

Heat Treatment Improves Glucose Tolerance and Prevents Skeletal Muscle Insulin Resistance in Rats Fed a High-Fat Diet

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OBJECTIVE—Heat treatment and overexpression of heat shock protein 72 (HSP72) have been shown to protect against high-fat diet–induced insulin resistance, but little is known about the underlying mechanism or the target tissue of HSP action. The purpose of this study is to determine whether *in vivo* heat treatment can prevent skeletal muscle insulin resistance.

RESEARCH DESIGN AND METHODS—Male Wistar rats were fed a high-fat diet (60% calories from fat) for 12 weeks and received a lower-body heat treatment (41°C for 20 min) once per week.

RESULTS—Our results show that heat treatment shifts the metabolic characteristics of rats on a high-fat diet toward those on a standard diet. Heat treatment improved glucose tolerance, restored insulin-stimulated glucose transport, and increased insulin signaling in soleus and extensor digitorum longus (EDL) muscles from rats fed a high-fat diet. Heat treatment resulted in decreased activation of Jun NH₂-terminal kinase (JNK) and inhibitor of κ B kinase (IKK- β), stress kinases implicated in insulin resistance, and upregulation of HSP72 and HSP25, proteins previously shown to inhibit JNK and IKK- β activation, respectively. Mitochondrial citrate synthase and cytochrome oxidase activity decreased slightly with the high-fat diet, but heat treatment restored these activities. Data from L6 cells suggest that one bout of heat treatment increases mitochondrial oxygen consumption and fatty acid oxidation.

CONCLUSIONS—Our results indicate that heat treatment protects skeletal muscle from high-fat diet–induced insulin resistance and provide strong evidence that HSP induction in skeletal muscle could be a potential therapeutic treatment for obesity-induced insulin resistance. *Diabetes* 58:567–578, 2009

Insulin resistance is associated with many related health complications, including type 2 diabetes and heart disease. A recent study demonstrated induction of the natural defense system of the body, heat shock proteins (HSPs), protects against obesity-induced insulin resistance (1). Earlier studies in patients with type 2 diabetes showed that hot tub therapy improved glycemic control (2) and an inverse correlation between HSP72 mRNA expression and the degree of type 2 diabetes (3).

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Currently, several HSP-inducing drugs are under investigation or in clinical trials for diabetic neuropathy and neurodegenerative diseases (4,5) and could be considered for prevention of insulin resistance. However, little is known about the mechanism behind this newly discovered role of HSP72, whether other inducible HSPs could be protective against insulin resistance, or the primary target tissue of HSP action.

Skeletal muscle is the major tissue responsible for whole-body insulin-mediated glucose uptake (6,7). HSPs are expressed in skeletal muscle and are strongly induced with exercise training (8,9). Overexpression of HSP72 has been shown to reduce skeletal muscle atrophy and oxidative stress with age (10). Therefore, skeletal muscle is a logical choice as the target tissue for the benefits of HSP overexpression. Previous studies indicate basal levels of HSPs differ between muscle fiber types with slow-twitch oxidative muscles having higher constitutive expression of HSPs than fast-twitch glycolytic muscles (11). In contrast, fast-twitch muscles possess greater capacity for HSP induction in response to physiological stressors and exercise (11,12). It is uncertain whether HSPs would be equally effective as mediators of insulin action in slow- and fast-twitch muscle.

The purpose of the present study was to determine whether weekly *in vivo* heat treatment could prevent skeletal muscle insulin resistance in rats fed a high-fat diet and elucidate mechanisms of HSP function in skeletal muscle. We hypothesized that heat treatment allows skeletal muscle to adapt and resist the development of insulin resistance as a result of increased HSP expression. Our findings indicate that heat treatment prevents skeletal muscle insulin resistance and stress kinase activation, whereas increased oxygen consumption and fatty acid oxidation in L6 cells suggest that heat treatment can improve mitochondrial function.

RESEARCH DESIGN AND METHODS

[¹⁴C]mannitol and 2-deoxy [1,2-³H]glucose were purchased from American Radiolabeled Chemicals (St. Louis, MO). Antibodies used include phospho-Thr183/Tyr185 and total Jun NH₂-terminal kinase (JNK), phospho-Ser473 and total Akt, and inhibitor of κ B α (IkB α) (Cell Signaling, Beverly, MA); HSP72, phospho-Ser82 and total HSP25, HSP60, and cytochrome c (Stressgen, Victoria, BC, Canada); tubulin (Sigma, St. Louis, MO); cytochrome oxidase IV subunits I and IV (Molecular Probes, Eugene, OR); citrate synthase (Alpha Diagnostic, San Antonio, TX); uncoupling protein-3 (UCP-3; Chemicon International, Temecula, CA); peroxisome proliferator-activated receptor (PPAR)- γ coactivator 1 α (PGC-1 α ; Calbiochem, San Diego, CA); phospho-Tyr612-IRS-1 (Biosource, Camarillo, CA); and IRS-1 (BD Biosciences, Franklin Lakes, NJ). [³H]palmitate was purchased from Perkin Elmer (Waltham, MA), insulin ELISA kits from Alpco diagnostics (Salem, NH) and all other reagents from Sigma.

Experimental animals and treatment. Male Wistar rats (100–130 g) from Charles River Laboratories (Wilmington, MA) were housed in a temperature-controlled (22 \pm 2°C) room with a 12:12 light/dark cycle. Animals were fed ad

libitum for 12 weeks with a standard chow diet (8604; Harlan Teklad, Madison, WI) or high-fat diet [60% calories from fat comprising lard and corn oil and 20% calories from carbohydrates (13)]. Experiments were conducted 48 h after the last heat or sham treatment, and rats were fasted 12 h before experimental procedures. All protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center.

In vivo heat treatment. Once per week, high-fat-fed animals were anesthetized with pentobarbital sodium (5 mg/100 g body wt), and the lower body was immersed in a water bath. Body temperature was gradually increased and maintained between 41 and 41.5°C for 20 min as monitored with a rectal thermometer. Sham treatment maintained core temperature at 36°C. After treatment, 5 ml 0.9% saline was administered to prevent dehydration. Preliminary experiments in our laboratory established that one heat treatment per week maintains an increase in HSP72 expression and avoids potential HSP inhibition by repeated heat treatment (14).

Intraperitoneal glucose tolerance test. An intraperitoneal glucose tolerance test (IPGTT) was performed in week 11, 48 h after the last heat/sham treatment. Overnight-fasted rats were anesthetized and given a glucose load of 2 g/kg body wt. To prevent dehydration, 5 ml 0.9% saline was administered after the GTT.

Immunoblotting, glucose transport, and kinase assay. In week 12, rats were anesthetized for the removal of soleus and extensor digitorum longus (EDL) muscles. Muscles were split longitudinally to allow adequate diffusion of substrates (11,15). Two muscle strips per rat were assessed for glucose transport, and two strips were incubated with or without 1 mU/ml insulin for 20 min and frozen for Western blot analysis as previously described (11). Western blots were first probed for phosphorylated proteins and then stripped for total protein expression. Glucose transport activity was determined using 1.5 μ Ci/ml 2[1,2-³H]deoxyglucose and 0.2 μ Ci/ml [¹⁴C]mannitol (11,16,17). Activity levels of inhibitor of κ B kinase (IKK- β) in whole-cell lysates were assayed as previously described (18). Phosphorylated I κ B α levels were detected by Western blot analysis.

KNK437 incubation. Soleus muscles were isolated from 3-month-old Fischer 344 rats and subjected to 42°C heat or 35°C sham treatment for 30 min in vitro. Subsets of muscles were incubated in 100 μ Mol/l HSP70 inhibitor KNK437 (Calbiochem) and stimulated with 10 μ g/ml anisomycin (Calbiochem). HSP72 and p-JNK/JNK were detected by Western blot analysis.

