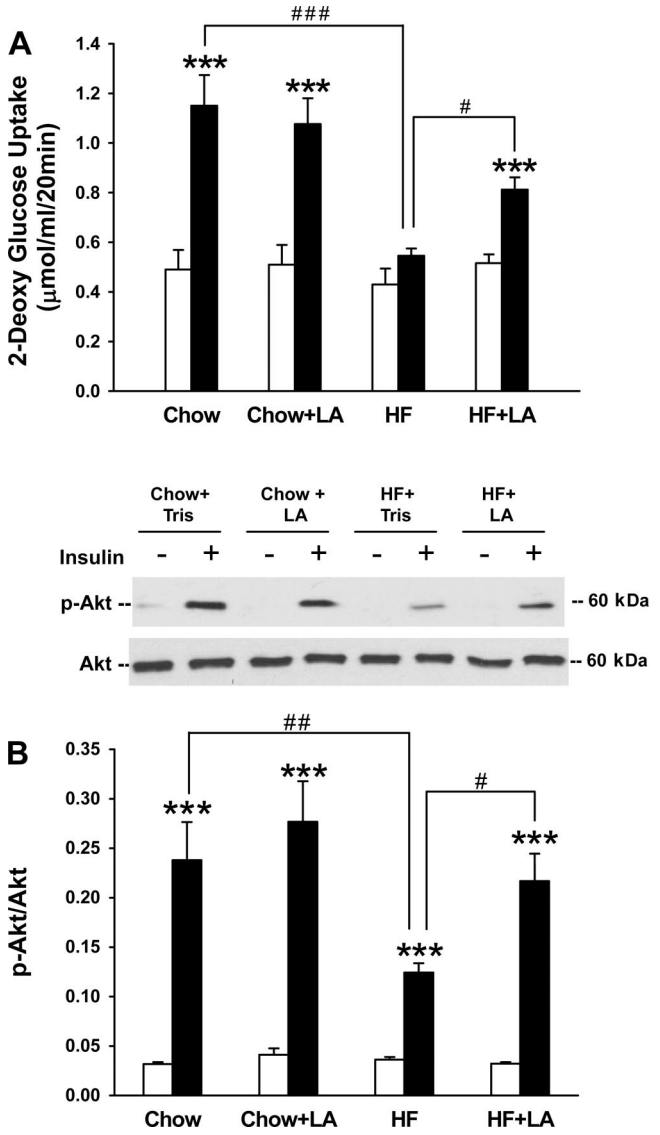

Fig. 2. LA improves glucose uptake and phosphorylation of Akt (p-Akt) in soleus muscles from HF-fed rats. *A*: insulin-stimulated glucose transport. Soleus muscles were incubated in the presence of insulin (1 mU/ml, solid bars) or in the absence of insulin (open bars), along with 2-[1,2-^{3}H]deoxyglucose and [^{14}C]mannitol. Insulin-stimulated 2-deoxyglucose uptake into the soleus muscles was determined. *B*: insulin-stimulated p-serine 473-Akt. Soleus muscles were incubated with insulin (1 mU/ml, solid bars) or without insulin (open bars) for exactly 10 min. Muscle lysates were separated with SDS-PAGE, and blots were analyzed for p-Akt. Blots were then stripped and probed for total Akt. Values are means ± SE for 6–8 (*A*) and 4–6 (*B*) muscles per group. ***$P < 0.001$, insulin vs. basal. ### $P < 0.001$, ## $P < 0.01$, chow vs. HF. #$P < 0.05$, HF vs. HF+LA.

LA induces activation of AMPK and p-38 MAPK. Apart from improving glucose metabolism through insulin-sensitive mechanisms, there is evidence that LA can improve glucose

uptake by activating insulin-independent mechanisms (20, 24). AMP-activated protein kinase (AMPK) is an enzyme that is activated in response to a decrease in cellular energy (18) and plays an important role in insulin-independent glucose uptake. AMPK activation has been reported in β-cells and in muscles with 3 days of LA treatment (31, 46), and we wanted to assess the effect of long-term LA treatment on AMPK in skeletal muscle. Our results indicate chronic LA treatment also induces AMPK activation, again only in HF-fed rats (57.6% increase in HF+LA over HF, Fig. 3*A*).

Studies in L6 cells have shown that acute LA treatment induces p38 MAPK activation, which is potentially important in activation of GLUT-4 (27). In our study, we report that even long-term LA treatment administered in parallel with a HF diet results in activation of p38 MAPK (26.4% increase in HF+LA over HF, Fig. 3*B*).