Mitochondrial enzyme activity. Citrate synthase activity was assessed in muscle lysates (prepared in the cell extraction buffer used for immunoblotting; Biosource), using a modified protocol (19) by Srere (20). The absorbance was recorded at 405 nm every 20 s for 3 min at 30°C, using a MRXII microplate reader and kinetic software package (Dynex Technologies, Chantilly, VA). The linear portion of the reaction curve was used to calculate activity levels of citrate synthase, normalized to citrate synthase protein expression levels, in micromoles per gram per minute.

For the cytochrome oxidase assay, 140 μ g muscle lysate in 20 mmol/l potassium phosphate buffer (pH 7.0) and 0.2 mg dodecyl maltoside was warmed to 30°C (21). The reaction was initiated by addition of 25 μ Mol/l reduced cytochrome *c*, and oxidation of reduced cytochrome *c* was followed for 2 min at 550 nm on a DU series spectrophotometer (Beckman Coulter, Fullerton, CA). Maximal oxidation of cytochrome *c* was determined by adding potassium ferricyanide. Activity was calculated and normalized to cytochrome *c* oxidase subunit 4 (Cox-4) protein expression levels (seconds per milligram protein).

Measurement of oxygen consumption rates. L6 myoblasts from American Type Culture Collection (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Five to six days after differentiation, L6 cells were treated with 30 ng/ml tumor necrosis factor- α (TNF- α) alone or in combination with heat treatment (43°C for 20 min), and experiments were performed 24 h later. O₂ consumption rate was determined using an Oroboros Oxygraph-2K high resolution respirometer (Innsbruck, Austria). After baseline stabilization, respiratory chain inhibitors were sequentially injected: 1 μ g/ml oligomycin, 3 μ Mol/l carbonyl cyanide 4-(trifluoromethoxy)-phenyl-hydrazone, 1 μ Mol/l rotenone, and 2 μ Mol/l myxothiazol. All values were normalized to protein content, and nonmitochondrial respiration rates were subtracted.

Fatty acid oxidation rates. Fatty acid oxidation was performed as previously described (22). Briefly, L6 myotubes were heat or sham treated and exposed to 200 μ Mol/l palmitate coupled with 7.5% BSA (wt/vol) for 72 h. A second heat treatment was given in the last 24 h of palmitate treatment. Cells were washed with PBS and incubated with 200 μ l 125 μ Mol/l [³H]palmitate-BSA solution (1 mCi/ml stock), supplemented with 1 mmol/l carnitine for 2 h at 37°C. After incubation, solution from each well was added to 200 μ l cold 10% TCA and centrifuged at 3,300 rpm for 10 min. After neutralizing with 6 N NaOH, the mixture was run through a DOWEX resin column to separate the fatty acid oxidation intermediates and ³H-labeled water. Scintillation fluid was

added to the flow-through, and ³H-CPM (counts per minute) was counted. Fatty acid oxidation rate was normalized to protein content and expressed as nanomoles per hour per milligram.

Statistical analysis. ANOVA and two-way ANOVA were used to study differences between groups. 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

Body composition, serum insulin, and glucose tolerance

Twelve weeks of high-fat feeding resulted in an increase in body weight compared with chow-fed rats (Fig. 1A). Weekly heat treatment in high-fat-fed animals did not significantly reduce body weight, and there was no difference in food intake between the high-fat-fed groups (chow 106.05 \pm 2.72 kcal/day; high fat 114.75 \pm 1.41 kcal/day; high fat + heat treatment 116.93 \pm 1.57 kcal/day, $P < 0.05$ for chow vs. high fat). Epididymal fat pad weight increased with high-fat feeding, whereas this increase was blunted in rats subjected to heat treatment (Fig. 1B). Fasting blood glucose levels did not change significantly with either diet or heat treatment (Fig. 1C). In contrast, fasting insulin levels increased robustly in high-fat-fed rats, and heat treatment significantly blunted this increase (Fig. 1D). Although high-fat-fed rats showed decreased whole-body glucose clearance (Fig. 1E), heat treatment effectively improved glucose clearance as further indicated by a decrease in glucose area under the curve (Fig. 1F).

Insulin action is improved with heat treatment. We examined the effect of heat treatment on glucose uptake, for the first time, in representative slow-twitch soleus (84% type I, 7% type IIA, and 9% type IIB) and fast-twitch EDL muscle (20% type IIA, 38% type IIB, 38% type IID, and 4% type I) (23). In rats fed a high-fat diet, insulin-stimulated glucose transport increased above basal (noninsulin stimulated) values in both muscle types (Fig. 2A, high fat + sham treatment). However, the insulin effect was significantly reduced compared with chow-fed insulin-sensitive muscles. With heat treatment, insulin-stimulated glucose transport was significantly increased above sham-treated soleus and EDL muscles from high-fat-fed rats.

Insulin phosphorylates and activates the insulin receptor substrate (IRS1) on tyrosine residues (rev. in 24). Phosphorylation of Tyr612 on IRS1 was reduced with the high-fat diet (not significantly in soleus muscle) but rescued fully with heat treatment in both muscles (Fig. 2B). Downstream of IRS-1, insulin-stimulated activation of Akt by phosphorylation on Ser473 was reduced in soleus and EDL muscles with high-fat feeding (Fig. 2C). Akt activation was fully rescued with heat treatment in soleus muscle and showed a nonsignificant improvement in the EDL. High-fat feeding reduced phosphorylation and activation of AS160 in EDL muscles (nonsignificant decrease in soleus muscle Fig. 2D), whereas heat treatment improved AS160 activation in both muscles.

Induction of HSP72 and inhibition of JNK with heat treatment. HSP72 expression was unchanged in soleus and EDL muscles with high-fat feeding. Heat treatment resulted in a much greater increase in HSP72 expression in EDL muscle compared with soleus (Fig. 3A). Heat treatment also induced an increase in HSP72 expression in adipose tissue and liver (adipose: chow 0.258 \pm 0.06, high fat + sham treatment 0.287 \pm 0.09, high fat + heat treatment 0.760 \pm 0.11, $P < 0.005$ for high fat + heat treatment vs. high fat + sham treatment; liver: chow 0.061 \pm 0.01, high fat + sham treatment 0.058 \pm 0.01, high

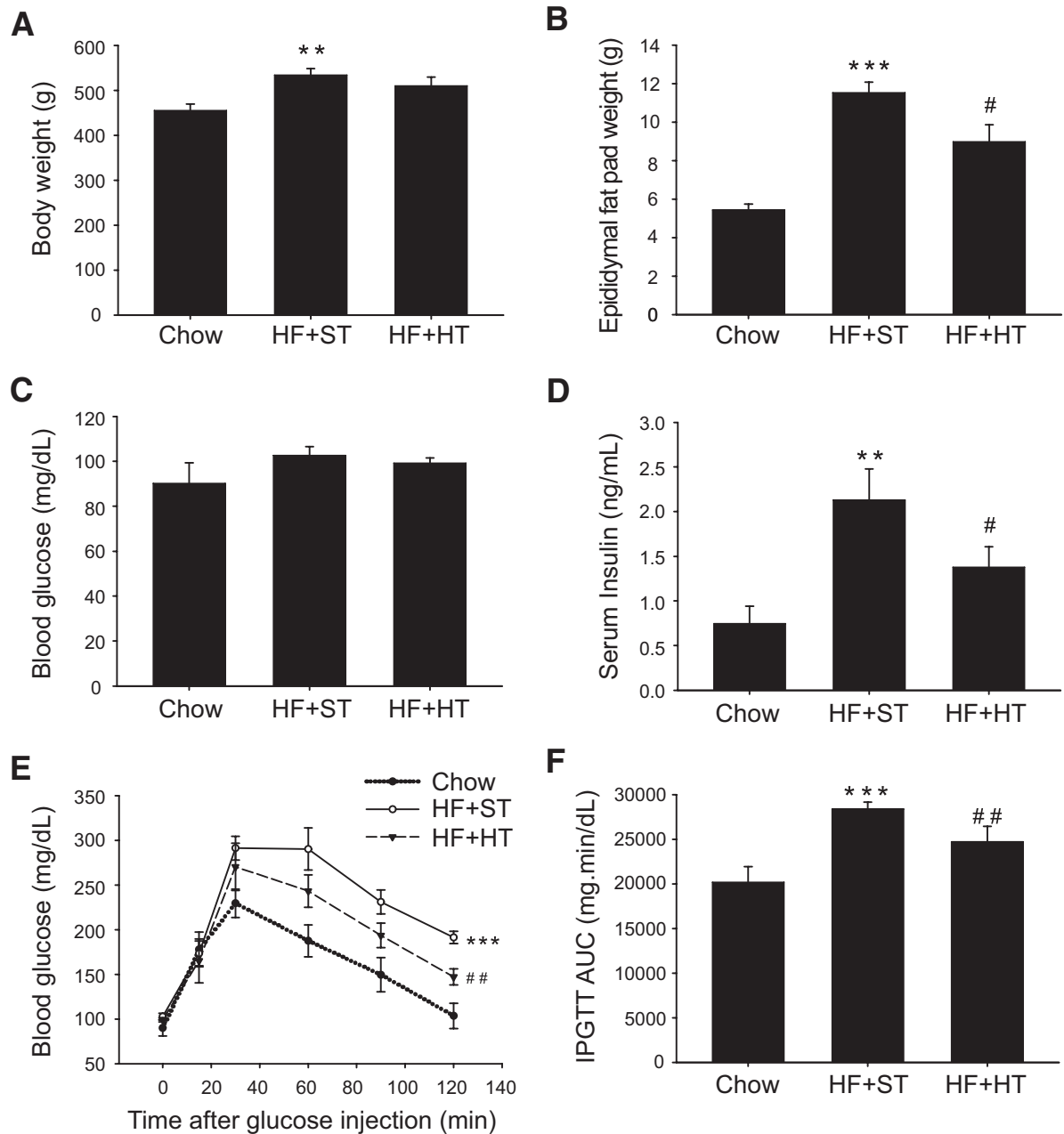


FIG. 1. Weekly heat treatment improves characteristics of high-fat diet-induced insulin resistance. Male Wistar rats were given a chow or high-fat diet for 12 weeks. The high-fat-fed rats received either a lower body heat treatment (HF+HT, 41°C for 20 min) or sham treatment (HF+ST, 36°C for 20 min) once per week during the diet regimen. At the end of the 12-week study, body weight (A), epididymal fat pad weights (B), fasting glucose (C), and fasting serum insulin (D) were measured. E: IPGTT. Overnight-fasted rats were injected with a glucose load of 2 g/kg body wt i.p. Blood glucose was measured at time 0, 15, 30, 60, 90, and 120 min after injection using a glucometer. IPGTT is expressed as glucose area under the curve in F. ^{***} $P < 0.001$, ^{**} $P < 0.01$ HF+ST vs. chow; and ^{##} $P < 0.01$, [#] $P < 0.05$ HF+HT vs. HF+ST. Values are means \pm SE for five to seven rats per group.

fat + heat treatment 1.157 ± 0.21 , $P < 0.001$ for high fat + heat treatment vs. high fat + sham treatment). JNK activation, as measured by phosphorylation levels, increased in both soleus and EDL muscles with a high-fat diet (Fig. 3B), although to a greater extent in EDL muscles. With heat treatment, there was a significant reversal of JNK activation in both muscles. As shown in Fig. 3C, the ability of heat treatment to increase HSP72 expression in isolated soleus muscle was blunted in the presence of the HSP72 inhibitor, KNK437 (25). KNK treatment alone did not inhibit HSP72 activation (data not shown). Muscle incubation in anisomycin results in an increase in JNK phosphorylation that can be directly inhibited by prior

heat treatment (Fig. 3D). The presence of the HSP72 inhibitor KNK437 in the incubation medium eliminated the ability of heat treatment to decrease JNK phosphorylation. **Activation of HSP25 and inhibition of IKK β with heat treatment.** Phosphorylation of HSP25 occurs in response to heat stress. (p)-HSP25 was unaffected with diet or heat in the soleus but was significantly increased in EDL muscles with heat treatment. (Fig. 4A). Phosphorylation of HSP25 was not increased in adipose tissue (chow 0.331 ± 0.1 , high fat + sham treatment 0.359 ± 0.09 , high fat + heat treatment 0.563 ± 0.08); however, there was a dramatic increase in the liver (chow 0.030 ± 0.00 , high fat + sham treatment 0.031 ± 0.00 , high fat + heat treatment $0.379 \pm$