Induction of HSPs with LA treatment. We examined levels of HSPs in the soleus muscles from chow- and HF-fed rats treated with either Tris or LA using Western blot analysis. The HF diet showed a slight trend for a reduction in HSP72 expression (14% reduction in HF compared with chow, $P = 0.1$, Fig. 4*A*). LA treatment did not affect HSP72 expression in the chow-fed rats, while LA induced HSP72 in rats fed a HF diet (44.9% increase in HF+LA over HF). Phosphorylation or activation of HSP25 also showed a significant increase with LA treatment in rats fed a HF diet (44.5% increase in HF+LA over HF, Fig. 4*B*). As shown in Fig. 3*B*, p38 MAPK, a kinase upstream of HSP25 (53), is activated by LA and could play a role in HSP25 activation. The most likely mechanism for HSP induction would be activation of the primary HSP transcription factor, heat shock factor 1 (HSF-1). In the present study, activation of HSF-1, as examined by its phosphorylation on serine 230, tended to increase with LA treatment (data not shown, $P = 0.07$).

Effects of LA treatment on stress kinase activation. JNK1/2 activity levels were measured with Western blot analysis using a phospho-specific antibody. While rats fed a HF diet showed a significant increase in phosphorylated JNK levels (35% increase in HF over chow), LA treatment reversed this to the levels seen in chow-fed rats (30.4% decrease in HF+LA compared with HF, Fig. 5*A*). No changes were detected in total JNK expression (representative blot in Fig. 5*A*, data not shown). IKKβ activation results in degradation of its downstream target IκBα, and, therefore, IκBα degradation is considered a marker of increased IKKβ activity (17). Figure 5*B* demonstrates that IκBα protein levels were reduced in the soleus muscles from rats fed a HF diet (19.44% reduction in HF compared with chow), but increased to levels in the muscles of chow-fed rats when treated with LA (44.8% increase in HF+LA over HF). High levels of diacylglycerol resulting from HF feeding are known to induce PKC-θ activity. PKC-θ is implicated in causing diacylglycerol-induced insulin resistance by phosphorylation of the IRS-1 on serine residues (16). In our study, a HF diet resulted in increased phosphorylation of PKC-θ in the soleus muscles (84% increase in HF over chow, Fig. 5*C*). However, LA treatment did not significantly reduce activation of PKC-θ. This suggests some specificity for LA to decrease JNK and IKKβ activation in skeletal muscle.