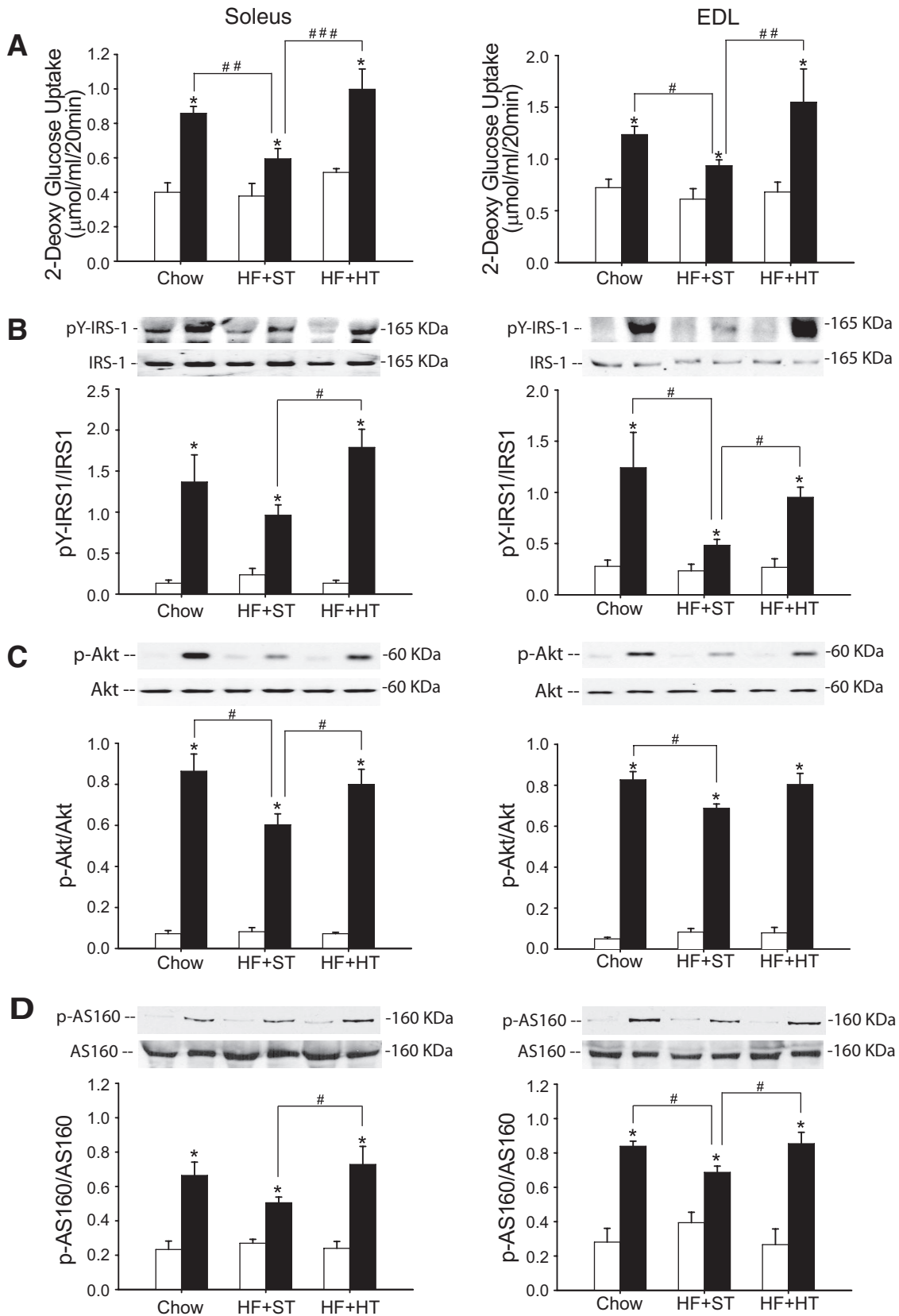


FIG. 2. Heat treatment increases insulin-stimulated glucose uptake and phosphorylation of IRS-1, Akt, and AS160. **A:** Insulin-stimulated glucose transport. Soleus and EDL from chow, high-fat diet-fed and sham-treated (HF+ST), and high-fat diet-fed and lower body heat-treated (HF+HT) rats were incubated in presence or absence of 1 mU/ml insulin with 2-[1,2- ^3H]deoxyglucose and [^{14}C]mannitol, and 2-deoxyglucose uptake into the muscles was determined. Values are means \pm SE for 3–5 muscles for the high-fat and HF+HT groups and for 11–12 muscles per group for chow. **Insulin signaling:** Soleus and EDL muscles were incubated in the presence or absence of 1 mU/ml insulin for exactly 20 min. Muscle lysates were separated with SDS-PAGE and blots were analyzed for (p)-Y612-IRS1/IRS-1 (**B**), (p)-S473-Akt/Akt (**C**), and (p)-T642-AS160/AS160 (**D**). \square , basal (noninsulin stimulated) measurements; \blacksquare , insulin-stimulated measurements. * $P < 0.05$ basal vs. insulin treated; ### $P < 0.001$, ## $P < 0.01$, and # $P < 0.05$. Values are means \pm SE for five to six muscles per group.

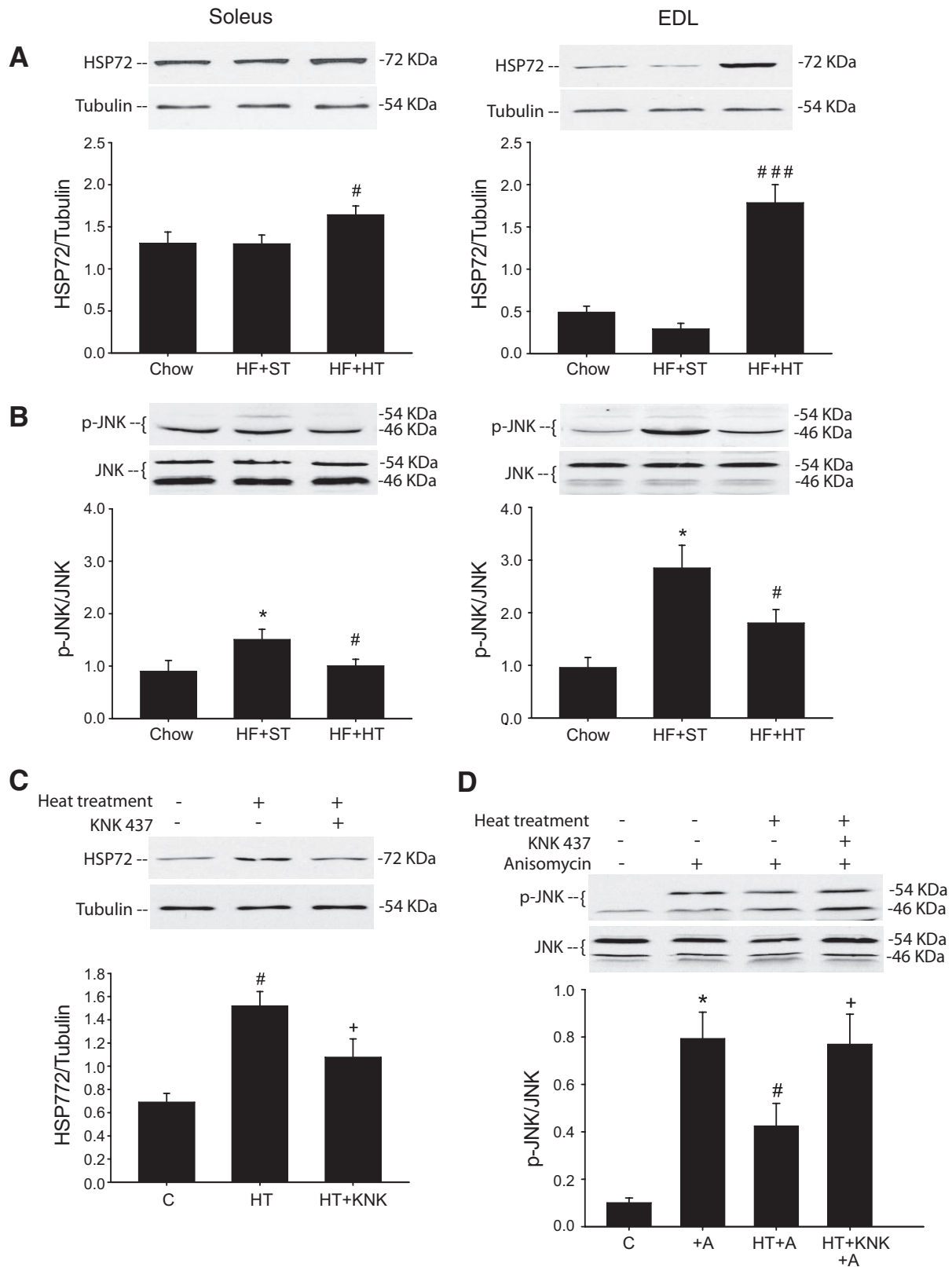


FIG. 3. Heat treatment induces HSP72 expression and a concomitant JNK inactivation. Soleus and EDL muscles were dissected from chow, high-fat diet-fed and sham-treated (HF+ST), and high-fat diet-fed and lower body heat-treated (HF+HT) rats and snap frozen in liquid nitrogen. Lysates were subjected to Western blot analysis for HSP72/tubulin (A) and (p)-JNK/JNK (B). * $P < 0.05$ HF+ST vs. chow; ### $P < 0.001$ and # $P < 0.05$ HF+HT vs. HF+ST. Values are means \pm SE for five to seven muscles per group. Pharmacological inhibition of HSP72 before heat treatment eliminates heat stress-mediated inhibition of JNK. Isolated soleus muscles from F344 rats were incubated in KHB in a water bath at 35°C and then exposed to sham (35°C) or heat treatment (HT, 42°C for 30 min). A reversible pharmacological inhibitor of HSP72, KNK437 (100 μ mol/l), was included in the incubation buffer before, during, and after heat treatment for a subgroup of muscles. C: After recovery for 12 h at 35°C, muscles were assessed for HSP72. D: JNK activation in response to anisomycin (A, 10 μ g/ μ l, 30 min), a potent inducer of JNK, was tested at the end of the recovery period. * $P < 0.001$ HT vs. C, # $P < 0.001$ HT+A vs. A, + $P < 0.001$ HT+KNK vs. HT and HT+KNK+A vs. HT+A. Values are means \pm SE for 7–20 muscles per group.

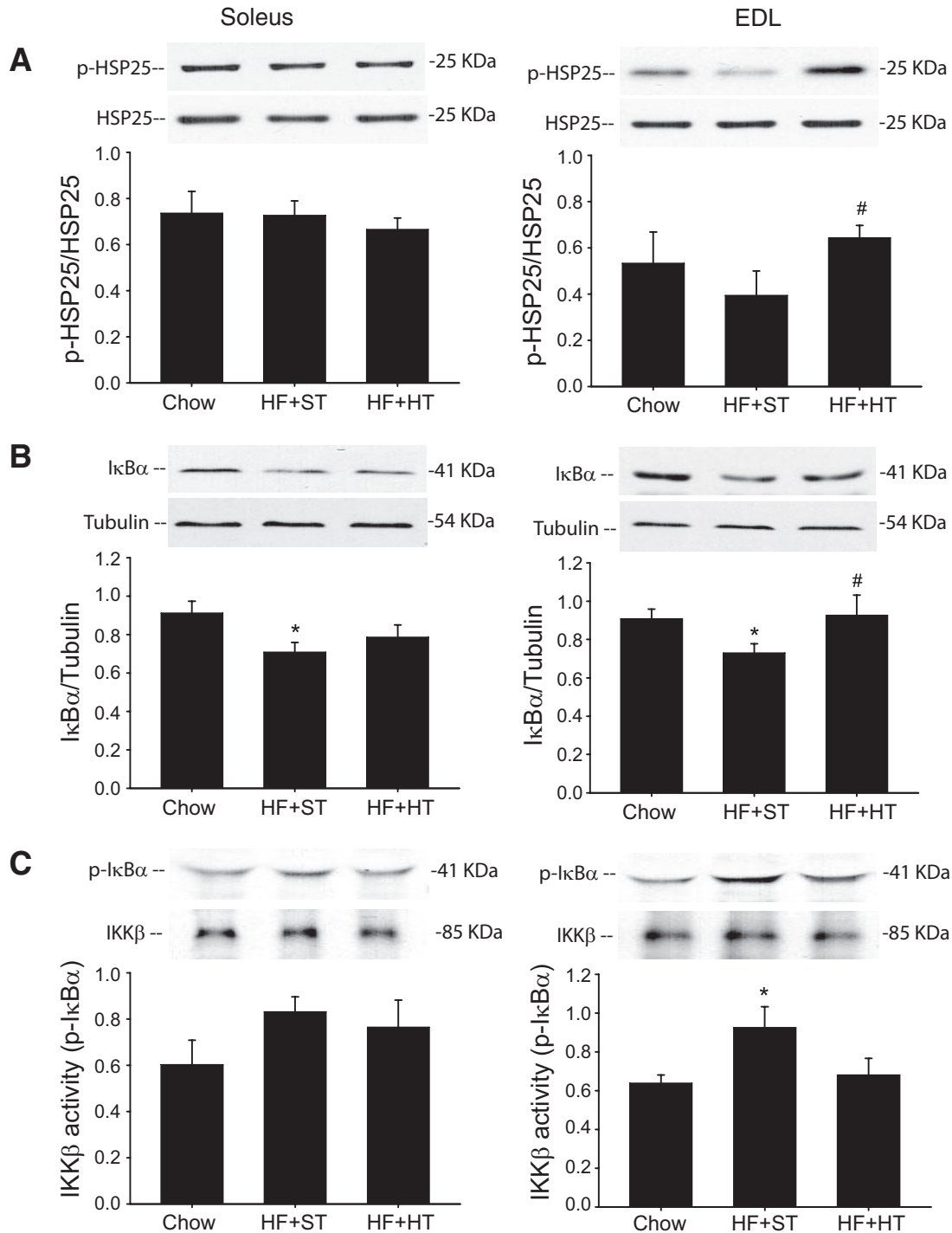


FIG. 4. Heat treatment prevents high-fat-induced IKK β activation in skeletal muscles in a fiber type-specific pattern. Snap-frozen soleus and EDL muscles from chow, high-fat diet-fed and sham-treated (HF+ST), and high-fat diet-fed and lower body heat-treated (HF+HT) rats were analyzed by Western blot for (p)-HSP25/HSP25 (A) and I κ B α /tubulin as a measure of IKK β activity (B). C: Kinase activity of IKK β was assessed by measuring phosphorylation of exogenous I κ B α substrate. Equal amounts of total IKK β were immunoprecipitated and subjected to a kinase assay in the presence of ATP and I κ B α substrate. * P < 0.05 HF+ST vs. chow and # P < 0.05 HF+HT vs. HF+ST. Values are means \pm SE for five to seven muscles per group.

0.15, P < 0.05 for high fat + heat treatment vs. high fat + sham treatment). Activity of IKK- β was assessed by measuring the protein levels of its downstream target I κ B α , which on activation of IKK- β gets phosphorylated and targeted for degradation (26). Protein levels of I κ B α were reduced with high fat in both muscles, indicating increased IKK- β activation (Fig. 4B). However, heat treatment reversed this activation only in the EDL muscle. Direct assessment of IKK- β kinase activity showed the

same pattern of IKK- β activation in soleus and EDL muscles (Fig. 4C).

Effects of heat treatment on mitochondrial protein expression and enzyme activity. Proteins involved in mitochondrial metabolism, including cytochrome c, citrate synthase, Cox-1 (encoded by the mitochondrial genome), and Cox-4 (encoded by the nuclear genome), were assessed in the current study. Our results indicate that these mitochondrial proteins increased with the high-fat diet