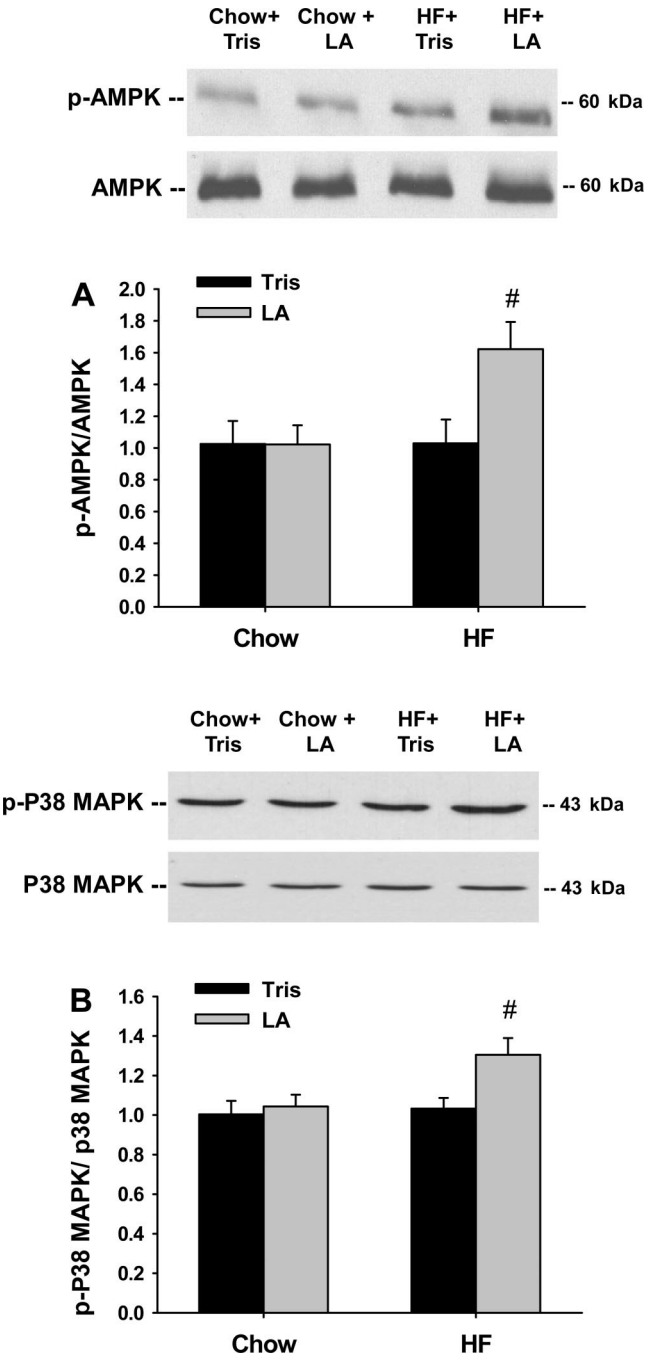

Fig. 3. LA leads to activation of p38 MAPK and AMP-activated protein kinase (AMPK) in soleus muscles from HF-fed rats. Soleus muscles from chow- and HF-fed animals, treated with either Tris or LA, were analyzed for p-AMPK and then stripped and probed for total AMPK (*A*) and for p-p38 MAPK and then stripped and probed for total p38 MAPK (*B*) with Western blot analysis. Values are means ± SE for 4 samples/group. #$P < 0.05$, HF+LA vs. HF.

LA pretreatment induces HSPs and prevents activation of JNK and IKKβ in L6 muscle cells. To verify the effects of chronic LA on induction of HSPs and inhibition of stress kinases seen in HF-fed animals, we examined the effect of acute LA treatment on L6 muscle cells. The cytokine TNF-α was used to induce stress kinase activation in muscle cells. Treatment of L6 myotubes with LA for 18 h, followed by

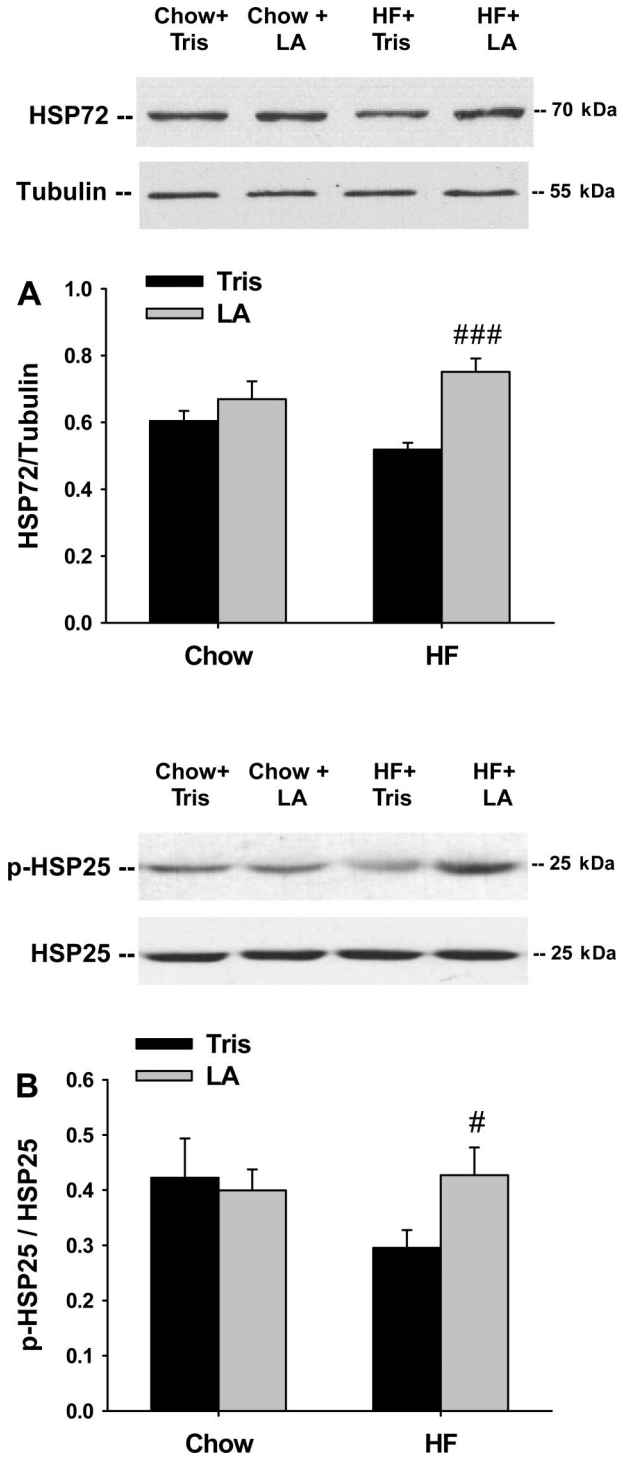

Fig. 4. LA induces expression of heat shock proteins (HSPs) in soleus muscles from HF-fed rats. Soleus muscles from chow- and HF-fed animals, treated with either Tris or LA, were analyzed for HSP72 and then stripped and probed for tubulin (*A*) and for p-HSP25 and then stripped and probed for total HSP25 (*B*) with Western blot analysis. Values are means ± SE for 8 muscles/group. HF+LA vs. HF: ###$P < 0.001$ and #$P < 0.05$.