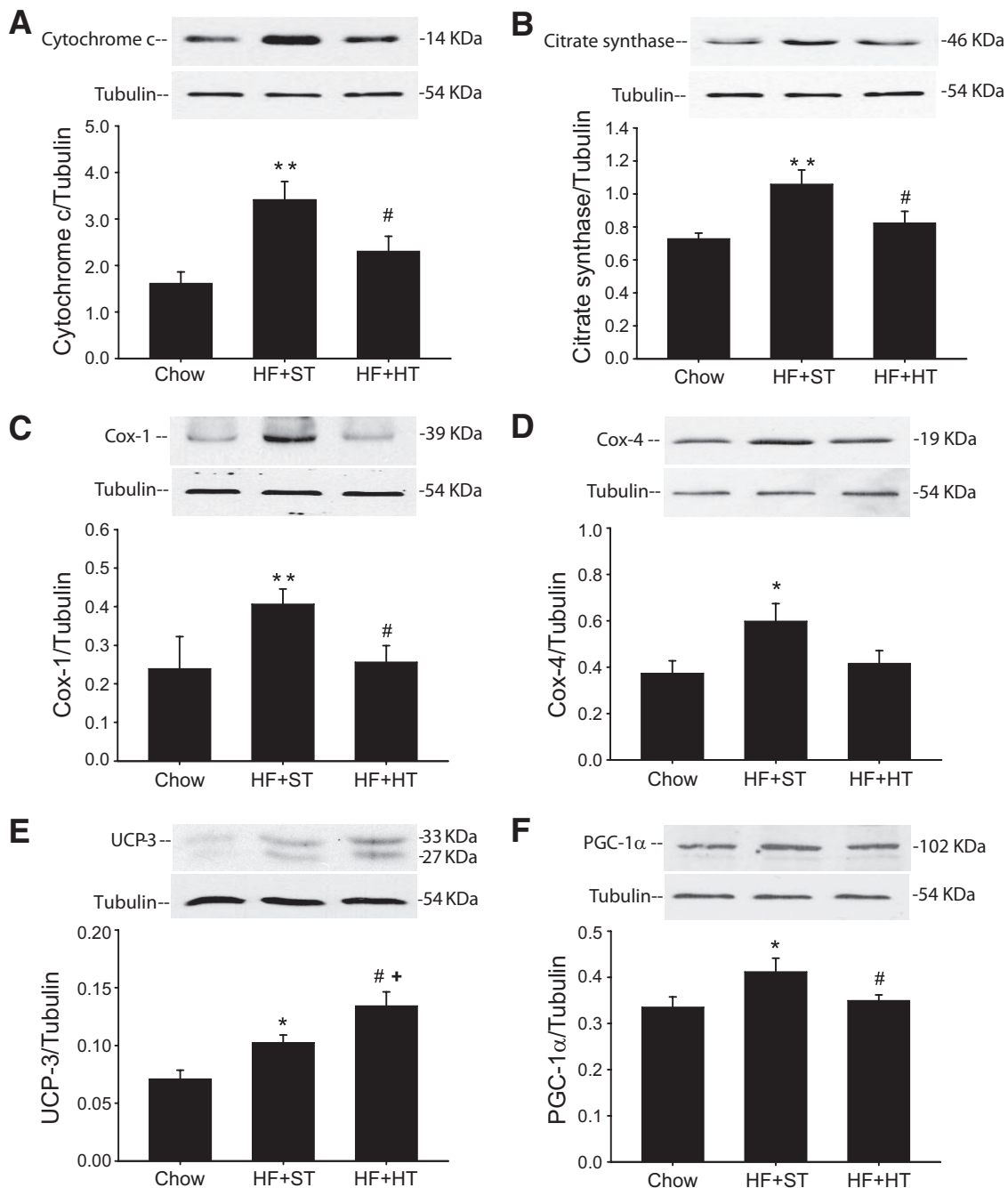


FIG. 5. High-fat feeding results in an increase in mitochondrial protein expression, but this increase does not occur when high-fat-fed rats are given heat treatment. EDL muscles from chow, high-fat diet-fed and sham-treated (HF+ST), and high-fat diet-fed and lower body heat-treated (HF+HT) rats were analyzed for mitochondrial protein expression using Western blot analysis. Cytochrome c (A), citrate synthase (B), Cox-1 (C), and Cox-4 (D) were increased with high-fat feeding in EDL muscles but not in rats given heat treatment. E: UCP-3 expression was increased with high-fat feeding and further potentiated by HT. The long form of UCP3 is detected at 33 kDa, and the short form is detected at 27 kDa. Expression of the master regulator of mitochondrial biogenesis, PGC-1 α (F), was also increased in EDL with high fat, but no increase was seen with heat treatment. ** $P < 0.01$ and * $P < 0.05$ HF+ST vs. chow; # $P < 0.05$ HF+HT vs. HF+ST; + $P < 0.05$ HF+HT vs. chow). Values are means \pm SE for five to seven muscles per group.

exclusively in the EDL muscle (Fig. 5A–D). However, an increase in mitochondrial proteins did not occur when heat treatment was administered in parallel with the high-fat diet. The mitochondrial HSP, HSP60, was slightly reduced with a high-fat diet and showed a significant increase with heat treatment (chow 0.971 ± 0.047 , heat treatment + sham treatment 0.834 ± 0.033 , high fat + heat treatment 1.117 ± 0.113 ; $P < 0.05$ for high fat + sham treatment vs. high fat + heat treatment). The HSP60 expression pattern differs from other mitochondrial pro-

teins but is consistent with the pattern of other HSPs in the current study. Expression of UCP-3, regulated by PPAR- δ (27), increased with the high-fat diet in EDL muscles, and heat treatment further potentiated this increase (Fig. 5E). Although high-fat feeding resulted in an increase in the master regulator of mitochondrial biogenesis, PPAR- γ coactivator 1 α (PGC-1 α), this effect was not observed in high-fat muscles given heat treatment (Fig. 5F). Mitochondrial protein expression was not significantly altered in the soleus muscle with diet or heat treatment (data not shown).

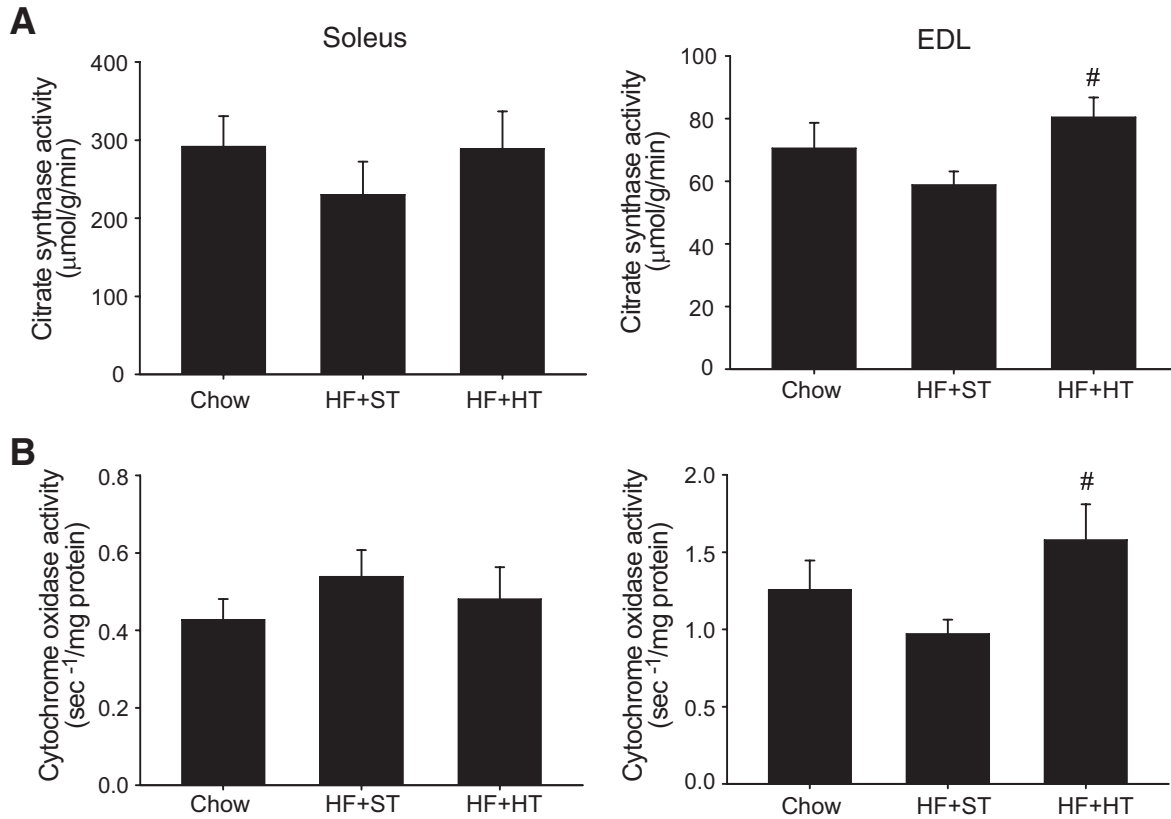


FIG. 6. Heat treatment maintains citrate synthase and cytochrome oxidase activities in the presence of a high-fat diet. Soleus and EDL muscle homogenates were assessed for citrate synthase and cytochrome oxidase activity levels as described in RESEARCH DESIGN AND METHODS. Citrate synthase activity was normalized to total protein levels of citrate synthase and cytochrome oxidase activity was normalized to total Cox-4 protein present in EDL and soleus muscles. [#] $P < 0.05$ high-fat diet-fed and lower body heat-treated (HF+HT) vs. high-fat diet-fed and sham-treated (HF+ST). Values are means \pm SE for five to seven muscles per group.

Despite the increase in mitochondrial protein expression seen with a high-fat diet, mitochondrial enzyme activity was not maintained in EDL muscle. A 12-week high-fat diet resulted in a slight decrease in citrate synthase and cytochrome oxidase activity in EDL muscles (Fig. 6A and B). In muscles given weekly heat treatment, enzyme activity levels were maintained. Soleus muscle mitochondrial enzyme activity was unchanged with diet or heat treatment.

Acute effects of heat treatment on glucose uptake and mitochondrial function. We measured, for the first time, the effects of a single heat treatment on skeletal muscle glucose uptake and insulin signaling. As shown in Fig. 7, 24 h after heat treatment, insulin-stimulated glucose uptake was increased in EDL muscles above that seen with insulin alone, with no effect of heat treatment in soleus muscle. Basal glucose uptake was slightly increased 24 h after heat treatment in both muscle types,