TNF-α for 6 h, resulted in an increase in HSP72 expression and phosphorylation of HSP25 (Fig. 6, *A* and *B*). Consistent with our findings for LA treatment in chow-fed animals, LA did not increase HSP expression/activation in control cells not exposed to TNF-α. Anisomycin, a potent inducer of JNK (1, 13), induced a robust activation of JNK in L6 myotubes; however, pretreatment with LA for 18 h suppressed this anisomycin-induced JNK activation (45.2% decrease from anisomycin, Fig. 6*C*). Similarly, TNF-α induced activation of IKKβ, as seen by reduced IκBα levels, but pretreatment with LA protected the myotubes from the TNF-α-induced IKKβ activation (Fig. 6*D*). These data further suggest a role for LA in inducing HSPs as a potential mechanism of stress kinase inhibition.

Inhibition of HSP72 abolishes the protective effective of LA on JNK. To examine the specificity of HSPs in the effects of LA, we inhibited HSP72 and assessed its effects on JNK activity. L6 myotubes were treated with the HSP72 inhibitor, KNK-437, along with LA for 18 h, followed by incubation with TNF-α for 6 h. KNK-437 inhibits the induction of HSP72 at mRNA levels and prevents transcription of new HSP72 (51). As seen before (Fig. 6*A*), LA treatment induced HSP72 expression, but this was blunted when incubated in the presence of 10 μM KNK-437 (24% reduction from LA+TNF-α, Fig. 7*A*). Higher concentrations of KNK-437 caused greater suppression of HSP72 but also resulted in a small, nonspecific decrease in JNK activation. In the presence of KNK-437, LA treatment did not prevent anisomycin-induced JNK activation (43% increase over LA+ anisomycin, Fig. 7*B*). This indicates that, in the absence of HSP72 induction, LA does not effectively suppress JNK activation.

DISCUSSION

The purpose of this study was to determine whether chronic LA treatment increases the expression of HSPs in skeletal muscle, and if this induction can effectively inhibit stress kinases and improve insulin action. We demonstrate the ability of LA to induce HSPs in skeletal muscle in vivo and in L6 cells in culture with a concomitant reduction in the activation of stress kinases JNK and IKKβ. While the effects of LA in skeletal muscle are likely multifactorial, HSP-mediated stress kinase inhibition is a previously unexplored mechanism for the improvement in insulin action with LA.

Kurucz et al. (29) were the first to establish that, in patients with Type 2 diabetes, subjects with impaired glucose tolerance, and in nondiabetic identical twins, very little HSPs are expressed in skeletal muscle. Low tissue HSP expression could play a critical role in the induction of insulin resistance and diabetes. Hooper (22, 23) proposes that Type 2 diabetes is a vicious cycle stemming from low-HSP expression that results in an impaired stress response, metabolic inflammation, and subsequent insulin resistance. Our results suggest that increasing HSPs with LA could be one of the important factors that forestall this cycle, thereby improving insulin sensitivity and diabetes. A number of in vitro and in vivo studies demonstrate the anti-inflammatory action of HSPs (25, 50). Previous studies have shown that NF-κB activation is suppressed by HSP inhibition of IKKβ and that HSP72 protects against TNF-α-induced IL-6 production, shock, apoptosis, and death. In addition, HSP72 has been shown to inhibit JNK, and low levels of HSP72 result in JNK activation (8). The recent study by Chung et al. (8) showed that overexpression of HSP72 was tightly associated with the prevention of JNK activation in models of diet- and obesity-induced insulin resistance. The activation of cytokines as a result of low HSP expression in insulin-resistant

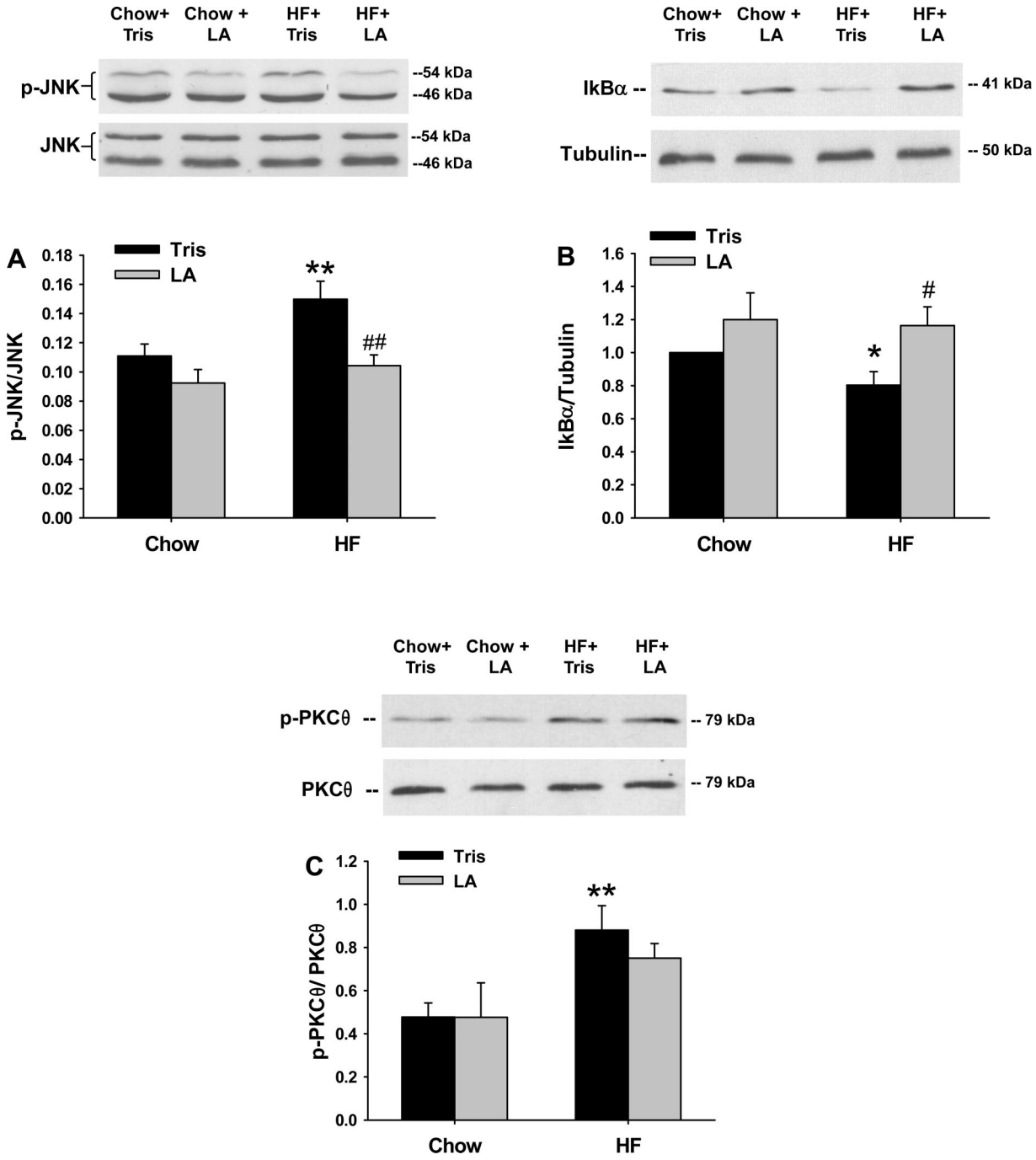

Fig. 5. LA prevents HF diet-induced stress kinase activation in soleus muscles from HF-fed rats. Soleus muscles from chow- and HF-fed animals, treated with either Tris or LA, were analyzed for the following. *A*: p-c-Jun NH_2-terminal kinase (JNK) and then stripped and probed for total JNK. $^{**}P = 0.01$, HF vs. chow. $^{\#\#}P < 0.01$, HF+LA vs. HF. *B*: inhibitor of κB kinase-β (IKKβ) activation. Western blots were analyzed for IkBα and then stripped for tubulin as a loading control. $^{*}P < 0.05$, HF vs. chow. $^{\#}P < 0.05$, HF+LA vs. HF. *C*: p-PKC-θ. Soleus muscles were analyzed with Western blotting using an antibody specific for p-T538-PKC-θ. Blots were normalized to total PKC-θ. $^{**}P < 0.01$, HF vs. chow. Values are means ± SE for 8 (*A* and *B*) and 4–5 (*C*) muscles/group.

tissue is a relatively new theory regarding the development of insulin resistance and Type 2 diabetes. Given the number of ways in which HSPs can be induced via physiological stressors (exercise, heat, calorie restriction), small molecules (BGP-15, geranylgeranylacetone, resveretrol), and antioxidants (LA), therapeutic induction of HSPs could be a promising new approach to the treatment of insulin resistance.