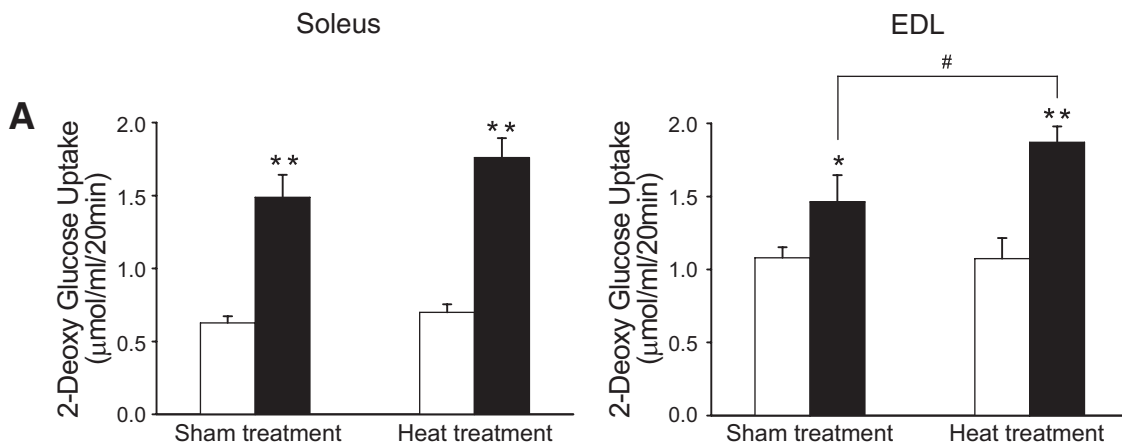


FIG. 7. A single bout of heat treatment improves insulin-stimulated glucose uptake in EDL muscle. Male Wistar rats received one heat bout of 41°C for 20 min or a sham treatment of 36°C for 20 min. Twenty-four hours after treatment, insulin-stimulated glucose uptake was assessed in isolated soleus and EDL muscles. ^{**} $P < 0.001$ and ^{*} $P < 0.05$ basal vs. insulin; [#] $P < 0.05$ sham vs. heat treatment. Values are means \pm SE for 7–10 muscles per group. □, basal (non-insulin stimulated); ■, insulin stimulated.

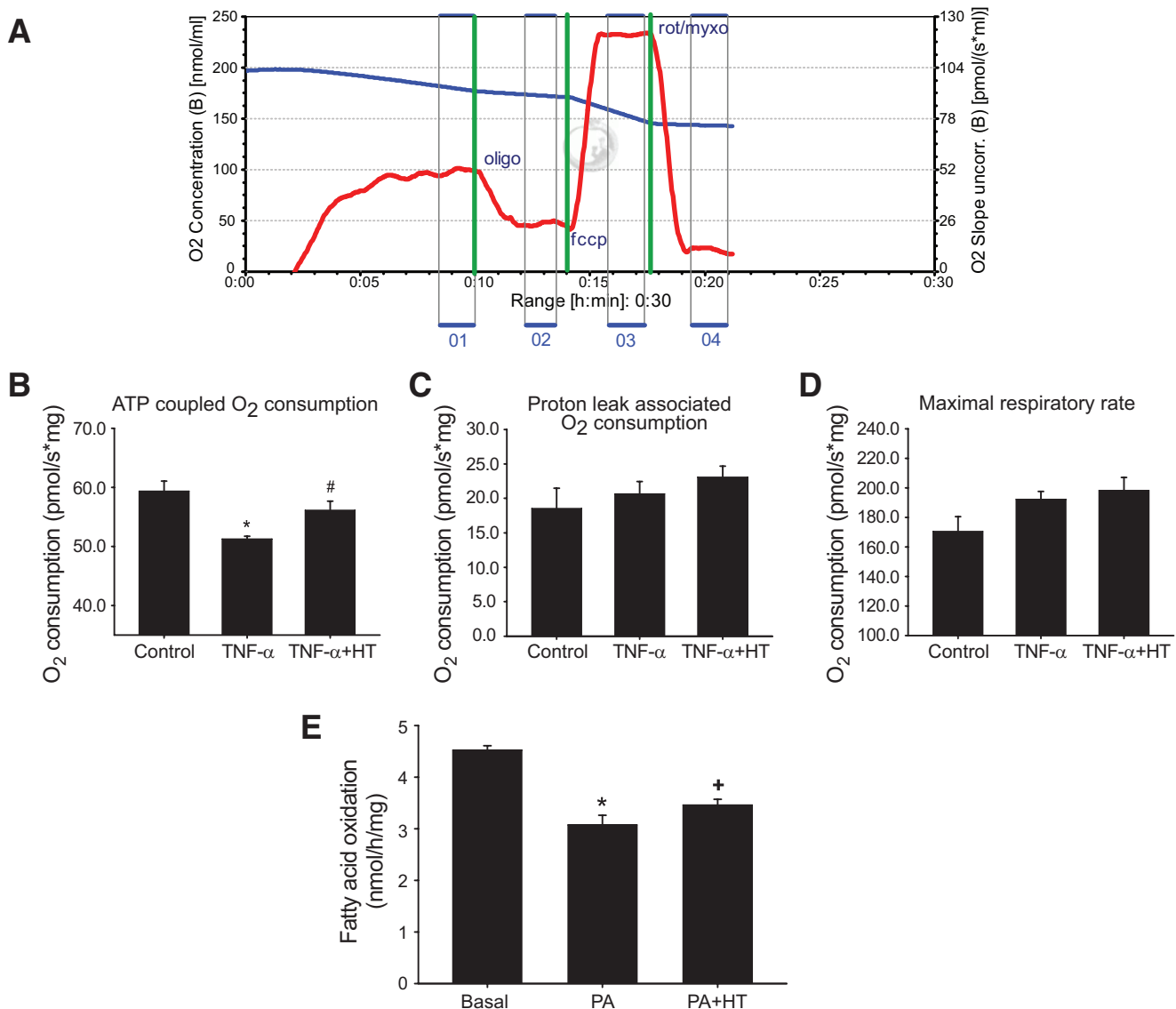


FIG. 8. Heat treatment improves oxygen consumption and fatty acid oxidation in L6 muscle cells. *A*: Representative tracing of O₂ consumption rates in L6 myotubes in a respirometer. Rates were determined by measuring O₂ consumption in time frames O1 for baseline, O2 for rates after 1 μ g/ml oligomycin addition, O3 for rates after 3 μ mol/l FCCP addition, and O4 for rates after 1 μ mol/l rotenone and 2 μ mol/l myxothiol addition. O₂ consumption rates of L6 cells were measured under control conditions, after treatment with 20 ng/ml TNF- α (24 h), and after treatment with both 20 ng/ml TNF- α (24 h) and one bout of heat treatment (43°C, 20 min, recovery 24 h). ATP-coupled oxygen consumption (*B*) was calculated as the mitochondrial rate sensitive to oligomycin, proton leak (*C*) as the mitochondrial rate insensitive to oligomycin, and maximal uncoupled respiration rate (*D*) as the O₂ consumption rate after FCCP addition (50). Rates were normalized to cellular protein, calculated in $\text{pmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$, and expressed as percentage of basal (* $P < 0.001$ TNF- α vs. control; # $P < 0.01$ TNF- α +HT vs. TNF- α). Values are means \pm SE for five to six samples per group. *E*: L6 cells were treated with 200 μ mol/l palmitic acid coupled with BSA for 72 h (PA), whereas control cells received BSA only (Basal). A subgroup of cells (PA+HT) received two heat treatments (43°C, 20 min), one right before the palmitate incubation and one after 48 h. Cells were washed thoroughly, and fatty acid oxidation was assayed using 125 μ mol/l [³H]palmitate-BSA. * $P < 0.001$ basal vs. PA and + $P < 0.05$ basal vs. PA+HT. Values are means \pm SE for four wells per group.

suggesting that a longer recovery interval after heat treatment may be optimal for glucose uptake. Phosphorylation levels of the insulin signaling intermediates Akt and AS160 were not increased in either muscle 24 h after one heat treatment (data not shown). Additional time points may be necessary to determine effects of acute heat treatment on insulin signaling.

To determine the impact acute heat treatment may have on mitochondrial function, we measured oxygen consumption in L6 muscle cells (Fig. 8A). L6 muscle cells treated with TNF- α , a cytokine known to induce mitochondrial dysfunction and insulin resistance (28), show reduced ATP-coupled oxygen consumption compared with untreated cells (Fig. 8B). In contrast, cells given one heat treatment in the

presence of TNF- α show preserved ATP-coupled oxygen consumption. Maximal respiratory rate and proton leak showed a nonsignificant increase with heat treatment (Fig. 8C and D). As another assessment of mitochondrial function, fatty acid oxidation in response to palmitate and heat treatment was measured in L6 cells. Exposing L6 cells to chronic palmitate results in decreased fatty acid oxidation and insulin resistance (29) and decreased mitochondrial enzyme activity (30). In the current study, chronic exposure of L6 cells to palmitate reduced the fatty acid oxidation rate, whereas heat treatment resulted in a small but significant increase in fatty acid oxidation (Fig. 8E). These findings suggest that acute heat treatment can positively enhance glucose uptake and mitochondrial function.