To our knowledge, this is the first study to report that LA can induce HSP expression in skeletal muscle. Although our findings did not demonstrate a significant increase in HSF-1 activation with LA, a strong trend was seen. Thus an effect of LA on HSF-1 cannot be ruled out. LA has been shown to increase HSF-1 mRNA in diabetic kidneys (36). Additional time points following LA treatment may be necessary to observe activation of HSF-1 in skeletal muscle. Also, HSF-1 activation is known to vary, depending on the muscle fiber type (35), and further studies will be needed to determine whether the effect of LA on HSF-1 is evident in fast-twitch muscles.

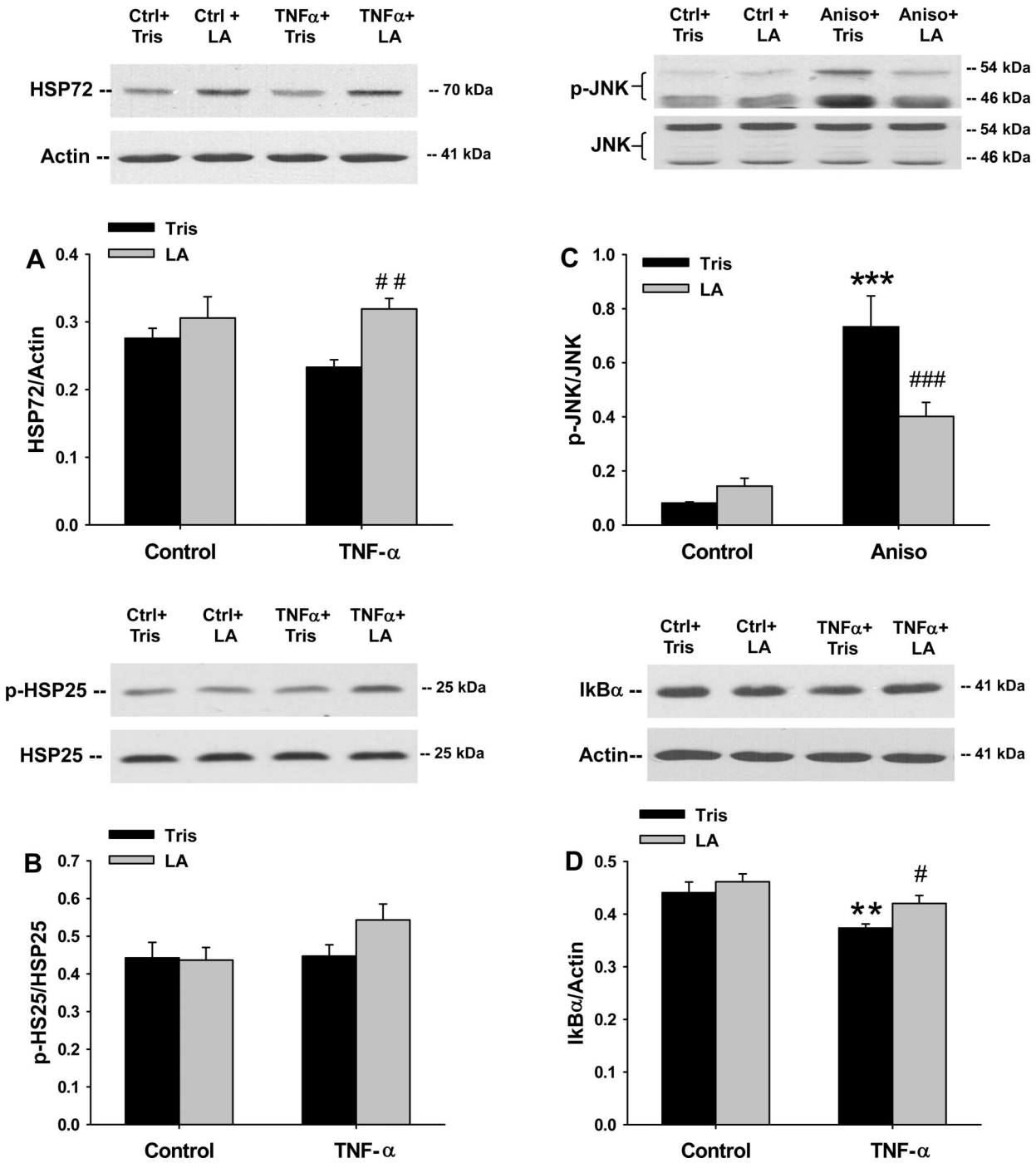

Fig. 6. LA induces HSPs and prevents stress kinase activation in L6 muscle cells. L6 cells were pretreated with LA (300 μM) or Tris for 18 h. Cells were then washed briefly and treated with TNF-α (20 ng/ml) for 6 h or with anisomycin (Aniso, 10 μg/ml) for 30 min. Homogenates of the cells were analyzed for HSP72/actin (*A*), p-HSP25/HSP25 (*B*), p-JNK/JNK (*C*), and IkBα/actin (*D*). Values are means ± SE for 6–13 samples/condition. ***$P < 0.001$, Aniso vs. control (Ctrl). ###$P = 0.001$, Aniso+LA vs. Aniso. **$P < 0.01$, TNF-α vs. Ctrl. #$P < 0.05$ and ##$P < 0.01$, TNF-α+LA vs. TNF-α.