DISCUSSION

New data in the present study demonstrate the ability of heat treatment to restore glucose uptake and improve insulin signaling in skeletal muscle from rats fed a high-fat diet. Our results indicate that heat treatment can prevent an increase in JNK phosphorylation and IKK- β activation, possibly through direct interaction with the inducible HSPs, HSP72 and HSP25, respectively. Heat treatment increases mitochondrial HSP60 and UCP-3 expression and maintains mitochondrial enzyme activity in the presence of a high-fat diet. Our findings in L6 muscle cells indicate that acute heat treatment increases mitochondrial oxygen consumption and fatty acid oxidation. These results provide strong evidence that HSP induction in skeletal muscle could be a potential therapeutic treatment for obesity-induced insulin resistance.

Twelve weeks of a high-fat diet resulted in increased overall body weight and epididymal fat pad weight. Epididymal fat pad weight was reduced with heat treatment, although body weight and food intake remained the same between the high-fat-fed sham and heat-treated rats. These changes suggest an increased level of metabolism in the animals receiving heat treatment. Whole-body hyperthermia has been shown to reduce plasma triglycerides and free fatty acids in *db/db* mice (31), suggesting that heat treatment improves fatty acid utilization and reduces their accumulation. Data from the current study demonstrating increased fatty acid oxidation in palmitate-treated muscle cells with acute heat treatment support this idea. Future studies are needed to measure the effects of chronic heat treatment on fatty acid oxidation in skeletal muscle. Heat treatment, like exercise, could increase the demand of the cells for energy such that fatty acid uptake and utilization are matched, reducing the accumulation of free fatty acids and adipose tissue in the presence of a high-fat diet.

We demonstrate for the first time that heat treatment can restore insulin-stimulated glucose transport and increase activation of insulin signaling intermediates in skeletal muscles from high-fat-fed rats. Increased oxidative stress has been strongly implicated as a cause of diet-induced insulin resistance (32,33). Activation of stress-induced kinases such as JNK, glycogen synthase kinase 3 β , IKK- β , and protein kinase C (34,35) can cause inhibitory phosphorylation of IRS-1 on serine 307. A decrease in muscle oxidative stress with heat treatment could result in decreased stress kinase activation and improved insulin signaling. Alternatively, HSP72 and HSP25 have been implicated as natural inhibitors of JNK and IKK- β , respectively (36,37).

In soleus and EDL muscles, an increase in HSP72 expression with heat treatment was accompanied by a concomitant decrease in JNK activity. Our findings, combined with Chung et al. (1), indicate that HSP72 protection is strongly associated with prevention of JNK phosphorylation. HSP25 phosphorylation was increased in the EDL muscles with heat treatment, and a corresponding reduction in IKK- β was observed. However, without an induction of HSP25 in soleus muscle, IKK- β was unchanged with heat treatment. This suggests IKK- β may not play a significant role in insulin resistance in the soleus muscle, because insulin signaling and glucose uptake were restored despite the lack of effect on IKK- β . Higher constitutive levels of HSPs in the soleus muscle may provide protection from stress kinase activation, as evidenced by much smaller activation of JNK in soleus compared with

EDL. Muscle fiber type differences and the potential role of HSP25 were not explored in the previous study by Chung et al. (1) and demonstrate the importance of fiber type and pre-existing HSP levels in determining HSP function in skeletal muscle.

Changes in mitochondrial number and function with obesity and insulin resistance are currently controversial in the literature (30,38–42). Our findings agree with previous studies demonstrating an increase in mitochondrial proteins with a high-fat diet (40,41,43). Proteins involved in mitochondrial metabolism increased with a high-fat diet in EDL muscle; however, this increase did not occur in high-fat-fed rats given heat treatment. It is hypothesized that skeletal muscle mitochondrial biogenesis occurs as an adaptation to an increased supply of fatty acids with a high-fat diet. An increased muscle energy demand, as a result of heat treatment given in parallel with a high-fat diet, could reduce the accumulation of free fatty acids and preclude the stimulus for mitochondrial biogenesis with a high-fat diet in the EDL muscle. Interestingly, mitochondrial biogenesis occurs in response to a high-fat diet in fast-twitch glycolytic and not in slow-twitch oxidative muscle. To our knowledge, this is the first study to make this direct comparison in fast- and slow-twitch muscles and could explain contradictory findings in the literature using mixed muscle types. Oxidative muscle has greater potential to fully oxidize lipid substrates (44) and therefore may not undergo adaptive mitochondrial biogenesis in the presence of a high-fat diet.

Despite high-fat diet-induced mitochondrial biogenesis, citrate synthase and cytochrome oxidase activities decreased in EDL muscle. Bonnard et al. (30) suggest a decrease in citrate synthase activity in mice with 4 weeks of high-fat feeding, despite any other indications of mitochondrial dysfunction, could reflect initiation of mitochondrial dysfunction subsequently observed with 20 weeks of high-fat feeding. Other studies have demonstrated an increase in mitochondrial function, as measured by oxidative capacity, with a high-fat diet in parallel with increased mitochondrial protein expression (40,41,43). It is unknown whether muscle oxidative capacity could increase due to enhanced fatty acid oxidation even in the presence of decreased citrate cycle and respiratory chain enzyme activity. Interestingly, Koves et al. (42) found that despite high rates of fatty acid catabolism with high-fat feeding, moderate depletion of several citrate cycle intermediates occurred in insulin-resistant muscle. These authors hypothesize that an increase in fatty acid oxidation that is not matched by increased flux through downstream mitochondrial pathways results in an accumulation of incomplete fatty acid oxidation byproducts and reactive oxygen species (ROS) that could activate stress kinases (42,45).

In this context, heat treatment, like exercise training (42), could result in a coordinated increase between fatty acid oxidation and downstream mitochondrial pathways in skeletal muscle. In support of this idea, citrate synthase and cytochrome oxidase activity levels were restored in EDL muscles of rats given heat treatment in the present study. In addition, data from L6 muscle cells suggest that heat treatment can increase fatty acid oxidation and mitochondrial oxygen consumption. These data are supported by previous studies demonstrating that HSPs can protect and enhance mitochondrial function (46,47). Future studies are needed to assess chronic heat treatment on specific steps in fatty acid metabolism in conjunction with a high-fat diet.

Mitochondrial proteins HSP60 and UCP3 were increased in response to heat treatment in the current study. HSP60 has been shown to protect complex IV activity in mitochondria (48), whereas heat treatment enhanced mitochondrial complex I–V activity (47). Based on previous findings and those of the current study, the mitochondria may be a primary target site for the protective effects of heat treatment from oxidative stress (46). UCP-3 expression increased in response to a high-fat diet and to a greater extent with heat treatment in the EDL muscle. Although efforts to define UCP-3 function are ongoing, a recent study suggests that UCP-3 induction may limit ROS emission in conditions of lipid excess (49). Increased UCP-3 with heat treatment could reduce ROS and enhance metabolic flux through mitochondrial pathways. Future studies are needed to investigate these potential heat shock-mediated mechanisms in the prevention of insulin resistance.

In conclusion, our study shows that weekly in vivo heat treatment can mitigate high-fat diet-induced skeletal muscle insulin resistance. Our findings indicate that heat treatment decreases JNK phosphorylation and IKK- β activation, significantly improves insulin signaling and glucose uptake, and preserves oxidative phosphorylation capacity. Exercise, resveratrol, the small molecule BGP-15, and lipoic acid have all been shown to induce HSP expression. Targeted induction of HSPs for the prevention of insulin resistance is a realistic approach for future therapeutic applications.

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