Activation of HSF-1 is tightly regulated by the inhibitory phosphorylation of HSF-1 by JNK, ERK, and GSK3 on serine 363, 307, and 303, respectively (7, 10, 48). Increased activation of these inflammatory kinases with HF feeding, diabetes, and aging is known to downregulate the HSF-1 machinery and thereby reduce HSP expression. In our hands, 6 wk of HF feeding was not sufficient to reduce HSP expression significantly in soleus muscles. Longer duration HF diet regimes may be needed to determine the role of LA on maintaining HSP expression.

Data in L6 cells demonstrate that acute treatment with LA also results in an induction of HSPs and a concomitant inhibition of JNK and IKKβ. These results support our observations in the animal model and validate our findings regarding the proposed mechanism of LA action. Several mechanisms of HSP-induced stress kinase inhibition have been suggested.

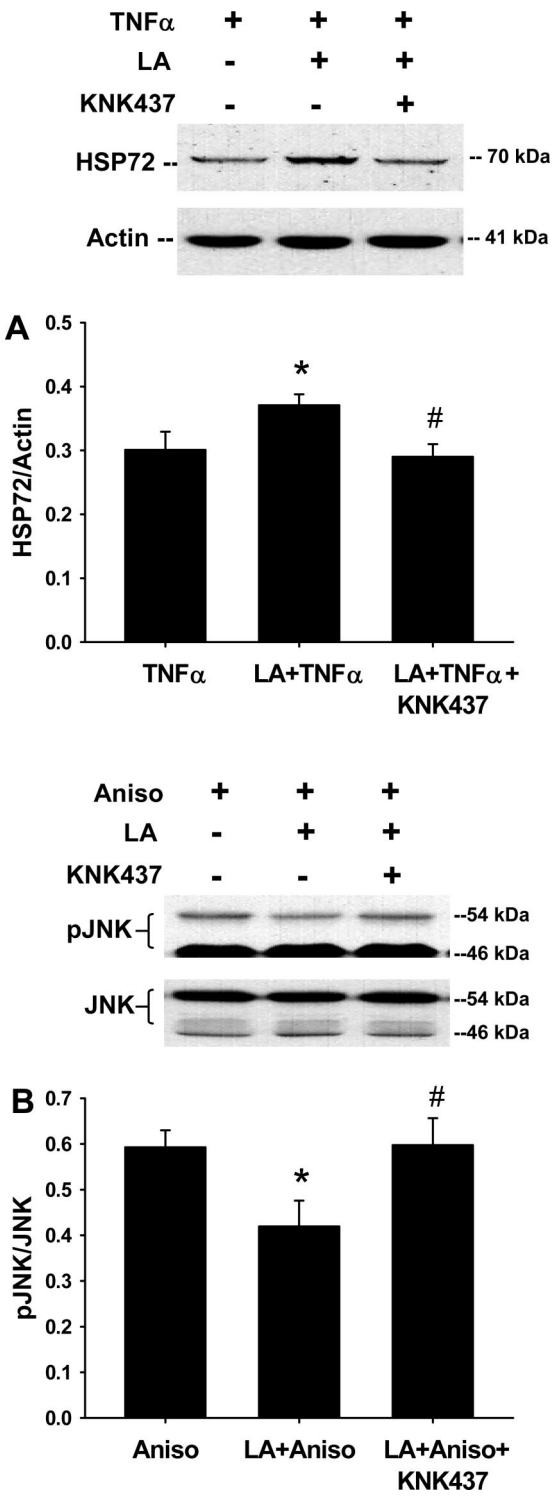

Fig. 7. In the absence of HSP72 induction, LA does not prevent JNK activation. L6 cells were treated with either Tris or LA (300 μM) or a combination of LA (300 μM) + KNK-437 (10 μM) for 18 h. Cells were then washed and treated with TNF-α (20 ng/ml) for 6 h or with Aniso (10 μg/ml) for 30 min. TNF-α-treated groups were examined for HSP72/actin (*A*), and Aniso-treated groups were analyzed for p-JNK/JNK (*B*). Values are means ± SE for 5–6 samples/condition for p-JNK and 9–10 samples/condition for HSP72. *A*: $*P < 0.05$, LA+TNF-α vs. TNF-α. $\#P < 0.01$, LA+TNF-α+KNK437 vs. LA+TNF-α. *B*: $*P < 0.05$, LA+Aniso vs. Aniso. $\#P < 0.05$ LA+Aniso+KNK437 vs. LA+Aniso.

HSP72 has been shown to bind to JNK and prevent its activation by the upstream kinases stress-activated protein/ERK kinase 1 and MAPK kinase 7 (38). Furthermore, interaction of HSP72 with a cochaperone CHIP is thought to negatively regulate activity of dual leucine zipper-bearing kinase, a kinase upstream of JNK (11). While other investigators suggest HSP72 prevents stress-mediated inactivation of a phosphatase that targets JNK (30, 34), both proposed mechanisms effectively inhibit JNK activation. Similarly, IKKβ could be inhibited by HSP25, either by direct binding or an as-yet uncharacterized signaling intermediate (39). Future studies are needed to determine the mechanism of HSP inhibition of stress kinases in insulin-resistant tissue.

It is possible that LA influences insulin signaling via oxidant effects, independent of HSP induction. However, research indicating LA has no effect on insulin-sensitive tissue argues against a direct activation of insulin signaling intermediates as the mode of LA action. LA treatment has little or no effect on cells that were not subjected to oxidative stress (33) or even on lean insulin-sensitive animals (31, 42). Consistent with these studies, we saw no significant changes with LA treatment of chow-fed rats in insulin sensitivity measures, stress kinase activation, AMPK/p38 MAPK activation, or HSP expression. AMPK plays an important role in insulin-independent glucose uptake and is activated with 3 days of LA treatment (31). In our study, although we did see a robust increase in phosphorylation of AMPK with LA in HF-fed rats, we did not see a statistically significant increase in basal glucose transport. It is likely that the observed increase in AMPK activation was not sufficient to result in increased basal glucose uptake or that chronic activation of AMPK with LA, as in the present study, might have a greater impact on fatty acid oxidation than glucose transport. Chronic AMPK activation can result in increased fatty acid oxidation and thus reduced accumulation of fatty acids and reactive oxygen species, contributing to the overall insulin-sensitizing effects of LA. Also, it has been reported that intramuscular triglycerides are reduced with LA treatment (43), potentially as a result of AMPK activation.

The ability of LA to display both reducing (antioxidant) and prooxidant properties makes its action in skeletal muscle multifactorial (26). A number of previous studies using LA suggest a mechanism of action primarily attributed to its antioxidant properties. For example, in vivo studies show that LA treatment reduces protein carbonyl levels in skeletal muscle and significantly improves insulin sensitivity in insulin-resistant animal models, such as the Goto-Kakizaki rats (3, 4). Additional antioxidant properties of LA include the capacity to scavenge ROS and to regenerate antioxidants, such as reduced glutathione and vitamins C and E (37). The antioxidant properties of LA could certainly improve the overall metabolic state in the muscle and indirectly improve insulin action. The prooxidant properties of LA include an ability to activate insulin signaling; cell culture studies have shown that high concentrations of LA (2.5 mM) can directly induce glucose uptake by activating insulin signaling intermediates in a PI3K-dependent manner (27, 49). The induction of HSPs in skeletal muscle can also be added to the list of LA prooxidant properties. The finding that LA had a slight but insignificant effect on PKC-θ levels suggests that the action of LA is targeting primarily JNK and IKKβ in soleus muscle, likely due to the induced HSP72 and HSP25, respectively. Our observation that

HSP72 inhibition with KNK437 abolishes LA's ability to inhibit JNK further supports the idea that HSPs constitute a key mechanism of LA action. Thus the beneficial effects of chronic LA treatment in skeletal muscle could result from both an overall reduction in tissue oxidative stress and, as our results suggest, from a complementary increase in the content of stress-protective HSPs in skeletal muscle.

Apart from just increasing glucose uptake into the muscle, LA may also facilitate the efficient oxidation of glucose, since LA is a cofactor of the PDH complex. Although we did not measure PDH activity in this study, increased LA treatment has been shown to increase PDH activity and improve glucose effectiveness in lean and obese patients with Type 2 diabetes (28). In conclusion, the present findings demonstrate, for the first time, that LA treatment increases expression of HSP72 and activation of HSP25 in skeletal muscle. Our study provides new information regarding the inhibition of JNK and IKKβ with chronic LA treatment and suggests HSP-mediated stress kinase inhibition is one mechanism by which LA improves insulin signaling in skeletal muscle. Future studies are needed to examine the direct mechanisms by which HSPs inhibit stress kinases.

GRANTS

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